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Candidate gene association testing in the dissection of genetic causes for depressive disorders and the response to antidepressant treatment

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List of symbols and abbreviations

CAMKK2 – calcium/calmodulin-dependent protein kinase
CEU sample – caucasian sample of the HapMap project[1]
CI – confidence interval
CRF – adrenocorticotrophic hormone-releasing factor
CRH – corticotropin-releasing hormone CRH
CYP2D6 – cytochrome P450, family 2, subfamily D
Dex – dexamethasone
DHW – deviation from Hardy-Weinberg equilibrium
FKBP4 – FK506-binding protein 4
FKBP5 – FK506 binding protein 5
GC – glucocorticoid
GR – glucocorticoid receptor (gene – *NR3C1*)
GRE – glucocorticoid responsive element
HPA axis – hypothalamic pituitary adrenal axis
HWE – Hardy-Weinberg equilibrium
LD – linkage disequilibrium
LMU – Ludwig-Maximilians University
MAF – minor allele frequency
MDD – major depressive disorder
MR – mineralocorticoid receptor (gene –*NR3C2*)
MTP – multiple testing problem
OASL – 2'-5'-oligoadenylate synthetase-like protein
OR – odds ratio
OPC – orthogonal polynomial coefficients
P2RX4 – purinergic receptor P2X, ligand-gated ion channel 4
P2RX7 – purinergic receptor P2X, ligand-gated ion channel 7
PS – population stratification
SD – standard deviation
SNP – single nucleotide polymorphysm
SSRI – selective serotonin reuptake inhibitor
TCA – tricyclic antidepressant

[1] http://www.hapmap.org

Chapter 1

Introduction

1.1 Objectives and structure of thesis

Both phenotypes major depressive disorder (MDD) and response to antidepressant treatment have been extensively investigated because of their social relevance. Chronic depressive disorder very often leads to disease related disability [120]. A mere 40-60 % of depressed patients enter full remission [18]. The remaining patients do not get essentially better, but they still have side effects by drug exposure. Identification of genes responsible for susceptibility to MD and being a non-responder is an essential step to individually adjusted treatment.

The main goal of this thesis is the identification of the susceptibility genes for MD and response to antidepressant treatment in order to gain a better understanding of its genetic causes. Currently, little is known about the nature of genetic variation underlying these complex traits. The main challenge in its investigation is the interplay of several genetical and environmental causes that obviously have small individual contributions in itself [136].

Many factors affect the outcome of an genetic association study. For this reason I address the particularities and problems relevant for the power and efficiency of association testing between the polymorphisms in candidate genes and clinical phenotypes in the first chapter of my thesis. There I describe the investigated phenotypes as well as known associations with candidate genes.

The data quality control and correction for false positive associations is a major and important part of each association study. False positive associations could be caused by technical problems as well as statistical fluctuations or systematical biases due to study design. Preliminary tests regarding population stratification and comparison of haplotype estimation algorithms are reported in appendix. Results of these tests are important for correctly performing of following analysis.

Because of the large number of tests in current studies it is necessary to address the multiple testing problem (MTP). Especially in the era of "whole genome association stud-

ies" the MTP has become a very important subject. For this reason chapter two includes methodical investigation on this research field that corresponds to the published manuscript [151]. In this part of thesis I compare the rapid method proposed by Nyholt [126] with the computationally intensive permutation based method.

In chapter three and four I test the association of candidate genes with MDD and response to antidepressant treatment. Chapter three details variability in the gene coding for a purinergic ligand-gated ion channel (*P2RX7*) and its association with MDD that corresponds to a recently published manuscript [109]. The *P2RX7* gene was chosen as a highly interesting candidate gene on the basis of published linkage and association within loci in 12q24.31 in a patient sample with bipolar depression [119, 161]. These loci contain the *P2RX7* gene as well as *P2RX4* and *CAMKK2*. The goal of this chapter is to determine if *P2RX7* or one of the neighboring genes are the susceptibility genes for MDD, and in the case it is true, to identify the causative genetic variant as well as a high risk genotype and to estimate its effect size.

Chapter four emphasizes the role of the *FKBP5* gene in response to antidepressant treatment. Polymorphisms in this gene were previously reported by our group to be associated with early response to antidepressant treatment [19]. The exact location of the causal polymorphism could not be identified because of extended linkage disequilibrium (LD) between markers [19]. In the present study I search for potential causal variants using fine mapping in an enlarged sample. In addition I resequence all potentially functional areas within that gene. I test for statistical interactions of genetic variants within *FKBP5* as well as between *FKBP5* and corticosteroid receptor genes. Moreover, I examine in this chapter whether interaction of polymorphisms in investigated genes interfere with previous disease history in predicting response.

1.2 Association studies of complex disorders

Disorders or traits with a lack of Mendelian inheritance patterns are referred as polygenic, multifactorial or complex. The contribution of variation of single genes on the phenotype is assumed to be only modest. The approach most often used these days for such studies is the case-control design in which a difference in allele frequencies between unrelated affected individuals and unrelated unaffected controls is tested for significance [79].

These association studies most commonly use single nucleotide polymorphisms (SNPs) for the dissection of the genetic determinants of complex diseases. They may be classified into two different groups: genome-wide association studies and candidate gene association studies. In our study we focus on the candidate gene approach. It includes two steps: the first one is the choice and/or discovery of the different polymorphic sites within the candidate gene region and the second one is the association testing *per se*. The data used to test for association are often presented in the form of contingency tables. In the contingency tables for our purposes rows describe an affection status and the columns refer to either specific alleles, genotypes, or haplotypes. A commonly used test statistic for association testing with such data is the χ^2 test for homogeneity of proportions. In case the expected number in any field of the contingency table is less than five, it is commonly advised to use the exact Fisher's test [61]. Sometimes it is necessary to consider gender or any other group-specific effects, which may be used as covariates in logistic regression [84]. Regression techniques may be also used in the case of quantitative dependent variables (quantitative phenotypes).

1.3 Factors affecting the power to detect genetic association

In this chapter several parameters are described that are crucial for association studies such as the extent and amount of linkage disequilibrium between marker and disease allele, the marker allele frequency and the disease allele frequency, and the effect size of the true disease causing allele.

1.3.1 Linkage disequilibrium

Most association studies of genetic variants are indirect. This means that observed genetic variants (markers) that are in proximity to a disease-causing variant on a particular chromosome will be more often co-inherited with the disease-causing variant than expected under independent assortment. This lack of independence among different polymorphisms is termed linkage disequilibrium (LD), and arises because the variants share a joint population history [108]. The amount of LD between any two markers is influenced by the classical forces of recombination, natural selection, mutation, genetic drift, ancestral population demographics and mating patterns [41, 141, 187, 208].

Patterns of LD are well known for being noisy and unpredictable [187]. In many studies it was shown that although the LD is significantly related to genetic distance, small physical distance does not guarantee a high level of LD [3, 63, 159, 165]. For example, pairs of sites that are tens of kilobases apart might be in complete LD, whereas nearby pairs of sites from the same region might only be in weak LD [130, 165, 187]. The probability of any particular pair of SNPs being in LD is not predictable, and, as a result, LD should be determined empirically for any specific genomic region in any population [165, 188].

Various statistical measures can be used to summarize LD between two markers, but in practice only two, termed D' and r^2, are widely used [49, 71]. Both measures are built on the basic of a pairwise-disequilibrium coefficient, D, which is the difference between the probability of observing two marker alleles on the same haplotype and observing them independently in the population: $D = f(A_1B_1) - f(A_1)f(B_1)$, where A and B refer to two genetic markers with alleles '1' and '2', 'f' is their frequency [108]. Then $D' = D/D_{\max/\min}$, where

$$D_{\min/\max} = \begin{cases} \max(-f(A_1)f(B_1), -f(A_2)f(B_2)) & D < 0 \\ \min(f(A_1)f(B_2), f(A_2)f(B_1)) & D > 0 \end{cases} \tag{1.1}$$

The second LD measure is

$$r^2 = \frac{D^2}{f(A_1)f(B_1)f(A_2)f(B_2)} \tag{1.2}$$

D' has a very simple interpretation: a value of 0 implies independence, whereas a value of 1 means that all copies of the rare allele occur exclusively with one of two possible alleles

at the other markers. The latter case is defined as complete LD [190]. The r^2 measure has a strong theoretical grounding in population genetics and more desirable statistical properties. An r^2 value of 0 also implies independence, but $r^2 = 1$ has a more strict interpretation than that of $D' = 1$: $r^2 = 1$ only when the marker loci have identical allele frequencies and every occurrence of an allele at each of the markers perfectly predicts the allele at the other locus: the markers are in perfect LD. These two measures behave very differently. High values of D' do not necessarily imply high r^2 and are not inconsistent with low values of r^2. In particular, there seems to be much more random variation in values of D' at a given recombination distance [130, 137, 187].

D' may vary between 0.0 and 0.4 for markers that are effectively independent, while r^2 tends towards 0.0 for independent SNPs [137].

The strength of LD between marker and disease locus in a given population directly influences the power of an association study using a given sample size. For example, if the required sample size for a given test size and power in the case of perfect LD (if r^2 between marker and susceptibility locus is equal to 1) were 1000 cases and controls. In the imperfect, although more realistic situation of, say, $r^2 = 0.2$, the sample size needed would be $1000/0.2 = 5000$ cases and controls for identical power and test size. In other words the sample size must be increased by a factor of $1/r^2$ [137, 208].

There are different opinions about what amounts of LD are required for association studies. The answer to this will vary in a any specific case as a function of disease and marker allele frequencies, sample size and effect size on the true disease causing allele, as well as power and specified test size.

1.3.2 Disease and marker allele frequencies

It is not yet clearly known whether causative mutations for complex traits are more likely to be common or rare [33, 137, 189]. If we argue that the fact that environmental factors have an important role in complex traits means that individual genetic variants have low attributable risks, than it is possible that these diseases are common because of highly prevalent environmental influences, but not because of common disease alleles in the population. On the other hand common complex disease may be caused by common variants. In reality, probably both mechanisms may exist in various combinations.

The second important point is that differences between the disease allele frequency and the frequency of either the single SNP or haplotype that is in LD with the disease allele dramatically changes the power to detect association. Power of an association study is greatest when the marker and disease allele frequencies match [2, 97].

Because the sample size in an association study is restricted, we only consider disease alleles that are of detectable frequency in the population, which is mostly greater than 5 %.

1.3.3 Genotype relative risk (effect size)

Genotype relative risk is easy to describe in the following expression: $r_1 = f_1/f_0$ and $r_2 = f_2/f_0$ [154]. f_0, f_1, f_2, are the probabilities of being affected in individuals who have 0, 1 and 2 copies of the disease gene, respectively.

From a clinical perspective, the larger the genotype relative risk, the more easily one can distinguish an individual's disease genotype based on their disease phenotype.

The standard measure for effect in a case-control study is the odds ratio (OR), defined as the odds of exposure among cases divided by the odds of exposure among controls.

The effect size depends on four parameters: the OR of the true disease causing allele, the amount of LD between marker and disease allele, the marker allele frequency and the disease allele frequency [147].

If the effect size of the true disease causing allele is small, for example is equal to 1.3, even in the case that disease and marker allele frequencies are about 0.5 and both are in LD($D' \geq 0.7$) at least 2100 cases and controls are required for a reasonable chance (power $\approx$ 80 %, $\alpha = 0.001$) to find this association [208]. By contrast, if the OR is equal to 3, $D' \geq 0.7$ and marker and disease allele frequency are about 0.05 we will need merely 360 cases and the same number of controls to be able to detect this effect [208].

1.3.4 Misclassification errors

Inclusion of incorrect data in genetic analysis can lead to the generation of false conclusions [1, 175] and a reduction of power [65, 64] to fine map trait loci. Additionally, genotyping errors can bias LD measurements [5].

The most frequently occurring genotyping errors are misclassification errors, for example if heterozygotes are interpreted as homozygotes and inverse. The misclassification may occur systematically or randomly. Systematic misclassifications are possible for example if signals of heterozygotes are not as intensive as of homozygotes, then heterozygotes will be under-represented. DNA contamination in samples lead typically to an excess of heterozygous genotypes in the sample. Quality of DNA, instability of genotyping reagents, pseudo-SNPs (i.e., ectopic sequence variants and paralogous sequence variants) and operator error contribute to the overall genotyping error rate [107].

For population- and family-based data, individuals can be genotyped repeatedly to determine the rate of genotyping errors. However, if a genotyping error occurred repeatedly, it will not be detected, and genotyping error rates will be underestimated. If it is suspected that DNA contamination has taken place, the samples should be genotyped at several microsatellite markers. In the case of DNA contamination, more than two alleles would be represented, but only two expected for diploid genomes.

Individuals from population-based studies, or controls from case-control studies, can be analyzed to determine whether their genotype data are in Hardy-Weinberg equilibrium

(HWE) to give clues as to whether or not genotyping errors at particular marker loci may have occurred [83]. It should be noted that deviations from HWE are not necessarily due to genotyping error and may be due to chance or other genetic factors such as a protective allele, population admixture/stratification, inbreeding, or deletions. Although testing for deviations from HWE is commonly used to detect potential genotyping errors, the power of this technique is low even for high error rates (i.e. $\alpha = 15\,\%$) [107].

1.3.5 Deviation from Hardy-Weinberg equilibrium (DHW)

As mentioned above, testing for HWE is commonly used for quality control of large-scale genotyping and is one of a few ways to identify systematic genotyping errors in unrelated individuals. Thus it is possible that investigators mistrust data with DHW and may sometimes ignore polymorphisms with DHW in their studies. This way of interpreting DHW is not necessarily consistent.

J.K. Wittke-Thompson et al. [197] suggested to assess markers with DHW in a more logical and systematic way by distinguishing those that could be attributed to an underlying genetic model at the susceptibility locus from those due to genotyping errors, chance, and/or violations of the assumptions of HWE, thereby improving the quality of scientific inferences. Associations between variation in candidate genes and complex disorders are often correlated with existence of DHW, because the associations are genotypic rather than allelic, and genotypic differences may result in DHW. Depending on the underlying genetic model DHW may occur in cases and/or controls with a lack or an excess of heterozygous genotypes. The observed deviations from the expected distribution in heterozygotes should be in opposite directions in cases and controls. DHW in cases and/or controls may be present in recessive, dominant and additive models. DHW in patients in contrast is never expected for a multiplicative model, and is less likely if the susceptibility-allele frequencies are extreme small or large [197]. Consistency of observed DHW with genetic models does not mean that errors, missing data patterns, or violations of HWE assumptions did not generate or contribute to the observed DHW. Admixture, assortative mating or natural selection of particular genotypes may cause DHW.

1.3.6 Population stratification

Case-control studies from different laboratories often yield contradictory results [78]. This lack of reproducibility is attributed to marked variation of disease prevalence and marker allele frequencies within subpopulations, so called population stratification (PS). PS can lead to false evidence for an association. If both the disease and a marker genotype have higher frequency in any of the subpopulations within the investigated population, there will be an apparent association between the marker genotype and the disease at the population level, even though there is no true association at the subpopulation level. In the case that

only two subpopulations are mixed the type I error rate will increase with a lower disease prevalence ratio and a higher marker frequency difference [73]. On certain conditions, by increasing the number of subpopulations, type I error rate can decrease, but in general PS biases association results.

PS may affect LD between a pair of SNPs. When subpopulations have significantly different allele frequencies LD between a pair of SNPs in the combined population can be stronger than in either subpopulation [24]. In some cases PS may lead to deficiency of heterozygotes, which in turn leads to DHW in the sample.

Recent methods, known as Genomic Control, have been proposed to adjust for population stratification. These methods use a panel of unlinked markers to determine if PS exists and if so, provide a means to adjust for it.

Genomic Control is conceptually simple: the method examines the distribution of association statistics (χ^2) between unlinked genetic variants typed in cases and controls. The statistic at a candidate allele being tested for association can then be compared with the genome-wide distribution of statistics for markers that are probably unrelated to disease to assess whether the candidate allele stands out. In the absence of stratification, the test statistic for association between an unlinked genetic variant and disease should follow a χ^2 distribution with 1 degree of freedom [50, 141]. In the presence of stratification, the distribution of association statistics should be inflated by a value termed λ, which becomes larger with an increased sample size [62].

1.4 Block concept

Regions with very high LD would be expected to be a consequence of a low population recombination rate. Actually, there is evidence of a striking negative correlation between the average recombination rate and average block length [39, 30, 56, 67, 95, 137, 188]. However, the rate of recombination is not a uniform function of the physical distance between two markers, leading to the concept of recombination hot spots and cold spots [132]. Several studies showed that patterns of LD are organized in block-like structures defined haplotype blocks [41, 89, 140]. Wang et al. [188] have demonstrated that randomly distributed recombination (i.e., no hot spots) with varying recombination rates across the chromosome can explain empirical data. At the same time many other authors concluded that block boundaries are largely shared across populations, which, however, supports the recombination hot spot hypothesis [30, 39, 56, 63, 95].

Against all expectation certain surveys found a small number of extremely long haplotype blocks. Among the published studies, the longest reported block is a region of low haplotype diversity on chromosome 22 that in individuals of European descent stretches across 804 kb [43]. LD block length described in the literature is mostly less than 5-20 kb in Europeans and Americans of North-European descent. By contrast, LD in Africans and African-Americans is markedly less [33, 56, 63, 95, 140, 188]. The latter two are 'older' populations, in which more recombination events have occurred that lead to shorter LD blocks than in 'younger' populations.

Ideal approach for haplotype block identification is one based on the distribution of observed recombination crossovers between loci. Unfortunately, on the genotypic data it is difficult to determine precisely where the recombinations have occurred. For this reason different computational methods are used for haplotype block estimation. Several are based on the concept of 'chromosome coverage', with a haplotype block containing a minimum number of SNPs that account for a majority of common haplotypes [130, 204] or a reduced level of haplotype diversity [6, 41]. Others are based on different subjectively determined thresholds [133], on the confidence-limit [63], or on the distribution of observed recombination crossovers between loci [39, 56, 188].

Ke et al. [95] and Schwartz et al. [157] compared three block definitions from publications [63, 130, 133, 188]. The authors demonstrated that there is a great instability in haplotype block boundaries and numbers of blocks when examining sub-samples of SNPs with different marker densities. Phillips et al [133] identified diverse block partitioning in the dynamic programming algorithm using different parameters.

I do not give here detailed descriptions of different methods. Considering published experience, I can conclude that the block concept has several difficulties including: the arbitrary nature of block definitions; the instability of blocks under different scenarios (especially different marker densities); the relatively limited extent of block coverage in the genome; the

high level of haplotype diversity and divergence in haplotype composition between different populations [33].

Thus, it seems likely that methods for LD testing that do not arbitrarily impose block boundaries among correlated SNPs might perform better than block-based methods [157].

1.5 Tagging-SNPs

In general, selection of non-redundant markers from a larger set has been called 'tagging'. A SNP or a set of SNPs that have been selected on the basis of LD patterns to represent other SNPs are referred as tagging-SNPs (tSNPs) [30].

Given an initial set of densely-spaced and potentially redundant markers, tagging aims at reducing the scale and cost of genotyping in subsequent studies, while retaining most or all of the information provided in the dense map [92, 130, 189].

Several algorithms and computer programs have been developed to define sets of tSNPs. It was demonstrated that tSNPs performed substantially better than randomly spaced SNPs [24, 30].

All existing algorithms can be divided into two general categories: haplotype block methods and block free methods. In the first category, genomic regions are partitioned into haplotype blocks within which sets of tSNPs are selected by measures of haplotype diversity [9, 92]. The second category of block-free methods was proposed with an added feature that the SNPs with high LD levels do not necessarily have to be adjacent to each other [24, 25, 115, 145, 168]. Taking into account the above mentioned difficulties in block concept the advantage of the second category is obvious.

Haplotype-based tagging methods have several disadvantages. A potential problem is that the frequencies of the haplotypes are not known and should be estimated based on genotype data from unrelated individuals. The estimation error increases with decreasing LD within a genomic region [200] or with the reduction of sample size [167, 168]. In large samples the certainty of haplotype frequencies is near 100 % [189], but not the individual's haplotypes. The idea of tagging implies that tSNPs should be chosen based on a small samples otherwise there is no reduction in genotyping costs. Several researchers have also showed that different methods for haplotype estimation yield different frequencies of estimated haplotypes and this inconsistency increases when data are not completely available [57, 167, 205].

Moreover, almost all the current approaches assume random union of gametes (or haplotypes), and any departure from HWE may lead to biased estimates of haplotype frequencies and indices biased on these frequencies [57, 189]. HWE may not be met in some samples.

It has been suggested that tSNPs selected based on haplotype diversity do not perform better than randomly selected markers in retaining power [206]. This is possible in case where the causal SNP is rare (MAF $< 10\,\%$) and the chosen tSNPs are common [189]. If a causal SNP occurs at a frequency that is different from the average of the observed set of markers in a region, the chance of this causal SNP being detected by any of the tSNP sets in the region would be low, especially if the SNP was rare [94]. Weale [189] et al. have demonstrated that a small tSNP set could be very effective in predicting the allelic state of causative mutation of unknown allele-frequency, given that such mutations are likely to be part of the same haplotype structure. Discrepant SNPs that distinguish recent haplotypes

will not generally be well tagged [189].

To avoid these disadvantages one can use r^2-based tagging suggested by Carlson at al [24]. Measures based on r^2 have the advantage that they approximately represent the effective units of sample size in a case-control association study [30, 137].

The idea of finding useful tSNPs does not in itself depend on the existence or quality of LD blocks [189]. Carlson's approach relaxes all block requirements and uses only the correlations between all markers, whatever their respective locations [24]. Imputation of the haplotype phase for each pair of SNPs in the region is required to compute r^2.

This r^2 method relies on a clustering technique in which a SNP is designated as tSNP for a cluster if a pairwise r^2 values between this SNP and all the other SNPs in the same cluster exceeds a specified r^2 threshold, for example 0.75. Different alternative clustering procedures are possible. The simple way to minimize the number of tSNPs is to look for the largest cluster in each iteration. In that case tSNPs will be the SNPs that had the highest r^2 with all SNPs in the corresponding cluster, defined 'bin'. If two SNPs could be tSNPs it is reasonable to select the one genotyped successfully in most individuals.

Carlson showed that LD-selected tSNPs are more powerful than an equivalent number of either haplotype-selected tSNPs or randomly selected SNPs. Moreover this method is robust to recombination history within the gene [24]. In fact, in the context of genetic mapping, it is more relevant to consider the r^2 measure of LD between the tags and the SNPs they are meant to represent [137], which will generally include the functional SNPs that are to be identified [30].

Recently, several genotype-based methods for tagging were suggested. Hu et al. proposed a new robust method based on power calculations from genotype data [85]. But this method requires the disease model to be specified in association study, because it greatly influences the power of the tSNPs for detecting unknown SNPs. Unfortunately, the underlying disease model is usually unknown. Another procedure proposed by Rinaldo [145] is a slightly modified Carlson's method with using Pearson's correlations between unphased genotypes instead of LD measures.

1.6 Haplotypes

A haplotype is defined as a sequence of polymorphic genetic attributes (in our study SNPs) characteristical for the single strand of the DNA-segment. Grouping SNPs in haplotypes leads to changing of dimension in association analysis. Haplotypes provide more information than the corresponding genotypes, since haplotypes include the information about genotypes as well as the phase information. Haplotypes may be directly responsible for the observed variation of the trait of interest, variants of promoter activity or protein structure and function. Several studies indicate that using extended marker haplotypes can provide additional power in detecting associations [106, 174, 207].

The diversity of haplotypes depends on the number of considered SNPs. A block of N independent biallelic SNPs could in theory generate 2^N different haplotypes. In reality, in the absence of recurrent mutation and/or recombination the number of observed haplotypes is often not greater than $(N + 1)$ [3]. Conventionally, haplotype phases have been resolved by tracing chromosomal transmission through extended families. Such extensive pedigree data are often not available in association studies where unrelated individuals or small nuclear families are used. Haplotype phases can also be determined by using molecular approaches, such as cloning, allele-specific polymerase chain reaction and single molecule dilution [31, 116, 148, 149]. These molecular methods are labour-intensive and expensive to use in haplotype determination and, therefore, are not suitable for high-throughput applications. A cheap and relatively straightforward alternative for haplotype estimation is the application of computational algorithms to predict haplotypes by using genotype data.

Different methods for haplotype reconstruction in a population are available that do not require assumption about LD between markers. I describe here only several of them.

1.7 Haplotype estimation methods

1.7.1 Clark's algorithm

The first algorithm for haplotype reconstruction (from genotype data) was described by Clark [29]. The algorithm starts by listing the haplotypes which exist with certainty in the sample; that is the haplotypes within individuals with no more than one heterozygous locus. The algorithm then iterates through a procedure which assigns ambiguous haplotypes to those in the known list. Solutions are dependent on the order in which the individuals with unresolved haplotype phase are entered. The algorithm does not assume HWE, unlike the other procedures for haplotype reconstruction, which are described below.

1.7.2 The expectation-maximization (EM) algorithm

This is an iterative maximum likelihood method to compute successive sets of haplotype frequencies $p_1, p_2, \ldots, p_h$, starting with initial arbitrary values $p_1^{(0)}, p_2^{(0)}, \ldots, p_h^{(0)}$. These initial values are used as if they were the unknown true frequencies in order to estimate genotype frequencies $P(h_k h_j)^{(0)}$ (the expectation step). These expected genotype frequencies are used in turn to estimate haplotype frequencies at the next iteration $p_1^{(0)}, p_2^{(0)}, \ldots, p_h^{(0)}$. (the maximization step), and so on, until convergence is reached (i.e., when the changes in haplotype frequency in consecutive iterations are less than some small value). This algorithm is based on the assumption of random union of gametes (or haplotypes), and any departure from Hardy-Weinberg (HW) equilibrium may lead to biased estimates of haplotype frequencies and indices based on these frequencies [57]. A potential problem of this method is that the variance of the estimates of haplotype frequencies is not known.

Depending on the initial conditions, the EM algorithm may not find the true maximum likelihood because it can lead to a local rather than global optimum, or the iterative process may stop before reaching the optimum [57].

In addition, the average performance of the EM algorithm is consistently worse for small samples, even when only two polymorphic loci are considered [189].

Nevertheless, the method often provides reasonably accurate estimates of haplotype frequencies. Good initial values are based on the product of the observed allele frequencies [57].

Fallin and Schork [58] have demonstrated via extensive simulation studies high accuracy in haplotype frequency estimation for biallelic diploid samples by using the EM algorithm. They have found that the estimation error is decreased by a number of factors: an increased sample size, a decreased ambiguity (proportion of the unphased heterozygous individuals), increased maximum haplotype frequencies, an increased LD between single nucleotide polymorphisms (SNPs) as wel as an increased number of rare SNPs.

1.7.3 EM algorithm based approaches for long haplotypes

Another approach is suggested by Clayton [138]. This method use the Markov chain Monte Carlo (MCMC) approach. It adds SNPs one at a time and estimates haplotype frequencies, discarding haplotypes with low frequency as it progresses. Clayton refers to the problem of 'culling haplotype assignments at early stages', which Qiu et al. described [138]. Clayton suggests including loci in different orders and observing whether the solution obtained varies. The algorithm works well, but it is possibly even more important to be careful when this algorithm is used. Clayton's program is called SNPHAP[1] and runs on Unix.

1.7.4 A coalescent-based Bayesian algorithm

Stephens et al. [167] proposed a MCMC approach for reconstructing haplotypes from genotype data. This involves a step where one samples from the conditional distribution $pr(H_i|G, H_{-i})$, where H_i represents the haplotypes for the i^{th} individual and H_{-i} the haplotypes for all the other individuals in the sample, and G represents the genotypes of all the individuals. Under most mutation models this conditional distribution is unknown. Stephens et al. [167] suggested to use an approximation to a general mutation model. In practice this approximation ensures that the haplotypes which are generated are similar to haplotypes that have been generated. This approach is implemented in the program PHASE[2]. According to Stephens and Donnelly [166] a new model that allows for recombination and decay of LD with distance has been implemented. The program also allows the user to estimate recombination rates, to identify recombination hotspots from population genotype data, and to perform a test for haplotype frequency differences between cases and controls.

[1] available at http://www-gene.cimr.cam.ac.uk/clayton/software/

[2] available at http://www.stat.washington.edu/stephens/software.html

1.8 Gene-gene interaction

Complex traits arise as a result of the interplay between many genetic variants and environmental exposures. The ubiquity of biomolecular interactions in gene regulation and biochemical and metabolic systems suggest that the relationship between DNA sequence variations and clinical endpoints is likely to involve gene-gene interactions. Thus, complex interactions are more important than the independent main effects of any single susceptibility gene [118].

The study of interlocus interactions in complex disease has been confused by differences in definition and terminology between biologists, epidemiologists and statisticians, and between quantitative and human molecular geneticists. The term 'epistatic' was originally introduced by Bateson [15] to describe a masking effect whereby a variant or allele at one locus (denoted at that time as an 'allelomorphic pair') prevents the variant at another locus from manifesting its effect. This was seen as an extension of the concept of dominance for alleles within the same allelomorphic pair i.e. at a single locus. The locus being masked is said to be 'hypostatic' to the other locus. This definition is actually closest to the mechanistic model of interaction of proteins.

The latter definition is applicable for quantitative traits, but it was not trivial to map the discrete segregation of alleles onto the continuous range of measured traits. R. A. Fisher [59] first had to deal with this essential problem. He used the term 'epistatic' in a different sense from its original usage. In Fisher's definition, epistasis refers to a deviation from additivity in the effect of alleles at different loci with respect to their contribution to a quantitative phenotype. Epistasis in the Fisher's sense is closer to the usual concept of statistical interaction: departure from a specific linear model describing the relationship between predictive factors (here assumed to be alleles at different genetic loci) [35, 118, 186] and is slightly more inclusive than what Bateson originally meant [134].

Biologically epistasis plays a key role in reproductive isolation – genes that function well in conspecific genetic backgrounds function poorly when combined in interspecific hybrids. Epistasis influences the evolution of genetic systems such as sex, diploidy, dominance, or the contamination of genomes with deleterious mutations [152, 186]. Simpler genomes such as those of RNA viruses display antagonistic epistasis (mutations have smaller effects together than expected); bacterial microorganisms do not apparently deviate from independent effects, whereas in multicellular eukaryotes, a transition toward synergistic epistasis occurs (mutations have larger effects together than expected) [152].

In human genetics, three main models of gene interaction for penetrance (the probability of developing disease given a certain genotype) are commonly considered [146]. The first is a heterogeneity model [123, 146], in which an individual becomes affected through possessing a predisposing genotype at either of two loci. The second is an additive model, which has been shown to approximate the heterogeneity model when modeling familial relative risks

[36, 146]; and the third is a multiplicative model [80]. The additive and heterogeneity models are usually assumed to represent non-epistatic models and to correspond to a situation in which the biological pathways involved in disease are at some level separate or independent. The multiplicative model is usually considered to be an epistatic model in which the loci and pathways involved are not independent. However, a multiplicative model can be considered to be an additive model when transformed to the logarithmic scale. In a statistical sense, therefore, the multiplicative model signifies independent additive effects on a logarithmic scale. A fourth model is one of additivity on a liability or probit scale, where loci contribute to an underlying, unobserved, continuous trait in an additive fashion and development of disease occurs if this trait exceeds a certain threshold [37, 60, 112, 131].

Mathematically, the quantitative genetic concept of epistasis may be represented for two loci by the linear model. In this model, alleles at loci, A and B (the independent variables), affect the phenotype (the dependent variable, P) in a manner described by the equation:

$$P = b_1 A + b_2 B + b_{12} AB + e \tag{1.3}$$

where b_1 and b_2 are the average effects of alleles at the A and B loci, respectively, on the phenotype P, whereas b_{12} describes the effect of interactions between alleles at the two loci (e reflects stochastic variations arising from the environment or from loci not being considered). The absence of epistasis means that the interaction coefficient b_{12} is zero; that is, the loci act additively (independently) [186].

Given case-control data we may compare this model with the null model, without an interaction term, using standard statistical software packages for logistic regression [37, 35].

Unfortunately, there is not a precise correspondence between biological models of epistasis and those that are more statistically motivated. We should like to perform a statistical test and interpret the outcome biologically, but this is in general not permissible. Statistical interaction does not necessarily imply interaction on the biological or mechanistic level [35]. Thus statistical modeling can only play a limited role in helping to understand biological interaction. Although, statistical modeling of interactions involving genes may be helpful in identifying genes influencing disease susceptibility which otherwise would remain unidentified. Moreover, if there is a multiplicative model, although there is considered to be no statistical interaction there may be still biological interaction. Adequate fitting to a statistical main effects model without interaction does not necessarily imply biological independence. Biological interaction can yield both a multiplicative and an additive statistical model. In particular cases, the statistical interaction can not be shown, because the power of interaction testing is limited. Breslow and Day pointed out that the sample size required to detect interaction is always at least 4 times what is needed to detect a main effect of the same size [21]. Only if the prior biological model can be postulated in some detail is it likely that statistical modeling of this kind will allow insight into the underlying biological mechanisms [35].

1.9 Multiple testing problem (MTP) in association studies

Case-control studies are used to test for association between a trait, e.g. disease, and candidate genes or regions. For this aim a number of SNPs in the genes or regions are genotyped. Association tests may be performed for each SNP individually or for sets of SNPs grouped into haplotypes or multiple genotypes. The number of tests performed has to be taken into account when judging their statistical significance. Otherwise, the probability of obtaining at least one false positive association will potentially be much greater than its nominal value (usually 0.05).

By setting the error rate on the individual test and not on the entire set of tests the likelihood of detecting true effects can be very low, because the false positive rate will greatly increase. For typical genotype-phenotype association studies many thousands of statistical tests will be examined. In this case we expect hundreds of falsely called associated genes among the set of genes exceeding the threshold. To control this multiplicity the family-wise error (FWE) methods for correction for multiple testing are adopted. The goal of multiple testing procedures (MTPs) is to control the 'maximum overall Type I error rate', which is the maximum probability that one or more null hypotheses is rejected incorrectly.

1.9.1 Definition of family-wise error rate (FWER) and false discovery rate (FDR)

Family-wise (FW) methods control the probability of committing one or more type I errors. 'Family' in this context is to be understood as a set of experiments, i.e. set of tests for dependent variables and the number of SNPs within one gene or a group of genes.

Suppose we have m (null) hypotheses H_0, of which m_0 are true. R is the number of hypotheses rejected. table 1.1 summarises the situation in traditional form. R is an observable random variable; U, V, C and T are unobservable random variables.

	Declared non-significant	Declared significant	Total
True null hypotheses	U	V	m_0
Non-true null hypotheses	T	S	$m - m_0$
	$m - R$	R	m

Table 1.1: Hypotheses definition

If each individual null hypothesis is tested separately at level α then $R = R(\alpha)$ is increasing in α. In terms of these random variables, the per-comparison error rate (PCER) is $E(V/m)$ and the family wise error rate (FWER) is the probability $P(V \geq 1)$ [16].

Another approach to controlling error in the multiple-testing situation which affords a greater power to detect true effects than the conventional FW methods is the false discovery rate (FDR) presented by Y. Benjamini and Y. Hochberg [16]. Applied to association studies the FDR estimates the proportion of genes that are falsely called associated among all genes called associated. It can be expressed as the random variable $Q = V/(V+S)$ – the proportion of the rejected null hypotheses which are erroneously rejected. Naturally we define $Q = 0$ when $V+S = 0$. Q is an unobserved random variable. Therefore the FDR is the expectation of Q.

$$FDR = E(Q) = E\{V/(V+S)\} = E(V/R) \tag{1.4}$$

where $V/R = 0$ when $R = 0$.

If all null hypotheses are true, the FDR is equivalent to FWER, otherwise FDR is smaller than or equal to the FWER.

1.9.1.1 Procedure

The Bonferroni inequality is often used when conducting multiple tests of significance to set an upper bound α on the FWER [117]. If $p_1, \ldots, p_m$ is a set of p-values for testing for corresponding $H_1, \ldots, H_m$ hypotheses, the classical Bonferroni multiple testing procedure is usually performed by rejecting $H_0 = \{H_1 \cap H_2 \cap \ldots \cap H_m\}$ (i.e. H_0 is the null hypothesis that include all individual hypotheses $H_1, H_2, \ldots, H_m$ are true) if the minimum p-value (denoted $\min p$ in the sequel) is less than α m. Furthermore the specific hypothesis H_i is rejected for each $p_i \leq \alpha/m$ $(i = 1, \ldots, m)$.

The Bonferroni inequality:

$$P(\cup_{i=1}^{m}(p_i \leq \alpha/m)|H_0 \leq \alpha(0 \leq \alpha \leq 1) \tag{1.5}$$

ensures that the probability of rejecting at least one null hypothesis when all are true is not greater than α.

Although several multivariate methods have been developed for multiple statistical inference the Bonferroni procedure is still valuable, being simple to use, requiring no distributional assumptions and enabling individual alternative hypotheses to be identified. Nevertheless, the procedure is conservative and lacks power if several highly correlated tests are undertaken.

An alternative formula is Šidák's correction, $p_{adj} = 1 - (1 - \min p)^m$ [162]. If H_0 is rejected when $p_{adj} \leq \alpha$, the FWER equals α when the tests are independent.

Simes [164] modified the Bonferroni procedure and proposed testing a composite hypothesis using the entire set of ordered p-values rather than just their minimum. Assuming the p-values are ordered as $p_1 \leq p_2 \leq \ldots \leq p_m$ for testing hypotheses $H_1, \ldots, H_m$, respectively. Then H_0 is rejected if $p_i \leq \alpha i/m$ for any $i = m-1, \ldots, 1$. This test procedure has Type I

error probability equal to α for independent tests. The power of this modified procedure is greater than the Bonferroni procedure at the same nominal significance level α.

Hommel [82] employed the closure principle to extend Simes's procedure for making statements on individual hypotheses so that it does control the FWER in the strong sense.

Hochberg [79] has suggested a different way to utilize Simes's procedure by offering the following modified procedure:

let m be the largest i for which

$$P_i \leq \frac{\alpha}{m - i + 1} \tag{1.6}$$

then reject all H_i with $i = 1, 2, \ldots, m$

Another method suggested by Benjamini-Hochberg [16] controls the FDR instead, and thereby also the FWER in weak sense.

Testing of the composite hypothesis $H_0 = \{H_1 \cap H_2 \cap \ldots \cap H_m\}$ is called weak control of the FWER. It can be expressed as P_{H_0}(rejecting any H_i) $\leq \alpha$ where 'rejecting any H_i' is equivalent to 'rejecting H_0'. The strong control of the FWER is the procedure only for a subset H' of $H = \{H_1, H_2, \ldots, H_m\}$ than P_{H_0}(rejecting H_i) $\leq \alpha$.

The power of FWER controlling procedures is highly dependent on the family size (i.e., number of comparisons) decreasing rapidly with larger families. Therefore, control of the FDR results in more power than the FWER controlling procedure in experiments with many test groups, yet provides more control over Type I errors than per-test controlling procedures. However, the larger the number of the non-true null hypothesis is, the larger is the difference between the FDR and the FWER. As a result, the potential for increase in power is larger when more of the hypotheses are non-true [16].

1.9.2 Permutation based MTPs

The above described MTPs are conservative for genetic association studies (H_0 is less likely to be rejected) because they do not account for correlations among the genotypes of SNPs in LD. Westfall and Young [191] suggested to compare the observed $\min p$ for a given composite null hypothesis to the actual α-quantile of the $MinP$ null distribution. That is, calculate the adjusted p-values $p_{adj} = P(MinP \leq \min p)$, where $MinP$ denotes the random variable for the minimum p-value for the given composite hypothesis $H_0 = H_1 \cap H_2 \cap \ldots \cap H_m$. Then, p_{adj} is simply compared to the error rate level α to decide whether to reject the composite hypothesis. Usually, the distribution of $MinP$ is unknown, but can be easily approximated via bootstrap resampling of the data vectors, as shown in Westfall and Young [191] or by using permutation of the tested group labels.

The resampling or permutation procedure should be repeated B times. The number of permutations B required depends on the true minimum p-value. For example, to correct a nominal true minimum p-value of 0.0001 for multiple testing the resampling procedure should

be repeated at least 100 000 times to get an adequate estimate of the distribution of Min$P$ and so achieve a reasonable accuracy. The p_{adj} obtained using the Westfall-Young method are usually smaller than those obtained using above mentioned methods like Bonferroni, since the former takes into account correlations. In other words the Westfall-Young method is less conservative.

1.9.3 Combined methods

Another way to calculate a p-value for a composite hypothesis are product methods. Fisher's product p-value method (FPM) [61], the truncated product method (TPM) proposed by Zaykin [203], and the rank-TPM of Dudbridge and Koeleman [53] are very similar. In these methods, a p-value for each individual SNP is calculated, and then a combined test statistic is obtained by multiplying together either all the p-values (in the FPM) or just those below some significance threshold (in the TPM), or the R smallest p-values (in the rank-TPM), where R is the preselected integer.

Historically, Fisher's method is the original one. Fisher [61] noted that for any continuous test statistic under H_0 p-values are distributed uniformly on the interval $\{0,\ 1\}$. Moreover, $-2\ln(p_i)$ has a χ^2_{2m} distribution with 2 degrees of freedom. Then the statistic

$$t = -2\sum_{i=1}^{m} \ln p_i = -2\ln\left(\prod_{i=1}^{m} p_i\right) \tag{1.7}$$

has a χ^2_{2m} distribution with $2m$ degrees of freedom when all m hypotheses are true and the m tests are independent. Therefore, the p-value for the hypothesis that all H_i are true is the probability of a χ^2_{2m} variable being greater or equal to the observed value t.

Experience shows that the ordinary FPM loses power in cases where there are a few large p-values. This can happen when tests are one-sided, with non-centrality in the wrong direction, or when there is a predominance of near-null effects. By TPM, these large components are removed, thereby providing more power, much like a trimmed mean gains efficiency in the presence of outliers. Therefore under certain conditions it is better to use TPM [53, 203].

If the individual tests are independent, exact analytic expressions exist for the significance of the combined test statistic. Zaykin et al. [203] and Dudbridge and Koeleman [53] proposed a number of approximate methods and permutation as an exact method. These tests involve permuting the treatment group or quantitative-trait vector and re-evaluating the *MinP* statistic for every permutation, then taking the combined p-value to be the proportion of permutations yielding a statistic smaller than observed in the original sample. Such an approach can be applied with the FPM, TPM and rank-TPM. The procedure is described in details below.

1.9.3.1 Procedure:

Suppose m null hypotheses, $H_1, \ldots, H_m$ are being tested by use of score tests. Let $T_1, \ldots, T_m$ denote the respective score test statistics, and let $t_1, \ldots, t_m$ denote the corresponding observed values. Let $p_{1,\text{real}} \leq \ldots \leq p_{m,\text{real}}$ denote the ordered p-values from m statistical tests on observed data, and $p_{1,\text{perm}} \leq \ldots \leq p_{m,\text{perm}}$ the ordered p-values from m statistical tests on permuted data. When we repeat the permutation N times with different random seeds, we may obtain the proportion of cases when the product of p-values from N permutations ($\prod p_{m,\text{perm}}$) is less or equal to the product of the p-values from the observed data ($\prod p_{m,\text{real}}$) as

$$p_{\text{adj}} = \frac{\#(\prod p_{j,\text{perm}} \leq \prod p_{j,\text{real}}) + 1}{N + 1} \qquad j = 1, \ldots, m \tag{1.8}$$

Here are three examples that could be of interest:

1. If we perform the procedure over all m tests, the corrected for multiple testing p-value will be obtained as shown in equation 1.8 over all m tests. We define this procedure permutation-based-FPM (PFPM)

2. The product is calculated only over $p_{j,\text{perm}}$ and $p_{j,\text{real}}$ below some significance threshold (permutation-based-TPM or PTPM).

3. The product is calculated only over R smallest $p_{j,\text{perm}}$ and $p_{j,\text{real}}$ (permutation-based-rank-TPM or P-rank-TPM).

1.10 Major depression

Major depressive disorder (MDD) and bipolar disorder (BP) are common psychiatric diseases with lifetime prevalence rates of 16–17 % for MDD (21 % in woman and 13 % in man) [20, 52, 102, 122] and 1–3.3% for BP [66, 88]. Both MDD and BP patients suffer from recurrent depressive episodes. They are characterised by symptoms such as: sad mood; loss of interest in activities that were once interesting or enjoyable, including sex; loss of appetite (anorexia) with weight loss or overeating with weight gain; loss of emotional expression (flat affect); a persistently sad, anxious or empty mood; feelings of hopelessness, pessimism, guilt, worthlessness, or helplessness; social withdrawal; unusual fatigue, low energy level, a feeling of being slowed down; sleep disturbance with insomnia, early-morning awakening, or oversleeping; trouble concentrating, remembering, or making decisions; unusual restlessness or irritability; persistent physical problems such as headaches, digestive disorders, or chronic pain that does not respond to treatment; thoughts of death or suicide attempts. Alcohol or drug abuse may be signs of depression. Bipolar patients in addition encounter episodes of mania (BP I) or hypomania (BP II), characterized by excessive elation, increased energy, decreased need for sleep, increased sexual desire and grandiose notions [153].

Depression is predicted to be the second leading cause of disease related disability, following ischemic heart disease in the year 2010 [120]. It is estimated that 85 to 90 % of individuals who die from suicide have a diagnosable psychiatric disorder, with the largest number suffering from severe depression. In the context of the high morbidity and mortality associated with depression, it is unfortunate that the psychological and neurobiological determinants of depression have not been precisely defined[3].

The causes of depression are thought to be due to both endogenous (genetic) or environmental factors. The genetic background of depression has been shown in familial studies. Depending on the assumed population risks estimates of heritability were between 34 % and 75 % for MDD [100, 112, 170] and 83–93 % for BP [99, 104]. The remaining part of the variance in liability was assigned to individual-specific environment. Kendler and Prescott [100] as well as McGuffin et al. [112] showed that MDD is equally heritable in men and women, and most genetic risk factors influence liability to MDD similarly in both sexes. Later, heritability of MDD was shown to be significantly greater in women than in men [96]. Probably, genes may exist that act differently on the risk for MDD in men *vs.* women.

The main known environmental risk factors for MDD are life stress events. There is compelling evidence that early life stress, such as childhood neglect, physical or sexual abuse, or early parental loss, constitutes a major risk factor for the subsequent onset of depression [72, 169]. However, despite a strong correlation between stressful life events and depression, part of this apparent association is non-causal, because genetic risk factors for some stressful life events are correlated with a genetic predisposition to major depression [98]. Stressful life events are not experienced at random; some individuals have a persistent

[3] http://webapp.cdc.gov/sasweb/ncipc/leadcaus10.html

tendency to place themselves in situations that have a high probability of producing stressful life events. Furthermore, genetic risk factors for stressful life events are positively correlated with genetic risk factors for major depression [101]. The type of stressful life event also affects vulnerability to a subsequent depressive episode [97]. Men and women are, in general, equally sensitive to the depressiogenic effects of stressful life events, but their responses vary depending on the nature of the event itself. Men are more likely to have depressive episodes after divorce, separations, or work difficulties, whereas women are more sensitive to events in their social network, such as difficulty getting along with an individual, serious illness, or death [101].

The neuroimaging and neuropathological abnormalities in major depression suggest that major depression is associated with activation of regions that putatively mediate emotional and stress responses (for example, amygdala), whereas areas that appear to inhibit emotional expression (such as posterior orbital cortex) contain histological abnormalities that may interfere with the modulation of emotional or stress responses [52].

The hypothalamic pituitary adrenal (HPA) axis is the main hormonal system involved in MDD, but the mechanisms underlying its abnormalities in these patients are still unclear. HPA axis activity is governed by the secretion of adrenocorticotrophic hormone-releasing factor (CRF) and vasopressin (AVP) from the hypothalamus, which in turn activate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, which finally stimulates the secretion of the glucocorticoids (GCs) (cortisol in humans and corticosterone in rodents) from the adrenal cortex. GCs interact with their receptors in multiple target tissues including the HPA axis, where they are responsible for feedback inhibition of the secretion of ACTH from the pituitary and CRF from the hypothalamus [129]. Hyperactivity of the HPA axis in major depression is one of the most consistent findings in psychiatry [121]. A significant percentage of depressed patients have been shown to hypersecrete cortisol, the endogenous adrenal glucocorticoid in humans, as manifested by increased 24-hour urinary free cortisol and elevated plasma and cerebrospinal fluid concentrations of cortisol [129].

The increased activity of the HPA axis is thought to be related, at least in part, to an altered feedback inhibition by endogenous GCs. Through binding to their receptors in HPA axis tissues, endogenous GCs serve as potent negative regulators of HPA axis activity including the synthesis and release of CRF in the paraventricular nucleus [44].

GCs mediate their actions through two distinct intracellular corticosteroid receptor subtypes referred to as the type I or mineralocorticoid receptor (MR), and the type II or glucocorticoid receptor (GR) [44]. The MR has a high affinity for endogenous corticosteroids and is believed to play a role in the regulation of circadian fluctuations in these hormones (especially the regulation of ACTH secretion during the diurnal trough in cortisol secretion). In contrast to the MR, the GR has a high affinity for dexamethasone (Dex) and a lower affinity for endogenous corticosteroids. The GR is therefore believed to be more important in the regulation of the response to stress when endogenous levels of GCs are high [44]. Because

patients with major depression exhibit impaired HPA negative feedback in the context of elevated circulating levels of cortisol, a number of studies have considered the possibility that the number or the function of *GR*, or both, are reduced in depressed patients. The few studies that specifically looked at *MR* mediated negative feedback in depression found that this pathway is intact (or possibly oversensitive) in depressed patients [93, 201]. EA Young et al. suggested that imbalance in the *GR*/*MR* ratio may play an etiologic role in serotonin receptor changes observed in patients with MDD [201]. Thus *GR* and *MR* are among the most important brain receptors involved in the pathogenesis of psychiatric disorders.

1.10.1 Polymorphisms in genes controlling activity of the hypothalamic pituitary adrenal (HPA) axis and major depression

In recent years, evidence has emerged that genetic variants contribute to the multifactorial genesis of depressive disorders and influence therapeutic outcome. As mentioned above, the HPA-axis is involved in major depression. This makes the *GR* a prime candidate gene for associations with susceptibility for depressive disorders as well as an altered clinical response to antidepressant drugs.

In our recent study we have shown the association of polymorphisms in *GR* with MDD. Significant differences in genotype frequency could be detected between healthy control subjects and depressive patients for BclI, weak association for ER22/EK23 and trend for N363S [182] (table 1.2). This finding strengthens the hypothesis of a causal involvement of the HPA-axis in the pathogenesis of depression.

A series of *in vivo* and *in vitro* studies have implicated *FKBP5* (FK506 binding protein 5) as an important regulator of *GR* sensitivity. The hsp90 co-chaperone *FKBP5* is part of the mature *GR* heterocomplex [135, 26]. Upon hormone binding, *FKBP5* is replaced by *FKBP4*, which then recruits dynein into the complex, allowing its nuclear translocation and transcriptional activity [42]. The function of the *GR* depends on a large molecular complex that is necessary for proper ligand binding, receptor activation and transcriptional regulation of its target genes [26, 135]. In a recent study we investigated whether polymorphisms in genes involved in HPA-axis regulation, and especially *GR* sensitivity, contribute to the susceptibility for developing depression and the onset of clinical response to antidepressant treatment [19]. To answer this question, we genotyped SNPs in the *GR* gene itself (*NR3C1*), in the target genes corticotropin releasing hormone precursor (CRH) and arginine vasopressin-neurophysin II (*AVP*) and in five co-chaperones of the *GR* (*BAG1, STUB1, TEBP*, *FKBP4* and *FKBP5*), as there is strong evidence that these genes tightly regulate *GR* activity [135, 156]. After correction for multiple testing over all investigated genes no association with affection status could be shown.

Angiotensin-converting enzyme (*ACE*) is assumed to influence the activity of the HPA system, which shows hyperactivity in the majority of patients with depressive disorder.

Polymorphism	Controls n (%)	Depressive Patients n (%)	RUD[g] Patients n (%)
ER22/23EK			
Non-Carriers	476 (95.8 %)	462 (94.3 %)	169 (92.3 %)
Carriers	21 (4.2 %)	28[a] (5.7 %)	14[a] (7.7 %)[c]
N363S			
Non-Carriers	336 (91.3 %)	395 (90.8 %)	142 (86.4 %)
Carriers	32[d] (8.7 %)	40 (9.2 %)	23 (13.6 %)[e]
BclI			
Non-Carriers	163 (43.6 %)	162 (37.0 %)	67 (39.4 %)
Heterozygous Carriers	174 (46.5 %)	208 (47.5 %)	75 (44.1 %)
Homozygous Carriers	37 (9.9 %)	68 (15.5 %)[b]	28 (16.5 %)[f]

[a] One patient homozygous for the carrier allele

[b] (Versus control) genotypic $p = 0.026$, allelic $p = 0.01$, OR = 1.3 (95% CI = 1.06–1.60)

[c] Allelic $p = 0.043$, OR = 1.98 (95% CI =1.01–3.08)

[d] One control homozygous for carrier allele

[e] Allelic $p = 0.11$, OR = 1.55 (95% CI = 0.89-2.67)

[f] Genotypic $p = 0.08$, homozygous carriers versus rest: $p = 0.03$, OR = 1.8 (95 % CI = 1.04–3.2)

[g] Patients with Reccurent Unipolar Depression

Table 1.2: Frequencies of three polymorphisms of the *GR* gene in healthy control subjects and depressive patients[182]

ACE interferes with the secretion of pituitary hormones such as ACTH and potentiates the stimulatory effects of corticotropin-releasing hormone (CRH) [90]. The *ACE* gene, known to be associated with cardiovascular disorders, which in turn are accompanied with an increased susceptibility for depression, is therefore a promising candidate gene for affective disorders.

In a recent study [10] we investigated the genetic association between 35 SNPs and an insertion/deletion (I/D)-polymorphism in the *ACE* gene and the susceptibility for unipolar major depression together with the genetic association with *ACE* serum activity and functional parameters of the HPA system. Two independent case/control samples with a total of 843 unrelated unipolar depressed patients and 1479 healthy controls were investigated. A case/control sample was screened to detect genetic associations with unipolar major depression. In addition, a replication sample was used to confirm the detected associations and to further investigate functional consequences of the genetic variants associated with MDD (table 1.3). In the screening sample, two SNPs within the *ACE* gene were significantly associated with the investigated phenotype.

The association with MDD of one SNP (rs4291) located in the promoter region of the *ACE* gene was confirmed in our replication sample. The T-allele of this SNP was associated with depression and patients carrying the T-allele showed higher *ACE* serum activity and HPA-axis hyperactivity. This variant of the *ACE* gene is suggested to be a susceptibility factor for unipolar major depression.

Patients homozygous for the T-allele showed a clear HPA system hyperactivity, whereas the cortisol response of A-allele carriers after CRH stimulation was blunted. Thus, the SNP rs4291 was shown to influence *ACE* activity and HPA-axis hyperactivity and might therefore represent a common pathophysiologic link for unipolar depression and cardiovascular disease [11].

Table 1.3: Association and genotype frequencies of rs4291 and rs4295 genotypes with unipolar depression in exploration and replication samples; HWE for patients, corresponding controls and the epidemiologic control group

Genotype	**Sample 1 (MPIP)**			**log. regr.**	**Sample 2 (LMU)**		log. regr.
	controls [1]	controls [e]	MDD	p	controls [2]	MDD	p
rs4291				0.45*			
HWE (p)	**0.0006**	0.17	0.62	raw:	**0.0043**	0.88	raw:
AA (n / %)	231 / 38.8%	146 / 41.3%	232 / 36.4%	**0.00076**	104 / 43.2%	72 / 36.2%	**0.0043**
AT (n / %)	315 / 52.2%	153 / 43.2%	300 / 47.0%	corrected:	123 / 51.0%	97 / 48.7%	corrected:
TT (n / %)	57 / 9.5%	55 / 15.5%	106 / 16.6%	**0.017**	14 / 5.8%	30 / 15.1%	**0.0086**
rs4295				0.16*			
HWE (p)	**0.001**	0.93	0.68	raw:	0.58	1.00	
CC (n / %)	229 / 37.7%	257 / 41.0%	228 / 36.0%	**0.0011**	97 / 40.1%	73 / 36.7%	0.765
GC (n / %)	319 / 52.6%	287 / 45.9%	300 / 47.3%	corrected:	109 / 45.0%	95 / 47.7%	
GG (n / %)	59 / 9.7%	82 / 13.1%	106 / 16.6%	**0.025**	36 / 14.9%	31 / 15.6%	
ACE I/D							
HWE (p)	0.62	1.00	0.87		0.24	0.66	
DD (n / %)	158 / 26.1%	78 / 21.8%	200 / 31.4%		60 / 25.1%	61 / 30.7%	0.201
ID (n / %)	309 / 51.0%	178 / 49.7%	311 / 48.9%	0.084	129 / 54.0%	102 / 51.3%	
II (n / %)	139 / 22.9%	102 / 28.5%	125 / 19.7%		50 / 20.9%	36 / 18.1%	

LMU — Ludwig-Maximilians University

[1]matched controls for sample 1

[e]epidemiologic control sample

[2]matched controls for sample 2

*p-value from logistic regression comparing sample 1 *vs.* controls[e]

bold are significant p-values

1.11 Pharmacogenetics and antidepressant drugs

Adequate therapy response to antidepressant drugs, i.e. full remission, to a single antidepressant drug is observed in only 40-70 % of patients, even when it is given in sufficiently high dose for up to 6 weeks [18]. Moreover, about half the active drug 'responders' are thought to be really placebo responders, who would have recovered without an active drug treatment. In pharmacogenetic studies, these individuals represent a large group of false positive observations. Nevertheless, antidepressants are still the most effective treatment for depressive disorders.

Family studies support a genetic basis of response to antidepressant drugs [18, 110]. These studies suggest that genetic factors may play a role in antidepressant drug response; however, it is possible that shared environmental factors may bias relative pairs toward similar response patterns.

Pharmacogenetic studies of antidepressants can be classified as addressing either pharmacokinetic or pharmacodynamic effects. Genetic variants affecting the metabolism of antidepressants may change pharmacokinetic factors, such as plasma drug concentration and half-life. Polymorphisms that affect the expression or function of receptors and signal transduction molecules in the brain may alter pharmacodynamics. Both pharmacokinetic and pharmacodynamic changes can affect the efficacy and side effects of antidepressants.

1.11.1 Polymorphisms in genes controlling the pharmacokinetics of antidepressant drugs

The majority of pharmacokinetic pharmacogenetic studies have focused on polymorphisms in liver cytochrome P450 isoenzymes that metabolize many antidepressant medications. The most intensively investigated gene is *CYP2D6*, which encodes debrisoquine hydroxylase. Many antidepressants, including tricyclics (TCAs), selective serotonin reuptake inhibitors (SSRIs), venlafaxine, and others, are metabolized primarily by debrisoquine hydroxylase [17]. The *CYP2D6* gene is highly polymorphic, with over 70 known variants[4]. Homozygosity for null alleles gives rise to the poor metabolizer phenotype for debrisoquine hydroxylase characterized by no enzyme activity. Null allele heterozygosity or homozygosity for intermediate metabolic alleles implies an intermediate debrisoquine hydroxylase metabolic phenotype characterized by impaired but not absent enzyme activity [111, 139]. *CYP2D6* gene duplications result in ultra-rapid metabolic activity of debrisoquine hydroxylase [91].

Another important protein for the transport of antidepressants is the *p*-glycoprotein located in the blood brain barrier. It is a member of the highly conserved superfamily of adenosine tri-phosphate (ATP)-binding cassette (ABC) transporter proteins. This P-glycoprotein is encoded by the *ABCB1* gene (earlier termed *MDR1*) and protects cells throughout the healthy organism against many drugs by acting as an efflux pump for xenobiotics [38, 155].

[4] http://www.imm.ki.se/CYPalleles

Results from our group suggest that common polymorphisms within *ABCB1* may alter the intracerebral concentration of antidepressants that are substrates of this transporter. We could show an association of an intronic *ABCB1* SNP with remission to antidepressant therapy but not to plasma drug levels (n = 286). This association was only seen in patients treated with antidepressants that proved to be substrates of P-glycoprotein in the mouse knock-out model (n = 105) [181]. It is therefore possible that certain *ABCB1* polymorphisms alter the efficiency with which *p*-glycoprotein transports substrate antidepressants at the blood-brain barrier and thus alter intracerebral concentrations of specific antidepressants. Prior knowledge of the patients relevant *ABCB1* genotypes could therefore prevent the administration of a drug that might never reach therapeutic intracerebral levels despite a plasma concentration believed to be sufficient.

Chapter 2

Evaluation of Nyholt's procedure for multiple testing correction

Nyholt [126] proposed a new method for adjustment of the $\min p$ obtained from a set of individual SNP tests in an association study, based on a procedure described by Cheverud [27] for multiple-testing adjustment in linkage studies. The advantage of Nyholt's method is its low computational requirement and hence rapidity. The procedure is as follows. The eigenvalues, $\lambda_1, \ldots, \lambda_M$, of a matrix of observed pairwise LD between the M SNPs are obtained by principal components analysis or, more generally, spectral decomposition. The variance of these observed eigenvalues, $\mathrm{Var}(\lambda)$, is then used in the formula

$$M_{\text{eff}} = 1 + (M - 1)\left(1 - \frac{\mathrm{Var}(\lambda)}{M}\right) \tag{2.1}$$

to calculate an 'effective' number of independent tests, M_{eff}. This is then used in Šidák's formula in place of M, the actual number of tests. Meng et al. [115] used a similar approach for the selection of haplotype tagging SNPs.

Nyholt showed that his method, when applied to two datasets, yielded $\min p$ approximately equal to those estimated by permutation. However, these datasets were small, one containing 10 SNPs, and the other 23 SNPs, and the latter dataset was of limited use, as the intermarker LD was so low that the estimated M_{eff} (22.53) was almost equal to M, the number of SNPs.

Dudbridge and Koeleman [53] investigated whether the assumption underlying Nyholt's method, that there really is an 'effective' number of independent tests, is true. When b independent tests are carried out, the $\min p$ has a $\beta(1, b)$ distribution. Using data on chromosomes 18 and 21 from the International Hap Map Consortium, Dudbridge and Koeleman fitted a $\beta(a, b)$ distribution to $\min p$ simulated from their null distribution by permutation and tested the null hypothesis that $a = 1$. By rejecting this hypothesis, they showed that the assumption of an effective number of independent tests is false.

The result of Dudbridge and Koeleman shows that Nyholt's procedure cannot be exact,

but does not indicate how well it performs as an approximate method in practice. Here, we describe a more extensive evaluation than that originally reported by Nyholt, using data on candidate genes from various studies in the Max-Planck Institute of Psychiatry (MPIP) on the genetics and pharmacogenetics of psychiatric disorders. We also present a theoretical investigation of the performance of Nyholt's procedure when certain patterns of LD are assumed.

2.1 Methods

We considered 31 candidate genes genotyped in 1360 individuals, of whom approximately half were cases and half controls. Choosing only SNPs with minor allele frequency above 10 % and in Hardy-Weinberg equilibrium, there were a total of 291 SNPs, i.e. an average of 9.4 SNPs per gene. None of the markers were significantly associated with case-control status, and hence we assumed that the LD patterns in the genes were the same in cases and controls. The program SNPSpD, described by Nyholt, was used to calculate M_{eff} for each gene.

Define, for each gene, M as the number of SNPs (and hence tests), the reduction factor as $R = M/M_{\text{eff}}$, and r^2 as the mean absolute pairwise Pearson correlation coefficient between the SNPs in the gene [49] (estimated using the function LD of the *R* Genetics package). Thus R is a measure of how much more powerful Nyholt's method is compared to the usual Šidák correction, and r^2 is a measure of LD within the gene. We examined the relation between r^2 and R in the 31 genes.

The type-I error rates of Šidák's and Nyholt's procedures were investigated using simulation. Each of 20 000 simulations was performed under the complete null hypothesis that none of the SNPs is associated with case-control. Half of the 1360 individuals were randomly assigned to be cases and half to be controls. The association between case-control status and genotype was tested for each SNP using a χ^2 test of the 2×3 contingency table and, within each gene, the $\min p$ obtained. These $\min p$ were then adjusted using Šidák's correction based on both M (Šidák's original method) and M_{eff} (Nyholt's method) for that gene. For each gene, the proportion of the 20 000 simulations in which the adjusted $\min p$ was less than 0.05 represents the 5 % type-I error rate, α, for the null hypothesis that none of the SNPs in that gene is associated with case-control status.

Next, we explored the effect of haplotype block structure on Nyholt's procedure, using the method of Gabriel et al. [63], implemented in the program HAPLOVIEW[1] [14], to detect blocks within each gene.

Finally, as Dudbridge and Koeleman [53] note, if the assumption that the $\min p$ corresponds to that of M_{eff} independent tests were true, it should be distributed $\beta(1, M_{\text{eff}})$ when all null hypotheses are true. We fitted a $\beta(a, b)$ distribution to the 20 000 simulated $\min p$ for each of the 31 genes in turn, to determine how close the maximum likelihood estimates, $\hat{a}$, of a were to 1. We also fitted the β distribution with a fixed at 1, and compared, for each gene, the maximum likelihood estimate, $\hat{b}$, of b with its corresponding M_{eff} value. A likelihood ratio test of the null hypothesis that $a = 1$ was also performed for each gene.

[1] http://www.broad.mit.edu/mpg/haploview/index.php

2.2 Results

The average intermarker spacing within a gene was 8.6 Kb, with standard deviation (SD) 7.6 and range 0.37–33 Kb. The mean of r^2 in the 31 genes was 0.44 (SD 0.20, range 0.11–0.84). There appeared to be an approximate quadratic relation between R and r^2 (figure 2.1).

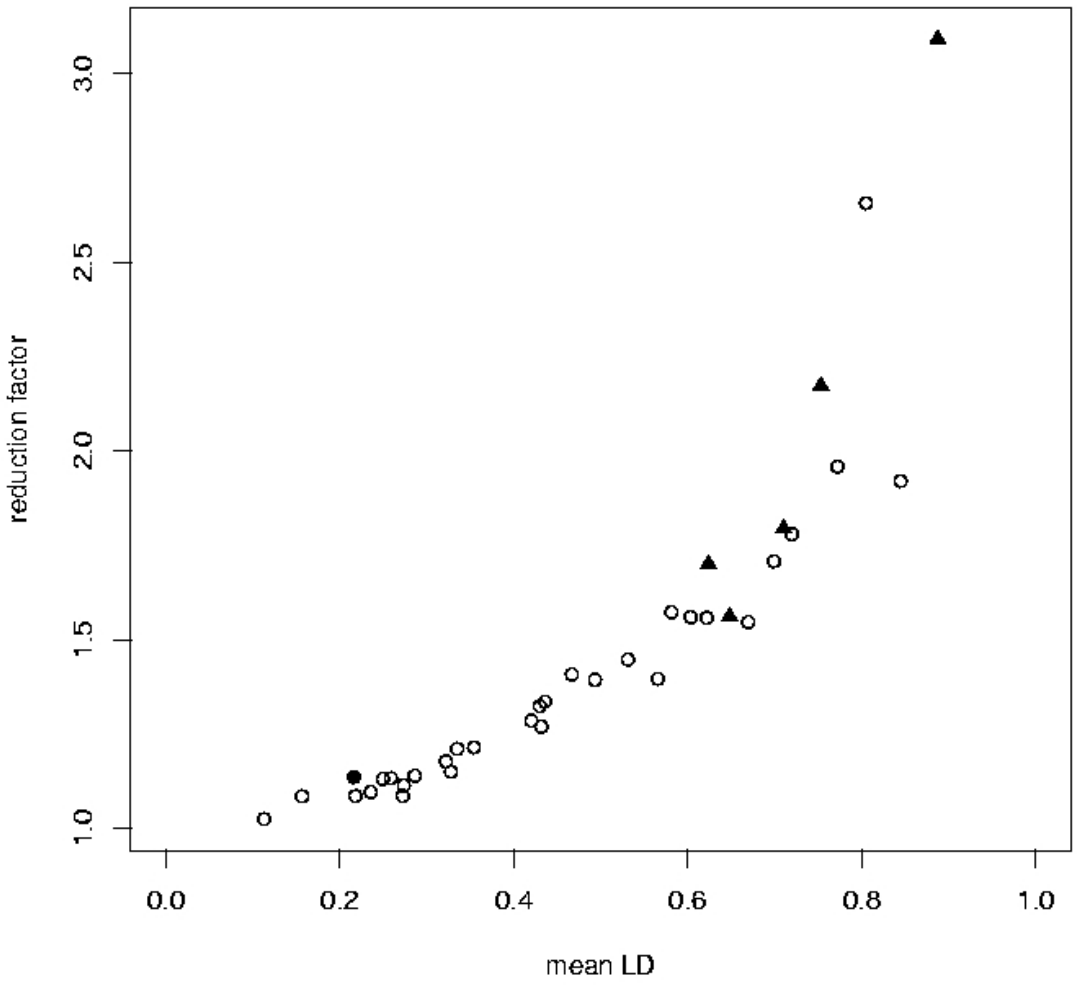

Figure 2.1: Relation between mean LD, r^2, within a gene and reduction factor, R, for that gene.Shown are 30 single-block genes (circles) and one multi-block gene (closed circle) containing 5 blocks (closed triangles).

Thus, the stronger is the LD within a gene the greater tends to be the increase in power of Nyholt's method compared to the simple Šidák correction.

Fugure 2.2 shows the type-I error rates for the 31 genes calculated using Šidák's procedure (open circles and cross) and Nyholt's procedure (closed circles and plus) plotted against r^2. As expected, for Šidák's method there is a negative correlation: the more (positively)

dependent the tests, the more conservative is Šidák's procedure.

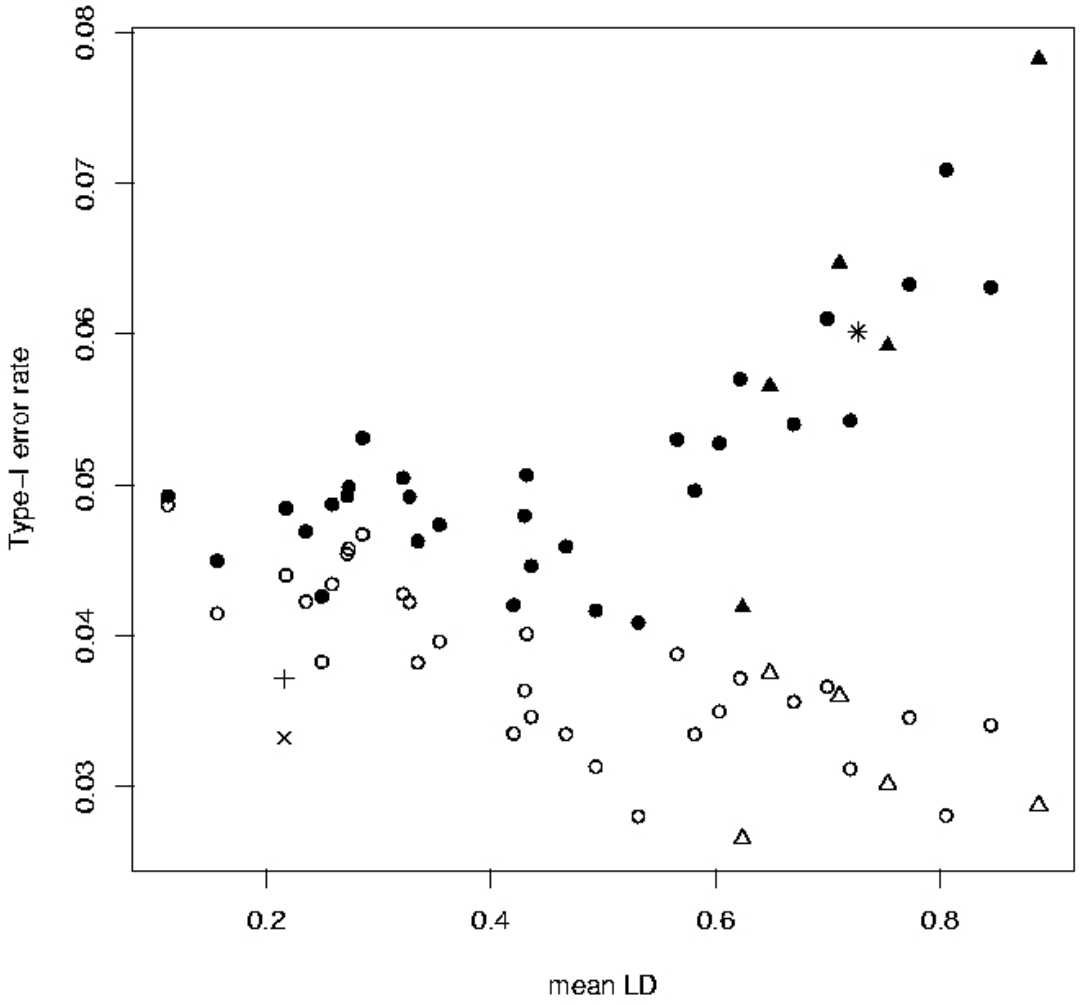

Figure 2.2: Relation between mean LD, r^2, within a gene and type-I error rate, α, for Šidák's and Nyholt's methods

Open circles represent 30 single-block genes and Šidák's correction; closed circles represent the corresponding Nyholt corrections. Cross, star and plus represent the multi-block gene, corrected using Šidák's, Nyholt method 2 and Nyholt method 1 respectively. Triangles represent the five blocks of the multi-block gene: open for Šidák; closed for Nyholt.

Using Nyholt's method all but five of the 31 genes had α between 4 % and 6 %. For the 23 genes with $r^2 < 0.6$, α was very close to 5 % or slightly less, whereas for genes with $r^2 > 0.6$, Nyholt's method was somewhat anti-conservative and became increasingly so as r^2 increased. The highest value of α was 7.1 % for a gene with $r^2 = 0.8$.

The standard error of the 5 % type-I error rate estimated from 20 000 simulations is

0.15 % when the true rate is 5 %. For 19 of the 31 genes, the 95 % confidence interval for the true type-I error rate excluded 5 %.

Similar results were obtained when the 1 % type-I error rate was examined.

Next, we used the program HAPLOVIEW, to detect blocks in each gene. Only one gene, containing 46 typed SNPs, had more than one block: there were five blocks and four SNPs that did not fit into blocks. This multi-block gene was the gene with the lowest α (3.7 %) and among those with the smallest r^2. Nyholt's procedure was then applied to the five blocks of this gene separately. r^2 within blocks was greater than r^2 in the gene as a whole, as LD between SNPs in different blocks is weaker than between SNPs in the same block, and it was found that the five α values for these blocks (closed triangles in figure 2.2) were all greater than that for the gene as a whole (cross in figure 2.2). For four of the five blocks the method was anti-conservative, and for one block α was as high as 7.9 %. The five blocks contained a total of 42 SNPs. The sum of the five M_{eff} values estimated in each block separately was 21.6. Adding the four SNPs not in blocks gave a total M_{eff} of 25.6. This compared with $M_{\text{eff}} = 40.5$ estimated for the 46 SNPs together. Thus the reduction factor, R, is greater when the blocks are considered separately. Based on $M_{\text{eff}} = 40.5$ (method 1) and 25.6 (method 2), respectively, α was calculated as 0.037 and 0.060.

Eight chromosomes contained 23 of the 31 genes. The procedure used above for blocks within genes was now used for genes within chromosomes. That is, the type-I error rate for each chromosome (for the null hypothesis that none of the genotyped SNPs in the chromosome is associated with case-control status) was estimated based both on the M_{eff} estimated for all the genes on the chromosome together (method 1) and on the sum of M_{eff} estimated for each gene on the chromosome separately (method 2). Table 2.1 shows r^2, R and α for methods 1 and 2 and the eight chromosomes. The value of r^2 calculated using all SNPs in a chromosome was naturally much smaller than the weighted average of the r^2s calculated within genes on that chromosome. Likewise, the eight R and α values calculated using method 1 were smaller than for those obtained from method 2. Using method 1 the mean values of R and α were respectively 1.08 and 0.042, not very much different from a simple Šidák correction based on M tests, where M is the number of genotyped SNPs in the chromosome. Method 2 was better: mean values of R and α were 1.27 (range 1.08–1.60) and 0.048 (range 0.041–0.055).

A $\beta(a, b)$ distribution was fitted to the simulated $\min p$ for each of the 31 genes. The maximum likelihood estimates, $\hat{a}$, of a. ranged from 0.91 to 1.01. The mean was 0.97 and the interquartile range 0.95 – 0.99. The same was done for the eight chromosomes. The mean of the eight $\hat{a}$ values was 0.95; the interquartile range was 0.94 – 0.97) and the range 0.92 – 0.99. For 20 of the 31 genes and seven of the eight chromosomes the null hypothesis that $a = 1$ was rejected at the 5% level.

Thus, the assumption that the $\min p$ have a $\beta(1, b)$ null distribution for some b is not supported by the data, but the maximum likelihood estimates of a are not very different

Chr no.[a]	No. genes[b]	No. SNPs[c]	Šidák	Method 1			Method 2		
			α	α	R	r^2	α	R	r^2
1	2	11	0.045	0.049	1.11	0.19	0.055	1.24	0.41
4	2	16	0.044	0.045	1.03	0.10	0.048	1.08	0.19
5	5	48	0.039	0.041	1.05	0.10	0.047	1.22	0.35
7	2	20	0.042	0.045	1.07	0.15	0.048	1.14	0.28
9	3	57	0.035	0.037	1.09	0.16	0.041	1.21	0.30
10	2	17	0.038	0.042	1.10	0.17	0.046	1.21	0.34
11	4	19	0.034	0.038	1.11	0.15	0.054	1.60	0.63
17	3	34	0.033	0.038	1.13	0.19	0.047	1.43	0.50

[a] 'Chr no.' is chromosome number;

[b] 'No. genes' is number of genes on that chromosome;

[c] 'No. SNPs' is the total number of SNPs genotyped in the chromosome's genes.

Table 2.1: Type-I error rates (α), reduction factor (R), and mean absolute LD (r^2) for eight chromosomes estimated using Šidák method, Nyholt's method applied to genes on same chromosome together (Method 1), and Nyholt's method applied to genes on same chromosome separately (Method 2)

from 1. Table 2.2 shows the maximum likelihood estimates, $\hat{b}$, of b for the 31 genes and eight chromosomes when a was fixed at 1. The $\hat{b}$ values for the 31 genes were on average 8% lower than the corresponding M_{eff} values, ranging from 40% lower (in the case of the multiblock gene) to 21% higher. For the eight chromosomes, $\hat{b}$ was on average 24% less than M_{eff}, ranging from 40% lower to 6% higher. Thus, M_{eff} estimated by Nyholt's procedure does not in general correspond to the best estimate of an effective number of independent tests.

2.3 Theoretical investigation

The results above show that Nyholt's method can perform poorly when applied to regions of DNA containing haplotype blocks, e.g. multiblock genes or multiple genes on the same chromosome. In this case, method 1 tends to overestimate M_{eff} and the power of the procedure is little different from that of a simple Šidák correction. An algebraic treatment of a very simple model example serves to illustrate why this happens. Suppose there are $2N$ SNPs with the first N in perfect LD ($r^2 = 1$) and the last N in perfect LD. Thus the effective number of independent tests is at most 2. Let C denote the correlation matrix for these

SNPs, and let δ be the Pearson correlation coefficient between a SNP in the first block and a SNP in the second block. The entries of the $2N \times 2N$ correlation matrix C are

$$C_{i,j} = \begin{cases} 1 & \text{if } i,j \leq N \text{ or } i,j \geq N+1; \\ \delta & \text{otherwise.} \end{cases}$$

The two largest eigenvalues of C are $N(1 \pm \delta)$. The eigenvalue $N(1+\delta)$ corresponds to an eigenvector whose entries are all 1, and the eigenvalue $N(1-\delta)$ corresponds to an eigenvector whose first N entries are 1 and whose last N entries are -1. Since the rank of C is 2, the remaining $2N-2$ eigenvalues are 0.

The sum of the eigenvalues of C equals $2N$, so the mean of the observed eigenvalues is 1. The variance of the observed eigenvalues is

$$\begin{aligned} \text{Var}(\lambda_{\text{obs}}) &= \frac{1}{2N-1}[(N(1+\delta)-1)^2 + (N(1-\delta)-1)^2 + (2N-2)(0-1)^2] \\ &= \frac{2N}{2N-1}[N(1+\delta^2)-1], \end{aligned}$$

and the estimate, M_{eff}, of the effective number of independent tests is, from equation (2.1),

$$\begin{aligned} M_{\text{eff}} &= 2N - \left(\frac{2N-1}{2N}\right)\text{Var}(\lambda_{\text{obs}}) \\ &= 2N - (N(1+\delta^2)-1) \\ &= (1-\delta^2)N + 1. \end{aligned}$$

However, the true effective number of tests is at most 2, independent of N.

The preceding calculation can be generalized to K blocks of SNPs when any two markers from different blocks are in linkage equilibrium. Suppose, the j^th block contains $2N_j$ SNPs with the first N_j in perfect LD ($r^2 = 1$) and the last N_j in perfect LD, and that the correlation between one of the first N_j SNPs and one of the last N_j SNPs is δ_j. Let $N = \sum N_j$. In this case

$$M_{\text{eff}} = 2N + 1 - \sum_{j=1}^{K} \frac{N_j^2}{N}(1+\delta_j^2). \tag{2.2}$$

We can now model a gene with 5 blocks and 8 SNPs per block by setting $K = 5$, $N_j = 4$, and $\delta_j^2 = 0.75$ (say) for $j = 1, 2, \ldots, 5$. Now $M_{\text{eff}} = 34$, but the effective number of independent tests is less than 10. Thus, the estimated effective number of independent tests, M_{eff}, is at least a factor of 3.4 too large.

Gene	M	Meff	$\hat{b}$	Gene	M	Meff	$\hat{b}$
1	13	11.5	9.6	21	5	2.6	2.9
2	6	5.1	4.8	22	14	12.4	10.2
3	5	4.5	4.5	23	7	4.5	4.1
4	3	1.6	1.9	24	4	2.3	2.3
5	4	2.6	2.6	25	8	7.3	6.7
6	6	4.2	3.3	26	6	5.3	5.1
7	4	2.6	2.8	27	17	13.2	9.2
8	3	2.8	2.8	28	46	40.5	24.4
9	9	6.5	5.1	29	9	6.7	5.4
10	7	6.1	5.9	30	12	4.5	5.0
11	10	8.3	7.1	31	18	12.8	9.8
12	5	2.9	3.1	Chr			
13	15	11.3	8.5	1	11	10.0	8.8
14	5	4.6	4.4	4	16	15.5	14.6
15	5	3.6	3.7	5	48	45.8	34.0
16	8	5.1	4.3	7	20	18.7	15.7
17	11	9.1	7.2	9	57	52.1	31.3
18	5	3.9	3.7	10	17	15.5	12.2
19	10	9.8	9.7	11	19	17.2	11.6
20	11	10.1	9.3	17	34	30.1	19.6

Table 2.2: For each gene and chromosome, numbers of SNPs (TP), effective number of independent tests estimated by Nyholt's method (M_{eff}), and maximum likelihood estimates of b when $\beta(1, b)$ distribution fitted to distribution of $\min p$ ($\hat{b}$).

2.4 Discussion

In this chapter we have reported an empirical and a theoretical investigation of the performance of Nyholt's method. Nyholt's method is less conservative than Šidák's correction (although when applied to a longer length of DNA containing several sets of SNP markers in different haplotype blocks, it may be only slightly less conservative than Šidák), but it may also be anti-conservative when there is strong LD between SNPs. In view of this observation, we recommend that any significant result obtained using Nyholt's procedure which is not already significant using Šidák's original correction should be confirmed by a permutation test.

Nyholt's derivation of the formula for M_{eff} (equation 2.1) is based on an interpolation of two extreme patterns of LD. When all SNPs are in linkage equilibrium, the eigenvalues, $\lambda_1, \ldots, \lambda_M$, of the correlation matrix C are all equal to 1, so that $\text{Var}(\lambda) = 0$ and $M_{\text{eff}} = M$. When all SNPs are in perfect LD, the eigenvalues are $M, 0, \ldots, 0$, so that $\text{Var}(\lambda) = M$ and $M_{\text{eff}} = 1$. Our theoretical investigation, however, shows that the correction is wrong when perfectly correlated and independent markers are mixed. in this situation, M_{eff} appears to be overestimated so that the method is conservative.

In addition, as Dudbridge and Koeleman [53] have shown for chromosomes 18 and 21 and we have shown for a sample of 31 candidate genes, the distribution of the $\min p$ from the permutation test is, in general, not equal to that of L independent tests for any integer L. A further sign that the theory underpinning Nyholt's correction is unreliable is that it yields different answers depending on whether it is applied to a set of markers S or to each disjoint subset of markers (e.g. in a haplotype block or gene) whose union is S.

Nyholt [126] mentions that his approach may be conservative in the presence of very strong LD. Our empirical results, on the other hand, show that it tends to become anti-conservative. The website of the SNPSpD[2] program contains a note describing a way of overcoming this. The suggestion is to exclude all SNPs but one from any set of SNPs that are in perfect LD. This is, however, somewhat contrary to the theoretical justification for the method, given that it is based on the formula for M_{eff} being correct when all SNPs are in perfect LD. Moreover, it does not work when SNPs are in high but not perfect LD. For example, when there are K pairs of SNPs with the two SNPs in any pair having correlation $\delta = 0.8$ and any two SNPs belonging to different pairs being in linkage equilibrium, equation 2.2 shows that $M_{\text{eff}} = 2K - 0.8$, which is unreasonably high when K is not small.

In the 31 genes we found only three genes containing SNPs in perfect LD. In each case, there was one pair of SNPs in perfect LD. When one SNP from each pair was removed, the type-I error rate improved slightly for two genes (from 3.7 % and 4.5 % to 3.8 % and 4.8 % respectively), but worsened for the other (from 7.1% to 7.3 %).

Nyholt's method is similar to that of Cheverud [27]. The latter was developed for linkage studies, but could equally be used for associations studies. Using Ceverud's method in our

[2] http://gump.qimr.edu.au/general/daleN/SNPSpD/

data we obtained very similar results to those reported here. For most genes, the 5 % type-I error rate was the same to an accuracy of one decimal place, and it never differed by more than 0.3 % (this was vor one gene where the type-I error rate was 7.1 % using Nyholt's method and 6.8 % using Cheverud's method).

In this study the largest number of genotyped SNPs in a gene or chromosome was 57, which is considerably smaller than the number of genotyped individuals (1360). In a genomewide study far more SNPs would be typed than individuals. In this case, Nyholt's method applied naively (we have termed this Method 1) would work poorly, since the number of non-zero eigenvalues of the matrix of pairwise LD can not be greater than the number of individuals. As most eigenvalues will then be zero, the $\mathrm{Var}(\lambda)$ term in equation 2.1 will be close to zero and M_{eff} will be close to M, so that the effective number of tests will be estimated as almost equal to the actual number of tests, i.e. no reduction. Nyholtś method clould still be applied to each haplotype block separately (we have termed this Method 2), but this would have several associated problems. First, this approach implicitly assumes that the haplotype blocks are independent, which is not necessarily the case in practice. Second, the boundaries of the haplotype blocks are not unambigious and different methods of estimating could yield different boundaries. Third, it is not clear what to do with SNPs that do not lie in any haplotype block.

Finally, although the principle of an effective number of independent tests was proposed for the Šidák correction, there is no formal connection between the two. There seems no immediate reason to believe it should work better for a Šidák correction than for other methods that account for multiple testing. We investigated whether it could be used with Fisher's product p-value method. For M independent tests, $T = -2\log\left(\prod_{i=1}^{M} p_i\right)$ has asymptotically a χ^2_{2M} distribution when all M null hypotheses are true [61]. Analogous logic to that which led to equation 2.1 suggests that $T \times M_{\mathrm{eff}}/M$ may have an approximate $\chi^2_{2M_{\mathrm{eff}}}$ null distribution. However we found that, for the 31 genes, this assumption yielded 5 % type-I error rates of between 5.4 and 12.1 %, with a mean of 7.9 %. Thus, the concept of an effective number of independent tests seems to be not at all applicable to Fisher's product method.

In conclusion, although Nyholt's method may be found useful as an exploratory tool, it is not a replacement for using a permutation test. It is worth noting that although permutation can be computationally intensive, methods are available for reducing the computational effort [53, 158].

Chapter 3

P2RX7, a gene coding for a purinergic ligand-gated ion channel, is associated with major depressive disorder

Genome-wide linkage analyses with BP patients yielded several regions of interest [70]. Two genome-wide scans on pedigrees from the Saguenay-Lac-St.-Jean (SLSJ) region of Quebec demonstrated the presence of a susceptibility locus on 12q24.31 administering both parametric and non-parametric analyses and using a broad affection status model (ASMII) that includes BPI, BPII and recurrent MDD. Four consecutive markers gave maximum sib-pair LOD scores close to or above 5 with empirical p-values of 0.0001 [119, 161].

Linkage analysis using tightly spaced microsatellite markers gave a LOD score 3.7 (p-value 0.0001) at marker NBG6 under ASMII and a case/control association analysis with the same marker showed positive allelic association with BP (p-value = 0.008) [160]. Since this marker is located within intron 9 of the *P2RX7* gene, coding for a member of the purinergic ligand-gated ion channels of the P2X family, 24 SNPs in *P2RX7* and the neighbouring genes, e.g. *P2RX4* and *CAMKK2* were genotyped in a bipolar case/control sample and 12 SNPs in the pedigrees used for the linkage studies. The strongest association (p=0.000708) was observed in bipolar families at the non-synonymous SNP *P2RX7*-E13A (rs2230912, Gln460Arg) lying in exon 13 of *P2RX7* [13].

The linkage results in the SLSJ population were strongest under a broad affection model including MDD patients. The significance of this chromosomal region in the susceptibility for MDD has been strengthened by further studies that reported linkage of this region in pedigrees consisting of BP and MDD patients [4, 40, 113].

Given the fact that there is a constant diagnostic conversion from MDD to BP of 1.25 % per year throughout the lifespan [7], we were interested if these heritable disorders share at least some genetic commonalities. Therefore, in our study we concentrated on *P2RX7* as a candidate gene for MDD.

ATP-gated P2X-receptors are cation selective ion channels with high calcium perme-

ability that open upon binding of extracellular ATP [103, 125]. In the brain, *P2RX7* has been shown to be expressed in glial cells [171], immuno-histochemical studies have suggested *P2RX7* to be also expressed in central and peripheral neurons and may regulate immune function and neurotransmitter release [48, 194]. However, expression studies of *P2RX7* in neurons have been inconsistent due to a limited quality of available antibodies [163, 194]. Polymorphisms in the gene are discussed to be relevant in the survival of chronic lymphocytic leukaemia [177] and *P2RX7* is considered to be a candidate gene for systemic lupus erythematosus and type I diabetes [54, 55].

To investigate the implication of *P2RX7* in MDD, we performed a case/control study in a sample of German Caucasian patients with recurrent MDD and diagnosed healthy controls from the same population. We report genotypic association in the *P2RX7* gene providing evidence that *P2RX7* might indeed be a susceptibility gene for major depressive disorder.

3.1 Materials and methods

3.1.1 Sample description

1000 patients (326 males, 674 females) with recurrent unipolar depression were recruited from in- and out-patients at the Max MPIP in Munich and psychiatric hospitals in Augsburg and Ingolstadt, located close to Munich. Each hospital contributed a third of the patients. Patients were diagnosed by WHO-certified raters according to DSM-IV using the Schedule for Clinical Assessment in Neuropsychiatry (SCAN). Only Caucasian patients over 18 years old with at least two moderate to severe depressive episodes were included. Exclusion criteria were the presence of manic or hypomanic episodes, mood incongruent psychotic symptoms, the presence of a lifetime diagnosis of intravenous drug abuse and depressive symptoms only secondary to alcohol or substance abuse or dependence, or to a medical illness or medication. Ethnicity was recorded using a self-report sheet for perceived nationality, first language and ethnicity of the subject himself, parents and all four grandparents. All included patients were Caucasian and 91.2 % were of German origin. Mean age was 49.35 14.09 years (males: 48.49±13.57, females: 49.86±14.38 years).

1029 controls (336 males, 693 females) matched for ethnicity (using the same questionnaire as for patients), sex and age (to 5-year intervals) were recruited at the MPIP. Controls were selected randomly from a Munich-based community sample and screened for the presence of anxiety and affective disorders using the Composite International Diagnostic Screener [195]. Only individuals negative for the above-named disorders were included in the sample. All included controls were Caucasian and 93.04% were of German origin. These subjects thus represent a group of healthy individuals with regard to depression and anxiety.

The study was approved by the ethics committee of the LMU in Munich, Germany, and written informed consent was obtained from all subjects.

3.1.2 DNA preparation

On enrollment in the study, 40 ml of EDTA blood were drawn from each patient and each healthy control. DNA was extracted from fresh blood using the Puregene® whole blood DNA-extraction kit (Gentra Systems Inc; MN).

3.1.3 SNP selection and genotyping

We genotyped 29 SNPs in *P2RX7* and in the neighbouring genes *P2RX4*, *OASL* and *CAMKK2*. Polymorphisms were detected by direct sequencing in a Canadian bipolar population where *P2RX7* was first implicated [13]. Some of the identified SNPs are available in the UCSC genome browser[1].

Genotyping was performed on a MALDI-TOF mass-spectrometer (MassArray® system) employing the Spectrodesigner software (Sequenom®; CA) for primer selection and multiplexing, and the homogeneous mass-extension (hMe) process for producing primer extension products [173]. All primer sequences are available upon request.

Genotyping for some SNPs, e.g. rs2230912, was verified by pyrosequencing (Biotage, Uppsala, Sweden).

3.1.4 Statistical analysis

3.1.4.1 Testing for deviation from Hardy-Weinberg equilibrium (HWE)

Deviations from HWE for each SNP were assessed in both samples, patients and controls, applying the exact test by Wigginton *et al.* [192]. For an adjustment for multiple testing, the false discovery rate correction was applied (table 3.2) [16]. All functions used are available in R.[2] For investigating whether deviations from Hardy-Weinberg equilibrium could be explained by an underlying genetic model we used the goodness-of-fit test by Wittke-Thompson[3] [197].

3.1.4.2 Case-Control analysis

Case/control analysis was performed using exact Fisher test. We used 2×2 and 2×3 contingency tables to perform genotype-wise analysis. To test for significance under different genetic models (e.g., dominant and heterozygote disadvantage model) and to calculate odds ratios with standard deviations we used 2×2 contingency tables.

Comparison of different genetic models was performed based on the method by Chiano and Clayton [28].

[1] http://www.genome.ucsc.edu/cgi-bin/hgGateway
[2] http://www.r-project.org
[3] http:/hg-wen.uchicago.edu/dhw2.html

3.1.4.3 Correction for multiple testing

In the confirmatory part of the study we analyzed three SNPs (rs1718119, rs11065501, rs2230912) of the *P2RX7a*nd *P2RX4* genes that have previously been reported to be associated with bipolar disorder [13]. In the exploratory part of the study, we considered the remaining 15 SNPs not supposed to be associated with BP, but turned out to be polymorphic with a MAF of larger than 1 %. Correction for multiple testing was preformed separately for the confirmatory and exploratory part of the study using the min p method of Westfall and Young [191] allowing for the linkage disequilibrium between genetic markers. After performing 30 000 permutations we determined the required threshold for controlling the type I error rate at 2.5 % to be equal to 0.0085 for the confirmatory analysis, and to be 0.0019 for the exploratory part of the study. The type I error rate was set to 2.5 % to allow for the two rounds of testing, confirmatory and exploratory, within this study. We compared the nominal p-values given throughout the text with this threshold instead of comparing it with 0.05.

3.1.4.4 Testing for LD

For the LD structure examination we used D' and r^2 measure [76]. Visualization of LD measures was performed using HAPLOVIEW[4]. Blocks were defined using the confidence interval method described by Gabriel [63].

3.1.4.5 Haplotype analysis

We estimated haplotypes using the SNPHAP[5] program for analysis of the highest associated SNP and for the three neighbouring SNPs that were in one LD-block. We tested all haplotypes with frequencies $> 5\,\%$ and a certainty of individual assignment of $> 95\,\%$. For association tests of each haplotype variant we used exact Fisher tests on 2×2 contingency tables.

3.1.4.6 Explorative genotype-phenotype correlations

To determine a possible association of age at onset and the number of previous depressive episodes we performed logistic regression analysis with genotypes treated as the independent variable and phenotypes as dependent variable. Age at onset was defined as the age at which diagnostic criteria for MDD were met for the first time.

[4] http://www.broad.mit.edu/mpg/haploview/index.php
[5] http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt

3.2 Results

3.2.1 Allele frequencies and quality control

23 of 29 genotyped SNPs were polymorphic. 18 of these SNPs had a minor allele frequency greater than 1 % and were included into the analysis. Reference numbers for SNPs are given when available; the remaining SNPs were named according to their position on the genomic reference sequence (table 3.1). Information about genes, location of polymorphisms within genes, function and MAF are presented in table 3.1. Genotyping was successful in 98 % of cases and controls.

We separately calculated the p-value for deviation from HWE in diagnosed controls and patients. One SNP in the patient group (rs3815990) and 3 SNPs in controls (29364 G→A, rs6489795, and rs2230912) showed nominally significant deviations from HWE (table 3.2). After correcting for multiple testing none of these deviations remained significant.

3.2.2 Case control association

The confirmatory analysis of the three SNPs that previously turned out to be associated with BP, showed a significant association with the exonic SNP, rs2230912 (Gln460Arg), of the *P2RX7* gene with a nominal p-value equal to 0.0019 (figure 3.1) remaining significant after correcting for multiple comparison. Homozygote A allele carriers were more frequent among controls while heterozygous carriers were overrepresented among MDD (figure 3.1). We tested different genetic models underlying this association. In case of a dominant model the odds ratio was equal to 1.3 with a nominal p=0.0081 (figure 3.2).

Under the assumption of a 'heterozygote disadvantage' model, i.e., contrasting both homozygous genotypes with the heterozygotes AG genotype, we observed an odds ratio of 1.402 with a nominal p-value of 0.00099. The power to detect an effect of this type in our study in the confirmatory analysis was 58 %, thus our finding is in keeping with the expectations. The 'heterozygote disadvantage' model appeared slightly better, but the difference was not significant ($p = 0.1407$). The exploratory analysis of the other 15 polymorphic SNPs did not show further significant case-control associations.

The distribution of observed genotypes of the associated SNP rs2230912 (Gln460Arg) showed a (nominally) significant deviation from HWE. This could be a consequence of genotyping error, but we could exclude this possibility by verifying the data using different genotyping methods, namely direct sequencing and pyrosequencing. All cases (n = 1000) and controls (n = 1029) were genotyped both by MALDI-TOF (at the Genetic Research Center in Munich, overall call rate 95.4 %) and pyrosequencing (at the MPIP, overall call rate 99.5 %) for rs2230912. Of these genotypes there were 12 discrepancies out of 974 valid genotypes for both assays in the controls (1.17 %), and 8 discrepancies out of 952 valid genotypes for both assays in the cases (0.80 %). In addition 500 randomly selected cases and 500

Gene	SNP	Position on hg17	Location	Function	MAF
OASL	rs12819210	119921120	exon 6	Ser503Ser	0.195
OASL	-14989 G→A	119924518	exon 5	Val348Met	0.004
OASL	rs3213545	119934057	exon 2	Leu136Leu	0.293
OASL	259 A→G	119939766	5'UTR	unknown	0.246
OASL	2529 C→T	119942036	5'UTR	unknown	n.p.
P2XR7	-2575 T→C	120030921	5'UTR	unknown	0.095
P2XR7	-1300 G→A	120032196	5'UTR	unknown	0.007
P2XR7	21749 C→T	120055245	intron 1	unknown	0.002
P2XR7	rs17525809	120055409	exon 2	Val76Ala	0.086
P2XR7	23160 C→T	120056656	exon 3	Arg117Trp	0.002
P2XR7	29364 G→A	120062860	intron 4	unknown	0.029
P2XR7	29463 G→A	120062959	exon 5	Gly150Arg	n.p.
P2XR7	32406 G→A	120065902	exon 6	Glu186Lys	n.p.
P2XR7	32422 G→A	120065918	exon 6	Leu191Pro	n.p.
P2XR7	rs7958316	120068093	exon 8	Arg276His	0.026
P2XR7	rs1718119	120077823	exon 11	Ala348Thr	0.407
P2XR7	rs6489795	120077851	exon 11	Thr357Ser	0.070
P2XR7	rs2230912	120084916	exon 13	Gln460Arg	0.147
P2XR7	rs3751143	120085024	exon 13	Glu496Ala	0.205
P2XR7	rs2230913	120085100	exon 13	His521Gln	n.p.
P2XR7	52857C→T	120086353	3'UTR	unknown	n.p.
P2XR4	-643 C→T	120110044	5'UTR	unknown	0.126
P2XR4	rs1653622	120110155	5'UTR	unknown	0.333
P2XR4	rs2303998	120117783	exon 2	Ala87Ala	0.006
P2XR4	rs7298368	120122404	intron 2	unknown	0.165
P2XR4	rs25644	120129366	exon 7	Ser242Gly	0.107
P2XR4	rs11065501	120134690	3'UTR	unknown	0.333
CAMKK2	rs3815990	120153808	exon 9	Ile365Ile	0.061
CAMKK2	rs3817190	120174797	exon 1	Thr85Ser	0.410

n.p. – not polymorph

Table 3.1: Information on genotyped SNPs.

SNP	HWE cases	FDR corrected	HWE controls	FDR corrected	Cases (n)	Controls (n)
rs12819210	1.0000	1.0000	1.0000	1.0000	985	1020
rs3213545	0.3182	0.7160	0.8201	1.0000	988	1012
259 A→G	0.8527	0.9593	0.0822	0.3699	987	864
-2575 T→C	0.3529	0.6352	0.5824	1.0000	992	1018
rs17525809	0.1105	0.9945	0.3097	1.0000	984	1002
29364 G→A	0.5639	0.7808	0.0477	0.2862	990	1023
rs7958316	0.1177	0.7062	1.0000	1.0000	984	1018
rs1718119	0.6484	0.8337	0.6032	1.0000	988	1016
rs6489795	0.3016	0.7755	0.0258	0.2322	985	1024
rs2230912	0.1753	0.7889	**0.0059**	**0.1062**	999	1029
rs3751143	0.9183	0.9723	0.5670	1.0000	1000	1029
-643 C→T	0.7769	0.9323	0.7770	1.0000	985	1018
rs1653622	0.3885	0.6357	1.0000	1.0000	989	1018
rs7298368	0.2354	0.7062	0.7329	1.0000	990	1016
rs25644	0.3238	0.6476	0.8691	1.0000	990	1017
rs11065501	0.2126	0.7654	0.6218	1.0000	991	1021
rs3815990	0.0171	0.3078	1.0000	1.0000	983	1018
rs3817190	0.4106	0.6159	0.8427	1.0000	937	977

Table 3.2: HWE-tests (p-values) and genotyped sample size in informative SNPs

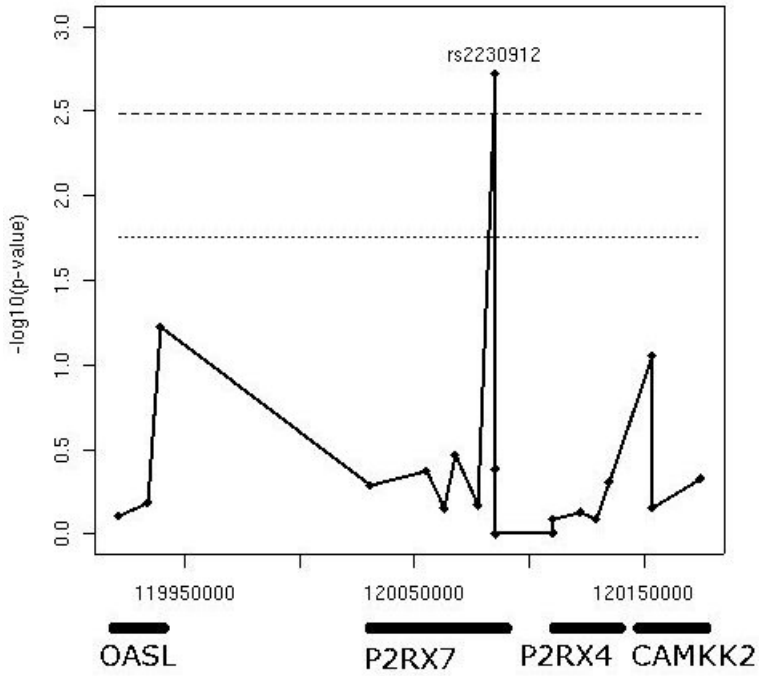

Figure 3.1: Association of investigated SNPs with MDD. Chromosomal positions are given on the x-axis and –log 10 (P-values) are on the y-axis. The dotted and dashed lines represent the permutation-based 5 % type I error rate for the three SNPs implicated in BP in the SLSJ sample [13] and the 17 remaining SNPs, respectively.

randomly selected controls were genotyped by direct sequencing (overall call rate 99.6 %) in the laboratory of Nicholas Barden at Laval University, Quebec, Canada. In these data there were 4 discrepancies with the pyrosequencing data and 7 discrepancies with the MALDI-TOF data (discrepancy rates of 0.40 % and 0.73 %, respectively). These discrepancies did not seem directed in any of the cases, and the influence on the various genotyping methods on the results of the analysis was minimal. To give an example, the result of the genotypic test in those samples where both the MALDI-TOF and the pyrosequencing genotypic data agreed, was 0.0014 for the genotypic model, 0.0026 for the dominant model, and 0.00039 for the heterozygote disadvantage model. For the analysis presented here we used the pyrosequencing data with missing values supplemented by MALDI-TOF data. This gave p-values of 0.0019, 0.0081, and 0.00099, for the respective genetic models. p-values when using the MALDI-TOF data only were 0.0013 for the genotypic test, 0.0033 for the dominant model and 0.00041 for the heterozygote disadvantage model. p-values based on the pyrosequencing data alone were 0.0028, 0.0094 and 0.0012, respectively. Thus the results of the analysis do not or only in a very minor fashion depend on the genotyping method used.

Another explanation for this deviation could be the fact of investigating a control sample selected for being negative for mental disorders. Provided that heterozygotes represent the high-risk genotype for depression, a lack of heterozygotes in the control group is expected. In that case the control group would not be in HWE, in contrast to the combined case and

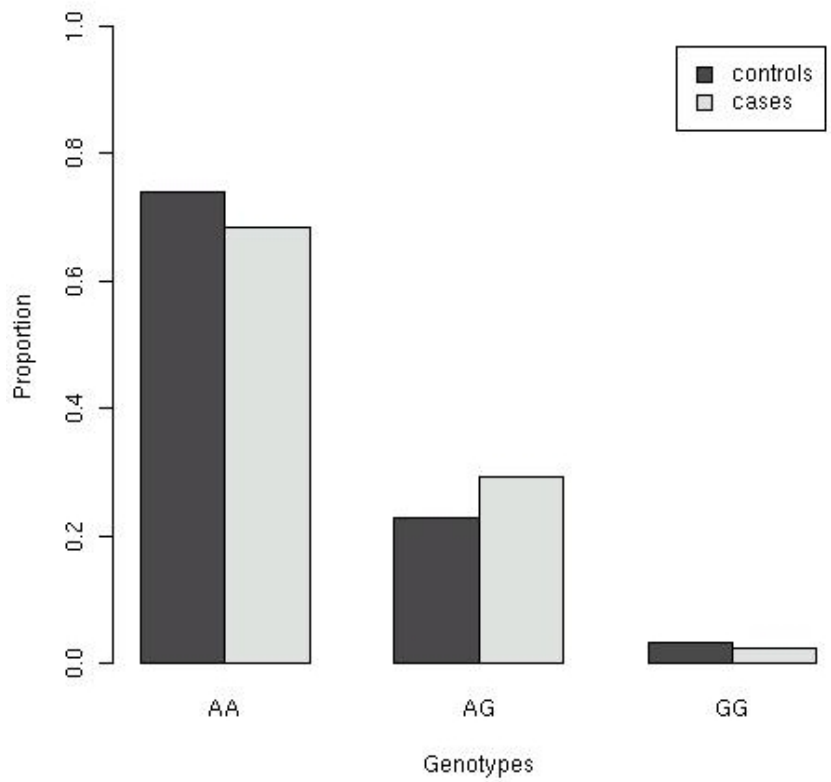

Figure 3.2: Distribution of genotypes of SNP rs2230912 in cases and controls. The odds ratio for a dominant model (genotypes AG and GG versus AA) was equal to 1.30 (CI = 1.07–1.59; $p = 0.008068$) and for a heterozygote disadvantage model (AG versus AA and GG) 1.40 (CI=1.14–1.72; $p = 0.0009938$).

control samples. In fact, if we merge diagnosed controls and depressive patients they are in HWE (nominal $p = 0.358$). To prove the statistical significance of the latter hypothesis we used the goodness-of-fit procedure of Wittke-Thompson *et al.* [197] testing the general, additive, dominant and recessive models. The data were found to be in agreement with the general model (p = 0.18). The estimated minor allele frequency for the susceptibility locus was equal to 0.16 and therefore nearly equal to the observed population frequency of 0.15 for rs2230912 (table 3.1). The relative risk for heterozygotes estimated from this model is 1.19, also in agreement with the results presented in figure 2.

3.2.3 Testing for LD

To get more insight into the pattern of LD between alleles at polymorphic loci, pairwise disequilibrium measures (D' and r^2) in controls were calculated using all SNPs with a minor allele frequency over 1 %. We detected 3 independent blocks of LD within the investigated region defined by the Gabriel method (figure 3.3). The first block spans 2 SNPs in the *OASL* gene, the second contains four SNPs in exon 11 and 13 of *P2RX7* and the third block spans five SNPs in *P2RX4* and *CAMKK2* genes. In the first and third block no SNP was

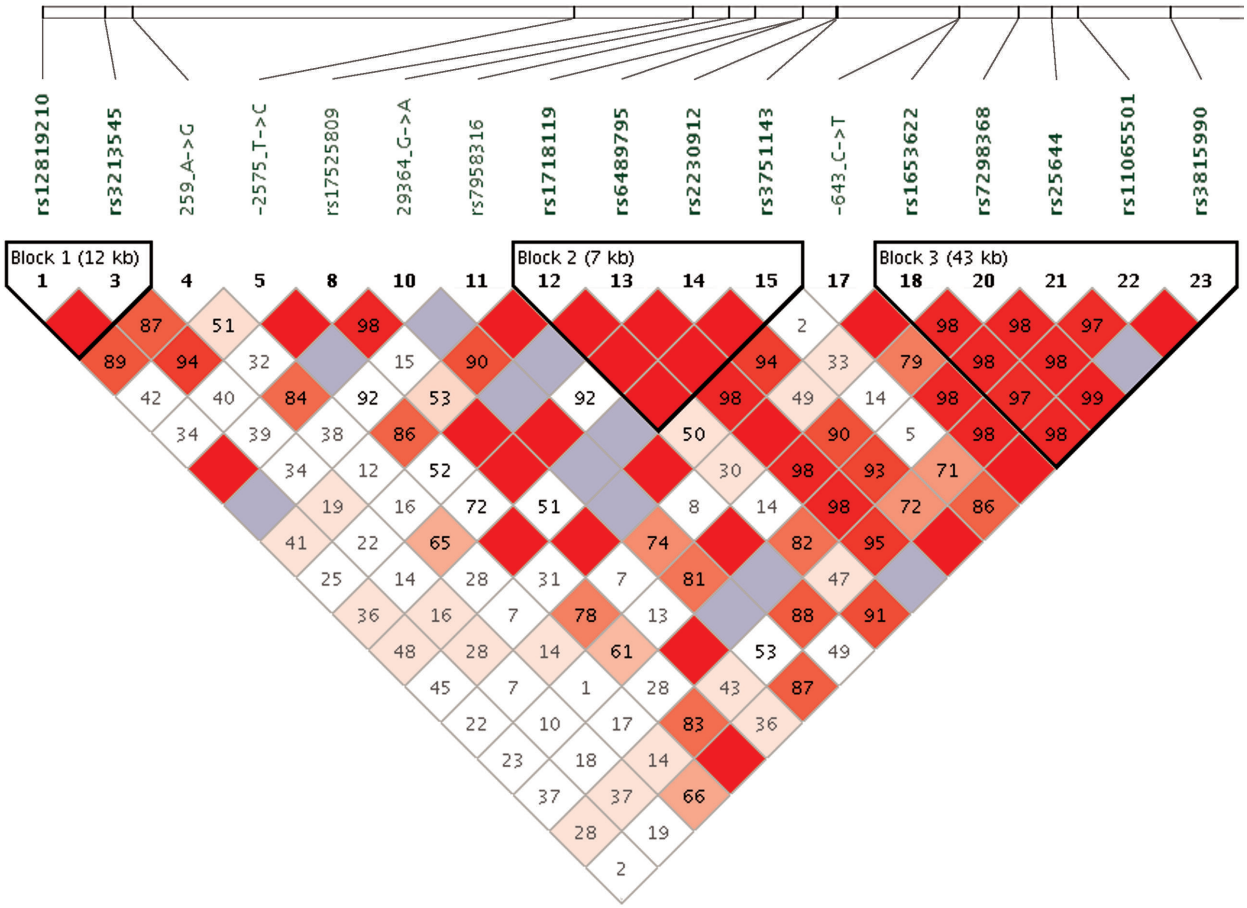

Figure 3.3: Linkage disequilibrium and block structure (D') of the P2RX7 region. SNPs lying within blocks are depicted in bold type

associated with MDD. D' within the second block was equal to 1, albeit r^2 was rather low (0.2, 0.01, 0.05 for pairs of the highest associated SNP rs2230912 with rs1718119, rs6489795, and rs3751143, respectively).

The presence of a single associated SNP within the second block is due to the low r^2 despite high D' and somewhat similar minor allele frequencies (0.1472 for rs2230912 and 0.2055 for rs3751143) and is coherent with the general recognition of r^2 as important for determining the power of association analyses.

3.2.4 Association of haplotypes

Using the SNPHAP program, we reconstructed haplotype alleles for each subject on the basis of the four SNPs in the LD block 2 (rs1718119, rs6489795, rs2230912, rs3751143), because one of these SNPs showed the highest association with MDD. We observed 5 frequent (> 5 % population frequency) and 2 less frequent haplotype alleles (data not shown). The 5 frequent haplotypes accounted for 99.1 % of the chromosomes in our sample. No differences in frequencies were observed between cases and controls. Only one haplotype allele 'ACGA'

showed a trend for association with a p-value = 0.0753, but that is a reflection of association of the 'G' allele in SNP rs2230912 with MDD.

3.2.5 Explorative genotype phenotype correlation

Differences in genotype distribution between cases and controls were independent from gender and age. Genotype variation had no influence on age at onset or the number of previous depressive episodes (data not shown).

3.3 Discussion

Here we report evidence that SNP rs2230912 in the *P2RX7* gene is associated with MDD. This functional polymorphism rs2230912 is located in exon 13 of the *P2RX7* gene resulting in a change of the amino acid glutamine to arginine at position 460 (Gln→Arg). Gln460Arg is positioned in the long intracellular C-terminal domain. This domain is unique among P2X receptors and is thought to be responsible for functions that are different from those observed in other ion channels, including alterations in cell morphology [32], intracellular signaling, and cytolysis/apoptosis [51].

In this region, several loss-of-function polymorphisms have been reported [69, 68, 193]. The Gln460Arg polymorphism has been described to lead to a functional decrease, albeit minor, when measuring Ca^{2+} influx in peripheral blood lymphocytes of patients affected with chronic lymphocytic leukaemia and in transfected recombinant human embryonic kidney cells [22]. Due to its position in the intracellular domain [45] and the fact that Gln460 residue is conserved in mammals, this residue is likely to be involved in *P2RX7* dimerisation as well as in other protein-protein interactions having effects upon *P2RX7*-mediated signalling.

Although so far little is known about the functional implications of the Gln460Arg variant, *P2RX7* receptors might well play a pivotal role in antidepressant action and the causality of mood disorders through their role in neuroprotection [172] and in neuroinflammatory responses [196] as well as through their influence on neurotransmission in the hippocampus [8].

In our study, we noted a nominally significant DHW for the associated SNP rs2230912. Since laboratory error is one of the most common reasons for DHW we verified our genotyping by pyrosequencing as well as by direct sequencing and found no discrepancies between results from different genotyping methods. This deviation is also unlikely to be due to hidden population structure since our study was restricted to Caucasians with 92 % of people originating from Germany. Formal assessment of population structure using STRUCTURE[6] gave no evidence of population admixture (data not shown).

In our sample a heterozygote disadvantage model was the most suitable mode of inher-

[6] http://pritch.bsd.uchicago.edu/software.html

itance, possibly being a reflection of *P2RX7* receptors having an oligomeric structure in the plasma membrane based on complexes of identical subunits [124, 178]. The same variant we found associated, rs2230912, was also the most highly associated SNP in Canadian bipolar families with an over-transmission of the (minor) G-allele in the affected offspring (p=0.000708) [13]. In our sample we observed the same direction of association with the G-allele being more frequent in patients. SNP rs3817190 (exon 1 in *CAMKK2*) as well as rs1718119 (exon 11 in *P2RX7*) and rs11065501 (3'UTR in *P2RX4*) were associated in Canadian bipolar families and in the case/control study, respectively, but showed no association in our MDD sample. These findings could reflect the clinical observation of MDD and BP being two different disorders that share some causal factors, with the variances in common conveying susceptibility to both disorders. Polymorphisms in other genes are likely to represent the discriminating factors, determining the ultimate clinical phenotype, unipolar or bipolar. This discrepancy might also be due to differing patterns of LD in the more isolated SLSJ as compared to the non-isolated Munich population. LD seems to have a longer reach in the SLSJ population [13] compared to the Munich population, which is in keeping with general knowledge about isolated *vs.* non-isolated populations [185].

In addition, epidemiological data indicate that there is a constant diagnostic conversion from MDD to BP of 1.25 % per year throughout the lifespan [7]. Accordingly, in a sample of MDD patients a substantial number of hidden bipolar cases are to be expected. However, when calculating odds ratios stratified by age, we observed no linear influence of age or age at onset on the association, which would be expected under the hypothesis that the association in the sample of MDD patients would be due to the hidden bipolar cases (data not shown). Therefore, the data suggest that the Gln460Arg variant might be a susceptibility factor for both disorders.

Our data, in combination with the association data in BP patients, suggest the implication of *P2RX7* in affective disorders and are consistent with the possibility that various mood disorders share some genetic commonalities. Being localized in the plasma membrane, *P2RX7* is a potential drug target and thus represents an example for a possible pharmacological drug discovery strategy emerging from an unbiased genetic approach.

Chapter 4

Variation in genes controlling HPA axis response to antidepressant treatment and depression severity

Several studies suggest that a normalization of the HPA axis hyperactivity and *GR* resistance that is observed in depression may be required for clinical response to antidepressant treatment [81]. Patients suffering from depression show elevated levels of plasma and urinary cortisol, an increased number of cortisol secretory episodes, and increased concentrations of CRH in the cerebrospinal fluid. These basal alterations are accompanied by an abnormal responsiveness of the HPA axis to stimulating (e.g., intravenous application of exogenous CRH) and suppressing (e.g., oral application of the synthetic corticosteroid Dex agents). These findings have led to the hypothesis of corticosteroid receptor resistance [81]. It could be explained by a changed expression pattern of *GR* and *MR* during acute depression, although the molecular mechanisms underlying these effects are not completely clear yet [44, 129]. An alternative explanation invokes changes in regulatory proteins of *GR* and *MR*. Endogenous GCs have differential occupancy profiles. *GR*s are thought to mediate negative feedback signals of elevated GC levels under stress conditions, whereas *MR*s control the inhibitory tone of the hippocampus on HPA activity [42, 129, 144]. Indeed, three genes within the stress hormone system, *GR*, *MR* and *FKBP5*, have so far been associated with antidepressant response.

Recently, our group investigated the influence of polymorphism in genes regulating the HPA axis on response to antidepressant drugs in the Munich Antidepressant Response Signature (MARS) sample. In this sample, we detected an association of a functional polymorphism of the *GR* gene leading to two amino acid substitutions in codons 22 and 23 (ER22/23EK) that results in partial *GR* resistance in non-depressed subjects, and with faster response to antidepressant treatment (n=367) [182]. The transcriptional activity of the EK22/23 *GR*-variant was decreased. While mRNA stability is not affected, this polymorphism seems to influence the ratio of expression of two isoforms, *GR*-A and *GR*-B [150].

The common variant V180 on polymorphism I180V in the *MR* protein permits enhanced responses in cortisol secretion and heart rate due to a psychosocial stressor. *In vitro* testing of the V180 allele revealed a mild loss of function using cortisol as a ligand, in comparison to the I180 allele. Therefore the V180 variant may represent a genetic vulnerability factor for stress related disorders such as depression [47].

Changes in *GR* and *MR* represent one potential cause of glucocorticoid resistance, but there are also complex interactions with cellular components involved in hormone signaling, such as chaperones and other transcription factors. For this reason we also linked polymorphisms within the locus of *FKBP5*, encoding the *GR*-regulating co-chaperone of hsp90, *FKBP5*, to response to antidepressant treatment [19]. We found a highly significant association ($p = 0.00003$) between polymorphisms in *FKBP5* and response to antidepressant drugs (n=280). Patients homozygous for the rare allele of the associated SNPs responded over ten days earlier to antidepressant treatment than patients with the other genotypes (table 4.1). This was observed in groups of patients treated with TCA, SSRI or mirtazapine, suggesting that this effect is independent of the class of antidepressant.

SNP	**Nonresponder**	**Responder**	***p*-value**
Max-Planck Institute of Psychiatry			
rs3800373	AA, 51; CA, 35; CC, 0	AA, 77; CA, 48; CC, 21	0.00003
rs1360780	CC, 49; CT, 36; TT, 1	CC, 75; CT, 50; TT, 22	0.00048
rs4713916	GG, 49; AG, 36; AA, 1	GG, 71; AG, 53; AA, 22	0.00031
Ludwig Maximilian University, Augsburg and Ingolstadt hospitals			
rs3800373	AA, 26; CA, 15; CC, 1	AA, 16; CA, 23; CC, 4	0.053
rs1360780	CC, 25; CT, 16; TT, 1	CC, 14; CT, 24; TT, 5	0.020
rs4713916	GG, 26; AG, 15; AA, 1	GG, 21; AG, 18; AA, 4	0.270

Table 4.1: Association of *FKBP5* SNP genotypes with response to antidepressants after 2 weeks of treatment [19]

This result could be replicated in a second sample of patients recruited at three different hospitals in Bavaria (n=80). The same genotypes were also associated with increased intracellular *FKBP5* protein expression which triggers adaptive changes in *GR* and thereby HPA axis regulation [19].

Patients carrying the associated genotypes displayed less HPA axis hyperactivity during the depressive episode, as measured by the combined Dex-suppression/CRH-stimulation test (Dex-CRH test). It is therefore possible that even though homozygotes for these SNPs are as severely depressed as the other patients at the time point of hospitalization, their HPA axis regulation is less impaired due to compensatory mechanisms elicited by increased intra-

cellular *FKBP5* levels, allowing a faster restoration of normal HPA axis function. Because of the lack of a placebo-treated group it was, however, not possible to rule out that these patients have an inherently shorter duration of their depressive episodes, independent of antidepressant treatment. The polymorphisms associated with faster response were located from the promoter region to the 3' end of the gene and all in very strong LD forming one risk haplotype. It was therefore difficult to pinpoint one of the polymorphisms as the causal variant. Nonetheless, rs1360780 located in intron 2 seems a promising candidate as it is only 400 bp downstream of a glucocorticoid responsive element (GRE) that has been shown to be functionally relevant [180]. We have observed a much stronger correlation between *FKBP5* mRNA expression in peripheral lymphocytes and serum cortisol levels in individuals carrying the genotypes associated with fast response to antidepressant than the two other genotypes, indicating an altered *GR*/*FKBP5* feedback mechanism associated with these genotypes [19].

Hence, increasing the level of *FKBP5* represents a direct autoregulatory loop to control GCs action. In depressed patients this negative feedback seems to be insufficient. The fact that long-term treatment with antidepressants leads to both normalization of HPA axis activity and an increase of *GR* and *MR* mRNA transcription [12, 23, 75, 87, 114, 127, 143, 142] suggests that antidepressant-regulated *GR*, *MR* and *FKBP5* gene expression renders the HPA system more susceptible to feedback inhibition by cortisol.

For the further elucidation of the role of the genes *FKBP5*, *GR* and *MR* in the susceptibility to depression and in response to antidepressants we enlarged the density of markers in the previously investigated *FKBP5* gene [19]. Additional polymorphisms were genotyped in an enlarged sample and we investigated statistical interactions between the three above-mentioned genes. In addition, we tested the predicted interaction between *FKBP5* and *MR* experimentally in a cellular reporter assay.

4.1 Materials and Methods

4.1.1 Patients

493 patients were admitted to the hospital of the Max Planck Institute of Psychiatry (MPI), Munich, Germany, for treatment of a depressive disorder, 422 (85.6 %) presented with a unipolar depressive episode (32.3 % first and 53.4 % recurrent episode), 61 (12.4 %) patients with BP disorder (6.1 % BP I and 6.3 % BP II), and 10 with other diagnoses of current depression (0.5 % dysthymia, 0.7 % schizoaffective disorder, 0.7 % adjustment disorder). To raise the power of the association tests we enlarged the sample size by 53 compared to the previous study [19].

Patients were included in the study within 1-3 days of admission, and the diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Patients with depressive disorders due to a medical or neurological condition were excluded. All included patients were Caucasian and 85.1 % were of German origin. Groups of other nationalities were smaller than 3 %. The study was approved by the local ethics committee and written informed consent obtained from all subjects.

All patients were treated according to the doctor's judgment with antidepressant drugs. For all patients plasma concentration of antidepressant medication was monitored to assure clinically efficient drug levels.

4.1.2 Psychopathological assessment and definition of response to antidepressant drug treatment

Psychopathological symptoms of depressed patients were assessed by trained raters using the 21-item Hamilton Depression Rating Scale (HAM-D) within 3 days of admission and then weekly until discharge. Patients fulfilling the criteria for at least a moderate depressive episode (HAM-D ≥ 14) entered the analysis. As in the previous pharmacogenetic study from our group [19], we used three common types of response definitions, each defining different aspects of antidepressant treatment outcome: early response, response, and remission. Early response was defined according to the change in HAM-D from study inclusion until after two weeks of treatment. Patients with a reduction ≥ 25 % from their score at admission were considered as early responders, while patients whose HAM-D score decreased less than 25 % were considered as early non-responders. We defined response as a reduction of at least 50 % of the HAM-D scores at 5 weeks compared to admission. All patients with a reduction of HAM-D scores less than 50 % at 5 weeks were considered non-responders. Remission was defined as reaching a total HAM-D score of less than 10 after five weeks. The weekly psychopathology ratings were available for 331 and 265 patients after 2 and 5 weeks of treatment after being included in the study respectively. The five-week time point was

chosen because this duration of treatment is considered sufficient for an antidepressant drug to display its clinical efficacy.

In addition to qualitative response evaluation quantitative changes in HAM-D scores during the first five weeks were evaluated by means of orthogonal polynomial coefficients (OPCs) describing depression severity level (0 order), linear improvement (1^{st} order), quadratic (2^{nd} order), and cubic (3^{rd} order) alterations during the observation period. Higher order polynomials were neglected as they did not provide additional information about changes of HAM-D scores.

4.1.3 Controls

Controls (N = 602) were enlisted from a randomly selected Munich-based community sample and screened for the presence of anxiety and affective disorders using the Composite International Diagnostic-Screener [195]. Only individuals negative for the above-named disorders were included in the sample. Recruitment of controls was also approved by the local ethics committee and written informed consent was obtained from all subjects. The control sample has been described previously in section 3.1.1) and in [109].

4.1.4 SNP discovery, selection and genotyping

We selected a set of SNPs for each of the investigated genes from dbSNP[1]. 32 SNPs were selected for the *FKBP5* gene (NM_004117). After fine mapping the average distance between markers with a genotyping success rate of at least 90% was equal to 6.1 kb compared to the previous paper [19] of 15.3 kb. For the genes *NR3C1* encoding the *GR* protein (NM_000176[2]) and *NR3C2* gene encoding the *MR* protein (NM_000901) we selected 11 SNPs each. SNPs included in the study had a MAF $> 2\%$. After correction for multiple testing, there was no significant evidence that any SNP was not in HWE. SNP IDs, positions on UCSC[3] genome built version hg17, MAF, p-values for HWE test and the number of successfully genotyped individuals in the sample are reported in table 4.2.

[1] http://www.ncbi.nlm.nih.gov
[2] http://genome.ucsc.edu
[3] http://www.genome.ucsc.edu/cgi-bin/hgGateway

Table 4.2: SNPs genotyped in the *FKBP5*, *NR3C1*, *NR3C2* genes which survived quality control and the number of successfully genotyped individuals in the case and control samples. 'nb' – no bin. p-values for the HWE test are for controls. SNPs in bold face have been described previously by Binder et al. (2004)

Gene	SNP	Position	Cases	Controls	MAF	HWE	Role	Alleles	LD bin
NR3C2	rs2871	149357629	326	464	0.30	0.91	3' UTR	A/G	nb
NR3C2	rs1879827	149383011	327	472	0.19	1.00	Intron	A/G	nb
NR3C2	rs3843413	149488406	325	506	0.33	0.92	Intron	C/T	nb
NR3C2	rs3846306	149488869	324	509	0.26	0.04	Intron	A/C	nb
NR3C2	rs3752701	149537915	326	510	0.21	0.42	Intron	C/T	nb
NR3C2	rs1355613	149613975	329	510	0.24	0.54	Intron	C/T	nb
NR3C2	rs2137331	149638301	326	472	0.44	1.00	Intron	A/T	nb
NR3C2	rs907621	149660326	330	509	0.16	0.75	Intron	A/G	nb
NR3C2	rs1490453	149678951	330	509	0.17	0.88	Intron	C/T	nb
NR3C2	rs5522	149715080	326	468	0.09	0.17	exon	A/G	1
NR3C2	rs1490464	149725352	347	504	0.11	0.16	Prom	A/G	1
NR3C1	rs258813	142654883	315	483	0.31	0.34	Intron	A/G	1
NR3C1	**rs6188**	142660537	315	487	0.31	0.60	Intron	G/T	1
NR3C1	**rs33388**	142677488	315	477	0.47	0.85	Intron	A/T	2
NR3C1	rs33383	142690179	350	164	0.45	0.53	Intron	C/T	2
NR3C1	rs2918416	142721220	344	493	0.31	0.40	Intron	A/G	1
NR3C1	rs2963155	142736197	355	161	0.23	1.00	Intron	A/G	nb
NR3C1	rs2963156	142738689	347	153	0.21	0.15	Intron	C/T	nb
NR3C1	rs1866388	142739978	351	504	0.31	0.40	Intron	A/G	1
NR3C1	rs6190	142760530	492	490	0.02	1.00	exon	A/G	nb
NR3C1	rs4582314	142760888	356	510	0.48	0.79	Intron	A/C	2
NR3C1	rs4634384	142760890	355	507	0.47	0.72	Intron	C/T	2
FKBP5	rs1883637	35602577	359	600	0.09	0.02		C/T	2

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Gene	SNP	Position	Cases	Controls	MAF	HWE	Role	Alleles	LD bin
FKBP5	rs4711420	35603789	331	341	0.23	0.03		G/T	nb
FKBP5	rs12526404	35608926	329	344	0.08	0.02		A/T	2
FKBP5	rs6909470	35619023	333	341	0.19	0.05		A/G	5
FKBP5	**rs3807050**	35620904	359	597	0.24	0.73		C/T	nb
FKBP5	rs1807142	35631772	332	324	0.08	0.46		C/T	2
FKBP5	**rs3800374**	35645384	357	599	0.17	0.15		A/G	5
FKBP5	rs10807151	35648846	337	345	0.17	0.44		C/T	3
FKBP5	**rs3800373**	35650454	355	163	0.26	1.00	3' UTR	G/T	nb
FKBP5	1095 C→T	35652768	337	346	0.03	0.22	exon	C/T	nb
FKBP5	**rs755658**	35657648	359	602	0.09	0.04	Intron	A/G	2
FKBP5	rs2294807	35663088	330	344	0.08	0.07	Intron	C/T	2
FKBP5	**rs992105**	35663161	356	593	0.15	0.05	Intron	A/C	nb
FKBP5	rs10498734	35667751	337	345	0.08	0.06	Intron	G/T	2
FKBP5	rs7753746	35673400	336	341	0.17	0.08	Intron	A/G	3
FKBP5	rs2395634	35675738	336	339	0.30	0.30	Intron	A/G	nb
FKBP5	**rs4713899**	35677259	360	599	0.16	0.01	Intron	A/G	6
FKBP5	rs7748266	35700722	336	345	0.17	0.09	Intron	C/T	3
FKBP5	**rs1591365**	35712085	360	597	0.29	0.62	Intron	A/G	1
FKBP5	**rs1360780**	35715549	359	602	0.29	0.49	Intron	C/T	1
FKBP5	rs6902124	35718286	318	345	0.30	0.37	Intron	A/C	1
FKBP5	**rs2143404**	35718659	353	600	0.15	0.02	Intron	C/T	6
FKBP5	rs12527329	35718729	315	344	0.09	0.09	Intron	A/T	2
FKBP5	**rs1334894**	35723108	357	509	0.09	0.08	Intron	C/T	2
FKBP5	rs9394309	35729759	334	332	0.29	0.18	Intron	A/G	1
FKBP5	rs9380525	35741016	333	343	0.32	0.39	Intron	C/G	1
FKBP5	rs9380526	35766305	334	316	0.31	0.29	Prom	C/T	1
FKBP5	rs943297	35775838	337	346	0.28	0.19	GRE	A/G	7
FKBP5	**rs4713916**	35777961	357	480	0.27	0.03		A/G	7

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Gene	SNP	Position	Cases	Controls	MAF	HWE	Role	Alleles	LD bin
FKBP5	rs9462104	35782823	318	334	0.30	0.15		C/T	4
FKBP5	**rs4713921**	35789755	359	598	0.29	0.07		C/T	4
FKBP5	rs4711429	35791526	336	344	0.29	0.19		A/G	4

Genotyping was performed on a MALDI-TOF mass-spectrometer (MassArray® system). For primer selection and multiplexing and the homogeneous mass-extension (hMe) process for producing primer extension products the Spectrodesigner software (Sequenom; CA) was used [173]. The polymorphism ER22/23EK (rs6190) in the *GR* gene was genotyped by pyrosequencing (Biotage, Uppsala, Sweden).

4.1.5 Bioinformatics

We examined whether any of the investigated SNPs were located within predicted transcription factor binding sites using the Genomatix software[4]. Additionally, the open access DNA sequence of *FKBP5* was examined for the hypothetical transcription factor binding site (GRE) by using the following sequence: DGNKCW N(1-4) WGKNCH .

4.1.6 *FKBP5* – mRNA sequencing

No exonic polymorphisms in *FKBP5* were available on open access genome browsers. Therefore, coding and untranslated regions of mRNA NM_004117 annotated by RefSeq (version: May 2004) were re-sequenced. For this purpose 32 healthy controls with various genotypes markers reported to be associated with response to antidepressants by Binder et al. [19] were selected.

The Human May 2004 Assembly as presented in the UCSC Genome Browser showed also a predicted mRNA from genomic sequence with an alternative first exon were available. According to our results from cDNA amplification analysis this isoform was not expressed in blood cells. Additionally, we re-sequenced 5 intronic regions: region one and two were described by T. R. Hubler at al. [86] and contained Dex and progestin responsive elements (positions: 35677508–35678308 and 35686487–35686940, respectively); region 3 was described by M. U [180] and contained 2 GREs (position: 35715729–35716706); and region 4 and 5 included several GREs, according to our screening search (35750268–35751039, 35741369–35741957). Sequencing reactions were performed with and according to the manual of the BigDye Terminator v3.1 Cycle Sequencing Kit and scanned with the ABI 3730 DNA Analyzer from Applied Biosystems. 500 ng of genomic DNA per subject and sequencing reaction were used. All sequencing primers are available upon request.

[4] http://www.genomatix.de/

4.1.7 LD-testing and tSNPs

For the examination of the LD structure we used a method similar to the one developed by Carlson et al. [24].

This method is described as being more powerful than long haplotype-based methods, because r^2 is directly related to the statistical power to detect disease association with an unassayed site [24, 167]. It requires the estimation of haplotype phase for each pair of SNPs in the region for the computation of the LD measure r^2. We allocated SNPs to bins in such a way that all pairwise r^2 within a bin exceeded a threshold of 0.75. With this method different alternative allocations are possible. We looked for the largest cluster in each iteration. After clustering we selected as tSNP that SNP that shared the strongest evidence for association.

4.1.8 Haplotype analysis

Individual haplotype assignments were determined using SNPHAP[5]. Only haplotype assignments with a remaining uncertainty of less than 5 % and haplotypes with a frequency over 1 % were included in the analysis. After individual haplotype estimation we built haplogenotypes for each person.

4.1.9 Statistical analysis

4.1.9.1 Phenotype correlations

There were no differences in ethnicity (assessed by a self-report questionnaire asking for native country, first language, and ethnic group of the individual and his/her four grandparents) or age distribution (mean age of 48.3, SD 14.5 in patients and mean age of 49.8, SD 14.4 in controls, $p = 0.78$) between cases and controls . Females were overrepresented in both samples, with a higher proportion of female controls compared with patients (62.3 % in controls and 55.8 % in patients, $p = 0.027$, Fisher's exact test).

Spearman's correlation coefficient between the number of previous depressive episodes (PDEs) and duration of disease (DD) was calculated. DD was calculated as the difference between the present age of a patient and their age at onset of the first depressive episode. Associations between phenotype variables with DD and the number of PDEs were investigated using logistic regression.

4.1.9.2 Genotype-phenotype association tests

Logistic regression analysis was also chosen for performing association tests of genotypes or haplotypes with phenotype variables. Because of an overrepresentation of females in our sample as well as a correlation of response phenotypes with DD and the number of PDEs, we used DD, number of PDEs and gender as covariates in association analysis.

[5] http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt

4.1.9.3 Interaction testing

We used stepwise logistic regression with the Akaike information criterion (AIC) to determine the best statistical model of interactions between different SNPs [84]. Because of the restricted sample size we included not more than 5 SNPs in a model and tested only main effects and pairwise interaction terms and compared global AICs between different statistical models.

On the basis of the LD structure and associations within genes we decided to compare AICs for combinations a total of 12 SNPs for *FKBP5*, 3 SNPs for *NR3C2*, 7 SNPs for *FKBP5–NR3C2*, 5 SNPs for *FKBP5–NR3C1* and 8 SNPs for *FKBP5–NR3C2–NR3C1*. The number of tested general models is easy to calculate as a sum of binomial coefficients for up to 2, 3, 4, 4 or 5 SNP-combinations of 12, 3, 8, 5 or 7 SNPs, respectively. Thus, we tested a total of 79 SNP-combinations for *FKBP5*, 8 for *NR3C1*, 120 for *FKBP5–NR3C2* interactions, 31 for *FKBP5–NR3C1* and 163 combination for *FKBP5–NR3C2–NR3C1* interactions. Gender, DD and the number of PDEs were used as covariates. The variance explained by investigated variables was calculated as multiple adjusted R^2.

4.1.9.4 Correction for multiple testing

Because of the large number of tests in our study we performed correction for multiple testing as described in section 1.9.2. For each phenotype separately we combined tests for all SNPs in the same gene by taking the product of their p-values. We repeated this procedure for the real data and for 1 000 000 permutations. Finally, we estimated permutation-based p-values for each phenotype using PFPM (section 1.9.3.1).

It was reasonable to separate quantitative changes in HAM-D-scores (OPCs) into two groups: the first one included a depression severity variable and the second one contained linear, quadratic and cubic trends in response over five weeks of treatment We combined p-values from the second group using FPM (equation 1.7), [61]. We also used FPM for estimating over all phenotype p-values. Single polymorphism tests were corrected using the Westfall and Young method (see section 1.9.2 and [191]) within each test-family (each phenotype separately). p-values for the tests for HWE were corrected using the FDR procedure (see section 1.9.1.1 and [16]).

Correction for multiple testing in interaction analysis was performed using the Bonferroni method, which is the most conservative adjustment providing the strongest protection against false positive results. The corrected p-values were calculated as a product of nominal p-values and the number of tested SNP-combinations. All calculations were performed in R.[6]

[6] http://www.r-project.org/

4.1.10 Reporter gene assays

Cultivation and transfection of human neuroblastoma SK-N-MC cells (ATCC # HTB-10) was performed as described by Wochnik et al. [198]. 2 days before transfection, cells were seeded in medium containing charcoal-stripped serum. Amounts of transfected plasmids per 10^{-7} cells were: 1.5 µg steroid-responsive luciferase reporter plasmid MTVLuc, 3 µg β-galactosidase expression vector pCMVβ-Gal (Stratagene) as control plasmid, 0.75 µg pRK7*MR* that expresses human *MR* from the CMV-promoter of the vector pRK7, and 3 µg of *FKBP5* expression vector or empty expression vector. After electroporation cells were seeded again in medium containing charcaol-stripped serum complemented with either hormone or the respective solvent.

Luciferase and β-galactosidase assays were described elsewhere [74]. After correction of the data by galactosidase activities, the stimulation in the absence of cotransfected *FKBP5* was set to 100 %.

4.2 Results

There was no association of response phenotypes with age or sex. Patients with a longer history of disease and more PDEs were more often non- responders after 5 weeks (p = 0.01 for both). The two variables DD and PDE were correlated: Spearman's correlation coefficient was equal to 0.55. Patients who did not respond to therapy had an average of 2.80 ± 4.06 PDE and had their first depressive episode on average 13.62 ± 12.67 years ago. Responders in contrast had 1.80 ± 2.40 depressive episodes, with the first episode 9.80 ± 12.26 years ago. Depression severity, remission after five weeks and early response were not dependent on previous disease history.

4.2.1 LD testing

After calculation of pairwise r^2 between genotyped SNPs we grouped them into LD bins. In *FKBP5* we found 7 bins and 5 SNPs (rs4711420, rs3807050, rs3800373, rs992105, rs2395634) that could not be fit in any bin and were assigned to 'one-SNP-bins'. The bin structure is presented in table 4.2. Eight out of 11 SNPs in *NR3C1* were assigned to two bins. The first bin included rs258813, rs6188, rs2918416 and rs1866388. Four SNPs, rs33388, rs33383, rs4582314 and rs4634384, were grouped into a second bin, and three SNPs, rs2963155, rs2963156 and rs6190, did not fit in any bin. Merely two SNPs (rs5522 and rs1490464) in the *NR3C2* gene had $r^2 > 0.75$, all remaining polymorphisms were not in LD according to our definition.

4.2.2 Sequencing *FKBP5* mRNA

Only one novel SNP was found in the re-sequenced regions of the *FKBP5* gene. This C→T polymorphism located in exon 9 (1095 C→T) and the T-allele occured with a frequency of 2.7 % and 2.5 % in controls and cases, respectively. 1095 C→T showed no association with any of the investigated phenotypes ($p > 0.05$).

4.2.3 Associations of polymorphisms in *FKBP5* with depression severity and response to antidepressant therapy

Association analysis of SNPs in *FKBP5* with response phenotypes yielded 40 % of nominal p-values less than 0.05 and 18.3 % less than 0.01. On the basis of the response data distribution we reconstructed genetic models for different bins (figure 4.1) and recalculated test statistics. For bins 1 and 3 - 7 the data were suggestive of a recessive model for improved response. The MAF for SNPs in bin 2 varied between 8 and 11 % in cases and controls, consequently only few patients were homozygous for the minor allele. For this reason we combined rare homozygotes with heterozygotes in this bin.

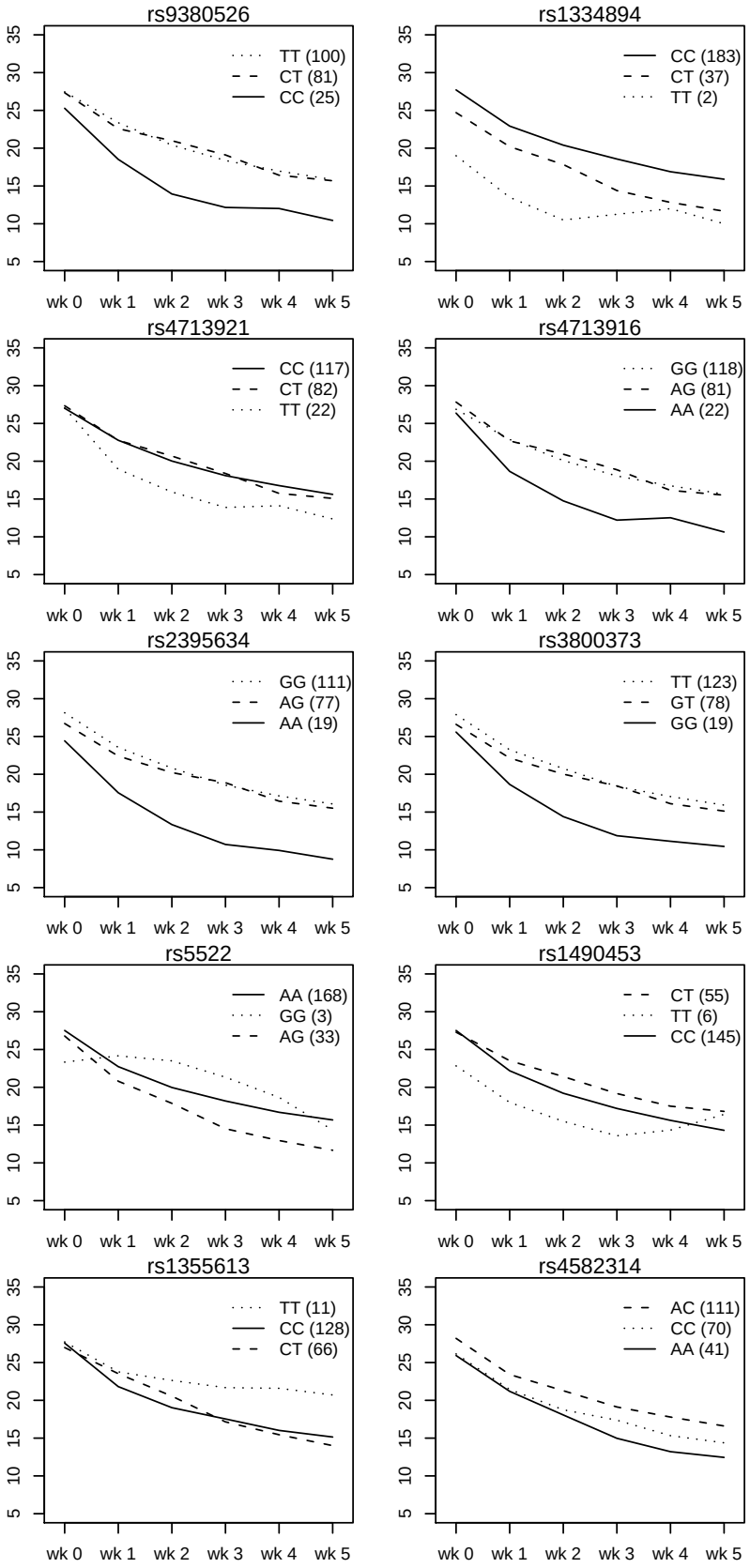

Figure 4.1: Distribution of HAM-D-scores during 5 weeks of treatment by different genotypes of SNPs from investigated genes

The most significant association with early response after 2 weeks was determined with polymorphism rs9380526 (nominal $p = 4.24 \times 10^{-5}$) (table 4.3). SNP rs4713916 had the most significant association with response after 5 weeks of treatment (nominal $p = 4.31 \times 10^{-4}$) and rs2395634 with remission after 5 weeks (nominal $p = 0.00133$). All 3 SNPs belonged to different bins, but r^2 between them was greater than 0.60 (table 4.4). The comprehensively high LD between these three SNPs makes it possible that their associations represent an effect of the same causative variant.

SNP rs4713916 has been described previously as being associated with early response after 2 weeks of treatment [19]. In the enlarged sample this association was still strong ($p = 1.55 \times 10^{-4}$). The same was true for two other SNPs from the previous study (rs3800373, $p = 1.08 \times 10^{-3}$ and rs1360780 $p = 3.08 \times 10^{-4}$, for details see table 4.3).

Bin 1 (table 4.2, figure 4.2 and 4.3) was associated with both depression severity and speed of response (table 4.5) and yielded combined p-value over all 3 quantitative response variables and depression severity equal to 4.52×10^{-5} for tagging-SNP rs9380526.

Table 4.3: Association of FKBP5 and NR3C2 SNP genotypes with response to antidepressants after 2 and 5 weeks of treatment, as well as with remission after 5 weeks of treatment.

SNP	Week of treatment	phenotype / genotype cross-tables			p	OR [95 % CI]
rs9380526	2	resp: 29 (CC)	*vs.*	non-resp: 3 (CC)	4.24×10^{-5}	7.60 [2.26; 40.00]
		resp: 56 (CT) + 73 (TT)		non-resp: 49 (CT) + 53 (TT)		
rs4713916	2	resp: 25 (AA)	*vs.*	non-resp: 3 (AA)	1.55×10^{-4}	6.45 [1.89; 34.18]
		resp: 62 (AG) + 82 (GG)		non-resp: 47 (AG) + 65 (GG)		
	5	resp: 16 (AA)	*vs.*	non-resp: 5 (AA)	4.31×10^{-4}	4.69 [1.57; 17.02]
		resp: 38 (AG) + 46 (GG)		non-resp: 47 (AG) + 77 (GG)		
rs2395634	5	rem:10 (AA)	*vs.*	non-rem: 8 (AA)	1.33×10^{-3}	5.14 [1.70; 16.04]
		rem:18 (AG) + 22(GG)		non-rem: 70 (AG) + 96 (GG)		
rs3800373	2	resp: 22 (GG)	*vs.*	non-resp: 3 (GG)	1.08×10^{-3}	5.60 [1.62; 29.96]
		resp: 57 (GT) + 89 (TT)		non-resp: 45 (GT) + 67(TT)		
rs1360780	2	resp: 87 (CC) + 58 (CT)	*vs.*	non-resp: 64 (CC) + 50 (CT)	3.08×10^{-4}	6.26 [1.83; 33.29]
		resp: 24 (TT)		non-resp: 3 (TT)		
rs1334894	5	resp: 75 (CC)	*vs.*	non-resp: 115 (CC)	7.54×10^{-3}	2.54 [1.20; 5.56]
		resp: 24 (CT) + 1 (TT)		non-resp: 15 (CT)		
	5	rem: 35 (CC)	*vs.*	non-rem: 161 (CC)	2.35×10^{-3}	3.11 [1.42; 6.75]
		rem: 17 (CT)		non-rem: 25 (CT) + 1 (TT)		
rs1490453	2	resp: 122 (CC)	*vs.*	non-resp: 61 (CC)	0.01	2.04 [1.16; 3.59]
		resp: 36 (CT) + 5 (TT)		non-resp: 39 (CT) + 3 (TT)		

'resp' – responders, 'rem' – remitters, 'non-resp' – non-responders, 'non-rem' – non-remmiters

OR [95 % CI] – Odds Ration with 95 %Confidence Interwal

Table 4.4: Pairwise r^2 (above the diagonal) and D' (below the diagonal) measures for the SNPs within the *FKBP5* gene.

SNPs	**rs3800373**	**rs1360780**	**rs4713916**	**rs9380526**	**rs1334894**	**rs2395634**	**rs943297**
rs3800373	-	0.87	0.52	0.74	0.25	0.88	0.51
rs1360780	0.98	-	0.64	0.85	0.23	0.96	0.63
rs4713916	0.74	0.82	-	0.76	0.23	0.62	0.97
rs9380526	0.96	0.98	0.95	-	0.20	0.85	0.79
rs1334894	0.98	0.98	0.97	0.97	-	0.21	0.23
rs2395634	0.99	0.98	0.80	0.98	0.94	-	0.62
rs943297	0.74	0.80	1.00	0.96	0.97	0.80	-

Table 4.5: Results of association tests ($-\log p$) between SNPs in *FKBP5* and response phenotypes

SNP	*OPC (0)	* OPC (1)	*OPC (2)	*OPC (3)	Early resp.	Resp. 5^{th} week	Rem. 5^{th} week	FPM
rs1883637	2.35	0.60	0.04	0.13	0.67	1.84	1.96	1.14
rs4711420	1.69	0.54	1.24	0.11	0.77	0.75	0.56	1.44
rs12526404	2.67	0.48	0.03	0.02	0.70	1.46	2.00	1.19
rs6909470	1.51	0.74	0.92	0.31	1.06	1.41	1.16	1.37
rs3807050	2.00	0.08	0.09	0.24	0.55	0.86	0.92	0.71
rs1807142	2.19	0.50	0.11	0.13	0.75	1.65	2.02	1.02
rs3800374	1.49	0.76	0.97	0.32	1.32	1.41	1.22	1.42
rs10807151	1.68	0.23	0.45	0.29	2.40	1.05	1.50	0.84
rs3800373	2.58	1.07	2.00	0.17	2.97	2.79	2.27	3.11
1095 C>T	0.28	0.99	0.36	0.71	1.45	0.68	0.01	0.66
rs755658	2.07	0.73	0.13	0.11	0.93	1.94	2.07	1.08
rs2294807	2.53	0.51	0.13	0.03	1.05	1.84	2.40	1.19
rs992105	1.81	0.24	0.50	0.30	2.50	1.11	1.60	0.96
rs10498734	2.32	0.56	0.10	0.03	1.04	1.83	2.26	1.07
rs7753746	1.47	0.55	0.59	0.16	1.90	1.58	1.34	0.92
rs2395634	4.09	1.27	1.54	0.24	2.81	2.71	2.88	4.19
rs4713899	1.51	0.56	0.61	0.15	1.68	1.61	1.39	0.96
rs7748266	1.47	0.55	0.59	0.16	1.89	1.58	1.34	0.91
rs1591365	4.04	1.38	2.39	0.68	3.48	3.30	2.81	5.30
rs1360780	4.12	1.38	2.39	0.68	3.51	3.36	2.87	5.40
rs6902124	4.26	1.17	1.64	0.29	2.88	2.70	2.83	4.37
rs2143404	1.52	0.60	0.62	0.15	1.73	1.65	1.45	0.99

continued on next page

*OPC - orthogonal polynomial coefficient of order (i). See chapter 4.1.2

'Resp.' – response to antidepressant treatment, 'Rem.' – remission

SNP	*OPC (0)	* OPC (1)	*OPC (2)	*OPC (3)	Early resp.	Resp. 5^{th} week	Rem. 5^{th} week	FPM
rs12527329	2.95	0.70	0.17	0.00	1.28	2.15	2.23	1.62
rs1334894	2.66	0.61	0.07	0.51	0.61	2.12	2.63	1.64
rs9394309	2.59	1.43	1.47	0.56	3.97	2.64	2.42	3.29
rs9380525	3.14	1.47	1.64	0.34	3.99	2.46	2.63	3.74
rs9380526	3.22	0.88	2.27	0.96	4.37	2.30	2.09	4.35
rs943297	2.23	1.17	2.02	1.15	4.09	2.45	1.75	3.72
rs4713916	2.38	1.26	2.60	1.37	3.81	3.36	1.59	4.59
rs9462104	1.90	0.92	1.78	1.36	2.82	1.98	1.66	3.22
rs4713921	1.14	0.55	2.35	2.18	3.16	1.30	1.03	3.43
rs4711429	1.10	0.51	1.59	1.48	2.61	0.93	1.01	2.24
haplo-genotypes	3.27	1.91	1.72	0.27	3.12	3.56	3.68	4.21

*OPC - orthogonal polynomial coefficient of order (i). See chapter 4.1.2

'Resp.' – response to antidepressant treatment, 'Rem.' – remission

Bin 2 was only associated with response and remission after 5 weeks of treatment and showed no association at the earlier time point. We decided to use polymorphism rs1334894 as the tSNP for this bin because of the strongest evidence for association with investigated phenotypes. Results of the association test for this SNP are presented in table 4.3 . For SNPs from bin 2, carriers of the rare allele had lower depression severity but there was no association with antidepressant treatment effectiveness. On the other hand, bins 4 (tSNP rs4713921) and 7 (tSNP rs4713916) were associated with the speed of antidepressant response rather than with depression severity (figure 4.1 and figure 4.4).

Thus, the carriers of the rare allele responded faster than the homozygotes for the alternative allele (combined p-value for association tests with OPC 1^{st}, 2^{nd} and 3^{rd} order on rs4713921 $= 6.68 \times 10^{-4}$, and on rs4713916 $= 5.01 \times 10^{-4}$).

The significance of association of *FKBP5* gene with early response had already been shown previously by Binder et al. [19]. In our study we additionally performed tests with quantitative response variables, for which we corrected for multiple testing. The genewide combined p-value (PFPM-method, see section 1.9.3.1), corrected for multiple testing after 1 000 000 permutations, for association of *FKBP5* with quantitative response variables was equal to 1.02×10^{-3}; with response after 2 weeks 1.84×10^{-3}; with response after 5 weeks

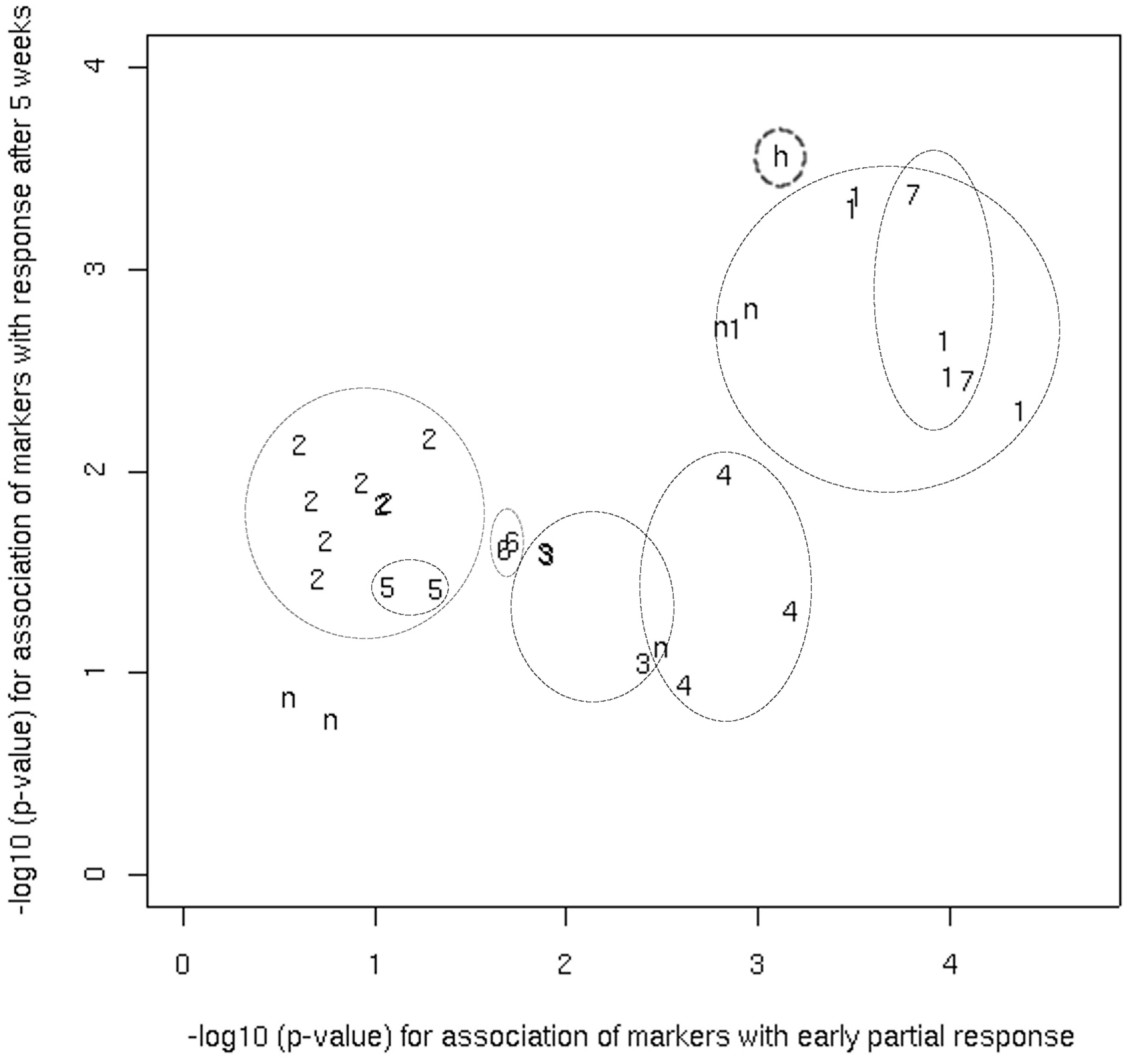

Figure 4.2: Coherence between LD bins of SNPs in *FKBP5* in association with early response and response after 5 weeks.

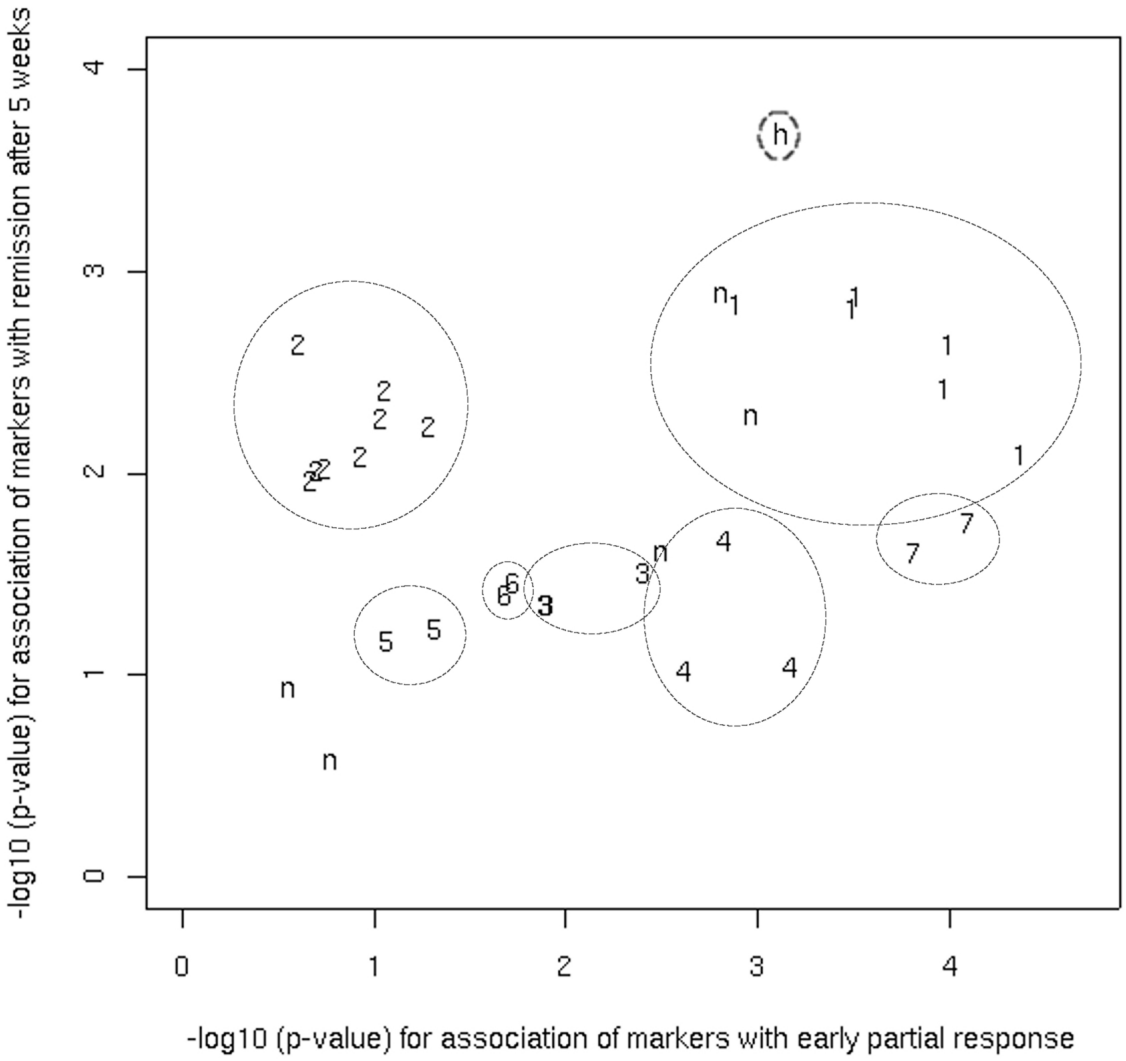

Figure 4.3: Coherence between LD bins of SNPs in *FKBP5* in association with early response and remission after 5 weeks.

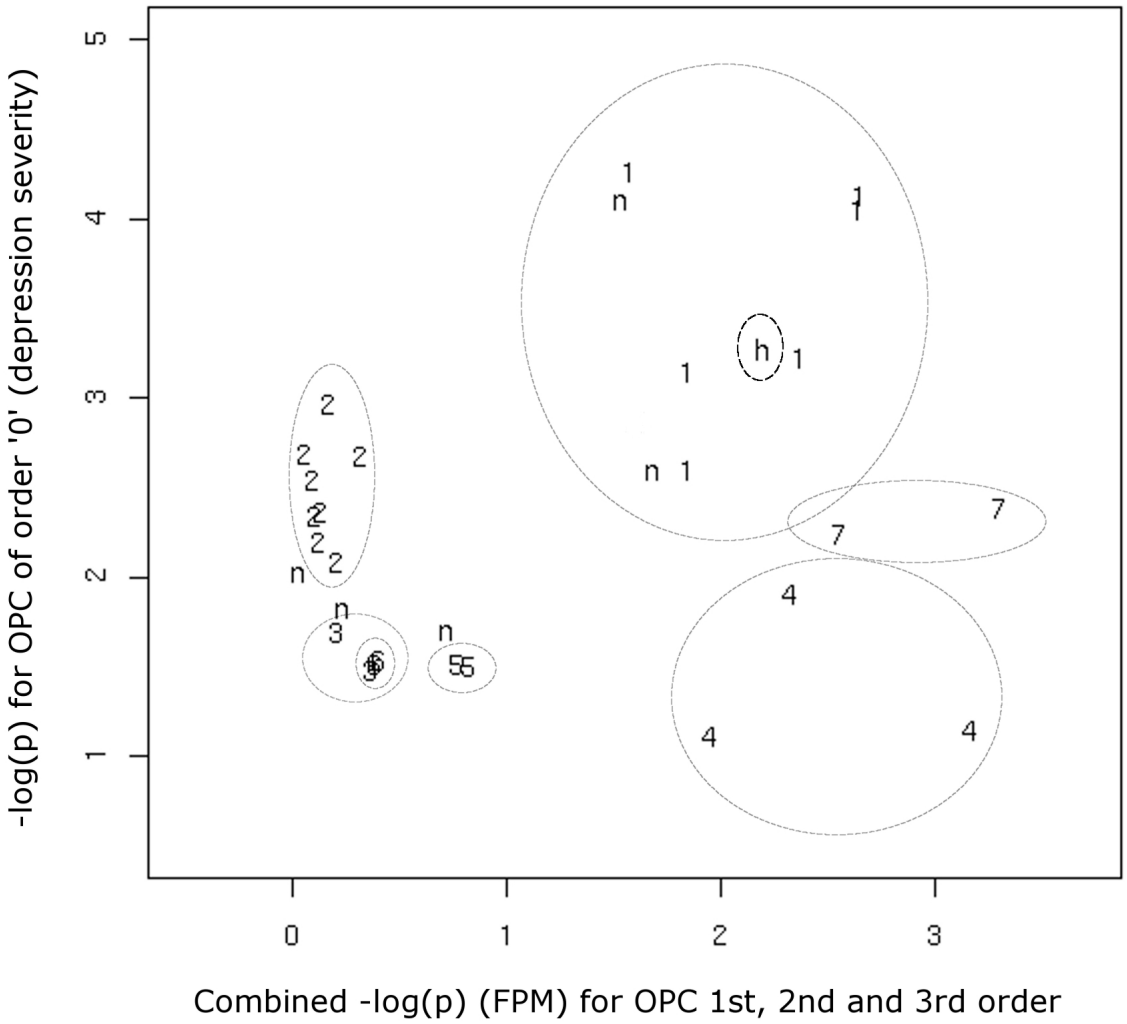

Figure 4.4: Association of SNP-bins within *FKBP5* with OPCs (quantitative variables of response).

5.23×10^{-4}; and with remission after 5 weeks 2.43×10^{-5}. In addition, when we performed Fisher's product method over all phenotypes, the genewide p-value was equal to 2.20×10^{-5}. Therefore the overall association of the gene *FKBP5* with response to antidepressant treatment was significant in our study.

4.2.4 Haplotype analysis in *FKBP5*

On the basis of the LD structure and p-values for association with response we picked out 6 polymorphisms from the *FKBP5* gene for the haplotype association analysis: rs9380526 (bin 1), rs1334894 (bin 2), rs4713921 (bin 4), rs4713916 (bin 7), rs3800373 (one-SNP-bin), rs2395634 (one-SNP-bin). The mean pairwise r^2 between these markers was equal to 0.45 with a maximum of 0.74 for the rs9380526 and rs3800373 pair and a minimum of 0.20 between rs9380526 and rs1334894. On the basis of the HAM-D score distribution we defined haplotypes TGCTGC, TGCCAT, TGCTGT, TACCAT as 'improved response' haplotypes and GACCAT, GATCAT, GACCGC, GACCAC as 'non-responder' haplotypes. The haplotype frequencies were 0.64, 0.03, 0.02, 0.02 and 0.12, 0.09, 0.04, 0.02, respectively. Haplotype-based association tests with response and remission after 5 weeks of treatment gave smaller p-values than the SNP-based tests, but not with early response (figure 4.1). This could be due to two or more independent causative mutations or polymorphisms in *FKBP5* for improved response to antidepressants at a later time point.

4.2.5 Best fitting interaction models for response and depression severity including the *FKBP5* gene

To verify the hypothesis of several causative SNPs in *FKBP5* we performed a logistic regression analysis of all possible 2-SNP interactions between 12 SNPs (rs9380526, rs4713916, rs4713921, rs3800373, rs2395634, rs1334894, rs4711420, rs3807050, rs992105, rs10807151, rs3800374, rs2143404) representing different LD bins. Different numbers of patients genotyped for all 12 SNPs were available for the investigated phenotypes (246, 208 and 199 for early response, remission after five weeks and response after five weeks, respectively).

The best model for association with early response included only one polymorphism, rs9380526 (AIC = 319.5). The estimated p-value was equal to 9.26×10^{-5}.

For response after 5 weeks two models showed fairly similar values of AIC. Both included rs1334894 as well as sex and DD. The first model with rs1334894 resulted in AIC = 254.04. Adding rs2395634 yielded AIC = 248.00. Hence the model of interaction of 2 SNPs (rs1334894, rs2395634) and DD fitted best (overall $p = 9.00 \times 10^{-7}$). The largest single effect in this model was the statistical interaction between rs1334894 and DD (table 4.6). Patients homozygous for CC on rs1334894 were more often non-responders after 5 weeks of treatment than T allele carriers. Non-responders with the CC genotype had more PDE and longer DD. In the responder group the relation was in the opposite direction, so that

non-responders with genotypes CT and TT had more PDE and longer DD. No statistical interaction between two SNPs for this phenotype have been shown (p = 0.30).

Explained variable AIC and p for the general model	Explanation term	p
Response after 2 weeks		
AIC = 319	rs9380526	9.26×10^{-5}
$p^* = 9.26 \times 10^{-5}$		
$p_{corr} = 0.007$		
Response after 5 weeks	rs1334894	0.059
AIC = 248	rs2395634	0.022
$p^* = 9.00 \times 10^{-7}$	DD	0.002
$p_{corr} = 7.11 \times 10^{-5}$	rs1334894 : DD	1.05×10^{-4}
Remission after 5 weeks	rs1334894	0.037
AIC = 202.1	DD	0.014
$p^* = 1.66 \times 10^{-4}$	rs1334894 : rs3800373	0.008
$p_{corr} = 0.013$	rs1334894 : DD	0.032
Depression severity		
AIC = 448.5	rs2395634	1.51×10^{-3}
$p^* = 3.02 \times 10^{-4}$	rs1334894 : PDE	0.017
$p_{corr} = 0.024$	rs2395634 : PDE	0.015

Table 4.6: Best fitting models explaining response phenotypes. 'p' are p-values for corresponding explanation terms from the logistic regression. p^* – nominal p for the general model,p_{corr} – corrected for multiple testing p^*. DD – duration of disease since the first episode, PDE – number of previous depressive episodes.

The statistical model for remission after 5 weeks as dependent variable and rs1334894, rs3800373 and DD as independent variables yielded a minimal AIC = 202.10 ($p = 1.66 \times 10^{-4}$). This was slightly better than the model including gender effect in addition (AIC = 205.20) and clearly better than the model with one SNP only (rs1334894, AIC = 212.9). The strongest effect in this model had an interaction term for two SNPs (table 4.6).

Two SNPs, rs2395634 and rs1334894 were included in the best model explaining the depression severity phenotype with AIC = 448.5 and global $p = 3.02\times^{-4}$. The effect of rs2395634, and the interaction between both SNPs and the number of PDEs were significant

(table 4.6).

These data suggest the presence of several independent polymorphisms in *FKBP5* that affect the ability to respond to antidepressant therapy. In our data response to antidepressant treatment was dependent on SNPs rs3800373, rs2395634 and rs9380526 (bin 1) as well SNPs from bins 4 and 7, that on the basis of LD structure (table 4.4) could potentially refer to one causative polymorphism. The SNPs from bin 2 (rs1334894) showed an additional independent effect on remission and response after five weeks and demonstrated statistical interaction with disease history.

4.2.6 SNP - associations in *NR3C1* and *NR3C2*

The permutation based over-all Fisher's product p-value for the *NR3C1* gene was equal to 0.027 and for the *NR3C2* gene equal to 0.003. If we considered single phenotypes, the association of *NR3C1* was significant only with severity of depression (genewide $p = 0.0034$), and was actually accomplished only due to association with bin 2 (table 4.7). All 4 SNPs in this bin (rs4582314, rs33388, rs33383 and rs4634384) survived the multiple testing correction with $p = 0.006, 0.007, 0.010$ and 0.032, respectively. Heterozygous carriers of these SNPs had higher average HAM-D scores at admission than homozygous carriers, but response curves paralleled (figure 4.1). Nevertheless, nominal p-values for association with remission after 5 weeks were below 0.05.

Table 4.7: Results of association tests ($-\log$ (p-values)) between SNPs in the *NR3C2* and *NR3C1* genes and investigated response phenotypes.

Gene	SNP	*OPC (0)	* OPC (1)	*OPC (2)	*OPC (3)	Early resp.	Resp. 5^{th} week	Rem. 5^{th} week	FPM
NR3C2	rs2871	0.02	1.62	0.09	1.32	0.00	0.00	0.00	1.04
NR3C2	rs1879827	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NR3C2	rs3843413	0.92	0.49	0.18	0.37	0.00	0.00	0.00	0.48
NR3C2	rs3846306	0.31	0.12	0.15	0.18	0.37	0.16	0.56	0.10
NR3C2	rs3752701	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NR3C2	rs1355613	1.44	1.47	0.24	0.25	0.00	0.00	0.00	1.19

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*OPC - orthogonal polynomial coefficient of order (i). See chapter 4.1.2

'Resp.' – response to antidepressant treatment, 'Rem.' – remission

Gene	SNP	*OPC (0)	* OPC (1)	*OPC (2)	*OPC (3)	Early resp.	Resp. 5^{th} week	Rem. 5^{th} week	FPM
NR3C2	rs2137331	0.64	0.10	1.37	0.19	0.90	0.08	0.33	0.55
NR3C2	rs907621	0.09	0.08	0.10	1.13	0.66	0.37	0.01	0.22
NR3C2	rs1490453	1.15	0.90	0.79	1.02	1.77	1.09	0.83	1.91
NR3C2	rs5522	1.53	1.33	0.01	0.19	0.00	0.00	0.00	1.05
NR3C2	rs1490464	1.34	1.40	0.18	0.03	0.00	0.00	0.00	1.00
NR3C1	rs258813	0.77	0.43	0.77	0.91	0.09	0.85	0.03	0.99
NR3C1	rs6188	0.78	0.40	0.78	0.88	0.08	0.80	0.03	0.96
NR3C1	rs33388	2.86	0.42	0.21	1.13	0.11	0.48	1.62	2.19
NR3C1	rs33383	2.74	0.41	0.30	0.62	0.16	0.53	1.54	1.79
NR3C1	rs2918416	0.96	0.24	0.81	0.82	0.11	0.98	0.15	0.95
NR3C1	rs2963155	0.70	0.05	0.13	0.33	0.29	0.65	0.30	0.16
NR3C1	rs2963156	0.86	0.29	1.23	0.49	1.19	0.21	0.17	0.98
NR3C1	rs1866388	0.85	0.28	0.94	0.78	0.03	1.01	0.13	0.97
NR3C1	rs6190	0.52	0.43	0.07	0.48	0.96	0.80	0.38	0.26
NR3C1	rs4582314	2.95	0.46	0.27	0.45	0.18	0.62	1.37	1.83
NR3C1	rs4634384	2.26	0.28	0.07	0.47	0.13	0.40	1.18	1.11

*OPC - orthogonal polynomial coefficient of order (i). See chapter 4.1.2

'Resp.' – response to antidepressant treatment, 'Rem.' – remission

The *NR3C2* gene in contrast was associated with both response and severity of depression. The genewide p-values for association with severity of depression, combined quantitative response variables, and early response were slightly less than 0.05. Dichotomous response and remission variables after 5 weeks were not significantly associated with variation in the *NR3C2* gene.

In *NR3C2*, individuals carrying the rare allele for rs5522 and rs1490464 (belonging to the only bin, 33 (AG) + 3 (GG) against 170 (AA) ; 41(AG) + 3 (AA) against 177 (GG), respectively), and the major allele for rs1355613 (129 (CC) + 68 (CT) against 11 (TT)) showed improved response to antidepressant treatment. Nominal p-values for association tests of these polymorphisms with depression severity were below 0.05. The number of PDEs, rs1355613, and interaction of rs5522 with the number of PDE and of rs1355613 with rs1490453 in a logistic regression were found to be independent explanatory terms ($p < 0.05$)

Explained variable AIC and p for the general model	Explanation term	p
Depression severity	PDE	0.018
AIC = 415	rs13355613	0.032
$p^* = 1.76 \times 10^{-4}$	rs5522 : PDE	0.021
$p_{corr} = 0.001$	rs1355613 : rs1490453	0.039

Table 4.8: Interaction within *NR3C2*. 'p' are p-values for corresponding explanation terms from the logistic regression. p^* - nominal p-value for general model, p_{corr} - corrected for multiple testing p^*. PDE – number of previous depressive episodes.

(table 4.8). The AIC for the global model was equal to 415 (overall $p = 1.76 \times 10^{-4}$), which was significantly lower than AIC = 421.7, 421.4, and 426.3 for the general models including one SNP at a time (rs5522, rs1355613 and rs1490453, respectively), likelihood ratio tests yielded p-values of 0.0010, 0.012 and 0.0013 for comparison of the 3-SNP model with each 1-SNP model, respectively.

Patients in the early responder group more often had the genotype CC for rs1490453 than patients who did not respond so rapidly (p = 0.01, for details see table 4.3). Even though several SNPs in *NR3C2* yielded nominal p-values under 0.05 in association tests (table 4.7) none survived correction for multiple testing. The best result was obtained for rs1490453 with a corrected p over all investigated phenotypes equal to 0.052.

We performed haplotype testing for *NR3C1* and *NR3C2*, but estimated haplotypes did not show better association with response phenotypes than single SNPs.

4.2.7 Interactions between *FKBP5*, *NR3C1* and *NR3C2*

As mentioned above all three investigated genes were associated with response to antidepressant treatment and depression severity. We asked whether their effects are multiplicative. First we tested interaction between two genes, *FKBP5* and *NR3C2*. On the basis of the LD structure and associations of SNPs in the investigated genes we included 7 SNPs in statistical interaction testing: four SNPs from *FKBP5* (rs9380526, rs3800373, rs2395634, rs1334894) and three SNPs from *NR3C2* (rs1490453, rs5522, rs1355613) despite only marginal significance of the latter three. We tested one-SNP models as well as all 2-, 3-, 4- and 5-SNPs combinations and model with covariates only. Overall 120 general models were tested.

Only two models explaining response after 5 weeks and depression severity survived the correction for multiple testing. The model explaining response after 5 weeks included 4 SNPs (rs5522 and 1490453 from *NR3C2*, rs1334894 and rs2395634 from *FKBP5*) with AIC = 204.6 and $p = 3.74 \times 10^{-4}$ (table 4.9). Three explanation terms had $p < 0.05$. One of them

demonstrated the interaction between *FKBP5* and *NR3C2* (rs1334894 with rs1490453).

Test for interactions on the depression severity phenotype resulted in AIC = 403.7 and nominal $p = 8.9 \times 10^{-5}$ for the global test (table 4.9). This model includes 5 SNPs (rs1490453, rs5522, rs1355613 from *NR3C2* and rs2395634, rs1334894 from *FKBP5*). The effects of two genes were multiplicative, the explanation term 'rs2395634 : rs5522' gave $p < 0.05$ (table 4.9).

Table 4.9: Interaction between three investigated genes. 'p' are p-values for corresponding explanation terms from logistic regression. DD – duration of disease since first episode, PDE – number of previous depressive episodes.

$^{*}FKBP5$ + $^{\ddagger}NR3C2$		
Explained variable AIC and p for the general model	Explanation term	p
Response after 5 weeks AIC = 204.6 $p^* = 3.74 \times 10^{-4}$ $p_{corr} = 0.045$	PDE	0.018
	DD	0.058
	‡rs5522	0.425
	‡rs5522 : PDE	0.145
	*rs1334894 : *rs2395634	0.025
	*rs1334894 : ‡rs1490453	0.026
Depression severity AIC = 403.7 $p^* = 8.9 \times 10^{-5}$ $p_{corr} = 0.011$	PDE	0.012
	‡rs1355613	0.023
	*rs2395634	0.004
	‡rs1355613 : PDE	0.166
	*rs1334894 : PDE	0.045
	‡rs1355613 : ‡rs1490453	0.004
	*rs2395634 : ‡rs5522	0.032
$^{*}FKBP5$ + $^{\dagger}NR3C1$		
Response after 5 weeks AIC = 194.6	DD	0.004
	†rs33388	0.737
	*rs9380526	0.011

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Explained variable AIC and p for the general model	Explanation term	p
$p^* = 9.31 \times 10^{-6}$	*rs1334894	0.275
$p_{corr} = 2.88 \times 10^{-4}$	*rs9380526 : PDE	0.124
	*rs1334894 : PDE	0.002
	*rs1334894 : †rs33388	0.006
	PDE	0.030
Depression severity	DD	0.022
AIC = 410.8	*rs9380526	0.005
$p^* = 2.24 \times 10^{-4}$	*rs1334894	0.275
$p_{corr} = 0.007$	†rs33388	0.038
	*rs9380526 : PDE	0.200
	*rs1334894 : PDE	0.005
	*rs1334894 : PDE	0.143
	*rs1334894 : †rs33388	0.073
**FKBP5* + *†NR3C1* + *‡NR3C2*		
	DD	0.015
Response after 5 weeks	*rs1334894	0.039
AIC = 181.8	*rs2395634	0.041
$p^* = 6.99 \times 10^{-6}$	‡rs5522 : PDE	0.039
$p_{corr} = 0.001$	*rs2395634 : PDE	0.070
	*rs1334894 : †rs33388	0.050
	*rs1334894 : PDE	0.001
	PDE	4.49×10^{-3}
Depression severity	DD	0.055
AIC = 375.1	‡rs5522	0.048
$p^* = 1.19 \times 10^{-4}$	‡rs1355613	6.67×10^{-3}
$p_{corr} = 0.019$	†rs33388	0.057
	*rs9380526	0.025

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Explained variable AIC and p for the general model	Explanation term	p
	‡rs1355613 : sex	0.941
	‡rs5522 : PDE	0.282
	*rs9380526 : PDE	0.036
	‡rs13355613 : PDE	0.001
	‡rs5522 : †rs33388	0.056
	*rs9380526 : †rs33388	0.171

In the next step we repeated the analysis for interactions of SNPs in FKPBP5 and *NR3C2*. SNP rs33388 from bin 2 in *NR3C1* was chosen as tSNP because of its lowest p-value over all investigated phenotypes (table 4.7). Response after 5 weeks was best explained by 3 SNPs (rs1334894, rs9380526, rs33388), DD and interactions of the number of PDE with rs1334894 and rs9380526 with a nominal $p = 9.31 \times 10^{-6}$ and AIC = 194.6 (4.9). The interaction between rs1334894 and rs33388 was also significant. The depression severity phenotype was best explaned by the same model with PDE in addition ($p = 2.24 \times 10^{-4}$, AIC = 410.8).

At last, we tested interactions between all 3 genes with the new number of 8 SNPs (rs9380526, rs3800373, rs2395634, rs1334894, rs1490453, rs5522, rs1355613 and rs33388), including combinations of up to 4 SNPs, in the sum 163 general models.

The best model of statistical interaction between SNPs for response after 5 weeks yielded a global $p = 6.99 \times 10^{-6}$ (AIC = 181.8). The model included four SNPs from three investigated genes (rs5522 from *NR3C2*, rs33388 from *NR3C1*, rs1334894 and rs2395834 from *FKBP5*). Two SNPs from *FKBP5* (rs2395834, rs1334894) and DD showed significant main effects (table 4.9). Both interaction terms of the number of PDEs with rs5522 and with rs1334894 had significant effect as well as of rs1334894 with rs33388. The part of explained variance by this model was about 12.6 %, that was greater than the part of the variance explained by covariates only (5.1 %).

Four SNPs from the investigated genes (rs5522, rs1355613, rs33388 and rs9380526) were included in the best explanatory model for depression severity (global $p = 1.19 \times 10^{-4}$, AIC = 375.1). Main effects of the number of PDEs, DD, rs5522, rs9380526, 1355613 as well as two interaction exploratory terms (rs9380526 and rs1355613 with the number of PDEs) were significant ($p < 0.05$, table 4.9). Explanatory terms of DD, interactions of rs33388 with both rs9380526 and rs5522, and interactions of the number of PDEs with rs5522 yielded p-values greater than 0.05, but were included in the best interaction model because of the minimal

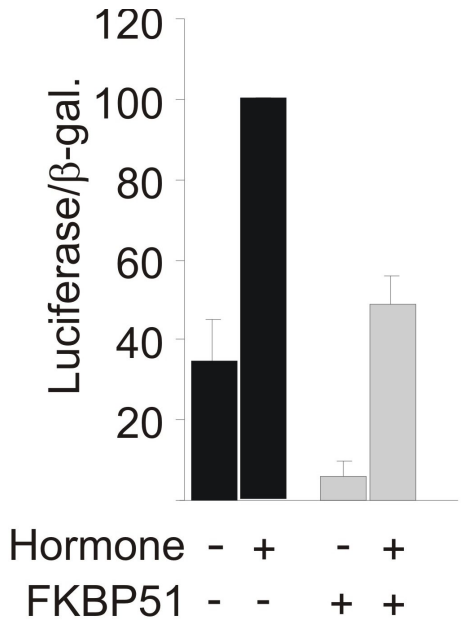

Figure 4.5: *FKBP5* influences the transcriptional activity of the *MR*. Neuroblastoma cells were transfected with plasmids coding for *MR*, *FKBP5*, the reporter gene firefly luciferase controlled by the *MR*-responsive MMTV-promoter, and the control enzyme β-galactosidase. Hormone exposure was 3 nM corticosterone for 16 h. The hormone-stimulated reporter activity in the absence of *FKBP5* was arbitrarily set to 100.

AIC. The part of explained variance by this model was about 22.9 %, that was greater than the part of the variance explained by covariates only (2.1 %).

The global p-values for the best interaction models explaining the remaining phenotypic variables did not survive correction for multiple testing, corrected $p > 0.05$.

4.2.8 Experimental test of the interaction between *FKBP5* and *MR*

Our statistical evaluations suggest a functional interaction between *FKBP5* and *MR*. Taking in addition the homology of *MR* and *GR* into consideration we were prompted to test for a potential effect of *FKBP5* on the transcriptional activity of *MR*. To this end, we employed a reporter gene assay using the *MR*-responsive MMTV promoter that drives firefly luciferase as reporter. There was a clear inhibition of the transcriptional activity of *MR* by cotransfected *FKBP5* (figure 4.5).

The inhibition of *MR* was already observed in the absence of hormone. It is not known whether the pronounced activity of *MR* already in the absence of added hormone was due to an intrinsic hormone-independent mode of action of *MR*, or due to unknown cryptic ligands in the medium. Whatever the explanation for this *MR* activity is, there is a strong functional interaction between *MR* and *FKBP5*, both in the absence and presence of added hormone, in this experimental set-up.

4.3 Discussion

Our original report of the association of the *FKBP5* gene with response to antidepressant treatment by Binder et al [19] raised the question of the function of the associated polymorphisms at the molecular level. For this reason, we resequenced the *FKBP5* mRNA. Only one novel exonic SNP was detected, but there were no associations with any of the investigated phenotypes. Resequencing intronic regions including transcription factor binding sites did not reveal novel SNPs. Nevertheless, SNPs investigated in previous studies were assigned to one large D' LD-block. In our study, we tried to localize the causative changes by detailed examination of the LD structure and by expanding the marker density. We also enlarged the sample size by 53 patients to raise the power of the association tests.

In our data, the *FKBP5* gene was significantly associated with all investigated response variables. LD bins showed independent statistical effects at different time points. The strongest association with response to antidepressant treatment was observed with SNPs within bin 1 (tSNP rs9380526). Bins 4 and 7 also showed high associations with early response, but their effects were not independent. Results of stepwise logistic regression suggested that markers of bin 1 actually showed the strongest main effect on early response to antidepressant treatment. Thus the association described in the paper of Binder et al [19] refers to the effect of one causative change in the *FKBP5* gene. This finding is in agreement with the LD structure within the gene. Pairwise r^2 between rs9380526 and SNPs described previously (rs3800373, rs1360780, rs4713916) was greater than 0.74 and D' was greater than 0.95.

At the same time, response and remission after five weeks probably are controlled by 2 independent changes in the *FKBP5* gene. Co-occurrence of these changes within one haplotype showed higher association with response and remission after five weeks. This could mean that the phase in which variants within the *FKBP5* gene might be important for any regulation processes of *FKBP5* expression.

One cluster of SNPs (bin 2) showed association only with response at the later time point and no association with early response. The genotypic distributions of SNPs in this bin were also correlated with DD, which itself is also correlated with the speed of response. Patients with longer disease histories tended to have more depressive episodes and, consequently more often received drug treatment. This repeated and extended action of antidepressants could have led to re-programming of the genes involved in response via epigenetic mechanisms. Intriguingly, rs1334894 localized in intron 1 of the *FKBP5* gene is part of a CpG dinucleotide in the C allele situation. It has been reported that the antidepressant imipramine has the ability to influence epigenetic chromatin modifications in the hippocampus in a mouse model of depression and antidepressant action [179]. Moreover, the actions of the GCs also entail epigenetic changes. For example, GCs have been shown to induce stable DNA demethylation within the rat liver-specific tyrosine aminotransferase gene [105, 176]. Alternatively, a simul-

taneous association of genetic markers with DD and with response could indicate a subtype of the MDD, characterised by a more unfavorable course of depression. This hypothesis is corroborated by data of Cook and colleagues [34], who showed that better clinical outcomes are achieved in those depressed subjects with less severe depression.

All associated SNPs in *FKBP5* are located in introns or in UTRs. SNP rs3800373 is located in the 3'UTR and is in LD with rs1360780, rs9380526 and rs2395634 (table 4.4). Polymorphisms in UTRs could potentially change the 3D structure of the mRNA. Alternatively, a change of G to A in rs943297 predicts the loss of a GRE and could thus lead to *GR*-resistance. rs943297 is located near the promoter in intron 1 of *FKBP5* and is in LD with rs9380526 and rs4713916.

The two other investigated genes that are involved in the regulation of the HPA axis also showed significant associations with the examined phenotypes. The *NR3C1* gene was nominally associated with remission after 5 weeks. After correction for multiple testing *NR3C1* in our data was associated only with severity of depression. Heterozygous carriers of four SNPs that are in strong LD had on average higher HAM-D scores during 5 weeks of antidepressive treatment. There was no difference in response speed. SNPs within bin 2 in *NR3C1* are located in intron 1 and 2 and their function is not known. We compared the LD structure in our data with the CEU sample available in the HapMap project[7] and found the structure in our sample to be virtually identical with the one in the CEU sample. No SNP in the bin in the CEU sample had a known function. Exonic SNPs in *NR3C1* (rs6192, rs6193, rs33391 and rs1800445) in our sample were not polymorphic, so we could not assign the association to changes in protein structure. SNP rs6190, described earlier as ER22/23EK [183, 184, 150] to be associated with response was not associated with the investigated response phenotypes, that have a slightly different definition as in van Rossum et al. [182]. Obviously, the ER22/23EK variant has a very small effect on response to antidepressant treatment if at all.

The *NR3C2* gene in contrast was associated with both response and severity of depression. Three SNPs (rs5522, rs1355613 and rs1490453) showed nominal significant associations with depression severity and with the linear trend in HAM-D scores during 5 weeks of treatment. One SNP (rs1490453) was associated with early response. All three SNPs were not in LD and had statistically independent effects on the response phenotypes. None of them survived the correction for multiple testing. Nevertheless, the interaction between rs1490453 and rs1355613 in the logistic regression was significant after correction. Both SNPs are located in intron 2 of the *NR3C2* gene and are not in LD with any known coding SNPs in the CEU sample. SNP rs5522 is a coding polymorphism in exon 2 and results in a I180V change in the *MR* protein. Carriers of the V180 allele in our sample showed faster response to antidepressants. Interestingly, the same variant was shown recently to be associated with psychosocial stress responsiveness. Carriers of the V180 allele were shown to have higher

[7] http://www.hapmap.org

saliva and plasma cortisol levels, as well as a higher heart rate response to the Trier Social Stress Test (TSST) than non-carriers (I180). *In vitro* testing of the V180 allele revealed a mild loss of function using cortisol as a ligand. Higher doses of cortisol were needed for cortisol dependent promoter induction by *MR*, but protein expression did not differ between the two *MR* variants [47]. Apparently, V180 allele carriers may have a more reactive HPA system that probably leads to an improved response to antidepressant treatment.

In our study we showed that all three investigated genes are associated with different aspects of response to antidepressant treatment. The *FKBP5* gene had the strongest association. All three investigated genes seem to have more than one causative polymorphism modifying response to antidepressant treatment. *FKBP5*, *NR3C1* and *NR3C2* probably have not only additive independent effects on the response phenotype, but interact epistaticly. Unfortunately, there is not a precise correspondence between biological and statistical models of interaction [35]. In addition, the power of interaction testing is limited. Breslow and Day showed that the sample size required to detect interaction is always at least 4 times what is needed to detect a main effect of the same size [21]. In our study we did not have sufficient power according to this estimation. Despite the insufficient power in our sample we could show significant statistical interaction between SNPs in 3 genes and disease history. Unfortunately, testing of interactions of an order higher than 2 were not possible because of the increasing number of tests and consequently the increasing multiple testing problem.

Given all these considerations it is important to note that a biological interaction of the *FKBP5* and *GR* proteins has been alredy shown experimentally. This lends support to the relevance of interactions found at the statistical level. Increased levels of *FKBP5* have been reported to be the common cause of *GR* resistance in New World primates. Overexpression of *FKBP5* have been shown to reduce the binding affinity of *GR*, and therefore to decrease the transcriptional activity of *GR* after hormone exposure [46, 199]. GC activated formations of *GR-GR* and *MR-MR* homodimers or *GR-MR* heterodimers can translocate to the nucleus and there bind to GREs and thus govern expression of steroid responsive genes [128, 144], one of them being *FKBP5* [86, 180]. Thus the biological interaction of *GR* and *FKBP5* represent an ultra-short negative feedback loop.

Our statistical data suggested a connection between *FKBP5* and *MR* as well. Indeed, we were able to demonstrate for the first time that *FKBP5* also interferes with the transcriptional activity of *MR*. Thus, the interactions found at the statistical level apparently have an equivalent at the biological level. This result further validates our statistical approach and the biological implications of the statistical interactions, even though the p-values for interaction terms were close to 0.05.

In summary, we provide evidence for statistically independent effects of variants in the *FKBP5, GR* and *MR* genes which are pivotal for HPA axis function. Moreover, we showed statistical interactions between them that might be responsible for changes in the HPA axis relevant for the therapeutic action of antidepressant treatment. Our finding may be

useful for the prediction of response to antidepressant treatment in depressive patients on the basis of the interplay of genetic and non-genetic determinants, in our case gene variants and disease history.

Chapter 5

Summary

In the current study I performed association testing of candidate genes with major depressive disorder (MDD) and response to antidepressant treatment. Unlike Mendelian traits, which are controlled by genes of large effects and show simple patterns of inheritance in families, the transmission of such complex phenotypes is governed by multiple factors. Probably, complex traits are controlled by more than one genes that are in turn interact with environmental factors.

Dozens of SNPs were tested in the current study for associations. For this reason minimizing of false positives was necessary. Sources of false positive associations can be divided into three main categories: statistical fluctuations that arise by chance and result in low p-values, underlying systematic biases due to study design, and technical artifacts.

The most widely discussed source of systematic bias is population stratification due to ethnic admixture. This can lead to the over-representation of one or more subgroups among the cases or control group. If a genetic marker has different frequencies in different subgroups, false positive associations can ensue. To avoid this problem an additional set of unlinked markers was genotyped and tested for admixture in the investigated data. No significant evidence for stratification was detected, consequently, no correction for population stratification was necessary.

To avoid false positive associations occurring due to genotyping errors I performed quality control including HWE and genotyping call rate checking. All conspicuous SNPs were re-genotyped or validated due to direct sequencing.

Different methods for accounting efficiently for multiple testing of many SNPs in an association study are available. One of them proposed by Nyholt [126] is very simple, but its performance was not extensively evaluated. Based on empirical results I evaluated this method for a simple model of haplotype block structure. Theoretical considerations show further that the method can be very conservative in the presence of LD blocks. In summary, although Nyholt's approach may be useful as an exploratory tool, it is not an adequate substitute for permutation tests. The permutation procedure is known to be computational intensive, and for this reason not applicable to large data sets, but fortunately, hardware and

software development allow current to permute even whole genome data sets[1, 2]. For this reason the permutation procedure still remains the best approach for correction for multiple testing and I used this approach in my calculations further on.

Candidate genes from two different systems were investigated. The first one is the *P2RX7* gene located within a region on chromosome 12q24.31 that has been identified as a susceptibility locus for affective disorders by linkage and association studies. *P2RX7* is a purinergic ATP-binding calcium channel expressed in neurons as well as in microglial cells in various brain regions. A non-synonymous coding SNP in the *P2RX7* gene (rs2230912), previously found to be associated with bipolar disorder, was significantly associated ($p = 0.0019$) with MDD in current study. This polymorphism results in an amino acid exchange in the C-terminal cytosolic domain of the *P2RX7* channel protein, suggesting that the observed *P2RX7* polymorphism might play a causal role in the development of depression. These findings could reflect the clinical observation of MDD and bipolar disorder being two different disorders that share common causal factors.

A heterozygote disadvantage model was the most suitable model of inheritance, possibly being a reflection of *P2RX7* protein having an oligomeric structure in the plasma membrane based on complex of identical subunits [124, 178]. This could explain the nominally significant deviation from HWE for the associated SNP rs2230912. The plasma membrane location renders *P2RX7* a potential drug target for a possible pharmacological drug discovery.

The second group of genes investigated in current study: *NR3C1* (coding GR), its close homolog *NR3C2* (coding MR), and its regulatory protein FK506 binding protein 5 (*FKBP5*), are key regulators of the stress hormone axis and, therefore, candidate genes for depression and response to antidepressant treatment.

The association of *FKBP5* and *NR3C1* with response to antidepressant treatment were shown previously, but the information about function of associated SNP and its interactions was insufficient. In present study I performed a fine mapping and re-sequencing of potential functional areas of the *FKBP5* gene. Only one new exonic SNP was found (1095 C>T) and it showed no association with depression or response to antidepressant treatment. The combination of association analysis and detailed investigation of LD structure provided evidence for two causal polymorphisms in *FKBP5* possibly controlling investigated phenotypes, however the exact location of them could not be detected. The first group of associated SNPs is distributed within the *FKBP5* gene from promoter to 3' end. The second identified association refer to rs1334894 localised in intron 1. In summary, it is still not clear whether associated SNPs have any function, that leads to response modification or are just markers for hidden causal mutation.

The GR and *FKBP5* are linked intracellularly via an ultra-short feedback loop, i.e. hormone-activated *GR* increases the expression of *FKBP5*, while *FKBP5* interferes with the

[1] http://www.wg-permer.de/

[2] http://pngu.mgh.harvard.edu/purcell/plink/

transcriptional activity of *GR*. The close homology of *GR* and *MR* raises the possibility that *MR* may also be regulated by *FKBP5*, but no experimental support has been published so far. Polymorphisms in the *MR* and *GR* genes were found to be associated in current study with the clinical course of depressive episodes or with depression severity, but not with depression. Moreover, several variants within the *GR* gene had statistically independent effects. In addition, I investigated interactions of variants within this genes and its benefit for predictability of depression as well as response to antidepressant treatment. I asked whether *FKBP5*, *GR* and *MR* effects are multiplicative in statistical sense.

Gene-gene interactions are thought to have an important role in complex traits, but the analysis of how this interactions contribute to complex disease is challenging [77]. Cordell [35] identified several reasons why establishing the biological importance of interactions that have been identified statistically might be very complicated. Nevertheless, I could show for the first time the evidence for statistically independent effects of variation in the *FKBP5*, *GR* and *MR* genes which are pivotal for the HPA axis function. The proteins coded by these three genes were shown to interact biologically, but its connection to the response to antidepressant treatment were not investigated yet. Due to the performed investigation I could emphasise the link between biological and statistical interactions. The variance explained by interaction of variants in these genes in investigated sample was equal to 12.6 % for response to antidepressant treatment and 22.9 % for depression severity during hospitalisation period. The results of this study could be useful for the prediction of response to antidepressant treatment in patients.

In addition, I identified that patients with a longer history of disease and more previous depressive episodes were more often non-responders. These non-genetic determinants showed considerable interplay with genetic variants in the investigated genes.

Considering the fact that association studies of complex traits rely on the LD between the marker and unknown causal variants the diagnostic use of an associated marker or markers would be of concern in any group that have a different ethnic composition from the one in which the association were first reported. It is possible that in population that differ from investigated one tested markers could be not informative for response prediction. For this reason it is necessary to reproduce described here results on other populations before drawing general conclusions.

Appendix A

Quantitative assessment of population stratification

Freedman et al. [62] proposed a simple method for Genomic Control. The procedure includes several steps. In the first step a set of SNPs should be chosen. The markers should be independent, preferably from unlinked loci and ideally from genes that have no influence on the investigated phenotype. For each successfully genotyped SNP with the expected minor allele count (based on the combined frequency in cases and controls) of at least 5 the χ^2 value for case-control allelic association is calculated [62].

In the second step likelihood analysis is needed to estimate the level of stratification consistent with the data. Defining c_j as the association statistic observed at marker $j, (j = 1, \ldots, K)$ genotyped in n_j cases and m_j controls, and f as the density of the χ^2 distribution with 1 degree of freedom, the likelihood of a given inflation factor $\lambda_{n_{ref},m_{ref}}$ for a reference sample size of n_{ref} cases, m_{ref} controls is

$$\lambda_{n_{ref},m_{ref}} = \prod \frac{f(c_j/\lambda_{n_j,m_j})}{\lambda_{n_j,m_j}} \tag{A.1}$$

where

$$\lambda_{n_j,m_j} = 1 + (\lambda_{m_{ref},n_{ref}} - 1) \times \left(\frac{1}{n_{ref}} + \frac{1}{m_{ref}}\right) \div \left(\frac{1}{n_j} + \frac{1}{m_j}\right) \tag{A.2}$$

Here we use $n_{ref} = 1000$ and $m_{ref} = 1000$. The inflation factor will differ from marker to marker because it scales with sample size according to the latter equality [50, 141]. We abbreviate $\lambda_{1000,1000}$ as λ_{1000}. The maximum likelihood estimate for λ_{1000} is simply the value for which likelihood L is maximized, with the requirement that $\lambda_{1000} \geq 1$. The likelihood surfaces may be represented by plotting the values of L for different λ_{1000}, normalizing it by the maximum likelihood. The upper bound of the one-sided 95 % confidence interval for λ_{1000} may be obtained by picking the value such that the likelihood ratio $2\ln(L_{max}/L) = 2.7$; that is, the point for which the likelihood is equal to 25.9 % of the maximum [62]. Note at

this point that Freedman [62] made an error in using the common logarithm instead of the natural logarithm in delineating the rule for deciding on the limits of the confidence interval.

A.1 Controlling for population stratification in the investigated data

In our study we investigated three samples (described in section 4.1.1 and 3.1.1): two samples of patients with depressive disorder and one control sample. In the first case sample (N=1000) all included patients were Caucasian and 91.2 % were of German origin. Recruitment of controls (N=1029) for the first sample was performed using the same questionnaires, matched for age, gender and ethnicity (all Caucasian, 93.04 % of German origin).

The second case sample (N=493, Munich Antidepressant Response Signature, termed MARS) included patients only that were recruited independently from the first case-control study. All patients were Caucasian and 85.1 % of German origin. No matching was done between groups of responders and non-responders to antidepressant treatment. For this reason it was necessary to control for PS within patients in the MARS sample.

Whole genome genotyping data were available from Sentrix Human-1 Genotyping BeadChips, (Illumina Inc., San Diego, USA). In total 109 000 SNPs were genotyped for other ongoing project. We have randomly chosen 7357 SNPs within the human genome (excluding the X and Y chromosome). Only SNPs with MAF 0.02 and in HWE were chosen. We assumed that the probability that any of them changes the response ability to antidepressant treatment is very low.

We performed Genomic Control as described above using the Freedman et al. method [62]. We built likelihood curves (figure A.1) for stratification for 3 binomial response outcomes: early response to antidepressant treatment, response after 5 weeks and remission after 5 weeks (detailed description of response phenotypes is in chapter IV).

In our data we did not found any significant evidence for stratification. The estimated inflation factor λ was close to 1. The increase of the type I error rate due to PS in the investigated sample was very small and numerically irrelevant. Thus, no correction for PS was necessary in our study.

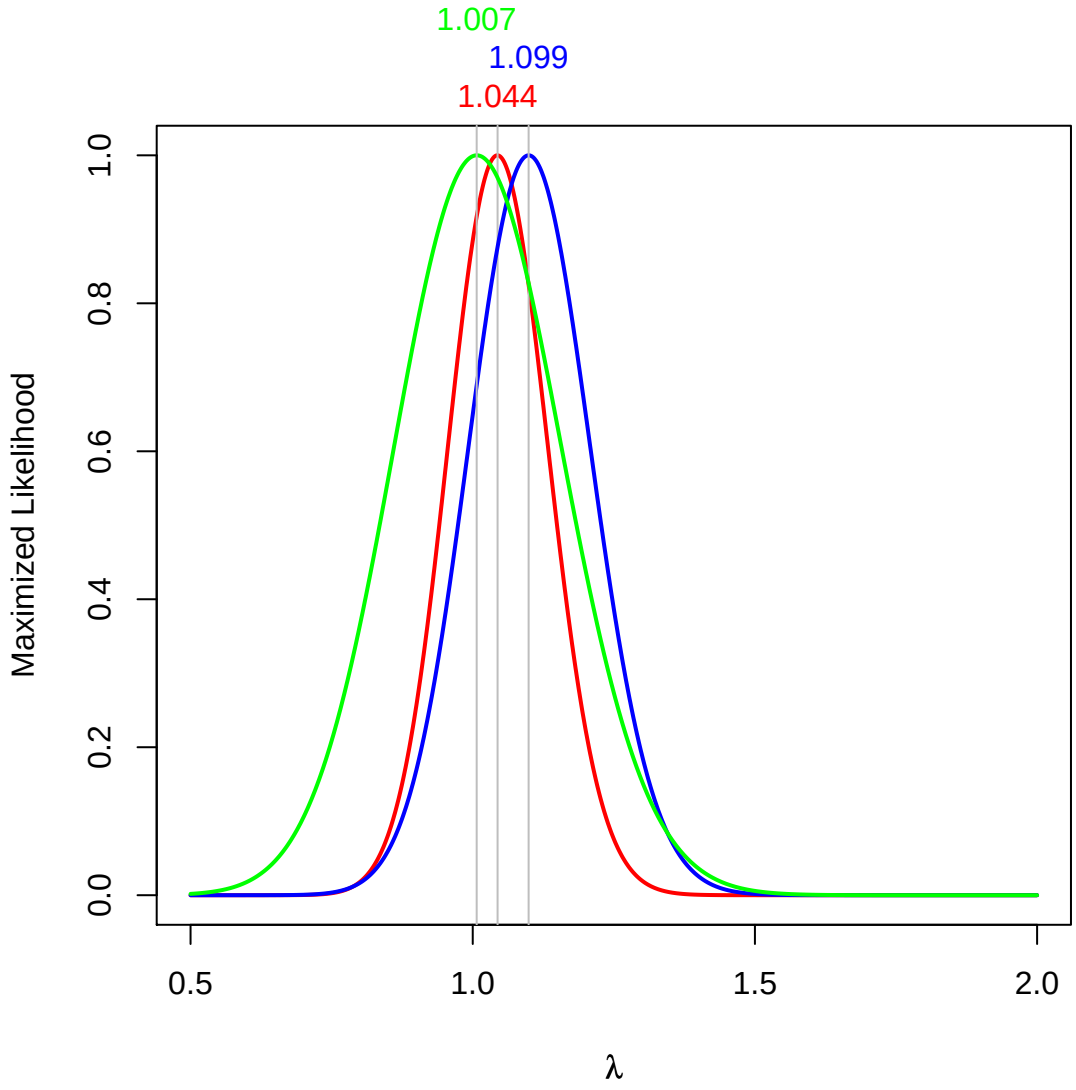

Figure A.1: Likelihood surfaces for stratification in patients (N=493) using 7357 randomly chosen genotyped SNPs. Red: early response phenotype, blue: response after 5 weeks, green: remission after 5 weeks of antidepressant treatment.

Appendix B

Comparison of haplotype estimation algorithms

We were interested in studying associations of haplotypes in candidate genes with susceptibility to depression and with response to antidepressant treatment.

In our sample of unrelated individuals haplotypes must be determined using statistical methodology. Several programs using different algorithms were available. Most of them were described to be 'good' in haplotype frequencies estimation, nevertheless, none gives a 100 % certain estimation [166, 200, 202].

Zhang et al. [205] recently compared EM and PHASE algorithms. On simulated data using empirical population haplotype frequencies, the PHASE method showed no improvements over the EM method, except for one data set of an African population. In analysis of totally nine African populations in which haplotypes were inferred through molecular methods, the EM method and the PHASE method yielded almost identical results in seven populations, and the PHASE method did outperform the EM method in the other two populations. Xu et al. [200] showed that there were no significant differences in haplotype estimation certainty between EM and PHASE algorithms. On the basis of their findings we could not decide *a priori* which of the two algorithms is better for our data. Moreover, the EM algorithm based approaches implemented in SNPHAP and proposed by Clayton are claimed to be better for long haplotypes, but it truly is not clear which algorithm is better. Consequently, we decided to compare two of the programs described above, SNPHAP and PHASE, on our data.

B.1 Material and methods

We performed a haplotype analysis in 3 genes related to the hypothalamo-pituitary axis, *FKBP5*, *NR3C1* and *NR3C2*. The number of investigated SNPs was 32, 11 and 11 from 545, 858 and 854 DNA-samples, respectively. DNA-samples and SNPs were the same as

Gene	Number of SNPs	Sample size	Missing data	Time in PHASE	Time in SNPHAP	Inter-error rate	Mean difference
FKBP5	32	545	0.03	36 min	3 sec	0.03	6.21
NR3C1	11	858	0.15	7 min	2 sec	0.03	1.67
NR3C2	11	854	0.04	46 min	1 min	0.21	2.79

Table B.1: Comparison of SNPHAP and PHASE algorithms for haplotype estimation in unrelated individuals

described in chapter IV (MARS-sample).

We calculated 'inter-error rate' and 'mean-difference'. The former we defined as the proportion of samples with differently estimated haplotypes by SNPHAP and PHASE algorithms. The latter one we defined as the mean number of differently called alleles of SNPs over the sample in diverse estimated haplotypes by SNPHAP and PHASE algorithms.

B.2 Results

The first remarkable difference between the two algorithms was the computational speed (table B.1). The algorithm implemented in the SNPHAP program was noticeable faster than the algorithm implemented in the PHASE program.

The certainty of estimation of haplotypes was dependent on the LD structure (detailed LD structure for the 3 genes is described in chapter IV). In the case of high LD (in the *NR3C1* and *FKBP5* genes) common haplotypes with frequencies above 0.35 were estimated with high similarity in both algorithms. The gene *NR3C2* had no common haplotypes because of low LD between investigated SNPs. The highest haplotype frequency in this gene was equal to 0.05 and the haplotypes estimated by the two programs were mostly different.

Both programs needed more processing time if LD was low. The number of considered SNPs correlated with computational speed. The proportion of missing genotypes did not affect the inter-error rate in our data.

B.3 Discussion

Results showed that the two algorithms seriously differed only in processing speed when the LD between the investigated SNPs was high and a small number of common haplotypes existed. In our data we could not show how well both algorithm perform if original haplotypic data are available, but according to the literature [205, 200] there were no significant difference in haplotype frequencies estimated by EM and PHASE algorithms.

The finding that the accuracy of computational methods was decreased with decreasing LD is in concordance with data described in literature [167, 205, 200].

On the basis of our results and results from literature we chose the SNPHAP program for haplotype estimation in our sample due to considerably higher computational speed. We also introduced a security threshold by only including those individual haplotypic configurations in the analysis for which the posterior probability was greater than 95 %.

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