GENETIC ANALYSIS OF NEUROPSYCHIATRIC DISORDERS IN A SOUTH AMERICAN POPULATION ISOLATE

BY

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To my family

"In sooth, I know not why I am so sad:

It wearies me; you say it wearies you;

But how I caught it, found it, or came by it,

What stuff 'tis made of, whereof it was born,

I am to learn."

Shakespeare, The Merchant of Venice

Statement of Authorship

I, Barbara Kremeyer, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

The sequencing of the *CLINT1* gene described in chapter 3 has been performed with the help of the undergraduate student Amy Roberts, who was carrying out a research project in our lab. Similarly, the genotyping of the SNP rs11955293, also described in chapter 3, has been carried out with the help of the undergraduate student Natalia Szypilow as part of a research project she performed in our lab. Both students worked under my supervision.

I have genotyped all microsatellite markers typed for the linkage scan in chapter 4, with technical assistance of Heike Müller, Émilie Boucher and Julie Pivard.

Most SNPs for the association analysis of the *NOS1AP* gene presented in chapter 5 were typed by our collaborators on that project at Rutgers University, U.S.A. SNP rs1415263 was genotyped by myself, with the help of the undergraduate student Hanna Kymäläinen who was carrying out a research project in our lab under my supervision.

With exception of the factor analysis of schizophrenia symptoms in chapter 5, which was done by Dr. Jenny García from Universidad de Antioquia in Medellín, Colombia, and of the haplotype analysis of the chromosome 5q region in chapter 3, which was done by Susan Service at UCLA, I have personally performed all statistical analyses in this thesis, and I have interpreted the results with the help of my supervisor, Professor Andrés Ruiz-Linares.

I have written this thesis myself without any help or use of materials other than that acknowledged in this thesis. Comments were made by Professor Andrés Ruiz-Linares.

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Abstract

Bipolar disorder (BP) and schizophrenia are severe neuropsychiatric conditions that are among the leading causes of morbidity and chronic disability world-wide. Both conditions are characterised by a substantial genetic heterogeneity, which has complicated the search for susceptibility loci. One strategy to tackle this difficulty lies in the study of population isolates that are characterised by a reduced genetic heterogeneity. In this thesis, I have therefore conducted genetic studies of BP and schizophrenia in the well-characterised South American population isolate of Antioquia, Colombia.

Our group has recently reported the results of a linkage scan of six Antioquian families segregating severe BP. Here, I performed a follow-up study of a candidate region on chromosome 5q33. I sequenced the *CLINT1* gene, a functional candidate that has also been implicated in schizophrenia, in affecteds from four BP pedigrees from the original linkage study and identified three single base pair variants, all of which had been previously described. A transmission distortion test of one of these variants, rs11955293, in a sample of 176 unrelated BP patients from Antioquia and their parents found no evidence of association with BP. Although these results do not rule out a minor effect of the *CLINT1* gene on susceptibility to the disorder in Antioquia, other loci are likely to be of greater significance. This includes other genes on chromosome 5q33, but also other candidate regions in the genome.

To further explore the latter possibility, I conducted a whole-genome linkage scan in an additional nine pedigrees with severe BP from Antioquia and analysed the obtained genotype data jointly with that of the initial linkage scan. Using parametric and non-parametric linkage approaches, I explored three different diagnostic models: a narrow model including only BP type I (BPI) as affected; a model including BPI and II and major unipolar depression; and a third model including only individuals who had experienced psychosis as affected. This second linkage scan found evidence for a number of candidate regions, including chromosome 13q33 for BPI, chromosomes 1p13-31 and 1q25-31 for mood disorders, chromosome 12ct-q14 for mood disorders, and chromosomes 2q24-31 and 16p12 for psychosis. Encouragingly, many of these loci had previously been pinpointed as BP susceptibility loci in other populations; on the other hand, we also identified a novel locus on chromosome 12q.

While the use of population isolates can help decrease the genetic heterogeneity of a complex disease, complementary strategies can be used to reduce this heterogeneity even further. In studying the *NOS1AP* gene, a functional candidate on chromosome 1q23 that is involved in glutamatergic neurotransmission, in a sample of 102 unrelated Antioquian schizophrenia patients and their parents, I have therefore used both categorical and dimensional approaches to the disease phenotype. In the categorical approach, I conducted an analysis for association between the *NOS1AP* gene and DSM-IV schizophrenia by TDT. For the dimensional approach, two clinical scales measuring positive and negative symptoms, SANS and SAPS, were applied to all patients and dimensional scores were obtained from these scales by factors analysis. I then performed quantitative TDT analysis of the dimensional scores. My analyses found association to both DSM-IV schizophrenia and a clinical dimension capturing negative symptoms, in line with a role of NOS1AP in glutamatergic neurotransmission. The results of these analyses also underline the usefulness of a dimensional approach in psychiatric genetics.

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CHAPTER ONE

INTRODUCTION

1. Introduction

This thesis aims at making a contribution to the genetics of two major neuropsychiatric disorders, bipolar disorder, also known as manic depression, and schizophrenia. In this introduction, I shall put the present work into the context of current efforts to understand the aetiology of psychiatric disease. First, I shall introduce the rationale of focussing on the genetics of psychiatric disease (chapter 1.1). In chapters 1.2 and 1.3, I will introduce different aspects of the methodology used in this thesis and elsewhere to localise and identify loci involved in the genetic susceptibility to disease. Chapters 1.4 and 1.5 shall give an introduction to the phenotypes studied here – bipolar disorder and schizophrenia –, and to their epidemiology and genetics. In chapter 1.6, I will present the ongoing psychiatric genetics project in the populations of Antioquia and Costa Rica, in the context of which this thesis has to be viewed. Finally, the aims of this thesis will be presented in detail in chapter 1.7.

1.1. Why Study Psychiatric Genetics?

Neuropsychiatric disorders are among the leading causes of morbidity and chronic disability world-wide, with unipolar depression as well as the two major psychoses, bipolar disorder and schizophrenia, ranking among the top ten causes of years lost to disability in both the developing and the developed world[1]. Severe psychiatric conditions do not only have devastating consequences for the patients' mental, social and economic well-being[2-5] and even their general health[6], they also represent a major cost to health systems and national economies alike. It has been estimated that the annual cost of bipolar disorder to society, including treatment as well as indirect costs due to factors such as unemployment and absenteeism, amounts to about £2 billion in the UK[7]. The figure for schizophrenia is even higher, with an estimated annual cost of £2.6 billion for England alone[8], and the yearly costs of major unipolar depression for adults in England are leading the list at a staggering £9 billion[9]. It is evident that there is a great need to reduce the burden of mental illness on both affected individuals and society as a whole.

1.1.1. Limitations of current diagnostic and treatment strategies

The successful and cost-efficient treatment of psychiatric illness (its cure not currently being conceivable) relies crucially on a meaningful and reliable diagnosis, as well as on an adequate choice of pharmacotherapy. In practice, however, both the correct diagnosis of psychiatric illness and the choice of treatment can represent a challenge. Because there are no indicators at a molecular, physiological or behavioural level that are at the same time necessary and sufficient for the diagnosis of any mental disorder (i.e., there are no biological or behavioural markers for mental disease), psychiatric diagnoses rely solely on constellations of clinical signs and symptoms as well as on the course of disease[10]. This is reflected by the diagnostic procedures specified in the main clinical manuals of psychiatry, the Diagnostic and Statistical Manual of Mental Disorders, published by the American Psychiatric Association (currently in its 4th edition: DSM-IV)[11] and the International Classification of Diseases, published by the World Health Organization (currently in its 10th edition: ICD-10)[12]. The classification system on which these manuals are based does most likely not represent true disease entities. Indeed, there is substantial clinical heterogeneity within the current diagnostic categories for many mental disorders. An extreme example is schizophrenia, where two patients with the same diagnosis might not share a single symptom[13]. This supports the idea that the current diagnostic categories include distinct diseases with different, but possibly related, pathophysiologies, resulting in a similar clinical phenotype. Conversely, disorders which have traditionally been regarded as distinct entities, namely the two main functional psychoses, bipolar disorder and schizophrenia[14], might share at least part of their aetiopathology[15,16]. As these examples show, there is still considerable uncertainty in psychiatric nosology, and although the diagnoses based on the current classification of mental disease are reliable and practical in many ways, they might not be valid from an aetiological point of view[17].

A valid and meaningful diagnosis should be based on the aetiology of the disease and will ideally inform the choice of treatment, with the ultimate goal to spare patients the tedious procedure of trial and error in order to find the right medication, as it is currently the case in psychiatric practice. This trial and error procedure is not only emotionally upsetting for patients and delays the onset of efficient treatment; it can even have an adverse impact on the treatment outcome, since the success of

pharmacological treatment may depend on an early intervention at the onset of the disease. This is thought to be due to neurotoxic effects of psychotic and depressive episodes, leading to structural changes in the brain and rendering subsequent treatment more difficult[18-20]. A quick start of the right course of pharmacotherapy is therefore essential, and a meaningful diagnosis is crucial to achieve this.

An additional factor influencing and often complicating diagnosis and treatment is comorbidity with other psychiatric disorders[21]. Mood disorders, for example, frequently co-occur with anxiety disorders[22,23]. There is also substantial comorbidity between bipolar disorder and panic disorder[24] and, in children, attention-deficit hyperactivity disorder[25], while substance abuse disorder is very common in patients with schizophrenia and mood disorders among others[26-28]. There are several possible mechanisms leading to psychiatric comorbidity: (1) one disorder could act as a risk factor for the development of another (e.g., cannabis abuse increases the risk of developing psychosis[29]); (2) the two disorders might share common risk factors (e.g., it has been suggested that schizophrenia and diabetes might share common genetic predisposition[30]); and (3) the two co-occurring disorders might in fact be different facets of the same disease. It is possible that all three mechanisms are truly relevant to psychiatric comorbidity. The distinction between them ultimately comes down to the distinction between true comorbidity, i.e., the true co-occurrence of two distinct disorders in the same patient, and the co-occurrence of symptoms in a single disorder. Since the aetiology of a disorder whose phenotype, for example, comprises both depressive and anxious symptoms might be different to the aetiology of both depression and anxiety disorder, this distinction might entail important consequences for the treatment and the prediction of course and outcome of mental illness[21].

As we have seen, there are several limitations to the current, descriptive classification system of psychiatric illness concerning the aetiological relationship between diseases, including comorbidity. Resolving these issues would lead to a better and more complete understanding of the epidemiology of psychiatric illness, including risk factors, course of disease, treatment outcome and patterns of true comorbidity, as well as to an aetiologically valid diagnostic system. To achieve a shift from a descriptive to a true taxonomic classification of disease (i.e., a classification system based on true disease entities), better insights into the pathophysiology of mental illness are indispensable.

While substantial advances have been made in the elucidation of disease mechanisms in other areas of medicine, research into the biological causes of mental illness has been much less successful, and our understanding of its aetiopathology remains poor[31]. This is due to the aetiological complexity of psychiatric disease, resulting from genetic heterogeneity, variable expressivity, pleiotropy and gene-environmental interaction among others[10,32], and reflecting the complexity of brain function. However, while we still have a long way to go in order to achieve a thorough understanding of mental illness that can be translated into better diagnosis and, ultimately, into better and more individualised treatment options, we have certainly started to move into the right direction. The past decades have seen important progress in the understanding of the neurobiology underlying many neuropsychiatric conditions, including Alzheimer's disease, schizophrenia, and bipolar disorder[33-37], and the field of psychiatric genetics has significantly contributed to these advances.

1.1.2. The relevance of genetics to psychiatry

From the early days of genetic research in psychiatry on, family, twin, and adoption studies have demonstrated the importance of genetic liability to mental illness[38,39]. With the advent of the first generation of psychotropic drugs, specific pathways involved in the aetiology of psychiatric disease, such as monoaminergic neurotransmission, could be pinpointed for the first time[40,41]. One of the major roles of genetic research since then has consisted in confirming the importance of these biochemical pathways for the pathophysiology of mental illness[42], the main methodological approach consisting in candidate gene association studies. In recent years, advances in high-throughput genotyping techniques and statistical analysis have made whole-genome association studies a reality[43-45], a development that raises the distinct possibility of inversing this relationship and uncovering new pathways involved in psychiatric disease through the identification of novel susceptibility loci. Since long before the era of genome-wide association, linkage analysis studies have pursued the same goal, although their success in psychiatric disease has been limited by the complex nature of mental illness[46,47]. The feasibility of whole-genome association approaches has been shown for other complex traits, such as obesity, where the discovery of a previously unknown susceptibility locus, the *FTO* gene, has opened up the possibility of detecting a whole new pathway[48]. At the same time, the

establishment of large-scale national and international collaborations allows the collection of samples that are large enough to detect susceptibility loci with small to moderate effects, such as they are expected for most psychiatric diseases[49]. This approach has been successful in other diseases including type 1[50] and type 2 diabetes[51,52] and breast cancer[53].

Through the uncovering of susceptibility loci and novel pathways leading to mental illness, progress in psychiatric genetics has the potential to catalyse advances in many areas of psychiatry. Some of the most important areas will be discussed in the following.

1.1.1.1. Identification of disease markers

As an essential step towards valid and meaningful diagnoses, as well as towards early detection and prevention of illness, genetics may contribute to the development of biological markers of disease. Because psychiatric diseases are thought to be caused by an accumulation of common genetic variants, each of which might only confer a small increase in disease risk, it seems unlikely that the test of a single genetic variant will be specific and at the same time sensitive enough to serve as a disease marker on its own[10,54]. However, the elucidation of disease mechanisms with the help of genetics should facilitate the identification of physiological markers with a higher predictive value than the genetic polymorphisms associated with disease alone. These might include neuroendocrinological factors and proteins involved in signal transduction, among others. Additionally, genetic polymorphisms could be incorporated in a panel of markers that together have a higher and more specific predictive value than any marker on its own[10]. A recent study on type 2 diabetes, for example, has shown that the combined information from three known risk loci allows the identification of population subgroups at risk for the disease[55]. Through simulation studies, Janssens et al. (2006) have recently shown that genetic profiling by typing a panel of up to 400 risk-associated polymorphisms can have high specificity and sensitivity in predicting the risk of developing common disease[56]. This is particularly true for rare diseases with a prevalence of around 1% and a high heritability, such as schizophrenia and bipolar disorder.

The development of biological markers of disease will be essential for the diagnostic process, as well as for the early identification of individuals at risk for psychiatric illness, thereby moving towards the ultimate goal of disease prevention. One setting in which early identification of individuals at risk might happen, is genetic counselling.

1.1.1.2. Genetic counselling and predictive testing

Genetic counselling is the process of educating patients and/or their relatives about principles of human genetics applicable to inherited disease, such as patterns of inheritance and the risk of disease attached to predisposing genes, thereby enabling them to make informed and autonomous decisions in all areas of their lives[57]. Importantly, this process should always be non-directive so that the decision making remains entirely with the consultand. Most counselling situations explore two basic scenarios: (1) in the case of affected individuals and their spouses, most questions evolve around the risk of disease in offspring, and (2) in the case of unaffected relatives of patients suffering from the disease, both the risk of disease in the offspring and the personal risk of developing the disease later in life are of concern[38,58]. Most genetic counselling occurs in the context of rare Mendelian disorders where risk estimation is relatively straightforward and is either based on the results of a genetic test or on the mode of inheritance. In psychiatry, where inheritance patterns are much more complex, genetic counselling is still a nascent field, but there is growing awareness of a need for such services[59]. This need might become more urgent as patients' awareness of genetic predisposition to mental illness is raised through the mass media.

Because there are no predictive tests for the vast majority of psychiatric diseases, and because of the complex inheritance of mental illness, recurrence risk estimates can only be given based on empirical epidemiological data. Currently, the main goals of genetic counselling in psychiatry are therefore to educate the patient about genetic factors in psychiatric disease, to provide empirical recurrence risks and to help the consultand cope with practical and psychological issues arising from this process[59,60]. While the first and the last point represent very important aspects of the genetic

counselling process, it is on risk prediction that advances in psychiatric genetics are most likely to have a direct impact.

Empirical recurrence risks are available for all major psychiatric conditions[38]. For example, the recurrence risk in the first-degree relative of a bipolar patient lies between 4 and 18%, while for the same relative, the risk of developing unipolar major depression can be as high as 25%[59]. These estimates can vary according to the number of affected relatives and their relationship to the consultand[38]. Naturally, empirical risk figures are not available for every possible family constellation, and it is therefore difficult to give consultands an estimate of their personal disease liability. The development of disease markers, as discussed in chapter 1.1.1.1, holds the – albeit still distant – promise of a more accurate disease prediction. This would enable mental health professionals to monitor specific individuals at high risk and intervene at the earliest stage of the disease, thereby significantly increasing the chance of effective treatment and management of the disease. Early recognition of individuals at risk for mental illness paired with innovative treatment approaches (see chapter 1.1.1.3), both fuelled by advances in psychiatric genetics, could constitute an important step on the way to disease prevention.

An aspect of special interest in the context of genetic counselling is the possibility of defining the interaction between genetic and environmental factors in a way that could enable counsellors in the future to give consultands personalised advice about which environmental risk factors best to avoid in order to keep their disease risk at bay. Given our current fragmentary level of knowledge, this scenario might seem even further away than risk prediction based on genetics alone, but its potential for disease prevention would make it well worth striving for. In recent years, evidence for the modifying effect of specific genotypes on the susceptibility to environmental risk factors for mental illness has accumulated (REF 61; see also chapter 1.1.1.4), and this knowledge could be used to inform life-style choices in individuals at risk for psychiatric disease.

While predictive testing in psychiatry has the potential to be of great benefit for psychiatric patients and their relatives, some important ethical considerations need to be made. Although these issues can only be touched upon in this thesis, it should be emphasised that it is crucial for every researcher in psychiatric genetics to realise and reflect on the profound impact the possibility of predictive testing might have on the

lives of those suffering from or at risk for mental illness. The availability of a genetic test for psychiatric disease introduces the possibility of prenatal or preimplantation testing for children of couples with a family history of mental illness. Although it might be justifiable to screen for the most severe conditions such as treatment resistant schizophrenia, it is not clear where the limit lies between disease prevention and screening for offspring with a certain behaviour, desirable to the parents or society as a whole. Only recently, a genetic test that helps predict physical endurance has become available[62], and while this test (which only analyses a single genetic polymorphism) might still be a long way from allowing precise predictions of physical performance, the possibility of tailoring one's offspring according to specific, non health-related ideals is conceivable. The prospect of this kind of physical performance-based selection seems sinister; a behaviour-based selection might seem even more so.

Furthermore, predictive testing in psychiatry can have a profound impact on the availability of medical insurance to individuals at risk, as well as on employment chances. These are just very few aspects of the stigmatisation resulting from being labelled as "at risk" for psychiatric disease, and a legal and ethical framework is needed before predictive testing in psychiatry can be put into practice.

1.1.1.3. Drug development and individualised pharmacological treatment

The first drug for the treatment of a psychiatric condition to become available was iproniazid, an antidepressant belonging to the class of monoamine oxidase inhibitors (MAOIs). Its discovery in the early 1950s was serendipitous – it had originally been developed for the treatment of tuberculosis but was then found to produce euphoria and enhanced activity in some patients –, and so were the discoveries of the first antipsychotic, chlorpromazine, the first tricyclic antidepressant (TCA), imipramine, and the mood stabiliser lithium[63]. Because they had not been specifically designed for the use in psychiatric disease, it was not surprising that this first generation of psychotropic drugs showed a broad spectrum of side effects, including cardiovascular and anticholinergic complications (TCAs), potentially life-threatening hypertensive crises through interaction with tyramine contained in food (MAOIs)[64], and debilitating extra-pyramidal side effects and tardive dyskinesia (first generation

antipsychotics)[65]. The side effects of lithium, an alkali metal about whose mechanism of action very little is known[31], include lack of coordination, cognitive effects, weight gain, hypothyroidism, and, in the case of lithium intoxication, renal failure and cardiac problems[66]. The most important innovation in psychiatric pharmacology in the last fifty years has therefore been the development of substances with fewer of these unwanted and often harmful effects, such as selective reuptake inhibitor antidepressants (SRIs) and atypical antipsychotics[41].

However, these second-generation psychotropic drugs are generally no more efficacious than older compounds[41,64,67]. Only 60-70% of patients with major depression respond to antidepressant treatment[41], and a recent review of published as well as unpublished clinical trials submitted to the U.S. American Food and Drug Administration (FDA) concluded that new generation antidepressants only performed significantly better than placebo in severe, but not moderate or mild depression[68]. An additional drawback of currently available antidepressants is the delay in the onset of therapeutic response, although, at least for selective serotonin reuptake inhibitors (SSRIs), this view has recently been challenged[69].

In schizophrenia, between 25 and 60% of all cases are classified as treatment resistant or partial responders. The only compound that has consistently been proven to be more efficacious than first-generation, or conventional, antipsychotics in the treatment of positive, negative and cognitive symptoms (see chapter 1.5.1), is clozapine. It is also characterised by the absence of extra-pyramidal side effects and tardive dyskinesia; however, it can cause other severe and debilitating side effects including weight gain, seizures, diabetes, and agranulocytosis, and is therefore never the first choice for treating schizophrenia[65].

Because of the severe limitations of currently available treatment options, there is a great need for the development of new psychotropic drugs with novel mechanisms of pharmacological action. Research in genetics and genomics holds great potential for this process. As discussed above, through the identification of susceptibility loci for mental illness, advances in psychiatric genetics can lead to the uncovering of novel pathways involved in the aetiology of disease (e.g., for depression, outside the monoaminergic pathway, which is currently targeted by all known classes of antidepressants[41]). Such novel pathways could then be targeted by new, and possibly more efficacious, therapeutic agents[31]. Importantly, this includes the possibility of

finding drugs that do not only treat the symptoms of psychiatric disease, but fix the underlying defect (current relapse rates upon treatment discontinuation indicate that the medications available at present do not achieve this)[41,70]. Genomic approaches could also contribute to the identification of novel targets for existing drugs, which could then pave the way to the development of new compounds aiming at the same pathways[41]. Furthermore, an improved understanding of the aetiology of psychiatric disease spawned by advances in genetics should help determine specific subgroups within the current diagnostic categories which will then allow the development of more specific drugs for each of these subgroups, with the ultimate goal of individualised treatment options[41].

A truly individualised treatment should take into account not only the patient's belonging to a specific subgroup within a diagnostic category (or, ideally, his aetiologically valid diagnosis once a true classification of disease has been established), but also his personal genetic makeup which might influence the efficacy of psychotropic drug action, as well as the patient's propensity to side effects[71]. The targets of pharmacogenetic studies include the site of drug action, as well as drug-metabolising enzymes[72]. A class belonging to the latter type, the cytochrome P450 (CYP) enzymes, has been studied extensively in the context of psychotropic drug metabolism. Many of its members have been shown to be involved in the metabolism of antidepressants and antipsychotics, and associations between specific alleles of the CYP-coding genes and adverse response to drug treatment have been reported (reviewed by Kerwin and Arranz 2004)[72]. As a taste of the potential use of pharmacogenetics for individualised drug response prediction, the FDA has recently approved the AmpliChip CYP450 Test (developed by Roche), which screens polymorphisms in the CYP2D6 and CYP2C19 genes. CYP2D6 plays a role in the metabolism of many antidepressants and antipsychotics, while CYP2C19 is important for the metabolism of some antidepressants, such as amytriptiline, citalopram and imipramine, among others[72,73]. The AmpliChip CYP450 Test is designed to identify ultrarapid and poor metabolisers to help choose a treatment for individual patients, and concrete suggestions for antidepressant and antipsychotic treatment according to metaboliser status have been made[73].

Finally, genetic research could help overcome an important limitation to current drug development strategies: the lack of adequate animal models reflecting the aetiology

of mental illness to test newly developed compounds on[70,74]. The development of such animal models is severely hampered by the paucity of our knowledge about the neurobiology and the genetics of psychiatric disease. Currently, it is therefore common practice to conduct tests in healthy animals, mostly rodents, in which symptoms resembling mental illness are induced externally, either through behavioural or pharmacological stimuli, or through neurosurgery. The forced swim test[75], the tail suspension test[76] and the learned helplessness test[77,78] all induce hypoactive behaviour similar to that seen in depressive patients and are therefore used as behavioural models of depression in the screening of antidepressants. In pharmacological models, drugs such as amphetamines and hallucinogens are used to produce specific effects similar to characteristic symptoms of mental disease (e.g. hallucinations as the hallmark of psychosis), and drug withdrawal can cause depressive or anxious symptoms. An example of the neurological approach is olfactory bulbectomy in rats, which causes a constellation of symptoms resembling major depression and has a high predictive value for the effectiveness of antidepressants[74,79-81]. However, there is considerable uncertainty about the validity of such models of psychiatric symptoms from an aetiological point of view. Often their validation is based on the model's susceptibility to currently available psychotropic drugs targeted at the modelled condition[79,80]. While this allows for a good prediction of the efficaciousness of compounds targeted at the same pathways as currently used drugs, it might not be a good strategy to screen drugs targeted at different pathways. This might be more successful if true aetiological models of the disease could be developed, and knowledge about the genetics of psychiatric disease is indispensable to achieve this.

In this context, a further crucial advantage of genetic models in psychiatry is the possibility to distinguish between substances that only target the symptoms of mental illness and those that address the underlying defect of the brain. This is based on the assumption that, while drugs that only cope with the symptoms can be tested in healthy animals with induced mental illness-like behaviour, drugs that target the underlying defect will only show an effect in aetiological animal models of disease[70].

In summary, progress in psychiatric genetics will have an important impact on the field of drug development and individualised treatment, and will therefore constitute

an essential step towards the management and, in the long term, perhaps even the cure of mental illness.

1.1.1.4. Impact on psychiatric research

By helping to cast light onto the "black box" of psychiatric aetiopathology, advances in genetics will be of great benefit for further research in the field of psychiatry. The identification of susceptibility loci and the biochemical pathways that link them should spark a new generation of neurobiological studies which should allow a much improved understanding of the mechanisms of disease[82]. Together, progress in genetics and neurobiology should then enable psychiatrists to move towards establishing a nosology of psychiatric illness that reflects true disease entities. This will not only be of great clinical benefit, it will also allow a more precise phenotype definition for future research into the neurology, epidemiology and genetics of psychiatric illness, thereby further catalysing our understanding of psychiatric aetiopathology.

Specifically, the identification of genetic risk factors will allow researchers to explore gene-gene and gene-environmental interactions, an approach that is increasingly being put into practice. Gene-gene interactions have been studied a number of psychiatric phenotypes, such as attention deficit hyperactivity disorder (ADHD), suicidal behaviour, anorexia nervosa, and autism, among others[83-87]. So far, the field of gene-gene interactions in psychiatry is still a nascent one. However, as the number of susceptibility loci identified in mental disease and our knowledge about the pathways they are involved in grows, the number of true gene-gene interactions being identified will increase as well. This will not least be due to the fact that more detailed knowledge about the aetiopathology of disease will enable researchers to formulate meaningful hypotheses which will inform research strategies better than it is possible at present.

Research into gene-environmental interactions has shown some interesting results in recent years. These include evidence that a functional polymorphism in the serotonin transporter acts as a modifier on life stress as a risk factor for depression[88], and that genetic variation in the *MAOA* gene influences mental health outcomes in children exposed to maltreatment[89,90]. Again, a lot of work remains to be done before the

effects of nature and nurture can be disentangled and incorporated into a comprehensive model of the aetiology of mental illness, but as with gene-gene interactions, this is likely to become increasingly feasible the more we learn about genetic susceptibility factors. It is also noteworthy that gene-environmental interactions can be studied in animal models once susceptibility loci have been established[61].

In summary, advances in psychiatric genetics will help shed light on disease aetiopathology, thereby enabling progress in many fields of psychiatry. Improvements in the diagnostic process through the establishment of disease markers and a meaningful nosology of mental illness, as well as advances in drug discovery will be of immediate clinical benefit to patients. Genetic counselling in psychiatry, on the other hand, is still in its infancy, but will improve with increasing knowledge about the aetiopathology of psychiatric diseases. Progress in psychiatric genetics will also reflect back on research in other areas of psychiatry, such as neuropsychiatry and psychiatric epidemiology, as well as on further research in genetics, and will therefore catalyse advances in all areas of psychiatry. The ultimate goal of this process is disease prevention.

1.1.3. The example of Alzheimer's Disease

An example of how advances in genetics can help decipher the underlying causes of a complex disease is provided by Alzheimer's Disease (AD), a severe neurodegenerative disease and the leading cause of dementia[91,92]. Although the brain lesions characteristic for AD had been known for several decades, it was the localisation and identification of the first AD locus, the gene encoding the Amyloid Precursor Protein (APP), in the late 1980s/early 1990s[93-96] that led to the formulation of a hypothesis explaining the aetiopathology of AD[97], the amyloid cascade hypothesis. This hypothesis has been further corroborated by the subsequent identification of additional loci for AD, *PSEN1*[98], *PSEN2*[99,100], and *ApoE*[101], which are all thought to act together in one pathway[102].

Although familial forms of AD constitute a minority of all cases[91], the *APP, PSEN1* and *PSEN2* genes were all identified through linkage analysis in extended pedigrees, thereby demonstrating the potential of the study of Mendelian forms of complex

diseases. While it is uncertain whether this approach can easily be transferred to other neuropsychiatric diseases – simply because true Mendelian forms of these disorders might not exist –, it shows that the identification of loci that have a direct impact on a small subset of affecteds only might represent an essential step towards the understanding of the molecular mechanisms of disease.

The identification of *ApoE* as an important risk factor for the common, late-onset, sporadic form of the disease, on the other hand, is a success story of association mapping approaches and provides hope for similar findings in other complex diseases. Additionally, the fact that *ApoE* has also been shown to act as a modifier of the age of onset in familial disease[103,104] is an example of the possibilities to study gene-gene (as in this case), but also gene-environmental interactions, which will then further enhance our understanding of the aetiopathological process.

The advances in AD also show how an improved understanding of disease aetiology can be translated into real therapeutic benefit for the patients. The formulation and subsequent corroboration of the amyloid hypothesis have made it possible for drug development strategies to move from targeting symptoms to targeting the underlying pathway of AD aetiology[105,106]. Although none of the approaches have yet led to the approval of an innovative, disease-modulating drug for AD, many compounds have entered clinical trials, and there is considerable hope that drugs targeting the aetiology of the disease will be available within the next five to ten years[106]. This development would be a very encouraging one for the vision of a disease-modifying therapy in other neuropsychiatric disorders.

Although there are clear differences between the elucidation of the aetiology of AD and that of other neuropsychiatric diseases – such as the fact that biomarkers for AD had been known for a long time before the first locus was identified, whereas the same is not true for other phenotypes –, the recent advances in AD can serve as an inspiring example of successes in neuropsychiatric genetics.

1.2. Strategies for Gene Discovery

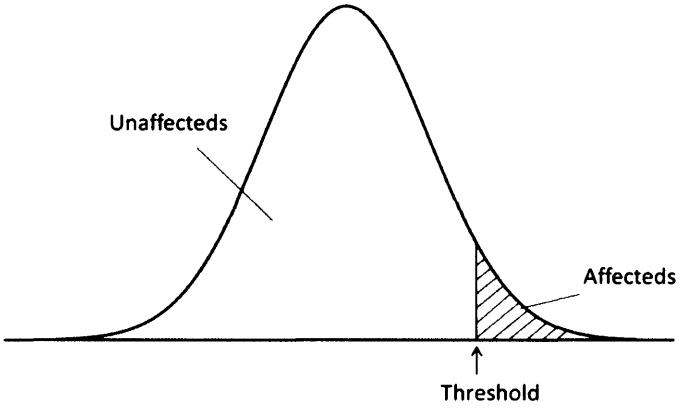
1.2.1. Monogenic vs. complex diseases

An important distinction for the purposes of genetic mapping is that between monogenic and complex diseases. As their name implies, monogenic diseases are caused by a mutation at a single locus, and their inheritance often follows simple Mendelian patterns. A mutation at the disease locus is necessary and in many cases sufficient to cause the phenotype. The probability of developing the disease given a certain genotype is referred to as the penetrance. At full penetrance, this probability equals 1 for homozygotes and heterozygotes in dominant disorders, and in homozygotes for recessive disorders. Several factors can render the inheritance patterns of monogenic diseases more complex, such as parental genomic imprinting, variable expressivity, and reduced penetrance, suggesting the interaction with additional, possibly genetic, factors. However, there is always one specific gene with a major effect. Monogenic diseases are often severe and debilitating from young age, and affected individuals may not reproduce. Therefore, even recessive disorders tend to be rare in the population. Examples of monogenic disorders include Huntington's Disease and familial Alzheimer's Disease, both autosomal dominant conditions, and cystic fibrosis, an autosomal recessive disease.

The relationship between genotype and phenotype is much more complicated in common diseases with a genetic component, such as diabetes, asthma, cardiovascular disease and psychiatric disorders. They are characterised by very complex, polygenic patterns of inheritance, where a combination of genetic variants at different loci influences the phenotype[107]. None of these variants seem to be sufficient, and most of them are probably not necessary to cause the disease. In contrast to monogenic disorders, mutations and polymorphisms implicated in the aetiology of complex diseases should therefore be thought of as risk or susceptibility factors, rather than causative variants. Additionally, the risk for complex diseases is influenced by environmental factors, and by the interaction of genes and environment. Complex diseases might be thought of as representing the extreme of a distribution of normal variation in the population, although this concept seems more intuitive for some phenotypes, such as cardiovascular disease, than for others, such as schizophrenia and bipolar disorder, where it is not clear of which normal variation they might

represent an extreme; however, the underlying concept is the same. Under this model, every individual has a certain liability for a complex disease. This liability is a function of the exposure to a variety of genetic and non-genetic risk factors. If the disease liability is higher than a certain threshold, the individual will become affected (see Figure 1.1 for illustration).

Figure 1.1: Liability distribution for a complex disease in the population.



Here, it is assumed that the underlying distribution of the disease liability in the population is normal, as it is typical for polygenic traits. Individuals become affected when their liability exceeds a certain threshold.

This thesis is a contribution to the genetic mapping of two complex neuropsychiatric diseases, bipolar disorder and schizophrenia. The discussion of mapping strategies in this chapter will therefore focus on complex disease, although the first method discussed, linkage analysis (chapter 1.2.3), is of great relevance for the discovery of genes in monogenic disorders.

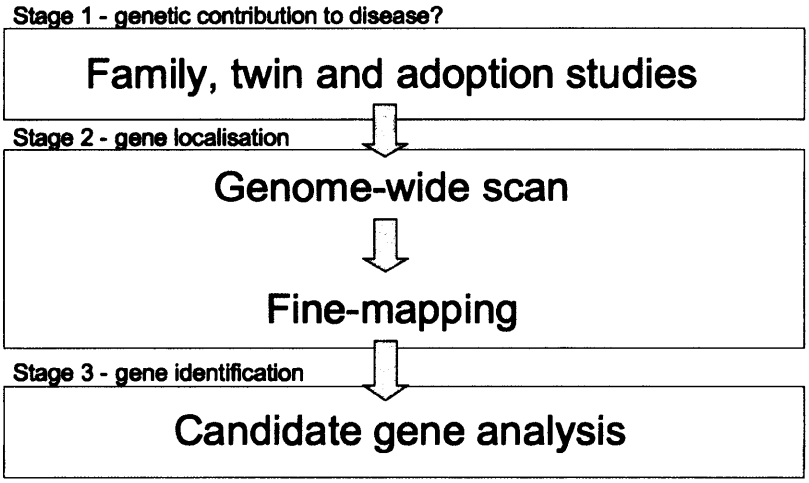
1.2.2. Genetic Mapping – a Conceptual Overview

In order to provide the conceptual context for the different gene mapping strategies presented in this chapter, this section will give an overview of the process of gene mapping and its different stages. Further details will be given in the subsequent sections.

Genetic mapping is the process of localising and identifying genetic variants that contribute to a phenotypic trait. In most cases, the trait of interest is a disease, and in

the following, I will refer to disease mapping. However, the same strategies can be applied to normal human variation. Genetic mapping involves three main stages – (1) establishing evidence for a genetic contribution to a disease (in practice, this is not part of the mapping procedure, but it represents an essential condition for mapping projects to go ahead), (2) gene localisation, and (3) gene identification. A schematic summary of the gene mapping procedure is shown in Figure 1.2.

Figure 1.2: Stages of gene discovery.



See text for details of the gene mapping process.

Stage 1 – establishing evidence for the genetic contribution to a disease

Before embarking on a mapping project, there has to be convincing evidence for a genetic contribution to the phenotype[38,108]. A first indication is familial accumulation of the disease; however, this might be due to a shared environment. Genetic factors can be disentangled from environmental ones through twin and adoption studies. These study designs shall not be explained in detail here; briefly, in twin studies, trait concordance is compared in monozygotic twins, who share genes and environment, and dizygotic twins, who also share their environment, but only 50% of their genes. In adoption studies, disease recurrence is compared between adoptive and biological families of affected adoptees, or between adopted-away offspring of affected and unaffected biological parents. The idea behind these approaches is that, in these settings, trait concordance patterns will vary with the relative importance of genetic

as opposed to environmental risk factors. The relative contribution of genetic factors to phenotypic variability is expressed as the heritability (h^2)[108].

$$h^2 = \frac{G}{V} = \frac{G}{G+B+E} \qquad (1)$$

Here, V denotes the total phenotypic variance, G is the genetic variance of the phenotype, B the within-family variance of the phenotype, and E represents the random environmental variance of the phenotype. It is important to note that heritability estimates are specific for the population in which they have been obtained; in a population with a very uniform exposure to environmental risk factors, the heritability estimate will be higher than in a population with large environmental variance although the genetic component might be the same[109].

Stage 2 – localisation of the disease gene

Once it has been established that the phenotype has a genetic component, the next step is a genome-wide screen for causal variants using genetic markers such as microsatellites and/or single nucleotide polymorphisms (SNPs). Traditionally, this used to be a linkage screen with highly polymorphic markers, usually microsatellites, an approach that proved very successful for monogenic Mendelian disorders, but less so for complex diseases, as will be discussed later in this chapter. Whole-genome association using SNPs has only recently become feasible but has already provided promising results for common diseases with complex inheritance[49]. Whole-genome analyses, be they based on linkage or association methods, are free of a prior hypothesis and are carried out to pinpoint candidate regions that might be involved in the aetiology of a disease.

The next step consists in fine-mapping the candidate regions identified in whole-genome scans, particularly linkage scans. This is normally done by adding additional markers in the region of interest to obtain a higher resolution image of that region. The genotyping of additional individuals or families in this step can also be useful. Sometimes, not all available samples can be included in the initial scan because of limits to the budget or the availability of DNA for specific samples; adding these samples to the study at the fine-mapping stage can help decide whether a linkage peak represents a genuine signal.

Stage 3 – identification of the disease gene

Finally, if there is good evidence for a specific candidate region, the last stage of the mapping project consists in screening genes located within that region. Those genes with a known biological function that fit best into the emerging picture of the aetiopathology underlying a disease should be considered first; they are often referred to as candidate genes. In the ideal case, candidate gene screening efforts will identify a clear sequence variant, such as a single-base change, an insertion or deletion, which will then have to be shown to co-segregate with the disease (in Mendelian disorders) or to be associated with the phenotype (in the case of complex diseases) before it can be considered as a candidate for the disease mutation, or, in complex disease, the susceptibility variant. In complex disease, a replication of the results obtained for a specific candidate region or gene in an independent sample is crucial. The ultimate proof that a variant is relevant to the aetiology of a disease, especially in diseases with complex inheritance, cannot be provided by studies at the DNA level. Instead, functional studies have to be carried out in order to establish a causal link between genetic and phenotypic variation.

Although genetic mapping is straightforward in theory, its practical implementation is often less simple. The genetic mapping of complex diseases in particular is riddled by difficulties, and successes in this field are still scarce, especially in the genetic dissection of psychiatric disorders[110]. However, if used correctly, the toolbox of genetic mapping described in detail in the remainder of this chapter should continue to yield important results in the discovery of genetic variation leading to disease.

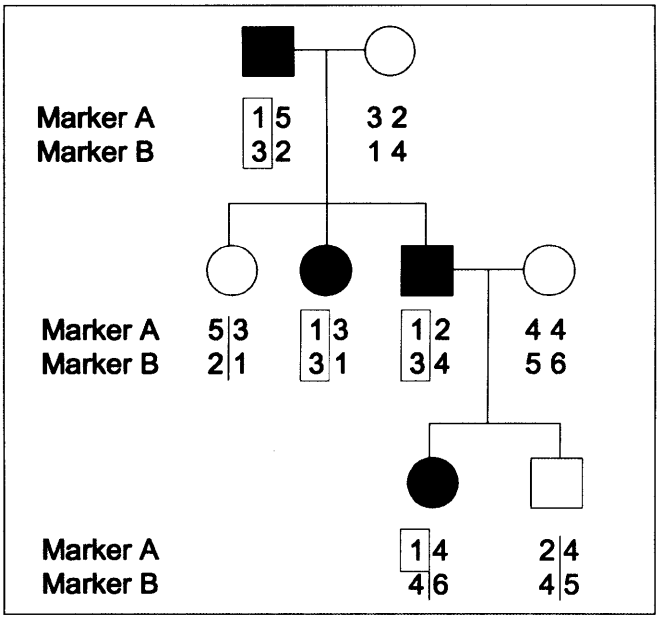
1.2.3. Linkage Analysis

The main gene mapping strategy in family studies is *linkage analysis*[111]. Linkage analysis tests for co-segregation within a family between a disease phenotype and genetic marker loci, such as microsatellites or SNPs. The principle of linkage analysis is illustrated in Figure 1.3.

Under the null hypothesis of no linkage – i.e., independent assortment –, the recombination fraction θ between a marker and the phenotype is expected to correspond to 0.5. This means that the inheritance of the phenotype is independent of that of a given marker allele. A recombination fraction of < 0.5, on the other hand,

means that the inheritance of the phenotype and of given marker allele are not independent events; there is genetic linkage between the marker and the locus causing the phenotype. It is noteworthy that the observed phenotype serves as a proxy for the underlying genotype at the susceptibility locus, which cannot be observed; the simpler the relationship between genotype and phenotype, the easier it is to infer the underlying genotype based on the phenotype. The complexity of this relationship in common, complex diseases is one of the reasons why the genetic mapping of these diseases is difficult.

Figure 1.3: Co-segregation of a disease phenotype and a two-locus haplotype in a three-generation pedigree.



Filled symbols indicate diseased individuals, blank ones unaffected individuals. The co-segregating haplotype is marked by a red box. Founders are phase-unknown, all other genotypes are phased. Note the recombination between marker B and the disease phenotype in the third generation. Based on this pedigree, it seems likely that the gene causing the disease is located on the same chromosome as the two markers, probably closer to marker A than marker B: everybody who has allele 1 at marker A, also has the disease.

The kind of linkage analysis described so far relies on the specification of a genetic model, which describes mode of inheritance, allele frequencies at trait and marker loci, penetrances (i.e., the probability of developing the disease given a certain genotype at the susceptibility locus), as well as the sex-specific recombination rates between the marker loci and the mutation rate at the marker loci (although the latter

is often assumed to be zero)[112]. It is therefore referred to as *parametric linkage analysis*.

The statistic generally employed to measure statistical significance in parametric linkage analysis is the LOD score[113]. It is equal to the logarithm of the likelihood ratio of the data under linkage ($\theta < 0.5$) compared to the data under free recombination ($\theta = 0.5$).

$$LOD = \log_{10} \frac{likelihood(data \mid \theta < 0.5)}{likelihood(data \mid \theta = 0.5)} \qquad (2)$$

For a fully informative pedigree,

$$LOD = \log_{10} \frac{\theta^R (1-\theta)^{NR}}{0.5^N}, \qquad (3)$$

where R is the number of recombinant meioses, NR the number of non-recombinant meioses, and N the total number of meioses.

A LOD score of 3.0 means that the likelihood of the observed pedigree data (including information on pedigree structure, phenotypes and marker genotypes) is 1000 times higher under linkage than under independent assortment. In genome-wide studies (in fact, in any study that is not hypothesis-driven, even if only few markers are tested), the prior probability of linkage at any one marker locus is low. Given this low prior probability, Lander & Kruglyak (1995) have shown that a LOD score of 3.3 needs to be attained in order to declare genome-wide significance of linkage, corresponding to an adjusted p-value of 0.05[114]. In practice, many studies use a threshold of LOD = 3.0 to declare genome-wide significance[112]. A LOD score $\leq$ -2.0 is considered to provide evidence against linkage[113].

The LOD score is a function of several parameters: the pedigree data, the genetic model specified and the recombination fraction, θ. By maximising the LOD score over the different parameters, it is therefore possible to get an estimate of these parameters[112]. This is one of the advantages of the LOD score method compared to other mapping methods. By determining the maximum LOD for a range of recombination fractions, for example, we can get a good estimate of the true θ between marker and disease locus. In addition to measuring statistical significance, the LOD score therefore also allows us to estimate how far the disease locus is from the marker.

The simple LOD score statistic assumes that the disease locus is the same in all families; however, in the analysis of complex diseases, this is an unlikely situation. This locus heterogeneity between families can be accounted for by incorporating an additional variable in the likelihood model. This variable is termed α (for "admixture") and corresponds to the proportion of families that are linked to a potential disease locus. The calculation of this "heterogeneity LOD score" (HLOD) provides a simultaneous test for linkage and heterogeneity[112]:

$$HLOD = \log_{10} \frac{likelihood(data \mid \theta < 0.5, \alpha > 0)}{likelihood(data \mid \theta = 0.5, \alpha = 0)}. \qquad (4)$$

A further extension of the LOD score method is the possibility to analyse co-segregation of the disease phenotype with a map of markers. This allows to overcome limitations imposed by missing genotype data and uninformative markers, and helps localise the disease locus on the marker map[112]. The multipoint LOD score is defined as

$$LOD = \log_{10} \frac{likelihood(data \mid x, \phi_0)}{likelihood(data \mid x = \infty, \phi_0)}, \qquad (5)$$

where φ_0 represents a specific genetic model and x is the location of the disease locus on a specified marker map. The null hypothesis, no linkage, is defined by assuming that the disease locus is not located on the marker map ($x = \infty$).

The LOD score method is a powerful tool for the genetic mapping of monogenic Mendelian disorders; however, in the context of complex diseases it is only of limited use. As explained above, parametric linkage analysis relies on the specification of a genetic model, which can be done with reasonable confidence for Mendelian disorders. For complex traits, the underlying genetic model is far more difficult to estimate. Since model mis-specification can produce misleading results in parametric analysis[112], the use of mapping strategies that are less dependent on model specification has become commonplace in the analysis of complex traits. Affected-only approaches measure the amount of allele sharing between affecteds within a pedigree[115]. These methods are based on the principle that, at a marker locus close to a disease-causing gene, affected individuals should have identical alleles more often than expected by chance. In this context, it should be distinguished between identity by state (IBS) and identity by descent (IBD). While IBS can occur in unrelated

individuals and simply refers to the fact that genotyping methods will produce the same result in both cases, IBD means that the shared allele is inherited from a common ancestor. By definition, this can only occur in related individuals. Allele sharing methods are generally aimed at investigating IBD in affecteds (since the disease locus is also assumed to be IBD); however, some methods use IBS status as a surrogate for IBD, and the distribution of IBS sharing probabilities between two affecteds approaches the IBD distribution for polymorphic markers, such as microsatellites[115].

Because they do not depend on the specification of a genetic model of disease inheritance, allele sharing methods are frequently referred to as *non-parametric linkage* approaches. However, this term can be misleading since these methods are not parameter-free in the statistical sense; they may still depend on the specification of certain parameters, e.g. marker allele frequencies and recombination fractions between marker loci.

A popular statistic measuring the significance of evidence in non-parametric linkage approaches is the NPL score[116]. It is calculated by first determining the amount of allele sharing, either between pairs of affecteds (NPL_{PAIRS}), or between all affecteds (NPL_{ALL}), and then evaluating whether there is significant evidence against independence of marker and disease loci. The NPL_{PAIRS} scoring statistic is defined as:

$$S_{PAIRS} = \sum S_{ij} \ , \qquad (6)$$

where S_{ij} is the number of alleles shared IBD by two individuals i and j. It can be 0, 1 or 2[115]. The significance of this statistic is evaluated by either normalising it and comparing it to a normal distribution, or by generating an empirical p-value by simulation.

The NPL_{ALL} scoring statistic is defined as:

$$S_{ALL} = 2^{-a} \sum_{h} \left[\prod_{j=1}^{2f} b_j(h)! \right], \qquad (7)$$

where a denotes the number of affecteds in the pedigree; h is the collection of alleles generated by taking one allele from each affected individual – there are 2^a possible collections –; $2f$ is the total number of founder alleles in the pedigree; and $b_j(h)$ is the

total number of copies of founder allele j in collection h[115]. Again, the significance of this statistic can be determined by a normal transformation or through simulation. Both the NPL_{PAIRS} and the NPL_{ALL} statistics depend on the inheritance pattern of marker alleles. For most pedigrees, this inheritance cannot be determined unambiguously; the statistics are then calculated and weighted across inheritance patterns. Genome-wide significance is reached for p-values that occur with a probability of 0.05 in a whole-genome scan, or in other words, that occur by chance once in every 20 genome scans[114]. For a microsatellite scan with 382 independent markers, this is equal to a p-value of $0.05/382 = 1.3 \times 10^{-4}$, or a NPL score of 3.88.

While non-parametric approaches are popular in the analysis of complex diseases, some authors have suggested that LOD score-based methods can be more powerful, especially when the LOD score is maximised over several inheritance models, thereby circumnavigating the difficulties caused by model mis-specification. This seems to hold even when accounting for multiple testing[117-121]. However, the reasoning of these authors is mainly based on simulations where complex diseases are modelled as two-locus traits, and it is not certain whether these conclusions can be extended to true polygenic inheritance, such as it is expected to underlie complex human disease.

1.2.4. Linkage Disequilibrium and Association Mapping

1.2.4.1. Linkage disequilibrium as a tool for gene mapping

Linkage disequilibrium mapping is based on allelic association and is a powerful tool for the genetic dissection of complex disease. Allelic association between two loci is observed when a combination of alleles at these loci is more frequent in the population than expected if the loci were independent. Therefore, alleles at two loci are associated when

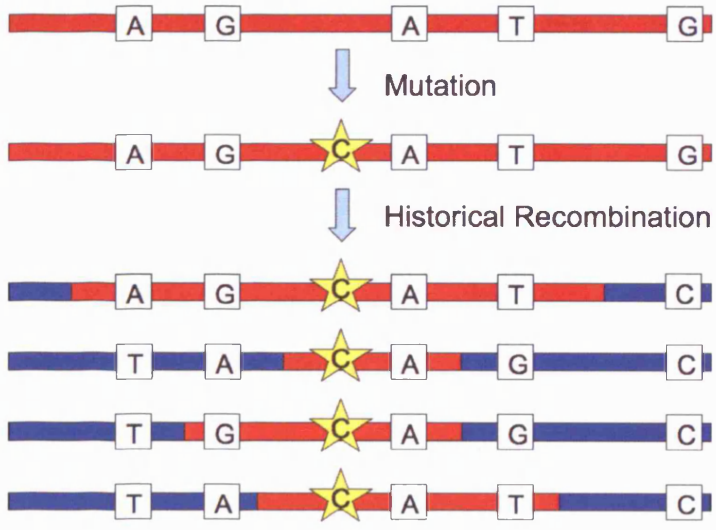
$$f_{AB} \neq f_A \times f_B, \qquad (8)$$

where f_A is the frequency of allele A at locus 1, f_B is the frequency of allele B at locus 2, and f_{AB} is the frequency of the joint occurrence of alleles A and B in a haploid genome (in most cases, and in all cases relevant to linkage disequilibrium mapping, this means the frequency of a haplotype carrying both alleles A and B). *Allelic*

association can occur in linked as well as unlinked loci; it should not be confused with *linkage* which refers to a recombination fraction $\theta < 0.5$ between two loci (see chapter 1.2.3). Allelic association usually arises through the occurrence of a mutation on a particular haplotypic background (see Figure 1.4), the ancestral haplotype. Because each mutational event only occurs once, and because the same mutation is unlikely to occur more than once on different haplotypic backgrounds, this event creates allelic association between the new mutant allele and SNP alleles on the ancestral haplotype.

Recombination between the mutation-carrying haplotype and other haplotypes present in the population gradually leads to the decay of allelic association (see Figure 1.4). This decay is a function of the time that has passed since the original mutational event, and the distance between loci (and the mutation rate; however, mutation rates are so low they are unlikely to contribute significantly to the decay of LD[122]). Because of this pattern of decline, allelic association naturally occurs more commonly between alleles at linked than at unlinked loci. Allelic association maintained by linkage is referred to as *linkage disequilibrium* (LD)[123].

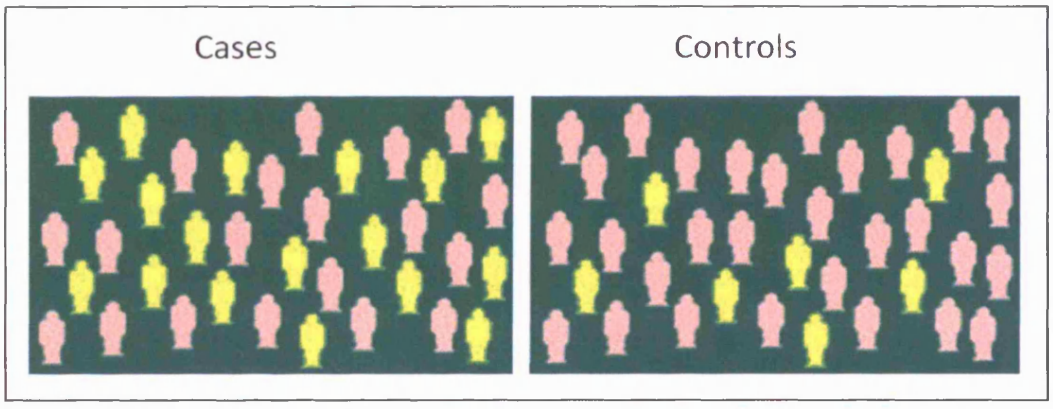
Figure 1.4: Linkage Disequilibrium.



Linkage Disequilibrium arises through the occurrence of a mutational event on an ancestral haplotype (represented by the red horizontal bar). The boxed letters represent alleles at biallelic SNP loci; the star, the mutation, in this case a single base substitution. Historical recombination events gradually lead to the decline of linkage disequilibrium, until in a contemporary population – represented by the four haplotypes, or chromosomes, at the bottom of the figure – complete association can only be observed between the mutation and allele A at the adjacent SNP. Varying degrees of association can be observed with alleles at the other loci.

Allelic association and LD can be exploited to map loci predisposing for common, complex diseases[47,124,125]. The principle of association mapping is that, while there is no straightforward relationship between genotype and phenotype in these diseases, genetic risk variants will occur more frequently in individuals with the disease than in ones without it. By collecting a large enough sample of affected (cases) and unaffected individuals (controls), it is possible to test whether a specific variant is more common in either of the groups and therefore increases or decreases the susceptibility to complex disease (see Figure 1.5). From the design of association studies, it results that, as opposed to linkage analysis, where a locus can be mapped using different families sharing the *same disease locus*, but not necessarily the same mutant allele, mapping strategies based on association rely on affected individuals sharing the *same susceptibility allele*.

Figure 1.5: The principle of association analysis.



Among both affecteds and unaffecteds, there are individuals exposed to a specific genetic risk factor, i.e., individuals possessing a specific susceptibility allele (shown in yellow). Because there is no straightforward relationship between genotype and phenotype, not all individuals carrying the risk-conferring allele will become affected, and not all affecteds will carry the risk allele. However, a significantly larger proportion of cases as opposed to controls will carry the risk allele. Picture courtesy of Andrés Ruiz-Linares.

Association analysis is relatively straightforward if the risk-conferring allele is genotyped and analysed directly. However, because of the very nature of gene mapping studies, it cannot be known whether the variants chosen for study include the risk allele. Additionally, although a vast amount of SNPs have been reported across the human genome (the current build of the NCBI's dbSNP database (no. 128) contains approximately 12 million SNPs; http://www.ncbi.nlm.nih.gov/SNP/;

accessed on 08/04/2008), the risk variant might not be among them, especially if it is rare, or not a SNP. This problem can be circumvented by typing densely spaced genetic markers across a region of interest. If there is a risk allele in that region and if the marker density is high enough, one of the typed markers will be in LD with the risk allele. This LD between marker and risk alleles will allow the detection of association between the marker allele and the disease. Because the marker allele is not the disease causing variant, this is an example of *indirect association*. In Figure 1.4, allele A of the SNP to the right of the disease mutation is in complete LD with the mutant allele; allele A will therefore be found more commonly in affecteds than in unaffecteds although it is not the risk-conferring variant. When association between a genetic variant and a disease phenotype is found, it remains unclear whether this variant is the risk-conferring allele until further studies (preferably of a functional nature) have been undertaken; however, the risk-conferring variant will be located very close-by.

LD mapping can be carried out in different contexts, either as a fine-mapping strategy after an initial whole-genome linkage scan, as a tool for candidate gene analysis, or, thanks to recent advances in genotyping technologies and statistical analysis tools, in a genome-wide approach. From a conceptual point of view, the first two strategies are hypothesis-based, while the last is free of a prior hypothesis (other than that the disease has a genetic component).

1.2.4.2. Measures of linkage disequilibrium

Several statistics have been proposed to measure the amount of LD between markers. One of the earliest statistics, and also the simplest, has been suggested in the 1960s[126] and can easily be derived from equation 8 (see chapter 1.2.4.1).

$$D = f_{AB} - f_A \, x \, f_B \qquad (9)$$

If two markers are completely independent of each other, D is expected to be 0. However, because D depends on the allele frequencies at both markers, its values for different pairs of markers are hard to compare, and it does not provide a very useful measure of LD. Therefore, several other measures have been introduced[127]. A popular measure is D', which describes the observed D in relation to the maximum possible modulus of D given the allele frequencies at both loci.

$$D' = \frac{D_{AB}}{\min(f_A f_b, f_a f_B)} \quad \text{for } D > 0 \qquad (10a)$$

$$D' = \frac{-D_{AB}}{\max(-f_A f_B, -f_a f_b)} \quad \text{for } D < 0 \qquad (10b)$$

D' can vary between 0 (no association) and 1 (complete linkage disequilibrium). A *D'* value of 1 means that there is no evidence of recombination between the two loci in the analysed sample. Because recombination between two bi-allelic loci always results in the creation of all four possible haplotypes (i.e., A-B, A-b, a-B, and a-b), a *D'* of 1 occurs when not all of these possible haplotypes are present in the sample.

The formula of the correlation coefficient r^2 shows a key property of this statistic: it is equal to 1 if, and only if, the two alleles that are in LD also have the exact same allele frequencies; i.e., if only two out of the four possible haplotypes occur in the population.

$$r^2 = \frac{D^2{}_{AB}}{f_A f_a f_B f_b} \qquad (11)$$

Like *D'*, r^2 ranges between 0 and 1; however, these two statistics, which are the most commonly used in the literature, measure slightly different properties of the data: because *D'* always equals 1 for a pair of loci unless there has been at least one recombination between them, the pattern of *D'* in a certain region can be interpreted as an indicator of recombination in that region, whereas r^2 focuses on measuring the correlation between the alleles at two loci. Pairs of loci including a rare allele tend to have a *D'* value of 1 because, due to the rarity of that allele, not all four haplotypes will tend to occur. They are, however, unlikely to have an r^2 value of 1, because the second locus is unlikely to have an equally rare allele.

If *D'* = 1 for a pair of loci, this is referred to as *complete LD*; if r^2 is = 1, this is known as *perfect LD*[122].

1.2.4.3. Patterns of linkage disequilibrium in the human genome

Since Risch and Merikangas predicted the future of complex disease mapping to lie in association-based rather than linkage-based strategies in 1996[47], there has been considerable interest in the extent and patterns of LD in the human genome. As

discussed before, untyped susceptibility alleles for complex diseases can be mapped through their LD with typed marker alleles. The greater the extent of LD in the genome, the lower the number of markers that need to be typed across a candidate region in order to capture the susceptibility variant, thereby reducing the cost of association studies. On the other hand, in fine-mapping studies greater stretches of LD result in a lower average resolution. In either case, the extent of LD levels in the human genome has important implications for genetic association studies.

Results from early studies of LD patterns varied a lot, with some studies providing evidence for high LD over hundreds of kilobases (kb), while others detected very little LD over distances as short as 10 kb. The latter results were corroborated by simulation studies, which predicted that levels of LD high enough to be used for mapping purposes would occur over distances no greater than 5-10 kb[128,129]. These discrepancies triggered a systematic attempt to empirically study LD in unrelated individuals across a range of independent genomic regions[130]. That study found high levels of LD at distances of up to 60 kb in North Americans of European ancestry, but a much shorter range of LD of < 5kb in the Yoruban population from Nigeria, thereby suggesting that the extent of LD in the genome might be population-specific. Like the findings from some of the earlier studies, the observation of long stretches of LD in European Americans found by Reich and colleagues (2001) were in marked contrast to the results obtained in simulations. Pritchard and Przeworski (2001) discussed several possible reasons for these discrepancies, including the fact that local variation in recombination frequency had not been taken into account in the simulations[129]. This point proved to be of great relevance when it was found that indeed, recombination rates vary greatly throughout the genome, and that the majority of recombination events are concentrated in very restricted areas along the genome, the so-called recombination hotspots. Direct evidence for the existence of such hotspots came from single-molecule analysis of the HLA locus in male sperm cells[131]. At the same time, it became evident that the human genome is organised in distinct haplotype blocks of limited diversity, i.e., with few distinct haplotypes accounting for the majority of observed chromosomes, but with high levels of recombination between adjacent blocks[132]. These findings are complementary and provide a simple and plausible explanation for the widely varying estimates of LD extent obtained from previous studies: long stretches with relatively low levels of

recombination and therefore high levels of LD, the haplotype blocks, are interspersed by short sequences with high recombinational activity, the hotspots, which account for the vast majority of cross-overs in the human genome (as much as 80% of recombination occurs in only 10-20% of the sequence)[133]. These hotspots are responsible for "re-shuffling" adjacent haplotypes and cause the abrupt, but not complete[134], decay of LD. Further investigation of LD patterns in the human genome showed the importance of hotspots in both female and male meiosis, the general similarity of LD patterns between different ethnic groups, although with significantly lower levels of LD in populations of African ancestry, and the genome-wide correlation of high LD levels and haplotype blocks[133,135].

The haplotype block structure of the genome has important consequences for genetic association mapping. Because of high levels of LD within blocks, combinations of single-marker analyses can be substituted by haplotype analysis, where each haplotype can be treated as a distinct allele at a multi-locus marker. In addition to reducing the complexity of analysis, this might reflect the underlying population structure better than any single-marker analysis[132]. It has been suggested to use a subset of SNPs in each haplotype block that are in strong LD with the remaining SNPs to capture the complete variation of that block. Such SNPs, which can be typed in representation of the whole haplotype, are called haplotype tag SNPs (htSNPs)[136], or tagging SNPs.

The completion of the first haplotype map of the human genome, the HapMap (http://www.hapmap.org), represented a major landmark on the way to understanding patterns of LD in the human genome[134,137,138]. The aim of its first phase was to characterise the haplotype block structure of the entire genome by typing at least one common SNP [minor allele frequency (MAF) $\geq$ 0.05] every ~5 kb in four different populations: the Yoruba from Ibadan, Nigeria (YRI), North Americans of European ancestry from Utah (CEU), Han Chinese from Beijing (CHB), and Japanese from Tokyo (JPT). Phase I of the HapMap project provided genotype and allele frequency information for these populations on ~1.3 million SNPs. The second phase of the project, completed in 2005, added information for the same populations on a further 3.1 million SNPs to this unique resource, increasing its resolution to ~1 SNP per kb, and the HapMap data is now thought to capture all common genetic variation with an average maximum r^2 between 0.8 (for Africans) and 0.95 (for non-African

populations). Although the project data has shown many similarities between LD patterns in different human populations, there are also important differences, e.g. in allele frequencies and fine-scale LD variation, due to differences in genetic population histories. A third phase of the project, which at the time of writing is ongoing, therefore aims at characterising an additional seven populations (Luhya and Maasai in Kenya, Italians from Tuscany, African Americans, and Gujarati Indians, Chinese and Mexicans from the U.S.A.). The extension of the HapMap to include a wider range of human populations will further promote the understanding of LD patterns in humans and facilitate the marker choice for association studies in populations not included in the current HapMap. Because of their high coverage of the human genome, enabling researchers to choose tagging SNPs capturing most of the variation in any of the available populations, the data from the HapMap project represent an invaluable and very powerful resource for the design of genetic association studies. It is also an important tool in the analysis of association data, where it is often useful to see how far LD extends from a site where association with a disease phenotype is detected, facilitating a more detailed interpretation of association signals in the context of the surrounding genes.

After LD is created by the occurrence of a new mutation on a particular ancestral haplotype, the single most important factor leading to a decrease in LD, and thereby shaping LD patterns in the human genome, is recombination, which, as discussed above, is governed by the occurrence of hotspots. Additional forces contributing to the creation of specific LD patterns are related to the genetic history of a population and include genetic drift, population growth, admixture, migration, population structure, and selection[122]. Some of these factors, such as drift, population growth and admixture, are of particular interest for the work presented in this thesis because of the choice of population in which this work has been carried out. This will be discussed in more detail in chapter 1.3.

1.2.4.4. The "common disease, common variant" and "common disease, rare variant" hypotheses

In complex disease, the effect size of each susceptibility variant is likely to be small, an estimate that has been supported by the recent results obtained in complex disease association studies (e.g., reference 49). However, disease variants might be present at high frequencies within the general population, and it has been pointed out that in spite of their small to modest effect sizes, their population-attributable risk (i.e., the proportion of people affected by the disease due to a specific variant) might be high because of their common occurrence[47]. This hypothesis is often dubbed the "common disease, common variant" (CD/CV) hypothesis[139-141]. A much-cited example of a common allele predisposing for a common disease is the ApoE ε4 allele in Alzheimer's Disease[139,142] (see chapter 1.1.3). A simulation-based approach has been used to explore the competing hypothesis, namely whether complex disease could instead be ascribed to the occurrence of combinations of rare variants (the "common disease, rare variant" hypothesis; CD/RV)[143]. The results of these simulations show that the allelic heterogeneity at putative disease loci depends on several parameters, such as the mutation rate at that locus and the selective pressure on the mutant allele. The results from that study do not contradict the CD/CV hypothesis for loci with a low mutation rate, for which a specific variant represents a major fraction of the disease-causing alleles at that locus; however, loci with a higher mutation rate are shown to be likely to exhibit a more complex spectrum of disease alleles, thereby leading to substantial allelic heterogeneity. Although to a lesser extent than for the CD/CV hypothesis, there is also some empirical support for the CD/RV hypothesis[144,145], and it is well possible that both common and rare alleles play a role in the aetiology of complex disease.

Whether a susceptibility variant is common or rare has important implications for the genetic analysis of complex disease. Because of the nature of association mapping, much larger sample sizes will be needed to detect the effect of rare variants, and allelic heterogeneity can therefore cause a significant loss of power in association studies[143]. The development of more advanced statistical methods for the analysis of association data might overcome this problem, but the methods currently available perform better at uncovering the effects of common alleles than rare ones.

Importantly, this might be one of the reasons why there seem to be more examples of common variants influencing complex disease as opposed to rare ones.

1.2.4.5. Candidate Gene Studies

Before the recent advent of genome-wide association studies, the most common use of LD-based strategies was for the fine mapping of candidate regions after an initial linkage scan, and for the association analysis of candidate genes in complex diseases, applications that remain highly relevant. Candidate genes are chosen for study because of their location within a candidate region identified in a genome-wide linkage scan, based on findings from previous studies, or based on prior hypotheses about their potential function for the aetiology of a disease. They are studied in a straightforward way by typing genetic variants, mostly SNPs, across the gene and testing these variants for association with the disease under study. Although candidate gene studies are very popular and have helped establish genetic risk factors for complex disease, such as *DTNBP1* in schizophrenia[146], they are often criticised for generating false-positive results[147,148]. This might be due to the relatively low thresholds of statistical significance and the small or medium-sized samples often used in these studies, which have been shown to contribute to false-positive results and the overestimation of the effect size, especially in the first report of a candidate gene association, where publication bias is a common issue (i.e., studies with positive results have a better chance of being published in a scientific journal)[149,150]. However, these are not inherent limitations of the candidate gene approach; they can be overcome by careful study design and strict and consistent standards for data analysis. Nevertheless, as with any association study, replication is crucial to lend credibility to the results of candidate gene studies[151].

1.2.4.6. Population stratification and family-based association

Association studies can be done in different settings, the simplest one consisting in a case-control approach where genotype, haplotype or allele frequencies (at a candidate locus, or genome-wide) are compared between a sample of unrelated cases and a sample of unrelated controls. However, a common issue with case-control

studies is the occurrence of undetected population structure within the sample. This is also referred to as *population stratification*; it describes a situation where samples within the same study are – unknowingly – ascertained from different genetic backgrounds, such as different ethnic or religious groups.

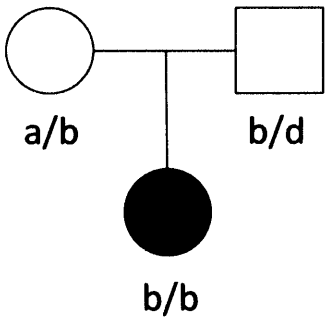
Figure 1.6: Spurious association due to population stratification in case-control studies.

From Marchini et al. (2004)[152]. See text for explanations.

Figure 1.6 shows how such sampling problems can lead to false positive association results. Assume cases and controls are sampled from a population that comprises two distinct underlying sub-populations. Assume furthermore that (1) these two sub-populations differ in their allele (and therefore, assuming Hardy-Weinberg, genotype) frequency distribution at the marker locus of interest, and that (2) the disease prevalence also varies between the two sub-populations, so that they will make up different proportions of case and control groups. In this case, frequencies between the combined case and control samples will vary, although there is no association between the locus tested and the disease[153].

One strategy to overcome this problem is the use of family-based approaches. In the simplest case, association analysis is carried out in a sample of trios consisting of affected individuals and their parents. Consider the trio shown in Figure 1.7.

Figure 1.7: Principle of trio-based association.



See text for explanations.

In this example, the affected daughter has received a copy of allele b from each parent, while alleles a and c, also present in the parents, have not been transmitted. In a large sample of trios, it will be possible to detect if some alleles are transmitted to the affected offspring more often than expected. If there is such preferential transmission of one allele over the other(s), this provides evidence for association between that allele and the disease. This is the principle of the trio design in association studies. At first, this approach might seem somehow counterintuitive because it seems to imply that the disease status of the child determines allele transmission from the parents, when the causal link should be the other way round. However, this is merely an artefact of the ascertainment strategy, which is based on the affected offspring. Importantly, family-based association analysis is robust to population stratification because every transmitted allele is matched to an untransmitted allele from the same individual. Even if the study sample contains individuals of different genetic backgrounds, "case" and "control" chromosomes will therefore always be perfectly matched.

One of the most well-known tests implementing this approach is the Transmission Disequilibrium Test (TDT)[154], which counts the occurrences of paired observations of transmission of alleles of one type and non-transmission of alleles of another type and compares them to the expected values. An extended version of the original TDT, which allows for missing data and makes it possible to analyse association with haplotypes is implemented in the program TRANSMIT [155,156].

1.3. Population isolates and the Paisa Community of Antioquia, Colombia

1.3.1. Population Isolates

The great difficulty in the genetic mapping of complex diseases lies in their heterogeneity at both locus and allelic levels. One of the possible strategies to help reduce this genetic heterogeneity and thereby increase the chances of mapping susceptibility loci for complex diseases consists in the use of population isolates.

In genetics, a population is considered to be isolated if it has not had any substantial admixture with neighbouring populations for a number of generations. This might be due to geographical, socio-political, or religious barriers[157]. Additionally, isolated populations often go back to a limited number of founding individuals, whose gene pool represented a sub-sample of that of their parental population, therefore leading to a reduced genetic heterogeneity in the newly founded population. This phenomenon is known as the founder effect. In the newly established population, genetic drift – the loss or fixation of alleles through random stochastic processes – will further decrease genetic heterogeneity, especially if the population is small, resulting in a noticeably less variable gene pool than that of outbred populations. A variety of studies have successfully exploited this reduced heterogeneity to map genes for Mendelian disorders[158], and as a consequence, a substantial interest has arisen in the use of population isolates for the mapping of complex diseases[159]. As outlined below, the advantages of isolates apply to both linkage-based and association-based methods.

The success of family-based linkage analysis strongly depends on the use of extended pedigrees with multiple affected individuals, or alternatively on the combined analysis of several families segregating the same disease locus (see chapter 1.2.3). Due to the genetic complexity of diseases like schizophrenia and bipolar disorder, it is highly unlikely to obtain a suitable, genetically homogeneous collection of pedigrees from an outbred population. This problem might be dealt with through the use of pedigrees from population isolates. The use of sub-isolates in particular can offer advantages for family-based studies[160]. Sub-isolates are local, often small, populations with restricted gene flow to and from other parts of the

isolate, such as the population of a village in an inaccessible mountainous region. Because of their restricted size, founder effect and genetic drift are stronger here than for the isolate as a whole, and there is often increased inbreeding due to a limited choice of mates. Coupled with a founder effect and genetic drift, inbreeding can lead to the accumulation of particular susceptibility variants in a sub-isolate, and a collection of disease-loaded pedigrees from such a population is likely to be genetically more homogeneous than a collection of pedigrees from an outbred population, thereby increasing the chances of gene localisation through pedigree analysis.

Similarly, population isolates offer advantages for LD mapping. A key prerequisite for association mapping is that affecteds share the same predisposing variants, that is, not only the same loci but more specifically the same allelic variants at those loci. Hence, the reduced heterogeneity found in population isolate can be crucial to the success of an LD study[160]. Additionally, it has long been suspected and recently been shown that isolated populations have increased levels of LD, especially if they have been founded recently ($\leq$ 20 generations ago) and by a limited number of individuals[161]. Longer stretches of LD make it possible to use a reduced number of markers in mapping studies[160], as well as to use the detection of shared (IBD) haplotype signatures in affecteds as an additional strategy in disease mapping[162].

Generally, it has been argued that different populations might be suitable for different purposes[160]. While younger isolates, such as the Colombian province of Antioquia (see below) and the Central Valley of Costa Rica (CVCR) [163], with their high levels of background LD, are thought to be useful for the initial mapping of a candidate region using genome-wide LD approaches, they might prove less useful to narrow down candidate regions. This is because their longer LD blocks offer limited resolution for fine-mapping studies. In this case, the use of older isolates ($<$ 200 generations), also with an increased genetic homogeneity compared to outbred populations, but with less background LD than younger isolates, might be of greater advantage. Examples of such populations include Finland, which has become well-known because of its unique disease heritage – another common characteristic of isolated populations –, and where many Mendelian disease genes have successfully been mapped[158]; Iceland, Sardinia and Japan[160].

Other factors influencing the choice of population for mapping studies are the number of founders – with smaller founder populations leading to an increased genetic homogeneity – and the current size of the population[160]. The latter can be of considerable importance for disease mapping because, if the population is too small, it might not be possible to collect a sufficiently large sample in order to carry out an association study. Linkage-based studies, on the other hand, are less affected by a small population size, as long as there are large disease-loaded pedigrees available for study.

An additional advantage of population isolates in the context of complex disease mapping is their higher environmental homogeneity compared to outbred populations[157]. Members of a population isolate are often exposed to a more uniform environment, including dietary, socio-economical and cultural factors, than members of an outbred population. Environmental factors influence the risk for complex disease and can act as confounders in genetic studies; greater uniformity in the exposure to environmental risk factors can therefore help disentangle environment and genetics in complex disease. This increased cultural homogeneity might apply particularly to sub-isolates.

Complex disease mapping has already yielded promising results in isolated populations. Family-based successes include the localisation of susceptibility loci for high-density lipoprotein cholesterol levels, a trait related to cardiovascular disease[164], and asthma[165] in Finland, for non-syndromic hearing loss in Bedouins[166], and for type 2 diabetes[167,168] and stroke[169,170] in Iceland. The two Icelandic studies used a two-step approach where linkage analysis was followed by population-based association analysis, showing that isolates can offer advantages for both strategies.

Population isolates have also shown their potential for research in psychiatric genetics. The *NRG1* gene, encoding neuregulin, a neuronal signalling protein, and the *DAOA* gene, encoding a protein involved in glutamatergic neurotransmission, both associated with schizophrenia and BP, were first identified in Iceland and French Canada, respectively[171,172]. A series of studies conducted in the CVCR has identified susceptibility loci for bipolar disorder on chromosomes 5q, 8p and 18p and q, using a combination of pedigree- and population-based approaches[173-179]. The studies in the CVCR are of particular interest for this thesis because of its close

genetic and demographic relatedness to the population of Antioquia, Colombia, studied here[180].

1.3.2. The Paisa Community of Antioquia, Colombia

The province of Antioquia is located in the Northwest of Colombia (see Figure 1.9) and lies embedded between the Central and Western ranges of the Andean Mountains. Its population has been founded in the 16th/17th century through admixture of mostly native women and European men of Spanish ancestry[181,182]. Historical and genetic evidence indicates that the admixture process was mostly completed by the mid-17th century, and that subsequent population growth from initially a few thousand individuals to the current size of ~4 million (see Figure 1.8) was fuelled almost entirely from within the population, with no substantial immigration taking place due to the rugged mountainous territory isolating the inhabitants of Antioquia from other local populations[181,182]. During this period of relative isolation, a strong sense of local identity developed in the region, reflected in the term *paisa*, derived from the Spanish word for compatriot, and used in Colombia to describe the inhabitants of Antioquia.

Figure 1.8: Population growth in the Province of Antioquia, Colombia, from 1780 to the 1990s.

Modified from Alvarez (1996)[183]

The original area of settlement of the *paisa* community is not entirely equivalent to the political limits of today's province of Antioquia, shown in Figure 1.9. Instead, *paisa* settlement was concentrated in the coffee-growing regions of today's

Antioquia and also extended to what is nowadays the province of Caldas (also shown in Figure 1.9), whose inhabitants are also part of the *paisa* community. In the following, the term "Antioquia" will be used as a synonym of the *paisa* community.

Figure 1.9: Map of Colombia and the Province of Antioquia

The province of Antioquia lies in the Northwest of Colombia. It consists of nine regions; the province's capital, Medellín, lies in the "Area Metropolitana" Region, the Metropolitan Area. The outlines of the province of Caldas, south of Antioquia and part of the region of original paisa settlement, are also shown on the map of Colombia. The map of Antioquia is taken from http://www.antioquia.gov.co/generalidades/mapas.htm; accessed on 20/05/2006.

Having been founded only ~20 generations ago, Antioquia is a young population isolate and is therefore expected to have increased LD in comparison with older isolates and outbred populations. Because of differences in allele frequencies between the parental populations – Spanish and Native Americans –, the admixture process is an additional source of LD in Antioquia. Increased levels of LD in this population have indeed recently been shown[161]. As discussed in chapter 1.3.1, this increased LD, together with the limited number of founder individuals and the initial small population size, giving rise to founder effect and genetic drift and therefore to a decrease in genetic heterogeneity, makes Antioquia a suitable population for the genetic mapping of complex diseases. Additional advantages of Antioquia in both

complex and Mendelian disease mapping include the relatively common occurrence of large sibships and tight family bonds that make it feasible to localise members of large extended pedigrees.

Within the *paisa* community, the region of Oriente (see Figure 1.9) has been established as a sub-isolate showing reduced mtDNA, Y-chromosomal and autosomal diversity than Antioquia as a whole[182]. The use of multigenerational pedigrees segregating severe bipolar disorder from this region, as well as from a village in Caldas, Aranzazu, whose founders had immigrated from the Oriente region (A. Ruiz-Linares, personal communication), therefore represents a promising approach to the identification of locally enriched susceptibility loci. This has been implemented in a genome-wide microsatellite scan of bipolar families described in chapter 4.

The population of the Central Valley of Costa Rica (CVCR) is demographically related to Antioquia: it originates from related ancestral populations, has been founded around the same time as Antioquia and has also shown a high demographic expansion based almost only on internal growth. Autosomal, Y-chromosomal and mtDNA analyses have confirmed this close relatedness[180], giving rise to the possibility that susceptibility loci for complex diseases might be shared between the two populations. The joint analysis of pedigree data from both isolates has identified a candidate susceptibility region on chromosome 5q31-34[184]. This promising result from the analysis of two closely related population isolates has been followed up within the frame of this thesis (see chapter 3).

1.4. Bipolar Disorder

1.4.1. Clinical Presentation and Classification

Bipolar disorder (BP) is a complex psychiatric condition characterised by severe mood disturbances manifesting as alternating episodes of depression and mania[185]. Depressive episodes in BP show great similarity to recurrent unipolar disorder; they are characterised by low mood and energy levels, loss of interest in activities otherwise enjoyed, decreased self-esteem, changes in sleep patterns (sleeping to much or too little), changes in appetite leading to weight loss or gain, decreased

psychomotor activity and difficulties to concentrate. These changes lead to a marked impairment of everyday functioning. Suicidal ideation is common, and 10-20% of bipolar patients take their own life[185]. Psychotic symptoms, such as hallucinations, paranoia, and delusions, may be present in depression, but are more characteristic of manic episodes. Mania is in many respects the opposite of depression. Its main symptom is extremely elevated mood, accompanied by inflated self-esteem, often leading to risk-taking behaviour, decreased need of sleep and increased energy levels, high activity levels and high levels of psychomotor activity. Patients may experience racing or crowded thoughts, and their ability to concentrate is decreased. During severe mania, everyday functioning is also markedly impaired[186]. While depressive episodes are characteristic of BP, it is the occurrence of at least one episode of mania that serves as a necessary diagnostic criterion for BP.

A commonly made distinction is that between Bipolar Disorder Type I (BP I), which includes at least one episode of full-blown mania, and Bipolar Disorder Type II (BP II), for which a milder form of mania, referred to as "hypomania", is typical. Hypomania shares many features with mania but is less severe and by definition never requires hospitalisation. Many patients describe an increase of goal-directed activities and creativity during hypomanic episodes[186], and artists and writers are over-represented among BP patients[187]. In contrast to severe mania, functioning in hypomania might be increased compared to the euthymic state (i.e., the state of normal mood in between affective episodes), and patients' mood is often characterised by irritability rather than the elatedness typical of mania. Mixed episodes, where symptoms of depression and mania co-occur, can occur in both BPI and BPII[185].

While the differences in the severity of manic episodes, along with further epidemiological evidence, seem to support BPI and II as different subtypes, it is still unknown whether these subtypes are a reflection of true aetiological differences[186,188]. A concept that has gained popularity in recent years views bipolar disorders as a spectrum, or continuum, of conditions with gradual rather than categorical differences between subtypes[189]. This spectrum concept can be applied to different dimensions. One dimension incorporates the different types of mood disorders and extends from BPI over BPII to unipolar depression, while another spectrum-based approach can be used to capture the severity of the disease[190]. A

severe subtype of BP in this dimension is that of rapid cycling, which is defined as the occurrence of four or more episodes of either polarity within one year is generally reported to occur in 10-20% of patients with bipolar disorder, especially BPII[185,191], while a less severe subtype is cyclothymia, in which hypomania alternates with depressive symptoms that do not meet the criteria for a major depressive episode[192]. A possible continuum between mood disorders and schizophrenia has also been suggested[193]. For none of these possible spectra, it has been established with certainty whether a dimensional or a categorical approach is a more accurate reflection of the underlying aetiology. This underlines the nosological and aetiological uncertainties discussed in chapter 1.1.1.

1.4.2. Epidemiology

The lifetime prevalence of bipolar disorder in the general population is often cited as ~1-1.6% [e.g., Müller-Oerlinghausen (2002)][185]; however, these estimates are based on a restricted definition of the disorder, taking into account only the most severe forms of the disease[194] and might therefore be interpreted as estimates of the prevalence of BPI only. This is in agreement with a more recent review of the prevalence of bipolar disorders across European countries, which found estimates between 0.2 and 1.8% for BPI lifetime prevalence[192]. The prevalence of BPII is less well studied. In the past, the administration of structured (and therefore possibly too rigid) diagnostic interviews by lay interviewers is thought to have led to common misdiagnosis of the disorder as unipolar depression, thereby leading to relatively low lifetime prevalence estimates of ~0.5% for BPII. Recent studies focusing on symptoms particularly characteristic for hypomania have resulted in much higher lifetime prevalence estimates of up to ~5% in community samples, while 50% of depressed outpatients are thought to be suffering from BPII[186,194]. The prevalence of bipolar spectrum disorders (including BPI, BPII and cyclothymia) has been estimated at up to 13.5%, although it is likely to be closer to 5%[186].

The prevalence of BPI is the same in both genders, while findings on BPII are more divergent. Some authors report a higher rate of BPII in females than males [e.g., Benazzi (2007)][186], whereas other epidemiological reviews find no differences between the two genders [e.g., Pini et al. (2005)][192]. While the comparison of

epidemiological studies is hampered by their use of different diagnostic instruments and the inclusion of different phenotypes, no evidence for a variation in prevalence across populations could be found in the literature [e.g., Pini et al. (2005)[192] and Wittchen & Jacobi (2005)[195] for Europe].

The age at onset of BPI varies, and it has been suggested that there are several peak ages of onset – 17, 27 and 46 years – for an early, intermediate and late age of onset group, respectively[196]. Other authors do not make this distinction and cite the average age of onset between 20 and 30 years[192,197]. While the average age at onset of BPII does not differ from that of BPI, there has been evidence for a possible gender-effect, with early-onset females more likely to suffer from BPII, and early-onset males more likely to be affected with BPI[197].

Bipolar disorder is often accompanied by other psychiatric conditions, such as alcohol and substance abuse. A study of nearly 400 bipolar patients from North Carolina, U.S.A., has found lifetime drug abuse rates as high as 60%[26], and conversely, significantly elevated rates of mood disorders, including bipolar disorder, have been reported in a cross-European study of drug and alcohol abusers[28]. High rates of comorbidity also exist between BP and anxiety disorders[22], BP and eating disorders[198] and BP and attention-deficit hyperactivity disorder, especially in children[25].

Relatively little research has been conducted on the role of environmental risk factors in BP, although some evidence points towards the importance of stressful life events in precipitating the disease[199,200]. The biggest risk factor for BP, however, is of a genetic nature.

1.4.3. Genetics of Bipolar Disorder

There is a wealth of studies showing that BP runs in families, and numerous twin and adoption studies have provided evidence that this familial aggregation is caused by shared genes rather than a shared environment[201,202]. Heritability estimates for BP are very high (80-90%)[203], and the greatest risk factor for developing BP remains a strong family history of the disease (λ_S, the ratio of the recurrence risk in siblings of bipolar probands to the one of the general population, is 5-10)[203]. In spite of the strong evidence pointing towards the importance of a genetic predisposition to BP,

no locus with a major effect on disease risk has been identified to date. This is almost certainly due to the complex nature of the disease (see chapter 1.2.1), where every predisposing variant is thought to have only a small effect on disease risk. In spite of these difficulties, there are now a range of interesting findings that have been confirmed through replication or in meta-analyses. Some of these findings have arisen from linkage-based studies, others from candidate gene analyses.

Several meta-analyses have been carried out on genome-wide linkage scans of BP. The first meta-analysis in the literature included eleven linkage scans and found genome-wide evidence for linkage on chromosomes 13q and 22q[204], while a subsequent analysis, based on 18 genome-wide scans and using a different methodology, identified suggestive evidence for linkage on chromosomes 9p, 10q and 14q[205]. The most recent meta-analysis for BP is based on eleven studies; the main regions identified here are chromosomes 6q and 8q, both of which reached genome-wide significance for linkage[206]. All three meta-analyses were based on a range of populations; however, the most recent one excluded studies from special populations (e.g., isolates).

While a review of candidate gene studies published as recently as 2006 still concluded that no locus had been consistently implicated in the genetics of BP[203], this picture seems to be rapidly changing. The most recent review of the genetics of BP has identified a number of genes that have repeatedly and consistently been found to be associated with the disease[207]. These include genes that are of importance in the metabolism of the neurotransmitter serotonin, such as the serotonin transporter gene *5HTT* on chromosome 17q11.1-q12 and the gene encoding the neuronal tryptophan hydroxylase (*TPH2*; chromosome 12q21.1), an enzyme involved in serotonin synthesis. Another pathway that might be implicated in the aetiology of BP is that of dopaminergic neurotransmission, with evidence for association between the disease and the *DRD4* gene on chromosome 11p15.5, encoding the dopamine receptor D4, and the dopamine transporter gene *SLC6A3* on chromosome 5p15.3. Promising results have also been obtained for the D-amino acid oxidase activator *DAOA(G72)/G30* locus on chromosome 13q34, which encodes a protein involved in glutamatergic neurotransmission, and the gene encoding the brain-derived neurotrophic factor (*BDNF*) located on chromosome 11p13[207]. Our group has performed a transmission distortion analysis of the *BDNF* gene in a sample of 224

BPI patients from the *paisa* community and their parents and has found an association of a *BDNF* functional variant, and of a two-marker haplotype comprising this variant and a nearby microsatellite, to BP[208] (our article is attached to this thesis in appendix 8.3). Further genes that have repeatedly shown association with BP include the dystrobrevin binding protein 1 (or dysbindin) locus (*DTNBP1*) on chromosome 6p22.3, the neuregulin 1 gene (*NRG1*) on chromosome 8p22-p11, and the *DISC1* (disrupted in schizophrenia) locus on chromosome 1q42.1. These last three genes have also been implicated in schizophrenia, a finding that lends support to a theory of common susceptibility to BP and schizophrenia[16,146,193]. Other promising candidate genes comprise the gene for the monoamine oxidase A (*MAOA*) on chromosome Xp11.23 and the gene encoding the catechol-o-methyltransferase (*COMT*) on chromosome 22q11.2, yet another gene which has also been shown to be associated with schizophrenia.

In spite of these very promising results, there is still a great deal to be learnt about the genetics of BP. Only those genes for which the evidence of association is currently strongest have been included here; this list of candidate genes is not exhaustive, and in the future, other genes might well receive further confirmation as candidates. Furthermore, the findings that are mentioned in this chapter need to be further replicated and confirmed, possibly in different populations, and additional loci remain to be discovered, so that an ever more detailed picture of the pathways of disease can be achieved.

1.5. Schizophrenia

1.5.1. Clinical Presentation and Classification

Schizophrenia is a severe and debilitating psychiatric condition, which often leads to life-long disability. Its symptoms can be grouped into three main complexes: positive symptoms, negative symptoms, and cognitive impairment[209,210]. Different groups of symptoms can dominate over others in different patients, making schizophrenia an extremely clinically heterogeneous disease and giving rise to the notion that it might represent a group of related conditions rather than one disease entity[211].

Positive, or psychotic, symptoms include delusions, hallucinations and bizarre behaviour. Delusions are irrational but firmly held beliefs that are not rooted in the patient's culture, such as delusions of control, where patients believe that their behaviour is controlled by, e.g., aliens, and paranoid delusions, where patients have a groundless fear of being persecuted, or the target of a conspiracy. Hallucinations, sensory perceptions that seem real to patients but cannot be perceived by others, are most commonly of an auditory nature ("hearing voices")[210], but other types of hallucinations, such as visual and olfactory ones, also occur[209].

The domain of negative symptoms is characterised by social withdrawal, lack of pleasure, poverty of thought, avolition (i.e., the lack of initiative and perseverance) and blunted affected[209,212]. While the symptoms of psychosis often occur in episodes, with significant inter-episode recovery, negative symptoms are more stable in nature and show greater persistence between psychotic episodes[209]. A subtype of schizophrenia, dominated by persistent negative symptoms and poor long-term outcome, has been suggested (the "deficit syndrome" of schizophrenia)[213], and more recently, it has even been put forward that the deficit syndrome might represent a distinct disease entity[214]. As it occurs so often in psychiatric nosology, this putative disease entity has neither been disproved nor confirmed, and the true situation might yet again best be approximated by a disease spectrum concept.

The third symptom complex in schizophrenia, cognitive dysfunction, includes working memory defects, problems with verbal and visual learning, deficits in concentration, and the impairment of problem-solving capacities and abstract thinking[209,212].

Taken together, these three symptom complexes affect almost every aspect of psychosocial functioning. Although the severity and features of the course of illness, such as age of onset, inter-episode recovery, and prominence of symptoms, may vary between patients, schizophrenia is always a very debilitating condition, which in most cases leads to chronic disability and the need of life-long psychiatric care[212].

Schizophrenia and BP are both characterised by the occurrence of psychosis, indicating that the two disorders might share part of their aetiology. This is further stressed by the fact that several genes have been found to be associated to both BP and schizophrenia[16,146,193] (see chapter 1.4.3), as well as by the occurrence of both schizophreniform and affective symptoms in a disease phenotype called schizoaffective disorder (SAD). Although SAD is often classified as a schizophrenia spectrum disorder, it has been suggested that it should be regarded as an affective disorder with psychotic features[215], thereby bridging the gap between affective disorders and schizophrenia and lending support to the idea of a spectrum of psychiatric illness encompassing both mood disorders and schizophrenia.

1.5.2. Epidemiology

The lifetime prevalence of schizophrenia lies at around 0.45%[216]. There is an ongoing debate about whether the prevalence varies between populations[216,217], and the figure cited above is the median of the prevalence estimates from nearly 200 studies conducted in 46 populations from all continents[216]. There are some well-documented cases of populations with a particularly high (e.g., second-generation Afro-Caribbean immigrants in the UK) or low (e.g., the Hutterites in North Dakota, U.S.A.) prevalence[211,217]; however, a lot of the inter-population variation is more subtle and might, at least partly, be the result of methodological problems.

Schizophrenia is equally common in both sexes, but male sex is associated with lower levels of pre-morbid functioning, a younger average age at onset, and a more severe course of illness[209,217]. Typically, the onset of the disease occurs in late adolescence or early adulthood, between 16 and 30 years of age, and onset after 40-45 years is rare[209].

A number of environmental factors have been found to influence the risk of schizophrenia. A higher risk of developing the disease has been associated with

winter/spring births[218,219], urban upbringing[218,220], and complications during pregnancy and birth[221,222], and an increased prevalence of schizophrenia is observed in immigrants[223,224]. As for BP, however, there is strong evidence for the importance of genetic factors in the development of schizophrenia.

1.5.3. Genetics of Schizophrenia

Family, twin and adoption studies have provided significant evidence for a genetic component to schizophrenia[225], and as for BP, the heritability of the disorder is in the order of 80-90%[226]. Like BP, schizophrenia is a complex disease – genetically as well as clinically –, and a polygenic susceptibility model with many loci conferring low to medium risk has been suggested as early as the 1960s[227].

Despite the polygenic nature of the disease, several candidate regions and genes have now received substantial support through replication and/or meta-analyses. Most candidate loci for schizophrenia have emerged from positional cloning – namely through the follow-up of linkage studies –, but association studies of functional candidates have also shown some success.

To date, there have been two meta-analyses of genome-wide linkage studies in schizophrenia. Badner and Gershon (2002)[204] included 18 linkage scans from different populations and found significant support for loci on chromosomes 8p, 13q and 22q. The second published meta-analysis included 20 genome-wide scans, also from a range of different populations, and used a different methodology[228]. It found significant evidence for a susceptibility locus on chromosome 2q. A range of other regions were also strongly supported, although they did not reach genome-wide significance. These included a second locus on chromosome 2q and regions on chromosomes 1, 3p, 5q, 6p, 8p, 11q, 14, 20p, and 22. The great amount of candidate regions supported in the latter meta-analysis underlines the amount of heterogeneity in schizophrenia susceptibility, as does the lack of agreement between the two meta-analyses.

A number of candidate genes in schizophrenia have been found through the follow-up of linkage signals. The two loci for which an implication in schizophrenia is currently most strongly supported, the neuregulin gene (*NRG1*) and the dysbindin

gene (*DTNBP1*), have been identified through such positional cloning approaches. *NRG1* is localised on chromosome 8p22-p11, a locus that was identified through linkage analysis in a collection of Icelandic pedigrees[171]. There is some evidence that *NRG1* might be involved in glutamatergic neurotransmission; however, the locus encodes ~15 different proteins with a variety of functions in the brain, so that it might influence the aetiology of schizophrenia through a different pathway[229]. The *DTNBP1* locus on chromosome 6p22.3 has been identified by following up a signal from a genome-wide linkage study of Irish schizophrenia families[230,231]. As for *NRG1*, its function remains unclear, but it has also been suggested to be involved in glutamatergic neurotransmission[229]. Additional genes identified through positional cloning include the *DAOA* gene on chromosome 13q33-34 (also called *G72*) and the gene for the nitric oxide synthase 1 associated protein (*NOS1AP* or *CAPON*) on chromosome 1q23.3. Interestingly, the proteins encoded by both these loci also play a role in glutamatergic neurotransmission, in agreement with several other lines of evidence pointing towards an implication of this pathway in schizophrenia[232]. An association analysis of the *NOS1AP* locus with schizophrenia in the *paisa* community has been carried out as part of this thesis; it is described in chapter 5, and the glutamatergic hypothesis of schizophrenia will be discussed in greater detail there. Finally, the regulator of G protein signalling 4 (*RGS4*) gene, located close to *NOS1AP* on chromosome 1q23.3 and encoding a GTPase activator involved in G-protein mediated neuronal signal transduction, is another interesting candidate locus identified through positional cloning that has been found to be associated with schizophrenia[233].

Schizophrenia has been associated with a number of chromosomal abnormalities. Individuals with Velo-cardio-facial Syndrome (VCFS), caused by a microdeletion on chromosome 22q11, have an increased risk for schizophrenia, an observation that has prompted the search for susceptibility genes in that region. Several genes located in the region of the deletion have been implicated in the disorder, most prominently so the catechol-o-methyltransferase (*COMT*) gene. It encodes a dopamine metabolising protein, a functional variant of which has repeatedly been found to be associated with schizophrenia[234]. Because of its location on chromosome 22q11 and its involvement in dopaminergic neurotransmission, another pathway implied in the aetiology of the schizophrenia, *COMT* is an excellent candidate gene, and the evidence for its

importance in disease susceptibility has grown in recent years[234]. Further genes identified in the 22q11 microdeletion region include the *PRODH* locus, which encodes a proline dehydrogenase that metabolises L-proline, and *ZDHHC8*, the gene product of which interacts with postsynaptic density protein 95 (PSD-95). Both gene products are involved in neurotransmission processes in the brain and therefore represent plausible candidate genes for schizophrenia[233].

Linkage analysis of a large Scottish pedigree found co-segregation between psychopathology including schizophrenia and a balanced translocation between chromosomes 1q and 11q (1q42.1;11q14.3)[235]. Sequencing of the translocation breakpoint identified the *DISC1* locus (disrupted in schizophrenia 1) on chromosome 1q42.1, a locus that has subsequently been found to be associated with schizophrenia. As for all of the loci mentioned here, there have also been negative association studies for *DISC1*; the overall evidence suggests, however, that the locus is a good candidate for causing susceptibility to schizophrenia[233]. The function of the DISC1 protein is not yet fully understood, but it is known that it is associated with a number of cytoskeletal proteins and might influence cell architecture[233] and thereby possibly synaptic function[236].

Most functional candidates have arisen from neuropharmacological studies[225]. Overall, they seem to have been less well replicated than positional candidates, but there are a few genes that have received support from meta-analyses. Among them are the serotonin receptor gene *HTR2A* on chromosome 13q14-q21 and the dopamine receptor genes *DRD2* and *DRD3* on chromosomes 11q23 and 3q13.3, respectively[146].

As for BP, the list of candidate genes in this chapter is not exhaustive. Association with schizophrenia has been reported (but not necessarily replicated) for many different candidate genes and only the most convincing findings are discussed here. As for BP, a lot of work remains to be done before we can even begin to understand the complexity of the genetics of schizophrenia, but the progress that has been made over the past decades is very encouraging for future work on the genetics of schizophrenia.

1.6. Psychiatric Genetics in Antioquia and the Central Valley of Costa Rica

Our group has been conducting psychiatric genetics studies in the population isolate of Antioquia for more than ten years. During this time, we have established an excellent collaboration with a team of clinicians at the Department of Psychiatry at Universidad de Antioquia (U de A) in Medellín, who in the course of, and inspired by, our joint work founded the Group of Psychiatric Investigation (GIPSI), with whom we continue to collaborate. Under the leadership of Drs Jorge Ospina, Jenny García and Carlos López, the clinical team has collected large case samples for different psychiatric disorders, including bipolar disorder, schizophrenia, alcoholism, and autism. All of these collections include several hundred cases and are constantly expanded. Both extended pedigrees and sporadic cases are available for most of the conditions studied by our groups, thereby enabling us to use both linkage and association approaches in our studies.

An integral part of the collaboration with Universidad de Antioquia is our special connection to the Molecular Genetics Laboratory (Genmol), which was established by Professor Ruiz-Linares whilst still in Medellín, and which continues to be under his leadership, in tight collaboration with Professor Gabriel Bedoya from U de A. The population genetics research conducted by our groups in London and Medellín has provided, and continues to provide, the basis for our research in psychiatric genetics[180-182,237,238]. Furthermore, as part of the studies of the population of Antioquia, our colleagues at Genmol have collected large samples of healthy population controls, which are available for use in case control studies.

Our groups in London and Medellín also have a longstanding collaboration with Professor Nelson Freimer and his research group at UCLA, who have established a psychiatric genetics project in the Central Valley of Costa Rica, an isolated population that is genetically very similar to that of Antioquia[173,180,239]. The parallel study of two closely related population isolates holds the promise of maintaining the advantages of a genetically relatively homogeneous population while at the same time facilitating the access to a greater amount of samples for both family and population-based association studies. Laying the foundations for our joint research in psychiatric genetics, our groups have collaborated on a number of projects aimed at

investigating the genetics of the populations of Costa Rica and Antioquia[180], the patterns of linkage disequilibrium in population isolates[161], and the genetics of admixture in Latino populations[240].

An important condition for the genetic study of psychiatric disorders is the availability of a reliable diagnostic tool, such as the Diagnostic Interview for Genetic Studies (DIGS)[241], a semi-structured interview for the use in research studies, which was originally developed in English. In order to be able to apply this valuable tool in the Spanish-speaking populations of Antioquia and the CVCR, our group has published a validated Spanish translation, which is now widely used[242].

For many years, the focus of our collaborations with U de A, UCLA and Costa Rica has been bipolar disorder. We have performed several association studies, linking a polymorphism in the promoter region of the 5-HTTL serotonin transporter, as well as the *BDNF* gene to the disorder[208,243], and our most important project so far in the genetics of BP is a genome-wide linkage scan of six extended pedigrees from Antioquia segregating severe bipolar disorder[184]. In this study, we have identified several candidate regions for BPI, including regions on chromosomes 1p, 3q, 21q, and chromosome 5q. This last region was also implicated in the genetic susceptibility to the disorder in a large Costa Rican pedigree ascertained and studied by our collaborators[178,244]. Follow-up studies, conducted in pedigrees and trio samples from both Antioquia and the CVCR, continue to support the presence of a BPI susceptibility locus on chromosome 5q[184,245], although it might be of greater importance in the CVCR than Antioquia. As part of this thesis, I have conducted a candidate gene study of the *CLINT1* gene on 5q33 in Antioquia, the results of which are presented in chapter 3. I have also carried out a genome scan in an additional nine BPI families from Antioquia and conducted joint analysis of both our previous linkage scan and the one performed as part of this thesis. This work is presented in chapter 4.

In an attempt to direct our studies towards a more clearly defined phenotype and thereby further reduce trait heterogeneity, our groups have now embarked on an ambitious project aimed at the identification and study of BP endophenotypes, involving both study sites, Costa Rica and Antioquia. We are currently also collaborating on a whole-genome association analysis of TS as part of the Tourette Syndrome Association International Consortium for Genetics.

The work conducted for this thesis therefore represents the continuation of a long and fruitful collaboration between researchers and clinicians from several continents and should be viewed in this context.

1.7. Thesis Overview and Aims

The central aim of this thesis is to make a contribution to the identification of genetic factors that confer susceptibility to two complex neuropsychiatric conditions, bipolar disorder and schizophrenia. The approach taken here is to perform gene mapping studies in a well-characterised population isolate, the *paisa* community of Antioquia, North West Colombia, thereby reducing the heterogeneity that complicates the genetic analysis of complex diseases.

Within this scope, the specific aims of this thesis are:

1. To follow up, by sequencing and association analysis, the most promising signal from a whole-genome linkage scan of six families segregating severe bipolar disorder (BPI) from Antioquia conducted in our group[184]. (Chapter 3)
2. To carry out a linkage scan of an additional nine BPI families from the *paisa* community, and to conduct joint analyses of the combined autosomal data sets of first and second linkage scans (i.e., the published scan[184] and the one undertaken for this thesis). (Chapter 4)
3. To carry out an association analysis of the *NOS1AP* gene and schizophrenia in a trio sample from Antioquia, incorporating a categorical as well as a dimensional approach to the phenotype. (Chapter 5)

CHAPTER TWO

SUBJECTS AND METHODS

2. Subjects and Methods

This chapter provides a general description of the subjects studied and the methods used in this thesis. Descriptions of specifically designed assays and specialised statistical methods that were of importance for specific projects can be found in the corresponding chapters, along with any reaction protocols deviating from the standard ones presented in this chapter.

2.1. Subjects

All patients studied for this thesis originate from the *paisa* community of North West Colombia[180-182] (see chapter 1.2.4). The gene mapping project in bipolar disorder is based on both linkage and association approaches; therefore, a sample of extended pedigrees as well as a collection of nuclear families (mainly trios) for family-based association analysis were studied. The schizophrenia project is based on an association approach only; here too, a study design based on nuclear families, mainly trios, was used.

2.1.1. Patient Ascertainment and Diagnostic Procedure

Patients with clinical diagnoses of bipolar disorder or schizophrenia were recruited in the municipalities of Medellín and Envigado (Antioquia, Colombia) at Hospital Mental de Antioquia, Hospital San Vicente de Paúl, Clínica Sameín, Clínica Insam, and the Mental Health Centre of Envigado.

Both BP and schizophrenia are clinically heterogeneous disorders, and schizophrenia in particular can present with so many different symptom constellations that two patients may not share a single symptom[246,247]. Because phenotypic variation might reflect underlying genetic heterogeneity, which can mask linkage and association signals, it is crucial for the success of a gene mapping study to limit this variation as much as possible. One approach is to use a narrow and clear-cut definition of the phenotype. In the case of bipolar disorder, only individuals affected with bipolar disorder type I, the most clinically homogeneous type of the disorder, were therefore

recruited as probands (however, relatives of BPI patients with different psychiatric diagnoses were studied in the context of the pedigree based linkage study, see below). In the case of schizophrenia, no other spectrum phenotypes were included.

To further ensure minimal diagnostic variation for both diseases, we aimed at assessing all patients following a standardised best estimate diagnostic procedure: As a first step, patients would be assessed by a psychiatrist using the Spanish version of the Diagnostic Interview for Genetic Studies (DIGS version 3) previously validated in Colombia[241,242]. During the DIGS interview, it would also be established whether the patient had experienced psychotic episodes. The DIGS as well as all available clinical records would then be revised by two further, experienced psychiatrists (the best estimators) who were each required to reach an independent diagnosis based on DSM-IV-TR criteria[11]. A final diagnosis would then be obtained through consensus between the best estimators. In case no consensus diagnosis could be reached, a third psychiatrist would be consulted; failing this, the subject would be excluded from further study.

While most patients involved in this study were diagnosed based on the strict best estimate procedure described above (including all sporadic samples used for association analysis in BPI and schizophrenia), for some patients from the extended BPI pedigrees, the diagnostic procedure has not yet been completed and a definite best estimate diagnosis has not yet been established. However, all living, genotyped patients studied have been seen and interviewed by an experienced psychiatrist involved in this study. In all cases, the diagnoses for these patients are based on the DIGS interview and follow DSM-IV-TR criteria. See Figures 2.1 and 2.2 for details on which patients do not have a best estimate diagnosis.

Two affected individuals included in the study (both part of the BPI pedigree collection) were deceased at the time of the analyses; however, they were interviewed when still alive and were diagnosed according to the criteria outlined above.

To ensure the population homogeneity of the sample, an additional inclusion criterion required at least six out of the patients' eight great-grandparents to be of Antioquian origin. For this reason, a genealogical interview was conducted with all patients. Furthermore, as part of the genealogical interview, the patients and/or their

family members were asked for information about possible further cases of the disease under study, or any other psychiatric diseases occurring in the immediate or extended family. For the bipolar study, families with at least three cases were then chosen for pedigree extension using the Family Interview for Genetic Studies (FIGS) [241], applied by an experienced social worker (a single family included in this study, ANT24, only has two BPI cases; it was included because it had an additional case of major depression and one case of schizophrenia). The other, sporadic BPI cases were included in the trio sample. For schizophrenia, only a trio sample was collected.

Following the FIGS, all potentially informative members of the extended pedigrees, possible cases as well as their relatives, were invited to participate in the study and, upon agreement to do so, assessed following the diagnostic procedure described above. Again, diagnoses were ideally based on a best estimate, but in some cases they were reached by a single experienced psychiatrist after revision of the DIGS and all available medical records (see Figures 2.1 and 2.2). All resulting psychiatric diagnoses were recorded, including bipolar disorder type I and II, major depression and any other mood disorders, schizophrenia and schizophrenia spectrum disorders, Gilles de la Tourette Syndrome, and substance abuse.

Any psychiatric diagnoses in patients with mental retardation and/or neurological lesions, as well as for cases of substance abuse, were disregarded, and such patients were excluded from the trio sample. For linkage analysis, even individuals with uncertain diagnostic status can be informative as they can provide haplotype phasing information and help infer missing parental genotypes. For this reason, patients with mental retardation and/or neurological lesions as well as cases of substance abuse could be included in the pedigree sample to help the reconstruction of missing data; however, their affection status was set to unknown. All schizophrenic patients were in remission during the entire assessment procedure.

A written informed consent was obtained from all subjects prior to enrolment in the study.

2.1.2. Extended Bipolar Pedigrees

Extended pedigrees from the Antioquian population segregating BPI were identified through probands with a clinical diagnosis of bipolar disorder as described in chapter 2.1.1. Altogether, patients from 15 families were studied for this thesis. While all probands were initially identified in the city of Medellín, 13 of these families originate in the Oriente region of Antioquia (family code beginning with ANT), and the two remaining families are from Aranzazu in the province of Caldas, Colombia (family code beginning with FAZU).

Of the 15 families studied in this thesis, six (all from the Antioquian Oriente region) had been genotyped and analysed as part of a previous genome-wide linkage scan for BPI[184] (see Figure 2.1). The data resulting from that first linkage scan were re-analysed together with the data for the nine remaining families (see Figure 2.2), which were obtained as part of this thesis (see chapter 4.1).

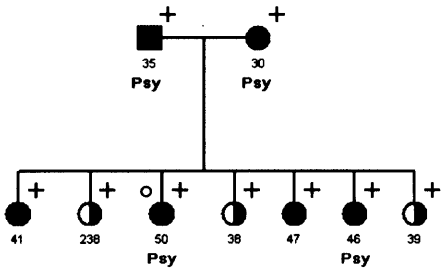
A total of 161 individuals were included in the pedigree sample. Although the families were ascertained through the recruitment of probands with a DSM-IV-TR diagnosis of BPI, there were a number of different psychiatric diagnoses amongst their relatives, most notably unipolar major depression (MD). The sample comprised 90 cases of BPI (37.8% males, 62.2% females), 22 cases of MD (18.2% males, 82.8% females), and one case each of BPII (one male) and schizophrenia (one female). Other diagnoses included alcohol and drug abuse and dysthymia; however, these phenotypes were not analysed in the linkage study and are therefore not discussed any further. Reliable information on age at onset was available for 62 out of the 90 BPI cases, and for 10 out of the 22 cases of MD. The average age at onset (± standard deviation) for BPI and MD were 22.2±7.6 years and 26.6±13.8 years, respectively. The age at onset for the schizophrenia patient was 27 years, and there was no reliable information about the age at onset for the BPII patient.

A summary of pedigree characteristics can be found in Table 2.1, and the pedigrees are shown in Figure 2.1 [pedigrees genotyped by Herzberg et al. (2006)[184]] and Figure 2.2 (pedigrees genotyped as part of this thesis).

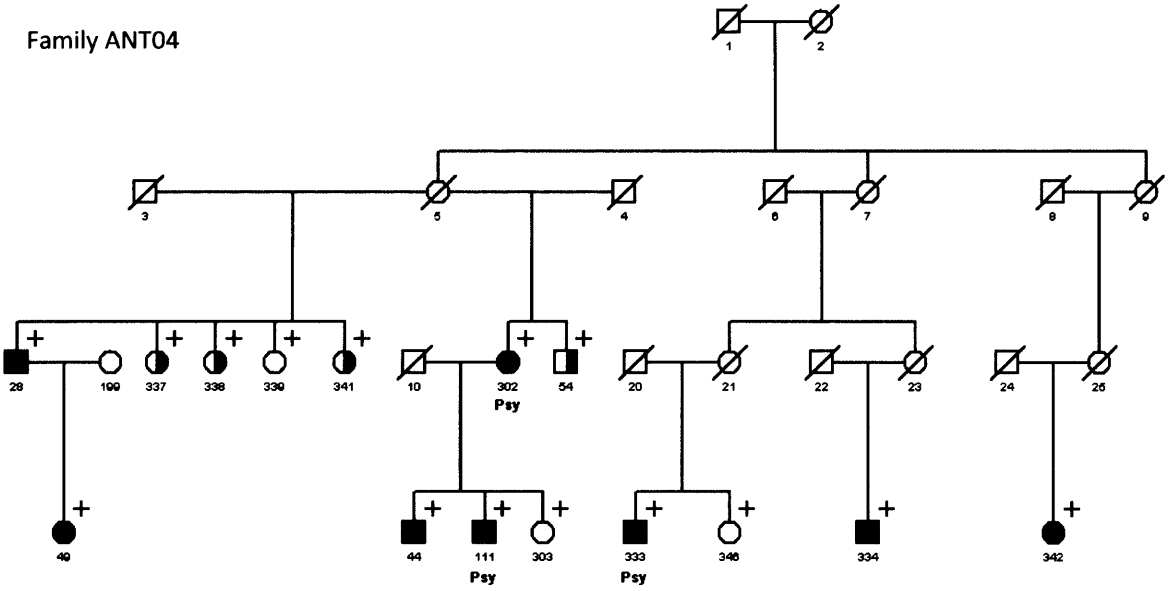
Table 2.1: Pedigree characteristics. For each pedigree, the number of individuals typed, and the number of patients with a specific diagnosis is shown. The number in brackets indicates how many of these cases have a best estimate diagnosis. MD, major depression; SCZ, schizophrenia. It is also indicated whether genome-wide genotype data were collected as part of this thesis.

Pedigree no.	# individuals typed	BPI	BPII	MD	SCZ	Psychosis	Genotype data collected as part of this thesis?
FAZU01	16	6 (3)	0	2 (1)	0	1	yes
FAZU28	12	7 (3)	0	0	0	2	yes
ANT03	9	6 (5)	0	3 (3)	0	2	no
ANT04	15	8 (8)	0	4 (4)	0	3	no
ANT07	26	13 (12)	1 (1)	5 (3)	0	8	no
ANT10	8	4 (2)	0	3 (3)	0	1	yes
ANT14	10	7 (7)	0	1 (1)	0	7	no
ANT15	5	3 (3)	0	0	0	2	yes
ANT18	14	6 (6)	0	0	0	3	no
ANT19	6	4 (4)	0	1 (1)	0	1	yes
ANT21	6	5 (3)	0	0	0	3	yes
ANT23	8	6 (5)	0	2 (2)	0	3	yes
ANT24	5	2 (1)	0	1 (0)	1 (1)	2	yes
ANT26	4	4 (1)	0	0	0	0	yes
ANT27	17	9 (9)	0	0	0	5	no
Total	161	90 (72)	1 (1)	22 (18)	1 (1)	43	-

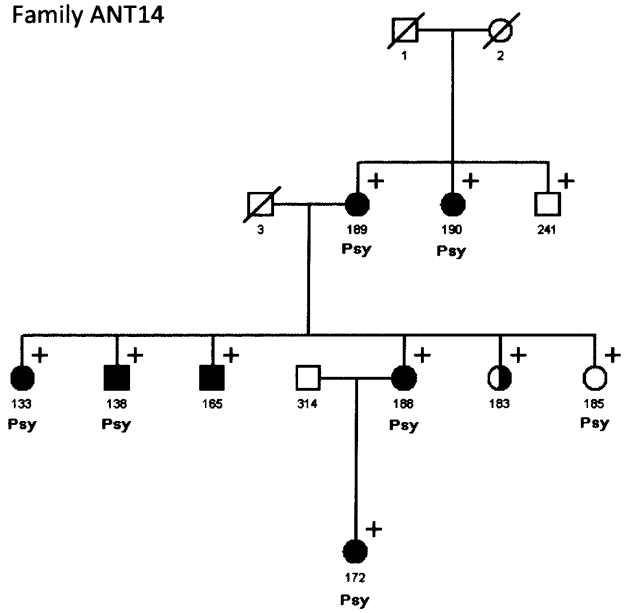
Family ANT03



Family ANT04



Family ANT14



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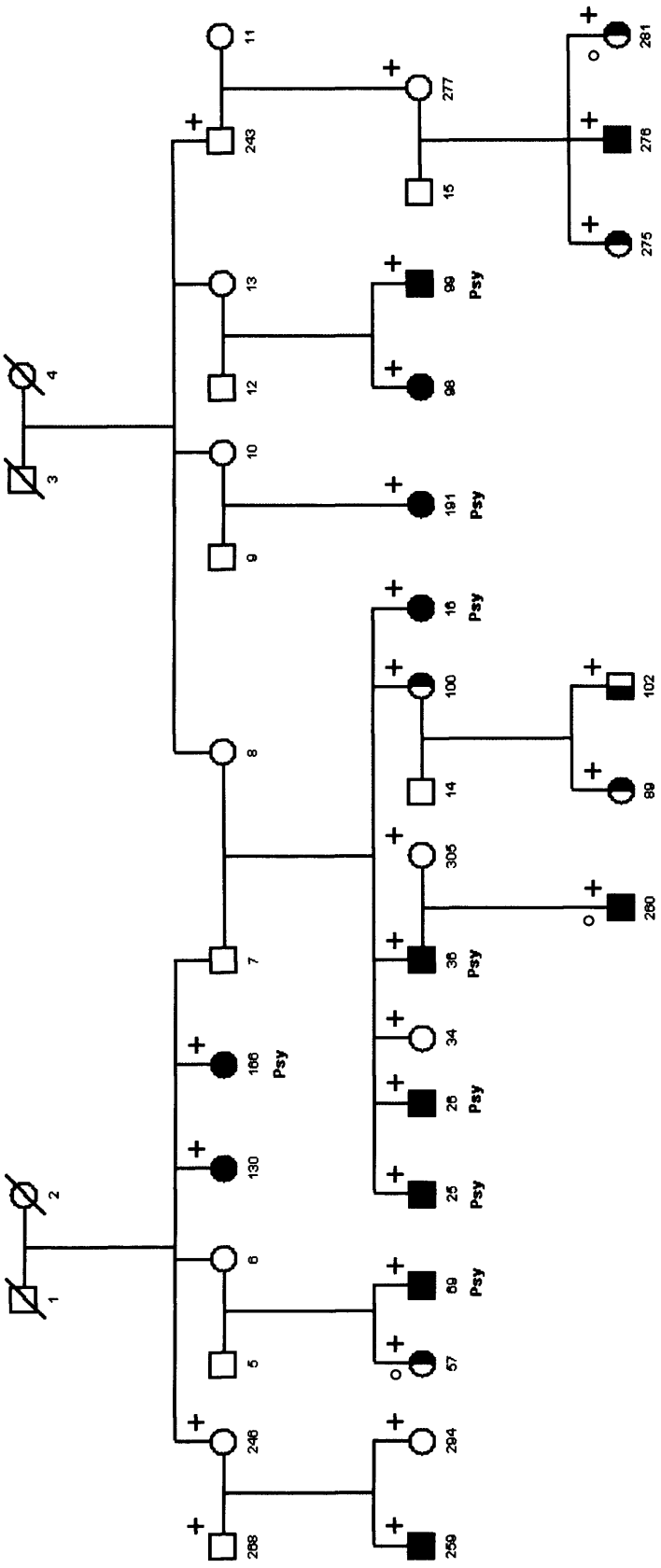
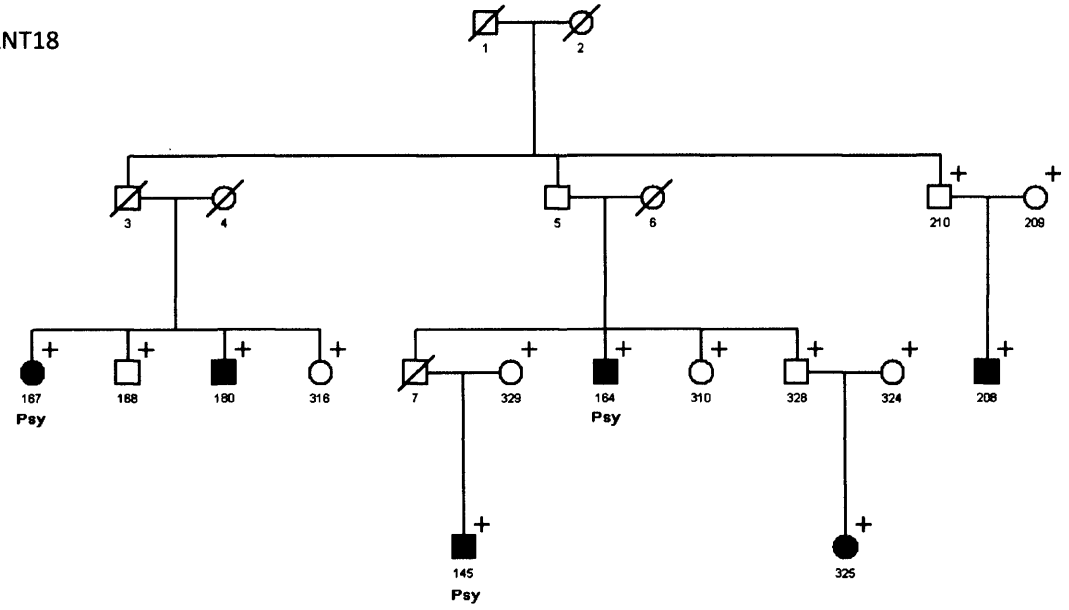


Family ANT18



Family ANT27

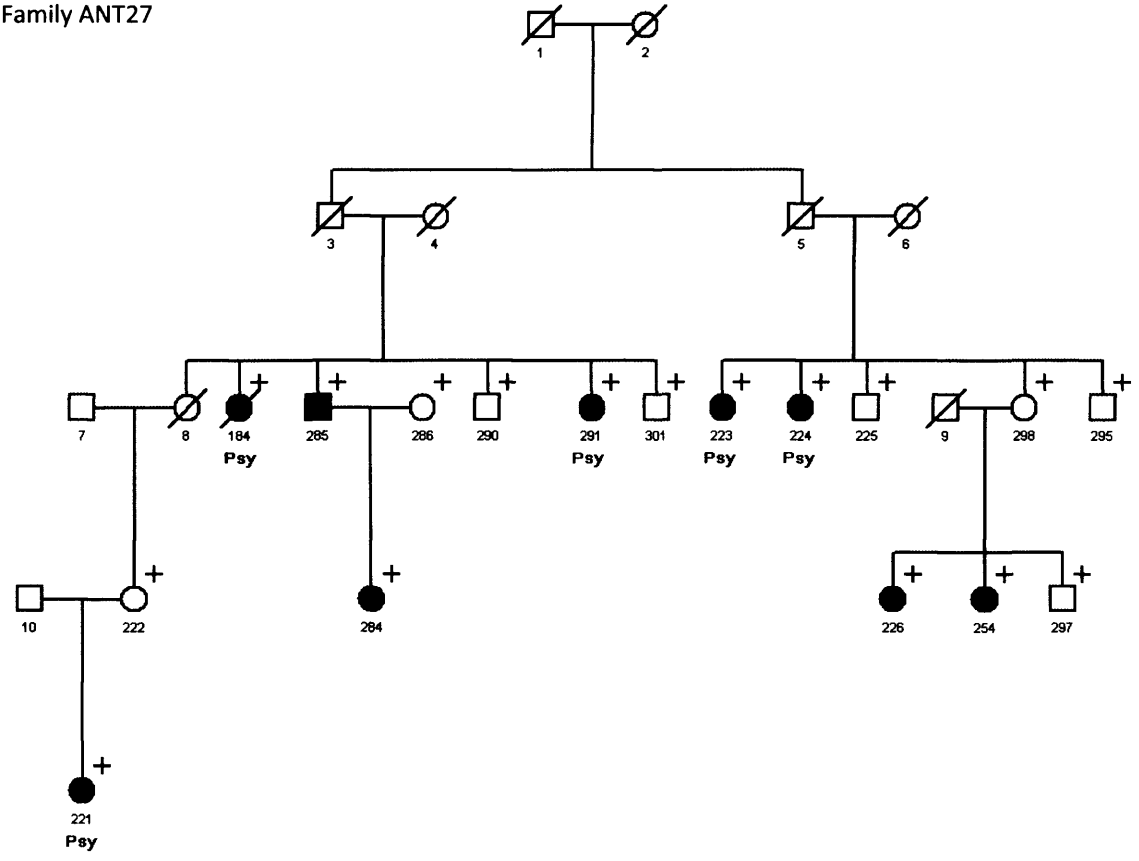
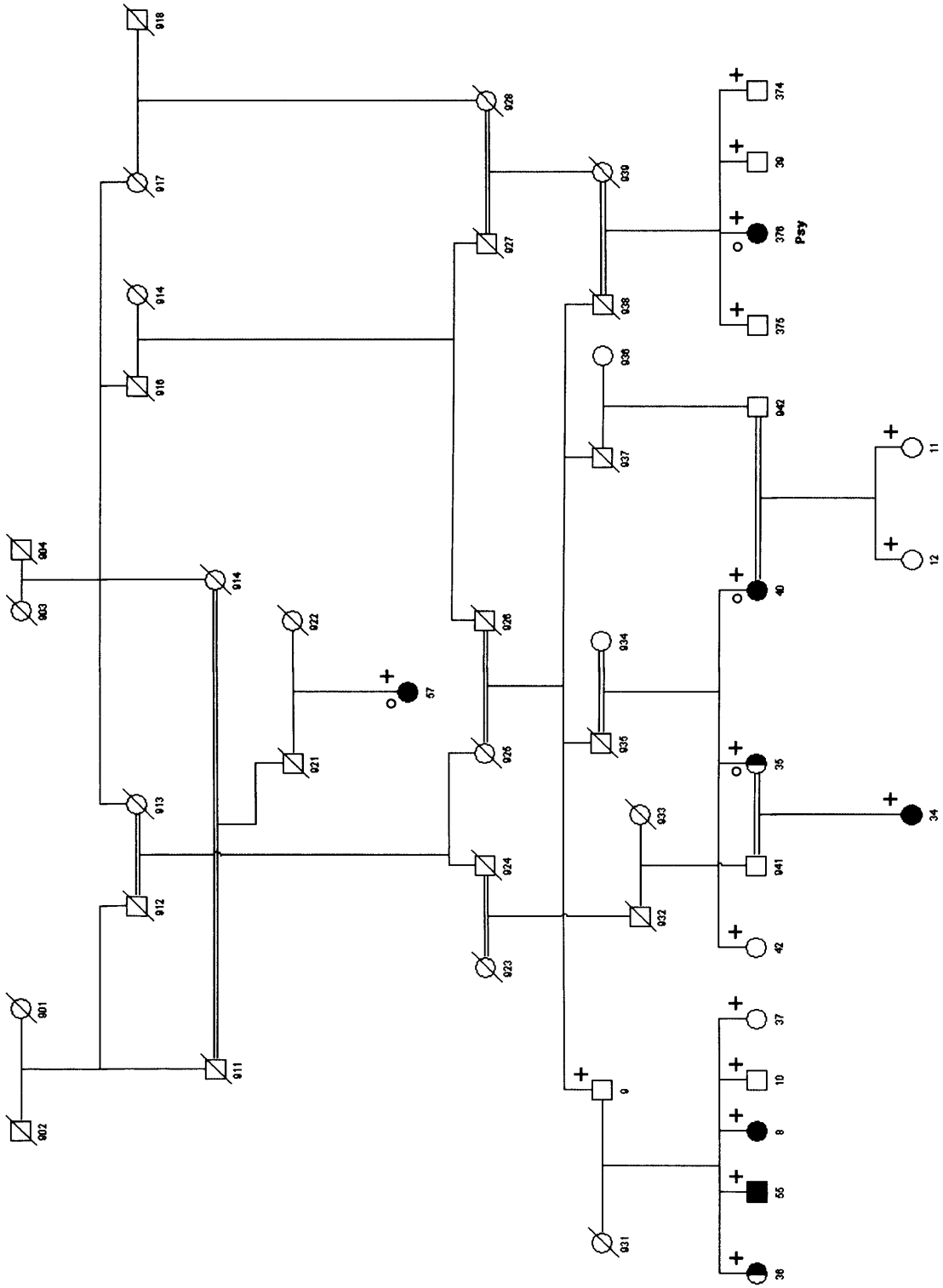
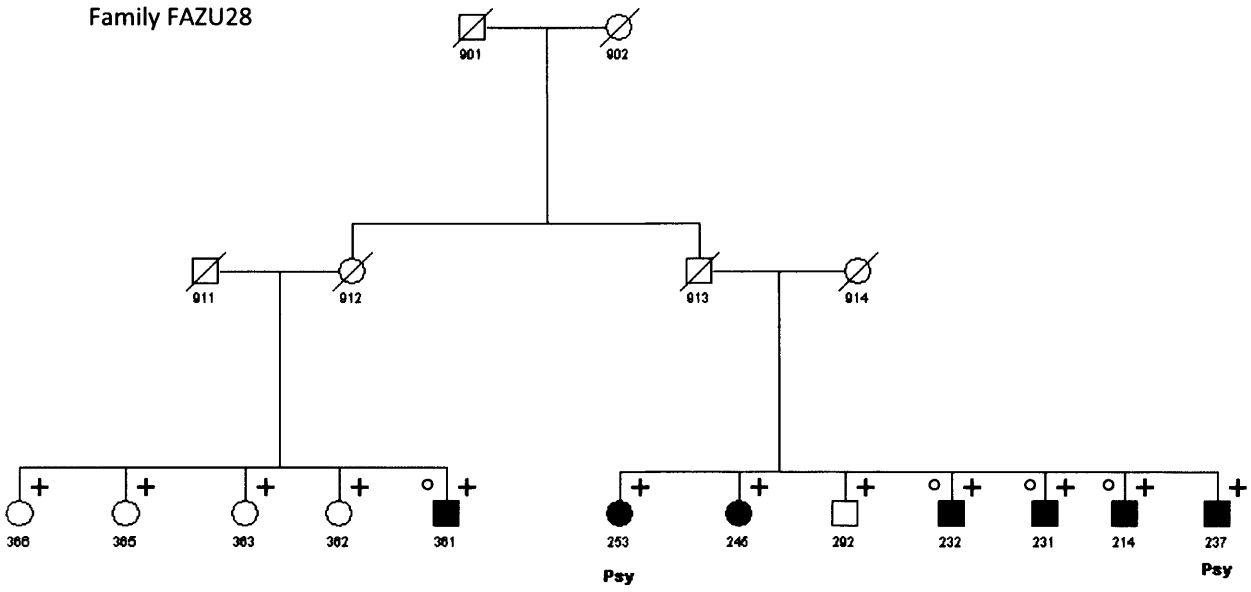


Figure 2.1 (extends over the previous three pages): Set of six Antioquian pedigrees genotyped as part of the whole-genome linkage study performed by Herzberg et al. (2006) and re-analysed in the frame of this thesis. Individuals marked by a cross were available for genotyping. Filled symbols indicate BPI, symbols with a filled right half MD, symbols with a filled left half BPII. Individuals suffering from psychosis are labelled "Psy". Individuals for whom no best estimate diagnosis is available are indicated by a circle (see chapter 2.1.1 for an explanation).

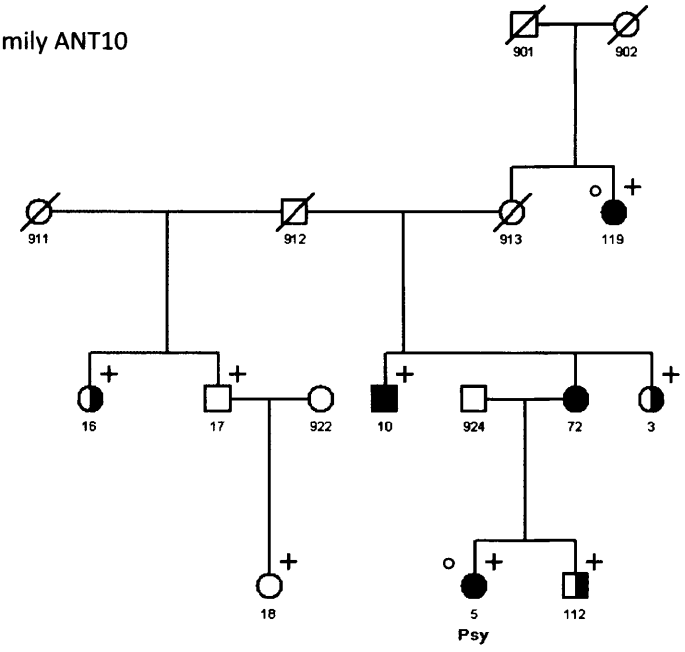
Family FAZU01



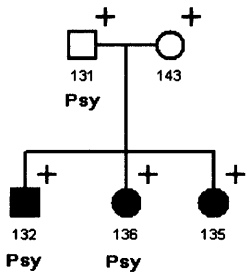
Family FAZU28



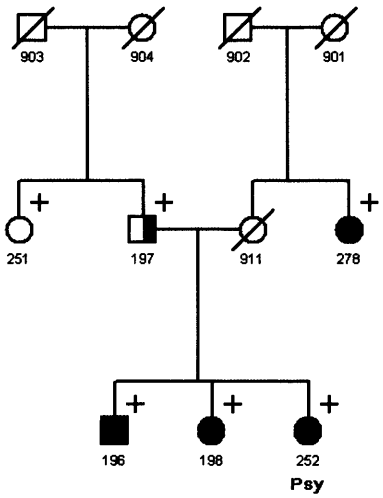
Family ANT10



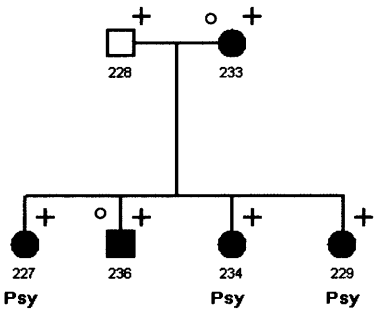
Family ANT15



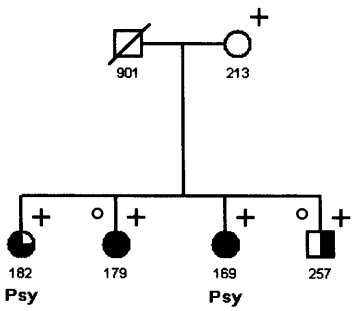
Family ANT19



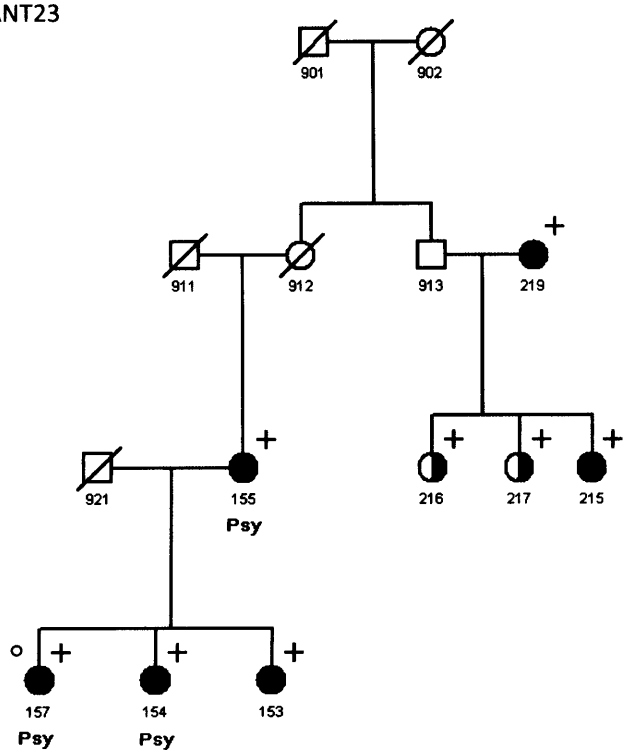
Family ANT21



Family ANT24



Family ANT23



Family ANT26

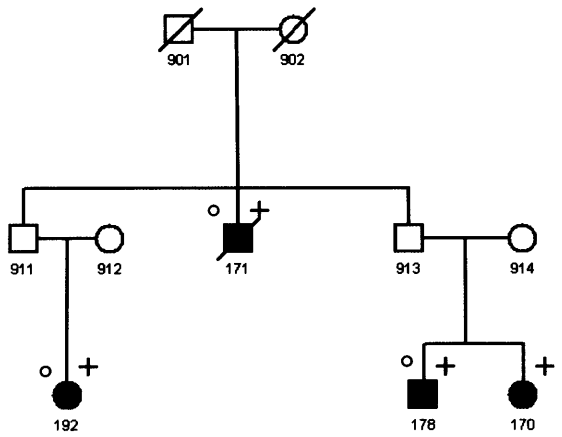


Figure 2.2 (extends over the previous four pages): Set of nine *paisa* pedigrees genotyped and analysed for this thesis. Individuals marked by a cross were available for genotyping. Filled symbols indicate BPI, symbols with a filled right half MD, filled symbols with the upper right quarter left blank indicate schizophrenia. Individuals suffering from psychosis are labelled "Psy". Individuals for whom no best estimate diagnosis is available are indicated by a circle (see chapter 2.1.1 for an explanation).

2.1.3. Bipolar Trio Sample

A sample of 176 unrelated BPI patients (64 males, 112 females; mean age 34.9 ± 9.8 years; mean age of onset 24.1 ± 8.2 years; mean duration of illness 10.7 ± 8.5 years) was recruited in the city of Medellín as described in chapter 2.1.1.

The sample recollection aimed at obtaining a sample of affected offspring-parent trios to carry out transmission disequilibrium analysis and is therefore referred to as the BPI trio sample; however, not all sporadic BPI cases had both parents available. The inclusion of siblings paired with the use of special analysis programs facilitates the reconstruction of missing parental genotypes; wherever parents were not available, siblings were therefore collected.

The characteristics of the nuclear families included in the sample are summarised in Table 2.2.

Table 2.2: Characteristics of the sample for family-based association in BPI. nf, nuclear family.

nf structure	#affecteds	#parents	#unaffected siblings	Total # individuals in nf	# nf with specified structure
Trio	1	2	0	3	81
Trio with sib	1	2	1	4	5
Duo	1	1	0	2	14
Duo with sib	1	1	1	3	68
Duo with 2 sibs	1	1	2	4	1
Three sibs	1	0	2	3	5
Two affecteds, one parent, one sib	2	1	1	4	2

2.1.4. Schizophrenia Trio Sample

The sample for family-based association analysis in schizophrenia consisted of 102 patients diagnosed as described in chapter 2.1.1 (79 males, 23 females; mean age 34.3 ± 10.2 years; mean age of onset 21 ± 7.2 years; mean duration of illness 13.4 ± 8.7 years), and their nuclear families.

As for the BPI trio sample, the schizophrenia sample was collected with the aim of performing transmission disequilibrium analysis. It was therefore attempted to collect parent-affected offspring trios, but again, not all patients had both parents available for study, and again, nuclear families of different structures were therefore collected, with an emphasis on collecting siblings where parents could not be obtained.

Table 2.3 shows an overview over the different types of nuclear families used in the schizophrenia association study.

Table 2.3: Characteristics of the sample for family-based association in BPI. nf, nuclear family.

nf structure	#affecteds	#parents	#unaffected siblings	Total # individuals in nf	# nf with specified structure
Trio	1	2	0	3	38
Duo	1	1	0	2	13
Duo with sib	1	1	1	3	45
Duo with two sibs	1	1	2	4	2
Three sibs	1	0	2	3	1
Two affecteds, one parent, one sib	2	1	1	4	1
Affected, sibling, aunt/uncle	1	0	1	3	2

2.1.5. Ethical Committee Approval

This study was approved by the Ethics Committees of all participating institutions.

2.2. Laboratory Methods

2.2.1. Sample Collection and DNA Extraction

Blood samples were collected from patients and their relatives by our clinical collaborators at the Department of Psychiatry at Universidad de Antioquia, Medellín. In some cases where blood samples could not be obtained a saliva sample was taken instead. DNA was extracted at Laboratorio de Genética Molecular (Genmol), Universidad de Antioquia, following a standard phenol-chloroform protocol. The DNA extraction was also done by our Colombian collaborators, and an aliquot of each DNA sample was then sent to our laboratory at UCL.

2.2.2. DNA Concentration Measurement and Adjustment

The DNA concentration of the samples was determined by photospectrometric measurement. Samples were diluted 1:100 with sterile de-ionized water (dH_2O) and their concentration was measured using a spectrophotometer (Biophotometer by Eppendorf), which records the absorption of the dilution at a wavelength (λ) of 260nm, where an absorption of 1.0 corresponds to 50μg of double stranded DNA. Prior to every measurement, the spectrophotometer was calibrated by performing a blank measurement using dH_2O only. After recording the concentration, samples were diluted to a working concentration of 20ng/μl. For some samples, the stock solution had a concentration of <20ng/μl; in these cases, the stock solutions were used for genotyping and sequencing.

2.2.3. The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR)[248] is a standard and well-characterised tool in molecular genetics, which allows the *in vitro* replication (amplification) of specific DNA segments.

Both the genotyping assays and the sequencing performed for this thesis relied on performing a PCR as a first step. Primers were obtained from different sources: the whole-genome linkage scan was carried out using the ABI Linkage Mapping Set v2.5 (Applied Biosystems); this set contained all primers ready for use. All other

PCRs for microsatellite markers were performed using primers published in the Genome Database (http://www.gdb.org). The remaining primers were designed using the Primer3 programme (available at http://frodo.wi.mit.edu/). The following well-established criteria were taken into account for primer design:

1. Primers should be between 18 and 24 bp long: the length of a primer is key to the specificity of the PCR; the shorter it is, the higher are the chances of unspecific annealing at other sites than the target of the PCR. If, on the other hand, the primer is too long, it might not properly anneal at all, and the efficiency of the PCR might be compromised. Deviations from the optimal primer length were possible, but it was attempted to keep primer oligonucleotides in the size range specified above.
2. The GC content of a primer should be around 60%. Guanidin and Cytosin are paired via three hydrogen bonds, and therefore require a greater amount of energy to be separated from each other than Adenine and Thymine. If the GC content of a primer is too high, it might form more stable secondary structures (see 3.) than primers with a higher AT content and therefore be unavailable for the PCR reaction, thereby lowering its efficiency. If the GC content of a primer is too low, however, it might not anneal stably to the DNA template, thereby also decreasing PCR efficiency.
3. To avoid the formation of primer dimers, the sequences of a primer pair should not be complementary to each other. Primer dimers capture primer molecules and make them unavailable for PCR, again decreasing the efficiency of the reaction. A similar problem occurs if a primer shows self-complementarity; it then forms a secondary structure, which keeps the primer from annealing at the target DNA.
4. The melting temperatures of the two primers constituting a pair should not lie too far apart as that might interfere with the annealing of at least one of the primers, thereby decreasing PCR efficiency. The annealing temperature of a PCR should be approximately 5°C below the primers' average melting temperature.

PCR conditions varied according to the primers used. A standard reaction set-up that was used as the starting point for the optimisation of many PCR assays is presented

in Table 2.4. Standard cycling conditions are presented in Table 2.5. For full information on PCR conditions for each assay refer to the corresponding chapters. All PCRs preceding genotyping were set up in 96-well plates, while PCRs preceding sequencing were set up in strips of 8 0.5µl tubes (both ABgene). PCR reactions were carried out in a 96-well plate DYAD™ DNA Engine Thermal Cycler or its disciple (MJ Research). Except for the primers that were part of the ABI linkage mapping set, all primers were purchased from Sigma Aldrich in lyophilised form. Primer stock solutions were prepared and kept at a concentration of 100µM; working solutions were 10µM. Each PCR included a negative and a positive control (dH_2O and a CEPH sample, respectively).

Table 2.4: Set-up of a standard PCR reaction (per reaction).

PCR component	volume (µl)	final concentration	function in PCR
Buffer (Bioline)	2.5	1x	Stabilisation of reaction environment
$MgCl_2$ (Bioline)	0.75	1.5 mM	Co-factor for oligonucleotides, primers, and enzyme
dNTPs (Bioline)	0.625	25 µM	Building blocks of nascent DNA
Taq polymerase (BIOTAQ™, Bioline)	0.1	0.5 U*	Catalyses DNA replication
forward primer	1.5	0.4 µM	The primers determine the fragment to be amplified and serve as starting point for elongation.
reverse primer	1.5	0.4 µM	
DNA	2	1.6 ng/µl	DNA template
dH_2O	16.025	-	Concentration adjustment
total	25	-	-

***As customary for enzymes, this figure does not denote a concentration but the total amount in the reaction.**

Table 2.5: Cycling conditions for a standard PCR.

PCR step	temperature	time
1 – Initiation	94°C	4 min
2 – Denaturing	94°C	30 sec
3 – Primer Annealing	55°C	30 sec
4 – Elongation	72°C	45 sec
5 – Repeat steps 2-4 29 x (for a total of 30 cycles)	-	-
6 – Final Elongation	72°C	5 min
7 – Cool-Down	4°C	15 min

2.2.4. Agarose Gel Electrophoresis

PCR amplification success was checked by means of agarose gel electrophoresis. In gel electrophoresis, DNA fragments are separated according to their size. This is achieved by applying an electric field to the gel. DNA molecules are negatively charged and will therefore migrate towards the plus pole of this field. Smaller molecules migrate faster through the gel matrix, while larger molecules are held back by the polymer structure of the gel. A base pair ladder, which consists of a mix of fragments of known sizes, is included in each run, allowing to determine the size of DNA fragments by comparison to the ladder. The resolution of an agarose gel depends on its concentration. PCR products in this thesis were between ~100 and ~500 bp long; an adequate agarose concentration to separate fragments in this size range is 2%.

DNA visualisation was achieved by staining agarose gels with ethidium bromide (EtBr), a reagent that intercalates between DNA base pairs. The region of intercalation shows intense fluorescence under a UV lamp, allowing the distinction of DNA bands.

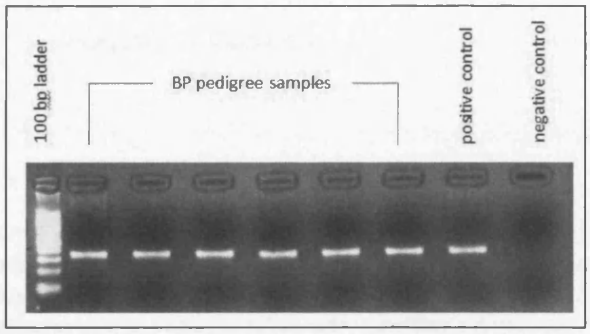
Agarose gels were prepared as follows:

1. 2g of agarose were mixed with 100ml of 1xTBE buffer (both Sigma).
2. The mix was heated in a microwave oven for approximately 2min at 800W, or until the agarose had completely dissolved and the solution appeared clear.

3. 80µl EtBr (Gene Choice) were added to the gel solution to make for a final concentration of 0.5µg/ml.
4. The mix was gently stirred to avoid the formation of bubbles and carefully poured into a previously prepared gel chamber (Electro-fast® *Stretch* System by ABgene). The combs were added, and the gel was then left to polymerise for ~45min.
5. After polymerisation, 1xTBE was poured into the chamber until the gel and the electrodes on both sides of the chamber were completely covered.

3µl of each PCR product were then mixed with 2µl loading buffer and loaded onto the gel. 2µl of a 100bp size standard (500µg/ml, New England Biolabs) were loaded into a separate well, and the gel was run for 35min at 80V. Finally, the DNA was visualised with the help of a UV transilluminator (BioDoc-It™ System, UVP), and a picture was taken using the built-in digital camera. An example of an agarose gel picture is shown in Figure 2.3.

Figure 2.3: Agarose gel with PCR products.



The gel picture shows the products of the amplification of STR marker D5S410 by PCR. A 100bp ladder is included. From comparison with this ladder, it can be seen that the PCR product is ~350bp long. A positive and negative control are also shown. Here, the positive control is the CEPH sample 1347-02.

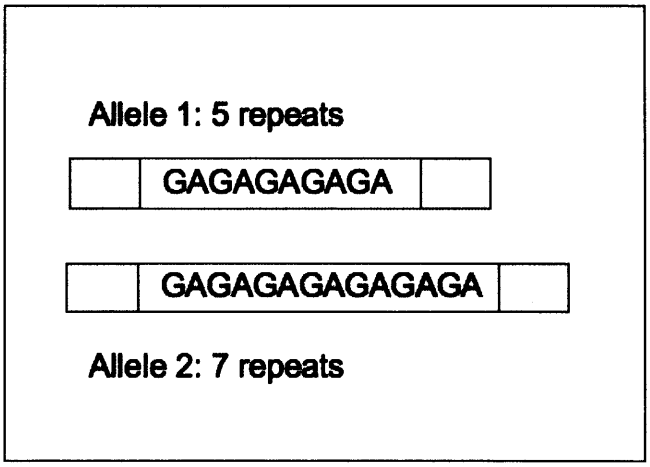
2.2.5. Genotyping

Two basic methods of genotyping were used in this thesis: fragment length analysis for the genotyping of microsatellite markers, and restriction fragment length analysis for the genotyping of SNP markers.

2.2.5.1. Microsatellite Markers

Microsatellite markers, also called short tandem repeats (STRs), are genomic DNA sequences consisting of a variable number of repeats of a short sequence motif[249,250]. The repeat motif is most commonly two to four bp long. The alleles of a microsatellite marker locus differ from each other in the number of repeat units (see Figure 2.4); amplification by PCR therefore results in differently-sized fragments, a property that can be used to separate the two fragments resulting from an individual's alleles by electrophoresis and to determine the genotype according to the size of the amplified fragments (fragment length analysis, see chapter 2.2.5.2).

Figure 2.4: Schematic representation of a microsatellite locus.



The microsatellite shown here has a dinucleotide repeat motif $(GA)_n$. The two alleles differ from each other by the number of repeat units (five vs. seven). The sequences on both sides of the microsatellite locus (shown in grey) are the same for both alleles, and PCR primers would be designed to be complementary to a section of this surrounding sequence.

Microsatellite loci occur spread throughout the whole genome. Some are located within genes (such as the trinucleotide sequence in that, through expansion, causes Huntington's Disease[251]), but most are located in non-coding regions and seem to evolve neutrally[252]. Partly because of their mechanism of mutation, which involves a process called "slippage", during which the DNA polymerase erroneously omits, or adds in, additional repeat units[252], partly because of their neutrality in evolution, microsatellites mutate relatively fast and are therefore highly polymorphic. Because linkage analysis depends on the identification of recombinants, for which

heterozygosity at both disease and marker loci is crucial, microsatellites are ideal markers for linkage analysis[253].

2.2.5.2. Fragment Length Analysis

Fragment length analysis consists in the electrophoretic separation of microsatellite alleles and the calling of the genotype based on the fragment lengths observed. Microsatellite alleles differ from each other by only a few base pairs, and the resolution of agarose gels is not high enough to separate them. For genotyping, microsatellite PCR products are therefore run through a high resolution electrophoresis that is able to detect size differences of less than a base pair. High resolution electrophoresis is either polyacrylamide gel- or capillary-based, but the principle of both methods is the same. Here, capillary electrophoresis was used. In both systems, DNA detection relies on a laser positioned near the plus pole, which records the signals emitted by a fluorescence labelled primer incorporated into the PCR product. Because the laser can detect a range of different fluorescence labels, an advantage of this system is the possibility of pooling several PCR products and running them in the same gel lane or capillary, thereby reducing genotyping costs.

The ABI linkage mapping set v2.5 is organised into 28 panels (i.e., groups of markers that can be run simultaneously owing to different combinations of size ranges and fluorescence labels). Panels comprised markers with three different fluorescence labels: FAM (blue), VIC (green), and NED (yellow). The ABI linkage mapping set ("ABI markers") had originally been purchased for the previous linkage scan carried out in our lab[184], and the fluorescence of the primers had diminished somewhat over time. Therefore, the dilution of PCR products that is necessary prior to fragment length analysis could not always be done according to the manufacturer's instructions. For example, ABI recommends diluting PCR products for FAM-labelled markers 1:20 before analysing it by polyacrylamide gel or capillary electrophoresis; however, to obtain a readable result, FAM-labelled markers with a weak PCR product had to be diluted 1:10. After PCR and agarose gel electrophoresis, each marker was therefore visually inspected to decide on a dilution. All dilutions were in the range of 1:20 – 3.5:20, and FAM- and VIC-labelled markers

were generally diluted more than NED-labelled ones, because the yellow fluorescence of NED is naturally weaker than that of the other fluorophores.

PCR product dilution is achieved as the different PCR products are pooled together in a fresh 96-well plate; if markers cannot be diluted as intended by the manufacturer, the panel structure suggested by ABI will not hold anymore. For this reason, and because of the relatively weak fluorescence of NED, with which stronger fluorophores can interfere, FAM- and VIC-labelled markers were pooled together, while NED-labelled markers were pooled separately for each panel. Because panel 28 contained X-chromosomal markers, which were not analysed in the BP linkage scan presented here, the ABI markers were run in 27x2 = 54 panels.

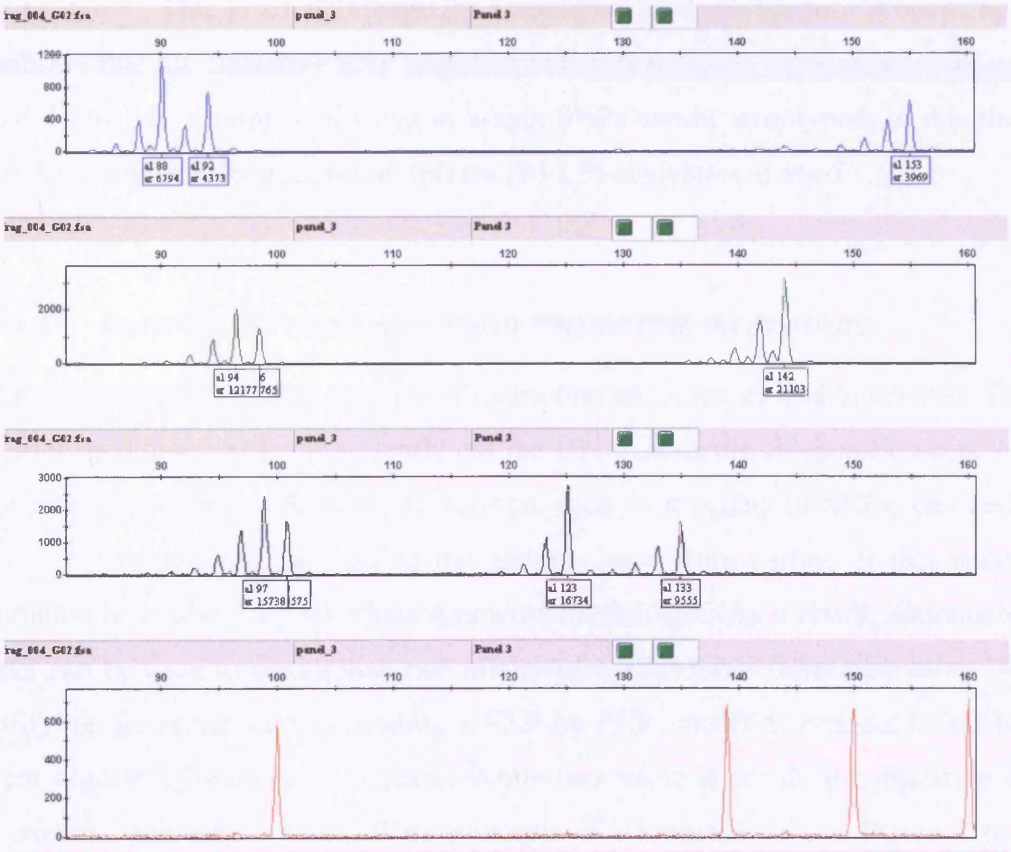
Non-ABI microsatellite markers were organised into panels by size ranges (as indicated on the Genome Database), and primers were ordered with different 5' fluorescence labels so as to make the genotyping as efficient as possible. For green fluorescence, HEX was used instead of VIC. For blue and yellow fluorescence, FAM and NED were used, respectively. Non-ABI microsatellite markers were used to follow up the 5q region, a candidate region that had emerged from the linkage scan by Herzberg et al. (2006)[184] (see chapter 3), and for some repeats in the BP linkage scan, where ABI primers had run out.

2.5µl of the pooling mix were transferred to a new 96-well plate, which was then handed over to the in-house genotyping service at the Centre of Comparative Genomics (CCG; http://www.ucl.ac.uk/biology/centre-for-comparative-genomics/), where both sequencing and genotyping are performed on a 96-capillary 3730*xl* DNA Analyzer (Applied Biosystems).

Raw genotyping data were returned by the CCG in ABI's .fsa file format and subsequently analysed using the GeneMapper® software v 3.7 (Applied Biosystems). Fragment sizes were determined by calibration to the LIZ500 size standard (Applied Biosystems), and alleles were called using the standard microsatellite analysis procedure implemented in the GeneMapper® software. See Figure 2.5 for an example of the GeneMapper® output for one sample. After automatic calling, peaks were checked by eye and any mislabelled peaks were corrected. Genotypes were then exported into a table.

This procedure, from importing the raw data into GeneMapper® to exporting the genotype data, was carried out by two independent researchers, including the author of this thesis, and the resulting genotypes were compared ("double scoring"). Any discrepancies between the results of the two double scorers were examined in detail by both researchers together. If the double scorers could not agree on a genotype, the allele calls were deleted and the sample repeated for the marker in question, until the maximum number of repeat rounds for a marker was reached (usually two, unless a new primer had been ordered or for any other reason it was sensible to perform more repeats).

Figure 2.5: Fragment length analysis with the GeneMapper® software



The figure shows an extract of an electropherogram for sample FAZU 01-H-376. The different size ranges and fluorescent labels of different microsatellite marker systems allow them to be run together in one capillary. The top panel shows markers labelled with FAM (blue; from left to right: D2S286 and D2S165); the one below shows markers labelled with VIC (green; from left to right: D2S125 and D2S206). The second lowest panel shows markers labelled with NED (yellow fluorescence, however, on screen, it is represented in black; from left to right: D2S2333 and D2S126). The LIZ500 size standard is shown in the bottom panel (orange peaks; the peaks shown in this extract correspond to the 100 bp, 139 bp, 150 bp and 160 bp peak). The analysed sample is homozygous for two systems and heterozygous for the remaining four.

2.2.5.3. *Single Nucleotide Polymorphisms*

Single Nucleotide Polymorphisms, or SNPs, occur when two alleles at a locus differ by a single base pair. In order to be called a SNP, such a single base pair substitution must occur with a minor allele frequency (MAF) of at least 1% in the population[254]. SNPs mutate at a much slower rate than microsatellites and, by definition, cannot have more than four alleles. Most SNPs have two alleles, meaning that their maximum possible heterozygosity is 0.5 (if both alleles occur at equal frequencies). As mentioned in chapter 1.2.4.3, the current build of the NCBI's dbSNP database (no. 128; http://www.ncbi.nlm.nih.gov/SNP/) contains approximately 12 million SNPs, most of which are located in non-coding sequences. As opposed to microsatellites, however, there are a great number of SNPs occurring in coding regions, too[255]. This is an advantage for association analysis because it opens up the possibility that the causative SNP might be amongst the ones typed in an association study. There are a number of ways in which SNPs can be genotyped; in this thesis, restriction fragment length polymorphism (RFLP) analysis was used.

2.2.5.4. *Restriction Fragment Length Polymorphism Analysis*

RFLP analysis relies on the activity of restriction enzymes, or endonucleases. These enzymes recognise, and subsequently cut the DNA molecule at, specific short, often palyndromic sequences. A base pair change, such as it occurs in SNPs, can destroy such a recognition site, preventing the endonuclease from cutting at that position. Alternatively, a SNP can introduce a new restriction site. As a result, endonuclease assays can be used to distinguish two alleles of a SNP locus from each other. After amplifying the sequence surrounding a SNP by PCR, the PCR product is left to be cut (or digested) by an endonuclease. While one allele is cut during digestion with the enzyme, the other is not. This principle is illustrated in see Figure 2.6. An important limitation of this method is that the SNP has to be located at an endonuclease recognition site. If there is no endonuclease that cuts at the location of a SNP, another way of genotyping has to be found, e.g. single strand conformation polymorphism (SSCP) analysis.

Figure 2.6: Principle of restriction fragment length polymorphism analysis.

From Syvänen (2001)[255]

The usual way of separating the different fragments resulting from an endonuclease digestion is by agarose gel electrophoresis. However, the assay used in this thesis (used to type a polymorphism in the *CLINT1* gene, see chapter 3) was designed in a slightly different way: because the DNA fragments resulting from the enzymatic digestion were too small to be distinguished by agarose electrophoresis, a fluorescently labelled primer was used, and the digestion product was run on a capillary sequencer instead. The design of the assay is explained in chapter 3.2. The reaction was set up in 96-well plates as shown in Table 2.6.

Table 2.6: Set-up of the Tsp509I digest (per reaction)

Reaction component	volume (µl)	final concentration
Buffer (New England Biolabs)	1.25	1x
Tsp509I Endonuclease (New England Biolabs)	0.4	4U*
PCR product	10	?
dH_2O	0.85	-
total	12.5	-

***As customary for enzymes, this figure does not denote a concentration but the total amount in the reaction.**

The reaction mix was incubated for 5h at 65°C in a DYAD™ DNA Engine Thermal Cycler or its disciple (MJ Research). Subsequently, 2µl of a 1:10 dilution of the digest were transferred into a new plate and sent to the CCG for electrophoresis in a

capillary sequencer. After receiving the raw data back from the CCG, the results were analysed using the GeneMapper® software version 4.0, using the standard analysis procedure for microsatellites, which could also be applied for this RFLP assay.

2.2.6. Sequencing

Sanger-sequencing was performed as part of the effort to identify new variants potentially associated with psychosis in the *CLINT1* gene (chapter 3). The sequencing procedure consists of five steps: (1) amplification of the segment to be sequenced by PCR, (2) clean-up of the PCR product, (3) the sequencing reaction, (4) clean-up of the sequencing product, and (5) the sequencing run. Step (1), the PCR, was carried out as described in chapter 2.2.3, with some adjustments to the protocol (see chapter 3.2 for details).

After checking the amplification success on an agarose gel, the remaining 22µl of the PCR product were cleaned up (step 2) by adding a shrimp alkaline phosphatase (SAP), used to eliminate unincorporated dNTPs, and an exonuclease (Exo-I), used to digest left-over primer molecules, so that neither of these PCR reagents could interfere with the sequencing reaction. 2.5µl (2.5U) SAP and 0.1µl (1U) Exo-I (both USB Corporation) were added to 22µl PCR product. The mix was then left to incubate for 1h at 37°C, followed by 15min at 72°C (enzyme deactivation step). This protocol was set up in strips of 8 0.5µl tubes.

1-1.5µl of the clean PCR product was then used in the sequencing reaction (step 3). Sequencing was carried out using the Big Dye Terminator v3.1 kit (Applied Biosystems). It was found that, instead of the 8µl/reaction of the Big Dye mix recommended by the manufacturer, 1µl/reaction was enough to give good sequencing results, and the resulting reaction protocol is shown in Table 2.7. A positive sequencing control was included as provided in the Big Dye kit, and sequencing reaction was carried out following the manufacturer's instructions.

Table 2.7: Sequencing reaction set-up (per reaction)

Reaction component	volume (µl)	final concentration
Big Dye v3.1	1	?
Buffer (from Big Dye kit)	1.5	1x
sequencing primer (5µM)	0.64	0.32µM
PCR product	1-1.5	?
dH_2O	5.81-5.86	-
total	10	-

The product of the sequencing reaction was subsequently cleaned up (step 4) by first adding 2.5µl of 125µM EDTA and 30µl of 100% ethanol to each sample and incubating the mix for 10min at room temperature. The samples were subsequently centrifuged for 1h at high speed (3870 rpm) in order to precipitate the product of the sequencing reaction. The supernatant was subsequently removed by inverting the samples and centrifuging at low speed (1000 rpm) for 1min. As a second cleaning step, 30µl of 70% ethanol were then added to each sample, and the mix was again centrifuged at 3870 rpm, this time for 10min. The samples were then again inverted and the supernatant removed by centrifuging at 1000rpm for 1min. After this final centrifugation step, the samples were left to dry at room temperature and subsequently sent to the CCG for the sequencing run (step 5).

The raw data received from the CCG was analysed using the Sequencher software v4.7 (Demo version; Gene Codes Corporation) and the ChromasPro software (Technelysium). The freely available online version of the MAFFT v5.8 software (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) was used for sequence alignments.

2.3. Data Analysis and Statistical Methods

A range of methods was used for the analysis of the genetic data collected for this thesis. The first method, presented in section 2.3.1, is not part of the actual data analysis but describes how the data, which was stored in the so-called "linkage

format", was recoded and converted into the different input file formats needed for the different analyses. In section 2.3.2, the SimWalk2 programme, a package for pedigree-based statistical genetics analysis, is introduced.

An important part of data analysis is concerned with the quality control of the genotyping data. Sections 2.3.3, 2.3.4 and 2.3.5, dealing with Hardy Weinberg equilibrium and the detection of genotyping errors, fall into this category. Section 2.3.6 describes the estimation of population allele frequencies from pedigree data, an important step prior to linkage analysis.

Finally, the analysis methods that are at the core of this thesis, the tests for genetic linkage or association in the clean data, are presented in sections 2.3.7 to 2.3.12.

2.3.1. The Linkage Format, Recoding and File Conversion

All genotype data were stored in the "linkage format", a straightforward computer readable format, which as well as assigning genotype data to each individual, describes the pedigree structure. In the linkage format, the data for each individual are displayed in one row: the first column contains the pedigree identifier, the second a unique personal identifier (PID), followed by the PID of the individual's father and mother in the third and fourth columns, respectively. These columns take the value of 0 for founder individuals. The fifth column contains the gender (1 = male, 2 = female) , the sixth the affection status (1 = unaffected, 2 = unaffected, 0 = unknown). The following columns contain the genotypes. For all microsatellite loci, genotypes were initially recorded in the form of either 2- or 3-digit alleles, according to the size of the corresponding DNA fragment. Because SimWalk2 does not handle 3-digit alleles, all microsatellite alleles were recoded to successive 2-digit alleles for all further analyses using the recoding facility of the programme Mega2, a file handling tool for linkage analysis[256].

While many programmes for the analysis of gene mapping data use input files in the linkage format, some do not, including both the Mendel software v8.0.1 (used to estimate population allele frequencies from the pedigree data, see chapter 2.3.6) and the programme SimWalk2 v2.9.1 (used for a range of different analyses, see sections 2.3.5 and 2.3.7 to 2.3.9). The linkage format input files were therefore converted into formats readable by these programmes using Mega2.

2.3.2. The SimWalk2 Programme

SimWalk2[257] is a software package that performs a range of statistical genetics analyses on pedigree data. Available options include parametric and non-parametric linkage analysis, haplotype inference, mistyping analysis and the calculation of identity by descent probabilities. Several of the options available in SimWalk2 v2.9.1 have been used to analyse the data from the whole-genome linkage scan for BP for this thesis (see sections 2.3.5 and 2.3.7 to 2.3.9). SimWalk2 operates in a maximum likelihood framework, where the likelihood of the observed pedigree data is represented as a function of a number of variables. These include allele frequencies, marker map and order, and a penetrance function linking genotypes and phenotypes. The different variables can then be varied until the point of maximum pedigree likelihood is reached.

The most distinct feature of the SimWalk2 programme, however, is that instead of performing an exact likelihood calculation, it relies on an approximation using a Markov Chain Monte Carlo (MCMC) algorithm. The MCMC method is a complex one; essentially, "Monte Carlo" refers to estimating an expectation by the sample mean of a set of simulated random variables. In the genetic analysis of pedigree data, the expectation represents the outcome of the pedigree likelihood function. A Markov Chain is a random walk procedure commencing at a (random) starting point and moving from one state to the next as determined by a matrix of transitional probabilities. Because of the inherent linearity of the process, the first steps will depend on the starting point, but as the Markov Chain continues, the state of the chain will become more and more independent of this starting position and will start to converge into a limiting probability distribution. The proportion of time the Markov chain spends in a certain state then tends towards the probability of that state. The first, starting position-dependent steps, referred to as "burn-in", are discarded. The principle of the MCMC method in the context of pedigree analysis is that the pedigree likelihood will be estimated by a Monte Carlo process that relies on a Markov Chain to sample from a distribution of random variables (essentially, every pedigree/data conformation consistent with the given data).

Although exact methods should be preferred if possible, the MCMC approach is extremely useful when dealing with a sampling space that is too big for an exact likelihood calculation. In the context of pedigree analysis, this refers to pedigrees

with a large amount of founder individuals, and to the simultaneous consideration of a large amount of marker loci. Other programmes available for linkage analysis are restricted either in the number of marker loci they can handle (programmes based on the Elston-Steward algorithm, e.g., Linkage[258-260] and Vitesse[261]), or in the number of founding individuals in a pedigree (programmes based on the Lander-Green algorithm, e.g., GeneHunter[116]). Even newer programmes that have implemented improved versions of the original algorithms, such as Allegro[262] and Merlin[263], cannot handle extended pedigrees of a complexity similar to that of two of the pedigrees analysed as part of the genome-wide linkage study of BP, ANT07 and FAZU01 (see Figures 2.1 and 2.2). Therefore, to facilitate the joint analysis of all pedigrees, SimWalk2 was chosen for most pedigree-based analyses. Furthermore, although SimWalk2 does not provide exact results, its results have been found to show excellent agreement with those of software packages that are based on an exact algorithm[264].

2.3.3. Hardy Weinberg Equilibrium

The Hardy Weinberg equilibrium (HWE) describes a situation, in which the genotype distribution at a genetic locus can be predicted for a population if the allele frequencies at that locus are known. For a biallelic locus with the alleles A and a and the allele frequencies f(A) = p and f(a) = q, the genotype distribution at HWE is:

$$f(AA) = p \times p = p^2 \; ; \; f(Aa) = p \times q + q \times p = 2pq \; ; \; f(aa) = q \times q = q^2. \qquad (12)$$

Equivalently, the allele frequency distribution can be inferred from the genotype distribution.

A number of theoretical conditions have to be fulfilled for the HWE to hold: (1) the population has to be of an infinite size, (2) there has to be random mating in the population, (3) there must not be any gene flow to and from the population, (4) no mutations must occur at the locus under consideration, and (5) there must be no selective pressure on that locus.

The Hardy Weinberg model is an idealised one, and no population can fulfil its theoretical conditions. In practice, the HWE is usually achieved in populations of moderate size, as well as for loci with a relatively high mutation rate, such as

microsatellites. More significant deviations can be observed if any of the other conditions are violated. Especially the presence of population substructure (i.e. the absence of random mating) and the action of a selective force on the locus under consideration can result in significant deviations from the HWE[265]. However, the most common cause of deviations from the HWE in genotyping studies are systematic genotyping errors, such as the consistent mis-scoring of certain heterozygotes as homozygotes, or the failure to score a specific allele (e.g. because it presents as an unusually small peak in fragment length analysis and is therefore taken for an artefact and ignored). For this reason, an important part of the quality control procedure in genotyping projects consists in checking for HWE. Because selection might play a role in causing deviations from the HWE, only unaffected founder individuals were tested (i.e., the patients' parents in the trio sample). Within an extended pedigree, genotypes are not independent of each other, and and the genotype distribution within a family is therefore likely to deviate from the HWE. One possible way to overcome this problem is to include only unrelated founders into the HWE test; however, there were too few of these available to obtain a meaningful result. Other means of genotyping quality control were therefore used in the families (see chapters 2.3.4 and 2.3.5).

The test for deviations from the HWE was carried out using the online version of the Genepop software v3.4 (http://genepop.curtin.edu.au/)[266]. Genepop can test for either excess or deficiency of heterozygotes (one-tailed tests), as well as for a general deviation from HWE (two-tailed test). Since there was no *a priori* reason to assume a deviation in any one direction, the two-tailed test was performed.

The test for deviations from HWE implemented in Genepop is a Fisher's Exact Test approximated using a Markov Chain Method, where the space of possible genotype tables given the allele frequencies is explored by a random walk procedure. The p-value for the test is obtained by comparing the observed genotype proportions with the distribution of possible genotype tables[267]. The Genepop run parameters were set to 1000 dememorisations (equivalent to the burn-in period in MCMC), 100 batches and 1000 iterations per batch.

Some basic parameters for the data collected in the trio samples (allele and genotype frequencies, and expected and observed amount of heterozygotes and homozygotes) were also calculated using the Genepop software.

2.3.4. Test for Mendelian Inheritance

When dealing with family data, an important part of the genotyping quality control procedure is to test for inconsistencies with Mendelian inheritance. Patterns of inconsistencies can help indicate whether there is a problem with the quality of the genotyping data, and of which kind the problem is. Sporadic inconsistencies indicate non-systematic genotyping errors; the occurrence of many inconsistencies for one marker might indicate systematic genotyping errors; and inconsistencies across markers between a child and one of its parents (or both) can uncover problems with the samples, such as false paternity and sample mix-ups.

Consistency with Mendelian inheritance was tested using the programme PedCheck[268]. As a first and basic step, this program carries out a comparison of genotypes within a nuclear family, checking whether the children's genotypes are consistent with their parents', as well as their siblings'. In a second step of the analysis, genotypes are then compared and tested for consistency across the extended pedigree.

PedCheck was used to test both pedigree and trio data. Genotypes that were inconsistent with Mendelian inheritance were removed, and the sample was re-typed for the marker in question. If a marker showed a considerable amount of inconsistencies, it was checked for systematic genotyping errors and, if necessary, re-typed for all samples using fresh and possibly newly designed primers. In cases of false paternity and sample mix-ups, the problematic samples were excluded from further analyses.

2.3.5. Test for Non-Mendelian Errors

Not all genotyping errors cause Mendelian inconsistencies. Simulation studies have shown that the percentage of genotyping errors consistent with Mendelian transmission can be up to ~50% for multiallelic markers, and as high as 87% for biallelic markers[269], and a check for Mendelian inconsistencies only is unlikely to identify all problematic genotypes. However, genotyping errors can mask linkage signals and distort marker maps (this includes producing an inaccurate estimate of the trait location on the marker map)[264], and it is crucial to obtain data as free of genotyping errors as possible before proceeding to linkage analysis. SimWalk2

v2.91[257] (see chapter 2.3.2) was therefore used to check for these "non-Mendelian errors".

The mistyping analysis (option 5 of the SimWalk2 programme) detects genotypes that introduce recombination in a small region of the chromosome and are therefore unlikely to be correct, even though they are consistent with Mendelian transmission. While haplotype analysis, which can also be carried out using SimWalk2 (see section 2.3.9) produces just one solution which might or might not contain double recombination around a problematic allele, there might be several equally likely solutions to the haplotype analysis, as well as solutions that are only slightly less likely. It is only by considering all solutions at the same time and weighting them according to their likelihood that it can be determined whether a genotype, or even a single allele, is likely to have been mistyped[264].

After either rectifying or removing genotypes that were inconsistent with Mendelian inheritance as described in chapter 2.3.4, the pedigree data collected for the whole genome linkage scan (see chapter 4) was tested for "non-Mendelian errors" using option 5 of the SimWalk2 programme v2.9.1 ("mistyping analysis"). Genotypes with a mistyping probability ≥ 0.5 were flagged by the programme and subsequently removed from the data set.

2.3.6. Estimation of Allele Frequencies from Pedigree Data

Linkage analysis relies on the availability of allele frequencies in the population under study. Because the genotypes within a pedigree are not independent from each other, the allele frequencies obtained from it do not provide an accurate estimate of the population allele frequencies. One possible way to overcome this problem is to calculate the allele frequencies from unrelated founder individuals only. The other possibility consists in typing all markers to be analysed in a sample of unrelated individuals from the same population. However, both approaches are unsatisfactory: the former wastes a lot of the available information, the latter leads to a considerable increase in genotyping costs and ultimately also wastes the information contained in the pedigree data.

A third possibility is to estimate the population allele frequencies using the data from the complete pedigree data, thereby neither wasting available information not

incurring additional genotyping costs. This can be achieved within a pedigree likelihood framework[270], where the likelihood of the observed data is maximised by varying the allele frequency estimates. An algorithm based on this approach is implemented in the programme Mendel[271], and analysis option 6 ("Allele Frequencies") of the Mendel software v8.0.1 was used in the genome-wide linkage study of BP to estimate allele frequencies from the pedigree data.

2.3.7. Parametric Linkage Analysis

Multipoint parametric linkage analysis (see chapter 1.2.3) was carried out using SimWalk2 v2.9.1 (see chapter 2.3.2). The whole-genome linkage scan data were analysed considering individuals diagnosed with BPI as affected and all other family members as of an unknown phenotype. No family members were considered as unaffected. The following parameters were used:

- frequency of the mutant allele: 0.003
- penetrance of the homozygous mutant genotype: 0.9
- penetrance of the heterozygous genotype: 0.81
- phenocopy rate: 0.01

This genetic model has been established through segregation analysis of BPI in the population of the Central Valley of Costa Rica, which is genetically very similar to that of Antioquia and has been used previously for the analysis of BPI pedigree data from the *paisa* community[173,184]. Population allele frequencies were estimated from the pedigree data using Mendel v8.0.1 as described in chapter 2.3.6. A genetic marker map for each chromosome was obtained from the Marshfield Mammalian Genotyping Service (http://research.marshfieldclinic.org/genetics/home/index.asp)[272], and sex-specific recombination ratios were used. The output of the parametric linkage analysis using SimWalk2 are so-called location scores, which correspond to multipoint LOD scores, and multipoint HLOD scores.

2.3.8. Non-Parametric Linkage Analysis

Multipoint non-parametric linkage analysis was also performed using SimWalk2 v2.9.1 (see chapter 2.3.2; analysis option 3). Evidence for linkage was measured using the NPL_{PAIRS} statistic, which has previously been used to analyse pedigrees from Antioquia and Costa Rica[178,184]. Allele frequencies and marker maps were used as described in chapter 2.3.7. Only individuals with the phenotype under study were considered as affected (see chapter 4.1 for the phenotype models used in the analysis of the Antioquian pedigree data). All other individuals were considered as of an unknown phenotype; no family members were considered as unaffected.

2.3.9. Haplotype Analysis

Haplotype analysis is the reconstruction of haplotypes based on genotype data. It is a useful tool in linkage studies because it allows the comparison of potential risk haplotypes within and across families. Haplotype reconstruction in family data is relatively straightforward in theory; however, when analysing complex extended pedigrees, it becomes too complex to carry out by hand, and a computer programme is needed to complete the task. SimWalk2 was used for haplotype reconstruction. Haplotypes were then imported into the Haplopainter software v024 beta[273] for easy visualisation.

2.3.10. Transmission Disequilibrium Analysis

The Transmission Disequilibrium Test (TDT)[154] for single markers was carried out as implemented in the TRANSMIT program, version 2.5.4[155,156]. TRANSMIT carries out a generalised transmission/disequilibrium test that can be applied to situations of uncertain allele or haplotype transmission, allowing the analysis of data from patients with only one available parent, and to multilocus data even under uncertain phase, thereby permitting the use of all available genotypic data, including all nuclear family structures (data from unaffected siblings are used to infer parental genotypes). The statistic estimated by TRANSMIT follows a χ^2 distribution and is calculated as

$$(O-E)^2 = Var(O-E), \qquad (13)$$

where O and E refer to the number of observed and expected transmissions of alleles/haplotypes. As an alternative method of determining the p-value, the program employs a bootstrap method which provides more accurate results than the χ^2 approximation. The minimal haplotype frequency for all TRANSMIT analyses was set to 2%.

As part of the candidate gene study of the *NOS1AP* gene (see chapter 5), haplotype-based TDT analysis was carried out using the WHAP programme v.2.09[274]. WHAP has the advantage of offering a flexible, regression-based statistical framework, allowing the user to perform a variety of haplotype- and SNP-conditional tests, as well as a global test of haplotype association. As part of the *NOS1AP* study, two tests were performed: an omnibus haplotype test (testing the effects across all haplotypes) and a haplotype-specific test (testing the effect of each haplotype against all others). For these analyses, the prevalence of the disease in the population was set to 0.55%[216], and the minimum frequency for a haplotype to be included in the analysis was fixed at 1%.

A further programme for single marker as well as haplotype-based TDT in unphased genotype data from nuclear families is TDTPHASE, which forms part of the UNPHASED suite of genetic association programmes[275]. While in this thesis, TDT analysis was performed using TRANSMIT and WHAP, TDTPHASE offers the advantage of producing an estimate of the allele frequencies of transmitted and untransmitted alleles/haplotypes in a trio sample. TDTPHASE v.2.4 was therefore used to estimate allele frequencies of the untransmitted alleles in TDT analysis.

2.3.11. Linkage Disequilibrium Analysis

LD patterns across the *NOS1AP* locus (see chapter 5) were evaluated using the programme Haploview v3.2[276]. This programme calculates and visualises patterns of LD, as measured by either D' or r^2, as well as haplotype blocks, and may also be used to choose htSNPs, making it a very useful tool for LD analysis. To evaluate LD patterns in a population, an sample of unrelated individuals is needed; this analysis was therefore based on parental genotypes only.

Haploview offers the two general options of loading one's own data set and of importing data from the HapMap project. This allows a quick and easy comparison

of LD patterns in the study population (here the population of Antioquia) and the HapMap populations.

2.3.12. Quantitative TDT Analysis

The TDT analysis can be extended to quantitative traits (i.e., continuous traits, such as height or weight, as opposed to dichotomous traits such as whether or not a person suffers from a disease). Quantitative TDT (qTDT) is based on a regression approach, where it is assumed that the dose of a certain allele will increase or decrease the trait value. In this thesis, the programme QTDT v2.5.1[277] was used to carry out a qTDT analysis of the *NOS1AP* gene with clinical dimensions in schizophrenia. The QTDT programme makes use of a variance components-framework, which distinguishes between-family and within-family association effects. In order to evaluate true genetic association effects without the confounder of population stratification, QTDT tests the within-family component.

CHAPTER THREE

ANALYSIS OF THE *CLINT1* GENE AS A CANDIDATE LOCUS FOR PSYCHOSIS

3. Analysis of the *CLINT1* Gene as a Candidate Locus for Psychosis

3.1. Background and Previous Work

We recently performed a genome-wide linkage scan of six extended pedigrees segregating severe bipolar disorder from Antioquia[184]. One of the most promising regions identified in that linkage scan was on chromosome 5q31-34, with a maximum NPL_{PAIRS} score of 1.92 for marker D5S410. The same region had previously shown suggestive evidence for linkage in a large Costa Rican pedigree[178,244], and fine mapping of chromosome 5q involving the original six pedigrees, eight additional pedigrees from Antioquia and three pedigrees from the Central Valley of Costa Rica, a population which is genetically very close to that of Antioquia[180] (see chapter 1.3.2), resulted in a maximum NPL_{PAIRS} score of 4.40 ($p < 0.00004$; see Figure 3.1). In a further follow-up study, two-point parametric linkage analysis was performed in the same collection of pedigrees for 1082 SNPs saturating the candidate region. The maximum LOD score obtained from these analyses was 4.9[245] (this study is included as a manuscript in appendix 8.3). The two follow-up studies of the original linkage scan therefore provide highly significant evidence for linkage of a locus predisposing to severe bipolar disorder on chromosome 5q31-33.

Figure 3.1: Results of the fine mapping of the candidate region on chromosome 5q31-34 in 17 pedigrees from Antioquia and the Central Valley of Costa Rica.

A, individual and study-wide NPL_{PAIRS} scores across the candidate region on chromosome 5q; B, location of some of the strongest candidate genes in this region. The black boxes represent genes; they are drawn approximately to scale with respect to gene size and position. From Herzberg et al. (2006)[184].

Interestingly, whole-genome linkage scans in several populations have repeatedly implicated regions on chromosome 5q31-35 in the genetic susceptibility to schizophrenia[278,279], including two studies from the Central Valley of Costa Rica[280,281]. Under the current classification of psychiatric disease, BP and schizophrenia are categorised as distinct disease entities, a concept that goes back to the German psychiatrist Emil Kraepelin, who introduced the syndromes 'dementia praecox' (schizophrenia) and 'manic-depressive illness' (bipolar disorder) in the beginning of the 20th century. This 'Kraepelinian dichotomy', however, has been much criticised, and there is an emerging picture of a shared genetic susceptibility and hence, possibly – at least partly – shared aetiology between the two disorders[15,16,193,282]. The locus on chromosome 5q identified in our bipolar families might therefore predispose to psychosis, a symptom that is common in severe bipolar disorder and characteristic of schizophrenia, rather than bipolar disorder as such. In line with this interpretation, Kerner and colleagues recently reported evidence of linkage to psychosis for chromosome 5q33-34 in a collection of bipolar pedigrees

from the NIMH Bipolar Genetics Initiative[283], and a Portuguese study including both schizophrenia and bipolar families and using psychosis as the phenotype also implicated the same region[279]. Furthermore, it is of interest that all of the schizophrenia linkage scans mentioned above used a phenotype definition including schizoaffective disorder, which is characterised by features of both schizophrenia and affective disorder.

Several possible candidate genes for psychiatric illness are located within the candidate region on chromosome 5q. The gene located directly under the linkage peak identified in the Antioquian study encodes the ENTH domain containing clathrin interactor protein 1, CLINT1 (see Figure 3.1; in the figure, the gene bears one of its alternative names, *ENTH*). Genetic variants in the *CLINT1* gene region have recently been found to be associated with schizophrenia in a sample of 450 British and Irish cases and 450 matched controls[284], a finding that was replicated in a family-based sample from the Han Chinese population[285]. Another, independent Chinese study failed to provide significant evidence for association after a promising signal did not survive the correction for multiple testing[286]; however, this might be due to low power of the study and does not exclude an effect of the *CLINT1* gene on genetic susceptibility to schizophrenia in that sample[287].

CLINT1, also known as EpsinR, Espin 4 and enthoprotin, is a member of a family of proteins containing an epsin NH_2-terminal homology domain (ENTH) and has been found to be enriched on clathrin coated pits and vesicles (CCPs and CCVs)[288-290]. The ENTH functional domain is thought to be involved in the formation of membrane curvature, and the Epsin proteins, after which the domain is named, play an essential part in the formation of CCVs and the endocytosis process[291,292]. Likewise, CLINT1 has been shown to participate in clathrin-mediated membrane budding and vesicular transport between intra-cellular compartments including the *trans*-Golgi network and the endosomes[288,289].

In the brain, clathrin-mediated endocytosis plays an important role in the trafficking and recycling of neurotransmitter transporters at the presynaptic membrane[293], and of neurotransmitter receptors at the postsynaptic membrane[294-296]. The availability of neurotransmitter transporters and receptors at the synaptic membranes influences the concentration of neurotransmitters in the synaptic cleft on the one hand, and the efficiency of signal transmission on the other. Alterations in the endocytic pathway,

potentially leading to a change in neurotransmitter transporter or receptor density, might therefore interfere with synaptic signal transmission, a process which is thought to be altered in psychiatric illness.

More recently, CLINT1 has been shown to interact with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Vti1b[297]. Through interaction with each other, SNARE proteins on vesicle and target membranes play a crucial role in the membrane recognition and fusion process of clathrin-mediated endocytosis, and different SNARE proteins therefore have to be sorted with great precision onto vesicle, cell organelle and plasma membranes, a process which is partly mediated by CLINT1[298,299]. Abnormalities in SNARE protein interactions have been found in post-mortem brain studies of psychiatric patients, and it has been suggested that these abnormalities could contribute to the disturbed neural connectivity characteristic of schizophrenia and other major mental illness[300].

Given the involvement of CLINT1 in clathrin-mediated endocytosis, a process which, if disturbed, might contribute to altered neurotransmission and neural connectivity, as well as the recent association findings and the gene location at the height of the linkage peak identified in the Antioquian and Costa Rican families, *CLINT1* is a prime candidate gene for susceptibility to psychiatric disease. We therefore sequenced the *CLINT1* gene in those families that had contributed most to the linkage signal on chromosome 5q, and followed up a polymorphism identified in these families by transmission distortion analysis in a collection of 176 BP trios from Antioquia.

3.2. Materials and Methods

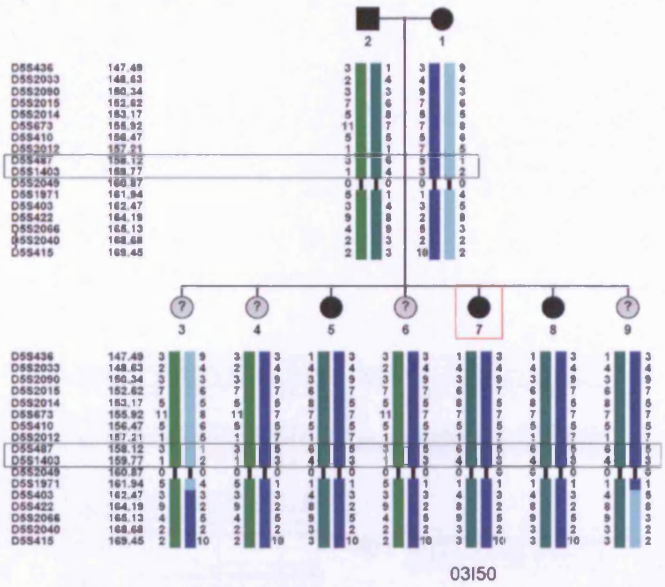
3.2.1. Sequencing of the *CLINT1* gene in the Antioquian families

The ascertainment of the BPI families recruited for the linkage scan and followed up here, and all diagnostic procedures are described in chapter 2.1. Figure 2.1 shows the pedigrees of all six families. Follow-up by sequencing of the *CLINT1* gene was conducted in those families with individual NPL_{PAIRS} scores ≥ 1 in the 5q region. These families were ANT03 (maximum NPL = 1.20), ANT04 (maximum NPL = 1.30), ANT07 (maximum NPL = 2.12) and ANT15 (maximum NPL = 1.81). The

choice of individuals for sequencing was based on haplotype analysis of the fine-mapped region on chromosome 5q. The haplotype analysis was carried out by Susan Service at UCLA using SimWalk2, based on the microsatellite fine mapping data which also formed the basis for Figure 3.1. For the results of this haplotype analysis, see Figure 3.2. For each family, an affected individual carrying the haplotype linked to disease was chosen for sequencing. This was judged by inspecting the haplotype between markers D5S487 and D5S1403, which frame the *CLINT1* gene on both sides and are highlighted by a black box in Figure 3.2. Where there were several potential disease-carrying haplotypes, several individuals were chosen from one pedigree so that each of these haplotypes would be represented in sequencing.

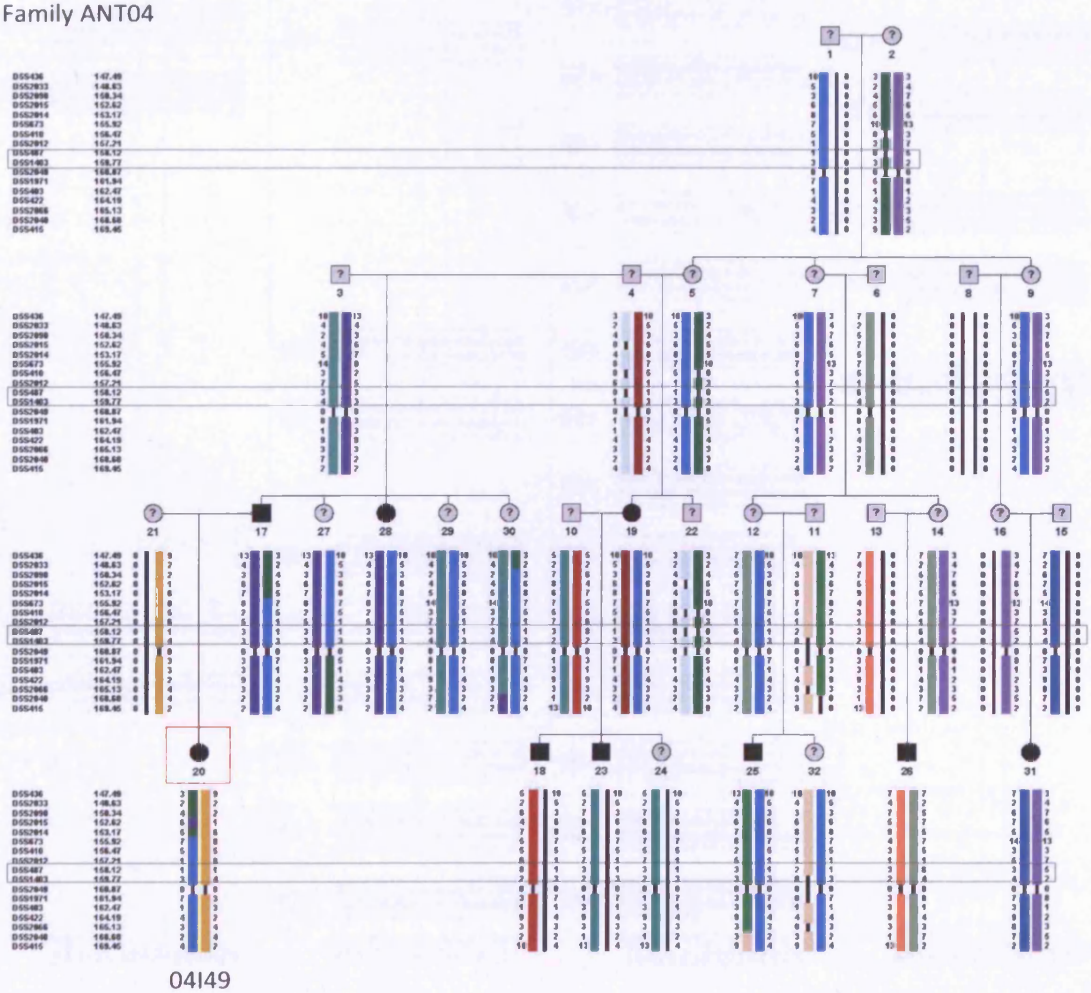
Altogether, six individuals from the Antioquian pedigrees were sequenced. In family ANT03, both the dark blue and the dark green haplotypes (see Figure 3.2) were present in all affected offspring; individual 03I50 had most DNA available and was therefore chosen for sequencing. In family ANT04, the majority of affected individuals shared the light blue haplotype; one of these, individual 04I49, was therefore chosen for sequencing. In pedigree ANT07, there were several potential disease-carrying haplotypes; three individuals were therefore sequenced from this family: 07T166 shares the green haplotype with several other affecteds, 07R259 additionally carries part of a haplotype displayed in pink shared by his affected cousin (individual 21 in Figure 3.2), and 07I1 shares the dark green haplotype with the majority of the remaining affecteds. From family 15, all affecteds again shared most of both haplotypes. Individual 15H135 had most DNA available and was therefore chosen for sequencing.

Family ANT03

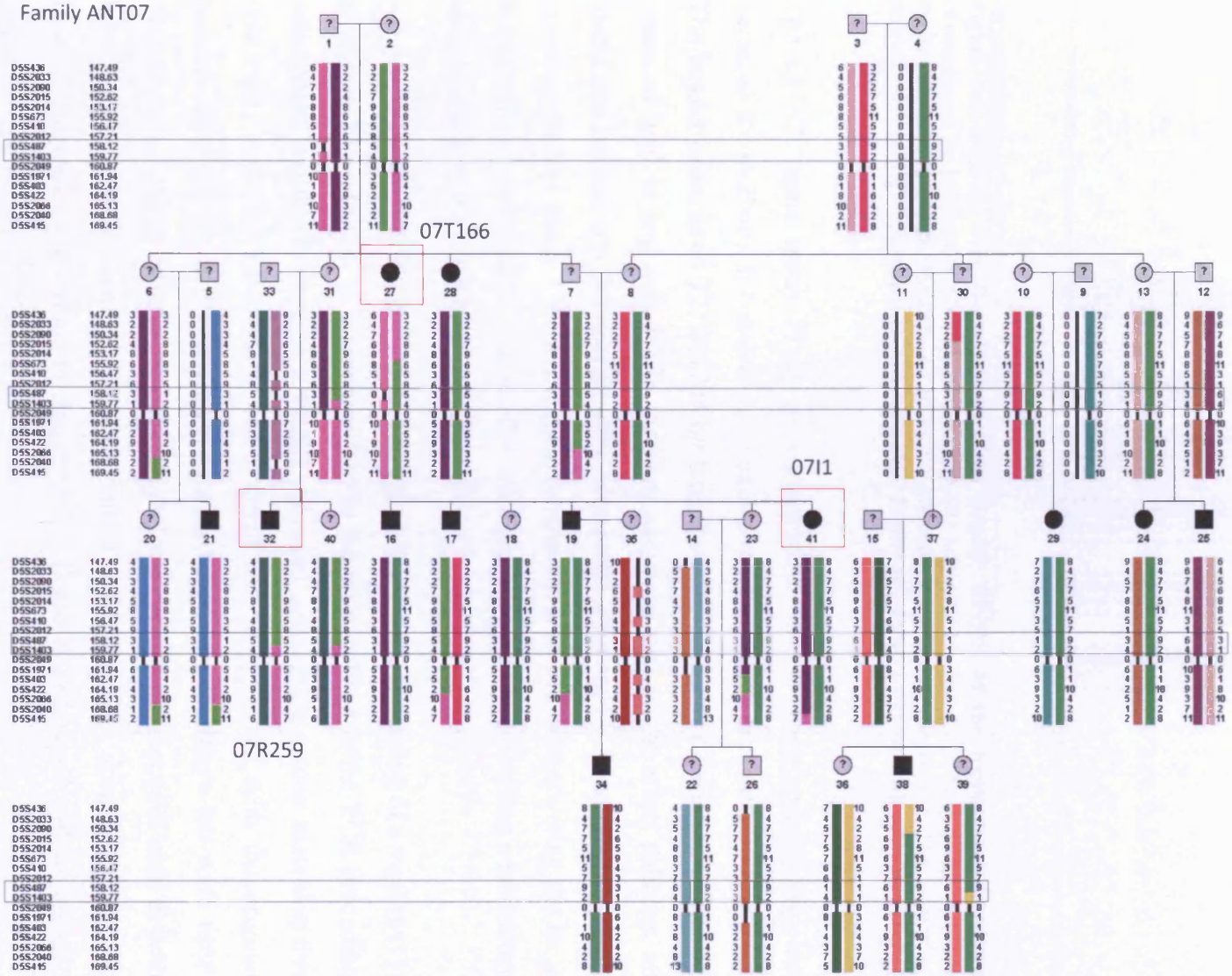


03I50

Family ANT04



04I49


Family ANT07
07T166
07I1
07R259

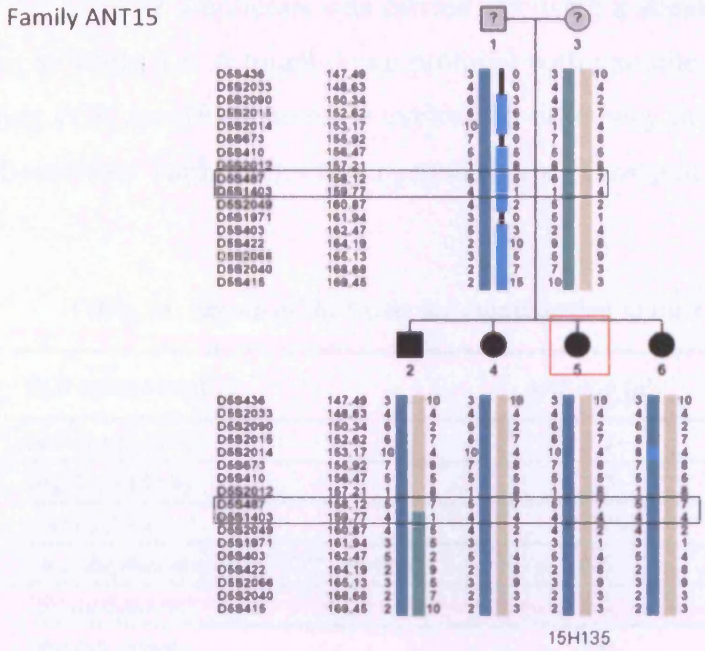


Figure 3.2 (extends over the previous three pages): Results of the haplotype analysis of chromosome 5q in families ANT03, ANT04, ANT07 and ANT15. The *CLINT1* gene lies between the highlighted markers D5S487 and D5S1403. Blackened symbols indicate BPI. The sequenced individuals are highlighted with a red rectangle. Figure produced together with Amy Roberts.

The *CLINT1* gene spans 73 kb on chromosome 5q33.3 (www.ensembl.org; last accessed 25/06/2008). It consists of 12 exons, most of which are under 300 bp long. The largest exon, exon 12, is 1704 bp long. In order to capture variation inside the exons as well as any changes in the gene sequence that might affect splicing, all exons and intron/exon boundaries were sequenced. Primers for 15 amplicons – one covering each of exons 1-11 of the gene (amplicons 1 to 11), four covering the large exon twelve (amplicons 12.1 to 12.4) – were provided by Hugh Gurling's laboratory at the Department of Psychiatry, UCL.

All primers were initially provided with a universal 5' tail consisting of a viral (M13) sequence. The addition of a universal tail to the primers used in the PCR preceding sequencing enables the use of a single, universal sequencing primer matching this tail. PCRs for *CLINT1* exons 1 and 9 could not be optimised with the original primers although the PCR conditions provided by Hugh Gurling's lab were used. This might be due to differences between thermal cyclers and reactives used in both laboratories. New primers for exons 1 and 9 were therefore designed using the Primer3 software as described in chapter 2.2.3. These were ordered without an M13 tail.

PCR of the *CLINT1* amplicons was carried out using a standard PCR cocktail set-up as shown in Table 3.1. A touch-down protocol with variable annealing temperatures, facilitating PCR specificity in early cycles and efficiency in later ones, was used for all amplicons (see Table 3.2). Primer sequences are shown in Table 3.3.

Table 3.1: Set-up of the PCRs for amplification of the *CLINT1* gene.

PCR component	volume (µl)	final concentration
Buffer (Bioline)	2.5	1x
$MgCl_2$ (Bioline)	1.0	2 mM
dNTPs (Bioline)	0.625	25 µM
Taq polymerase (BIOTAQ™, Bioline)	0.5	2.5 U*
forward primer	1.0	0.26 µM
reverse primer	1.0	0.26 µM
DNA	2	1.6 ng/µl
dH_2O	16.375	-
total	25	-

Table 3.2: Touch-down PCR programme used for the *CLINT1* amplicons.

PCR step	temperature	time	# cycles
Initiation	95°C	5 min	
Denaturing	94°C	30 sec	
Primer Annealing	63°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	60°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	57°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	54°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	51°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	48°C	30 sec	15 cycles
Elongation	72°C	30 sec	
Final Elongation	72°C	10 min	

Table 3.3: Sequences of the primers used for the amplification of the *CLINT1* gene. All sequences are noted in 5'-3' direction.

Primer Name	Primer Sequence	Product Length
Epsin4_Exon1F	CGGCACTCAGGGAAAACTAC	380 bp
Epsin4_Exon1R	CCAAGGCCAGCTCCTTCT	
Epsin4_Exon2F	cacgacgttgtaaaacgacAGTGTCCTTGGGGTAGCTTG	464 bp
Epsin4_Exon2R	ggataacaatttcacacaggGGAATGGATTGCATAAAA	
Epsin4_Exon3F	cacgacgttgtaaaacgacAACATAGGGAAAACATTTTCAAGG	419 bp
Epsin4_Exon3R	ggataacaatttcacacaggAATCTACTTCTTTCATGTGCTTTCG	
Epsin4_Exon4F	ggataacaatttcacacaggAGAAACCTCACCACCAGCAT	444 bp
Epsin4_Exon4R	cacgacgttgtaaaacgacGCCAGTCTTTTCTGCAAGTTC	
Epsin4_Exon5F	cacgacgttgtaaaacgacTAGCACAACTTGCCCCTTTC	526 bp
Epsin4_Exon5R	ggataacaatttcacacaggCTATTGCCGAACCCACAAGT	
Epsin4_Exon6F	cacgacgttgtaaaacgacGAATCTGCATTCCTGCCACT	570 bp
Epsin4_Exon6R	ggataacaatttcacacaggTTTCTGTGAGCTTAAATTGCTCT	
Epsin4_Exon7F	ggataacaatttcacacaggCCCCAGCGCACATATATCA	530 bp
Epsin4_Exon7R	cacgacgttgtaaaacgacGCCCAAGCCTACTAATGCAG	
Epsin4_Exon8F	cacgacgttgtaaaacgacGCTTGCTGCCTCTCCATAAG	369 bp
Epsin4_Exon8R	ggataacaatttcacacaggAAAAATGAACCCAATGCAAA	
Epsin4_Exon9F	AGCTTTTGTGAGGCATCTGG	373 bp
Epsin4_Exon9R	TTTGGCACATTTAAGGCATCT	
Epsin4_Exon10F	ggataacaatttcacacaggAATCTGGGGGACTCAGATCA	579 bp
Epsin4_Exon10R	cacgacgttgtaaaacgacAATGAAATGTGCAAATGCGTA	
Epsin4_Exon11F	cacgacgttgtaaaacgacTGTTGTGATTAAATTGCCATCTG	478 bp
Epsin4_Exon11R	ggataacaatttcacacaggTCAATGTGCCAGGAGGAATA	
Epsin4_Exon12.1F	ggataacaatttcacacaggTCAAGAGGAACCAGTGCAGA	606 bp
Epsin4_Exon12.1R	cacgacgttgtaaaacgacGCATTTTCCATCCCAACATC	
Epsin4_Exon12.2F	cacgacgttgtaaaacgacAACCCAAGCAAGATGCCTTT	722 bp
Epsin4_Exon12.2R	ggataacaatttcacacaggAAAAGTCCCATGGCAATCAC	
Epsin4_Exon12.3F	ggataacaatttcacacaggTGGCTAAATCAAAGGTAACTGGA	665 bp
Epsin4_Exon12.3R	cacgacgttgtaaaacgacCCAGCTTGAGGGTAAAACA	
Epsin4_Exon12.4F	cacgacgttgtaaaacgacTGAAAATGTTTTTGTTTTTCTGC	469 bp
Epsin4_Exon12.4R	ggataacaatttcacacaggAAGAATGGAGCAGGTCAGGA	

PCR product clean-up and sequencing were performed as described in chapter 2.2.6. Both forward and reverse sequencing were attempted for each exon, and for each of the samples. The CEPH sample 1344-03 was included as a positive control in both PCR and sequencing; in PCR only, dH_2O was included as a negative control.

All sequences were visually checked for the presence of mutations or polymorphisms, initially using the Sequencher software v4.7 (Demo version). A

license for the programme ChromasPro was purchased while this project was already underway, and analyses were continued using that software. The sequences obtained in this project were aligned with the human reference sequence using MAFFT v5.8 to identify the reference allele for any polymorphism encountered. The CEPH sample provided an additional healthy reference sample.

3.2.2. Transmission Distortion Analysis of a SNP in Exon 12 of the CLINT1 gene in a Bipolar Trio sample from Antioquia

Sequencing of *CLINT1* in BP cases from the pedigrees, we identified a SNP in intron 11 of the gene, close to the intron/exon boundary with exon 12 (see section 3.3). This SNP had been previously described as rs11955293 (NCBI dbSNP; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi? rs=11955293). In order to investigate whether this SNP showed association with BP in the Antioquian population, we genotyped the SNP rs11955293 in a collection of 176 unrelated BP patients and their parents (or, if unavailable, their siblings) and performed a transmission distortion analysis in this sample. A detailed description of the sample can be found in chapter 2.1.3.

The SNP rs11955293 was genotyped using an RFLP-based assay. The T allele introduces a recognition site for the restriction enzyme Tsp509I at the SNP; this enzyme was therefore used in the RFLP assay. A difficulty lay in the fact that the recognition motif for Tsp509I, AATT, recurs several times in close proximity of rs11955293; the next one lies only 15 bp downstream of the SNP (see Figure 3.3).

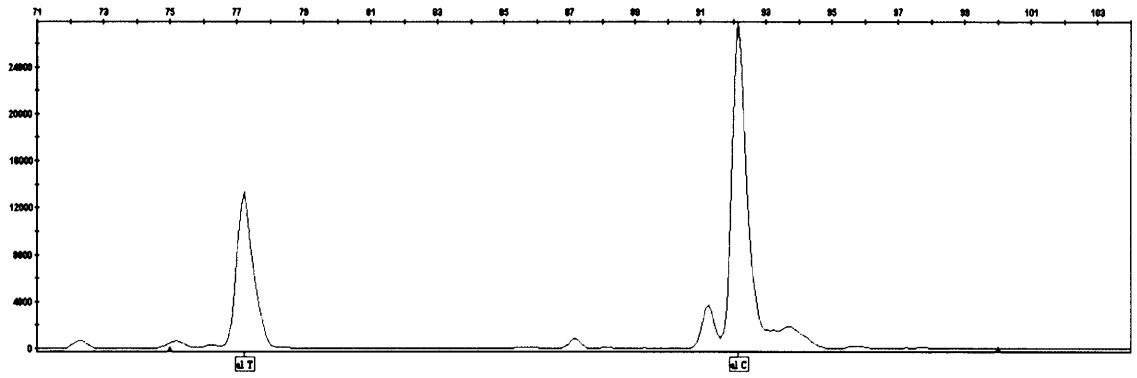
Figure 3.3: Sequence of the amplicon designed for rs11955293 genotyping.

FAM-TGAGGTTCACAGCTCCAAAACTTTGAGTCATCACATTCATAGGCTGCTGCATATCTACCAGACGA
AAACAGACAGAAGAAC/TTTTACCACAATAAATTTTTAGGTTCATAGAACAGAGAACTAATAACTTCTT
GACCATCTATCTATACTTCTTTAGCCCCAATAAAATTCCTTTACAGTGATTTTATTAGAAATCAATTAC
TCTGCACTGGTTCCTCTTGA

The two alleles of the SNP are represented in red. Tsp509I recognition sites are highlighted in yellow. PCR primers are shown in bold type. For detection of the digestion product on a capillary sequencer, the forward primer was labelled with FAM.

Because a size difference of 15 bp cannot be reliably detected on an agarose gel, a suitable agarose electrophoresis-based assay could not be designed. Instead, the forward primer used to amplify the genomic DNA around the SNP was labelled with the fluorescent dye FAM, allowing the detection of the size-variable digestion product through electrophoresis on a capillary sequencer: while complete digestion of the 221 bp-amplicon shown in Figure 3.3 results in five short DNA fragments, only the fluorescently labelled fragments can be detected in capillary electrophoresis: a 78 bp long fragment for the T allele (cut at rs11955293), and a 93 bp long fragment for the C allele (uncut at rs11955293).

Figure 3.4: Electropherogram showing a heterozygous genotype for SNP rs11955293 (from the GeneMapper® software).



The fluorescent dye at the 5' end of the forward primer allows the detection of the size-variable digestion product by capillary electrophoresis. The 15 bp-difference between both alleles is easily detectable using high-resolution electrophoresis.

As a first step, a 221 bp-fragment containing the SNP was amplified by PCR using a standard protocol as shown in Table 2.4. Modifications made to this protocol were:

- 1.5 µl $MgCl_2$ were used per sample (final concentration 3.0 mM).
- 0.13 µl (0.65 U) Taq polymerase were used per sample.
- The volume of dH_2O was adjusted accordingly to maintain a total volume of 25 µl.

PCR primers were designed using the Primer3 programme as described in section 2.2.3. Primer sequences were as follows:

forward primer: 5'FAM-TGAGGTTCACAGCTCCAAAA-3'

reverse primer: 5'-TCAAGAGGAACCAGTGCAGA-3'

A touch-down cycling protocol was used, as shown in Table 3.4. A negative (dH_2O) and a positive control (CEPH sample 1347-02) were included in each PCR.

Table 3.4: Touch-down PCR programme used for the amplification of the genomic sequence around rs11955293.

PCR step	temperature	time	# cycles
Initiation	95°C	5 min	
Denaturing	94°C	30 sec	
Primer Annealing	63°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	60°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	57°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	54°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	51°C	30 sec	3 cycles
Elongation	72°C	30 sec	
Denaturing	94°C	30 sec	
Primer Annealing	48°C	30 sec	15 cycles
Elongation	72°C	30 sec	
Final Elongation	72°C	10 min	

After checking the amplification success by agarose gel electrophoresis (see chapter 2.2.4), the PCR product was digested with Tsp509I following the standard protocol described in section 2.2.5.4. After the enzymatic digestion, the samples were sent to the Centre of Comparative Genomics, where they were run on an ABI 3730xl® genetic analyser (Applied Biosystems). The resulting raw data was sent back to our

lab, and genotype analysis was performed with the GeneMapper® v3.7 software. All genotypes were visually checked by two independent researchers ("double scoring"). Upon disagreement between the two scorers, a single attempt was made to re-genotype the sample in question. If re-genotyping was unsuccessful, the sample was excluded from the study.

The methods used for the statistical analysis of the trio data have are presented in detail in chapter 2.3. Briefly, the genotype data were checked for Mendelian inconsistencies using PedCheck[268]. Hardy-Weinberg equilibrium was evaluated separately in founders and cases using the Genepop program[266]. The Transmission Disequilibrium Test (TDT)[154] was carried out as implemented in the TRANSMIT program, version 2.5.4.[155].

3.3. Results

3.3.1. Sequencing of the *CLINT1* gene in the Antioquian families

Amplification success

Amplification by PCR was attempted for fifteen amplicons covering the twelve exons and the intron/exon boundaries of the *CLINT1* gene. Amplicons 1-11 correspond to exons 1-11; the large exon 12 was covered by amplicons 12.1-12.4.

Despite various attempts at optimisation of the PCR, including the design of a new primer pair, amplicon 1 could not successfully be amplified. This amplification failure might be due to the high GC content of this amplicon, which was 70%. Amplicon 9 could not be amplified using the original primers provided by Hugh Gurling's lab; after re-designing the primers, however, amplification was successful. PCR for all other amplicons was successful in all six samples from the BP families, as well as in the CEPH sample 1344-03.

Sequencing success

Both forward and reverse sequencing were attempted for all successfully amplified amplicons in all samples, making for a total of 196 sequences (14 amplicons x 2 x 7 samples). For most samples, at least one readable sequence, either forward or reverse, could be obtained for every amplicon (see Table 3.5 for details). For the following amplicons and samples, no readable sequence could be obtained: amplicon 4 (sample 04I49), amplicon 8 (07T166 and 15H135), and amplicon 12.4 (07R259).

Table 3.5: Sequencing success for the *CLINT1* gene in Antioquian BP family samples.

Sequence	03I50	04I49	07I1	07T166	07R259	15H135	CEPH*
Amplicon 2-F	sequenced	x	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 2-R	sequenced	sequenced	x	sequenced	sequenced	sequenced	sequenced
Amplicon 2	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**
Amplicon 3-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 3-R	sequenced	sequenced	sequenced	sequenced	sequenced	x	sequenced
Amplicon 3	**complete**	**complete**	**3'-gap**	**complete**	**complete**	**complete**	**complete**
Amplicon 4-F	sequenced	x	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 4-R	sequenced	x	x	x	x	sequenced	x
Amplicon 4	**3'-gap**	**x**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**
Amplicon 5-F	sequenced	x	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 5-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 5	**complete**	**complete**	**complete**	**complete**	**complete**	**complete**	**complete**
Amplicon 6-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 6-R	x	x	x	x	x	x	x
Amplicon 6	**GBE**	**GBE**	**GBE**	**GBE**	**GBE**	**GBE**	**GBE**
Amplicon 7-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 7-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 7	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**GBE**
Amplicon 8-F	sequenced	sequenced	x	x	sequenced	x	sequenced
Amplicon 8-R	sequenced	x	sequenced	x	x	x	sequenced
Amplicon 8	**complete**	**5'-gap**	**3'-gap**	**x**	**5'-gap**	**x**	**3'-gap**
Amplicon 9-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 9-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 9	**5'-gap**	**5'-gap**	**3'-gap**	**5'-gap**	**5'-gap**	**5'-gap**	**complete**
Amplicon 10-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 10-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 10	**complete**	**complete**	**complete**	**complete**	**complete**	**complete**	**complete**
Amplicon 11-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 11-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 11	**complete**	**complete**	**3'-gap**	**complete**	**complete**	**complete**	**complete**
Amplicon 12.1-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	x
Amplicon 12.1-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 12.1	**complete**	**complete**	**3'-gap**	**complete**	**complete**	**complete**	**complete**
Amplicon 12.2-F	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 12.2-R	sequenced	sequenced	sequenced	sequenced	sequenced	sequenced	x
Amplicon 12.2	**GBE**	**GBE**	**GBE**	**GBE**	**GBE**	**GBE**	**GBE**
Amplicon 12.3-F	sequenced	sequenced	sequenced	sequenced	sequenced	x	x
Amplicon 12.3-R	sequenced	x	sequenced	sequenced	sequenced	sequenced	sequenced
Amplicon 12.3	**complete**	**3'-gap**	**3'-gap**	**5'-gap**	**complete**	**complete**	**3'-gap**
Amplicon 12.4-F	sequenced	sequenced	sequenced	sequenced	x	sequenced	sequenced
Amplicon 12.4-R	sequenced	sequenced	sequenced	x	x	sequenced	sequenced
Amplicon 12.4	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**	**3'-gap**

Sequencing of amplicon 1 was not attempted because of PCR failure. For each amplicon, success of forward and reverse sequencing is indicated separately; a third row then shows the overall success for the amplicon in each sample (i.e., whether the sequence is complete, or whether there is a gap at either of the two ends; 5' and 3' refer to the forward sequence). x, sequence could not be obtained/sequence not readable; GBE, gap at both ends. *CEPH 1344-03

As shown in Table 3.5, many sequences have gaps at either or both ends, meaning that for none of the samples, the entire sequence was obtained. However, these gaps were typically only a few bp long. Because the PCR products were designed to include intron/exon boundaries, in most cases, the complete exon and the sequence immediately adjacent to it was therefore covered whenever sequencing in either direction was successful. The only exception was the large exon 12, which was amplified in several fragments, and where sequencing gaps therefore led to gaps in the assembly of exonic DNA.

Among all amplicons, the most sequences were missing for amplicon 8 and for reverse sequencing of amplicon 6. This might be explained by the fact that both amplicons contained long repeat stretches. In amplicon 6, a long mononucleotide T-repeat was located next to the reverse sequencing primer. Such long repeat stretches are extremely difficult to sequence because of polymerase slippage: during sequencing, template and product strands might denature and subsequently re-hybridise in the wrong position, forming a loop on either strand and thereby shortening or lengthening the repeat stretch. Because the repeat stretch was located next to the reverse primer in amplicon 6, forward sequencing worked without problems; reverse sequencing, however, was messed up from the beginning by the mononucleotide T-run. Several shorter repeat runs occur throughout the sequence of amplicon 8. The fact that the repeat runs were shorter than in amplicon 6 might explain why part of the sequences could be obtained.

In summary, exon 1 could not be sequenced in any sample. The remaining exons of the *CLINT1* gene were successfully sequenced in samples 03I50, 07I1, and in the CEPH sample 1344-03; although not for all amplicons, both forward and reverse sequences could be obtained. For the remaining samples, the majority of the amplicons were successfully sequenced, leaving only small segments of the coding region unsequenced.

Identification of sequence variants

We identified three sequence variants in the *CLINT1* gene region in the BP samples from Antioquia. In individual 07I1, a T > C change was observed in the 3' untranslated region of the gene, which forms part of exon 12 (amplicon 12.2). This

SNP has been previously described as rs12284 (NCBI dbSNP; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=12284, last accessed 27/06/2008). This change did not occur in any other individuals sequenced in this study, in line with the low frequency of the derived C allele in Europeans (~4-10%, dbSNP; the Antioquian population has highly predominant European ancestry, and allele frequencies in Europeans are a proxy for those in Antioquia[182]).

The second sequence variation we identified was a C > T change, which occurred in intron 11 of the *CLINT1* gene, 27 bp away from the intron/exon boundary with exon 12 (amplicon 12.1). This change was found in individuals 03I50 and 15H135. It has been previously described as rs11955293, and the frequency of the rare T allele has been reported to be ~8% in Europeans (NCBI dbSNP).

Finally, a third change was found in the same two individuals who carried the rare allele for SNP rs 11955293, 03I50 and 15H135. This SNP, which has equally been previously described and bears the number rs6682224, is a C > T change in intron 3, just four bp away from the intron/exon boundary with exon 4 (NCBI dbSNP, http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=6882224). As for rs11955293, the frequency of the rare T allele in Europeans is around 8%.

3.3.2. TDT Analysis of a marker rs11955293

Test for Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium at SNP locus rs11955293 was tested for parents and offspring separately. In none of the two groups, the genotype distribution showed a significant deviation from that expected under Hardy-Weinberg (parents: p-value = 0.16; affected offspring: p-value 1.0).

Transmission Distortion Analysis

The results of the TDT analysis of rs11955293 in a collection of 176 unrelated BPI patients and their parents/siblings are shown in Table 3.6. The observed allele transmissions correspond almost exactly to the expected transmission under Mendelian inheritance; thereby indicating that there is no transmission distortion at the SNP locus analysed here.

Table 3.6: Transmission distortion analysis of alleles at locus rs11955293.

Allele	O	E	Var (O-E)	χ^2 (1d.f.)	p-value	bootstrap p-value[c]
C	313	312.45	5.848	0.05	0.819	0.816
T	15	15.55	5.848	0.05	0.819	0.816
		Global		**0.05**	**0.819**	**0.816**

O, observed allele transmission; E, expected allele transmission under Mendelian inheritance; Var(O-E), variance of O-E; d.f., degrees of freedom.

3.4. Discussion

Sequencing of the *CLINT1* gene in six BPI patients from four extended pedigrees showing evidence of linkage for a region on chromosome 5q identified three sequence variants. These changes had been previously described as rs6682224, rs11955293 and rs12284.

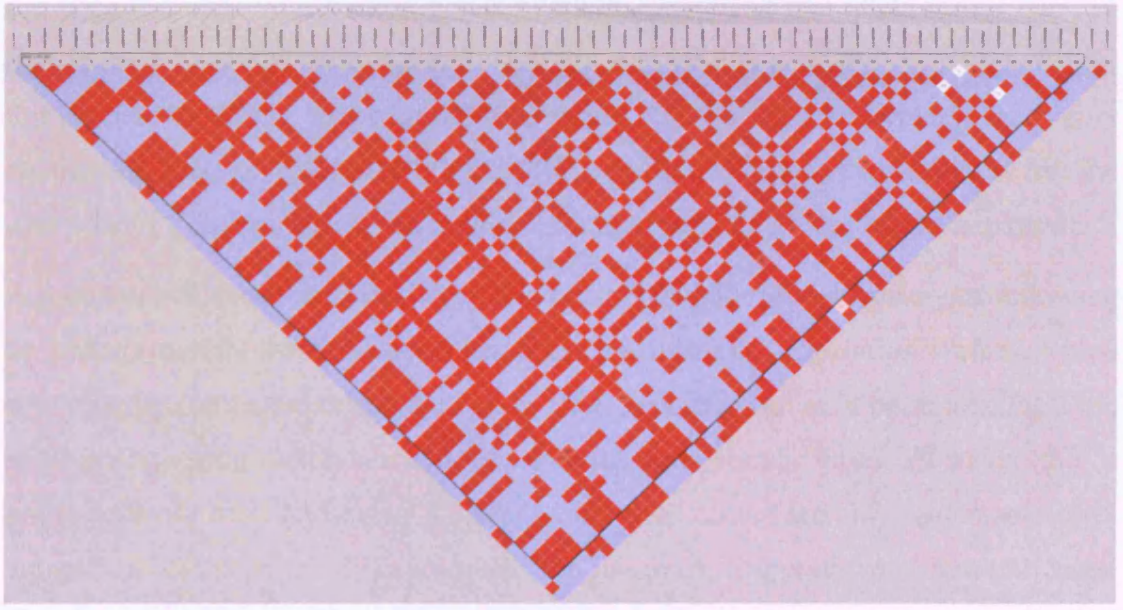
Rs12284 is located in the 3' untranslated region of the *CLINT1* gene and is not predicted to have an effect on gene expression. Furthermore, this change was only seen in a single individual, 07I1. Because the number of individuals sequenced for this study was very small, this fact alone does not exclude a potential role for SNP rs12284 in bipolar disorder in the population of Antioquia; however, it was not a prime candidate for follow-up by association analysis.

The identification of the two SNPs rs6682224 and rs11955293 in two apparently unrelated individuals, 03I50 and 15H135, seemed of greater interest. Both SNPs have a minor allele frequency of around 8% in Europeans (NCBI dbSNP, last accessed on 27/06/2008). The population of Antioquia is of predominantly European ancestry[182]; allele frequencies in that population are therefore a good approximation of those in Antioquia. The shared occurrence of a rare allele in two out of four unrelated BPI families might indicate a possible role for this variant in the genetic susceptibility to bipolar disorder. Furthermore, individuals 03I50 and 15H135 share a rare allele not just at one, but at both SNP loci, thereby opening up the distinct possibility of IBD sharing in families ANT03 and ANT15. Inspection of the reconstructed haplotypes for the region on chromosome 5q (shown in Figure 3.2) additionally reveals that affected individuals from families ANT03 and ANT15 also share alleles at the microsatellite markers on both sides of the *CLINT1* gene: allele 5 at marker D5S487, and allele 4 at marker D5S1403. Although these alleles are not uncommon, with estimated frequencies of 14 and 23%, respectively, in the population of Antioquia (Susan Service, personal communication), this finding is in line with possible IBD haplotype sharing between the two families.

Both rs6682224 and rs11955293 are located in intronic sequence – introns 3 and 11, respectively – and are therefore not expected to influence the amino acid sequence of the CLINT1 protein directly. However, because they are relatively close to the intron/exon boundary, they might affect the splicing of the CLINT1 transcript. It was

therefore of interest to investigate a possible association between these SNPs and BPI in the population of Antioquia. The TDT analysis of the SNP rs11955293 in the BPI trio sample, however, revealed no transmission distortion of alleles at that locus. Failure to detect association at a single SNP locus does not exclude the possibility of finding association for other markers in the same gene because there may be several LD blocks within a gene. However, HapMap data show that LD across the entire *CLINT1* gene is extremely high, with a single haplotype block covering the whole gene region in all HapMap populations (Figure 3.5 shows the data for Europeans only; patterns in the Asian and Yoruba populations are the same).

Figure 3.5: LD structure across the *CLINT1* gene region the European HapMap population.



Shown are pairwise D' values for position 157145 – 157218 kb on chromosome 5q, covering the *CLINT1* gene region. The vast majority of D' values between marker pairs are 1.0; for D' values < 1.0, values in the boxes refer to % D' Red boxes indicate a LOD score of ≥ 2.0 (D' significant); blue and boxes indicate a LOD score of < 2.0 (D' not significant).

Our analyses provided no evidence for an implication of the *CLINT1* gene in genetic susceptibility to bipolar disorder. The haplotype sharing between affected individuals from families ANT03 and ANT15, which prompted the association study in the trios, might be a chance event. Alternatively, and independently from the question whether *CLINT1* is a predisposing locus for bipolar disorder or psychosis, it might indicate that these families are distantly related. Given that both families stem from the

Antioquian sub-isolate of Oriente, this is a distinct possibility. Such a connection between the two pedigrees might prove useful for future gene mapping studies because it increases the probability that individuals from these families will share predisposing variants, which might be localised through the identification of shared haplotypes in candidate regions.

The lack of even a trend towards association in our BPI trio sample seems surprising, given the fact that *CLINT1* is located at the height of the highly significant linkage peak detected in the Antioquian families. There are several possible interpretations for our findings. The simplest of these is that our study did not have sufficient power to detect an existing weak association. Given the modest size of the trio sample examined here, this possibility cannot be disregarded. However, as noted above, we did not even observe a trend towards overtransmission of one allele in our data, so that, if there is indeed an association between rs11955293 and BPI, the effect size for this variant is likely to be small and might not be able to explain the highly significant linkage peak obtained in the families. Further explanations for the discrepancy between the results in families and trios should therefore be explored.

A fundamental question emerging in this context is that of the adequacy of following up linkage signals from extended pedigrees by means of association studies, which are typically conducted in sporadic cases. This approach can only be successful if the same predisposing factors are acting in familial and sporadic cases. However, this is not necessarily true. Alzheimer's disease and breast cancer are only two examples of complex diseases where different genes are involved in sporadic and familial cases. These examples are different from familial psychiatric disease in that the familial forms are truly Mendelian, and the distinction between susceptibility to familial and sporadic forms of psychiatric illness might not be as clear-cut as that seen in Alzheimer's and breast cancer. However, these examples do show that different loci might be involved in familial and sporadic disease, and this might at least partly explain why the follow-up of the linkage peak in a collection of unrelated sporadic samples was unsuccessful.

CLINT1 was chosen for candidate gene analysis because of functional evidence linking it to psychiatric disease, because of evidence for association to schizophrenia, and because of its location directly beneath the linkage peak on chromosome 5q in the BPI families. However, several other interesting candidate genes for major

psychiatric illness are located within this linkage peak (see Figure 3.1). Amongst them are a cluster of gamma-aminobutyric acid receptor subunit genes (*GABRA1, GABRA6, GABRB2, GABRG2*), the gene encoding the glutamate receptor 1 subunit of the ionotropic AMPA receptor (*GRIA1*), and the serotonin receptor gene *HTR4*[184]. *GRIA1* has been linked to both schizophrenia and affective disorder by mRNA expression analysis in the cells of the dorsolateral prefrontal cortex[301], and a fine mapping study in pedigrees from the NIMH Genetics Initiative which had produced evidence for linkage of psychosis to chromosome 5q33-34[283], found association of that phenotype to polymorphisms in the *GRIA1* gene[302]. Findings of association of the major psychoses to the *HTR4* gene, and to the cluster of GABA receptor subunit genes found on chromosome 5q have also been reported[303-306]. Therefore, it seems possible that one of these genes, rather than *CLINT1*, could be at the root of the linkage signal obtained in our genome scan for chromosome 5q, and it would be of great interest to further explore a possible role of these genes in predisposition to bipolar in our Antioquian sample.

It is noteworthy that a whole-genome linkage scan of bipolar disorder performed as part of this thesis and incorporating a further nine Antioquian families in addition to those included in our original study, did not provide evidence for linkage on chromosome 5q. The results of this linkage scan are presented in chapter 4 of this thesis. As noted in the discussion of that chapter, the lack of a signal for chromosome 5q in the new study does not invalidate our original report of linkage, but it might nevertheless indicate that the locus on chromosome 5q is of greater importance in the Costa Rican families than in those from Antioquia. This is in line with the fact that our collaborators had previously reported linkage for that region in a very large Costa Rican pedigree, which was also included in the finemapping efforts preceding the present study of the *CLINT1* gene[178,244]. One final interpretation of our failure to find association between BPI and *CLINT1* is therefore that the 5q-locus might not be as important in the Antioquian population as appears from the results of our first linkage scan[184], and that the exploration of other candidate regions might be of greater interest.

3.5. Conclusion and Future Work

In this study, we have followed up the results of a previous linkage scan by sequencing and association analysis of the *CLINT1* gene on chromosome 5q33 as a possible candidate gene for psychosis. We have found no evidence for a contribution of this gene to disease susceptibility in the population of Antioquia.

In our effort to sequence the *CLINT1*, some portions of the genomic sequence, including the entire exon 1 and small segments of exon 12, could not be covered. It might be of interest to obtain the missing parts of the *CLINT1* sequence in our samples in order to ensure that no sequence variants that might be of importance in the families have been missed. Sequencing of the promoter region, which plays an important part in the regulation of gene expression, is already underway. However, in the light of the results presented here, it might be of greater interest to focus on follow-up studies of other genes in the 5q region, including *GRIA1*, *HTR4*, and the GABA receptor subunit genes. In an approach equivalent to that taken in the *CLINT1* study, these genes could be sequenced in the Antioquian families first. Interesting variants could then be further investigated in the BPI trio sample. It would also be interesting to conduct follow-up studies in the Costa Rican pedigrees, which might have been the main contributors to the 5q linkage signal.

CHAPTER FOUR

GENOME-WIDE LINKAGE SCAN IN 15 EXTENDED BIPOLAR PEDIGREES FROM THE PAISA COMMUNITY

4. Genome-Wide Linkage Study in 15 Extended Bipolar Pedigrees from the Paisa Community

4.1. Background and Previous Work

As discussed in previous chapters of this thesis (see chapters 1.6 and 3), we have recently conducted a whole-genome linkage scan of six extended pedigrees segregating severe bipolar disorder from the *paisa* community[184]. In that study, we conducted both two-point parametric and multipoint non-parametric linkage analysis using bipolar disorder type I as the phenotype definition.

Our analyses identified several candidate regions for BPI, which are presented in Tables 4.1 (two-point parametric analysis) and 4.2 (multipoint non-parametric analysis). The most important of these regions are located on chromosomes 6q22, 15q26, and 21q21 in parametric analysis, and on chromosomes 3q28, 5q33 and 21q21, and 1p22 in non-parametric analysis.

Table 4.1: Markers with heterogeneity LOD scores ≥ 1.3 in two-point parametric linkage analysis of genome scan data from six Antioquian BPI pedigrees.

From Herzberg et al. (2006)[184]. Families CO3 to CO27 correspond to the families named ANT03 to ANT27 in this thesis. See Figure 2.1 for the pedigrees.

Table 4.2: Markers with the highest combined NPL scores ($p < 0.05$) in the genome scan of six Antioquian pedigrees.

From Herzberg et al. (2006)[184]. Families CO3 to CO27 correspond to the families named ANT03 to ANT27 in this thesis. See Figure 2.1 for the pedigrees.

We initially chose to follow up the signal on chromosome 5q33-34, because our collaborators had found the same region to be implicated in susceptibility to BPI in a very large pedigree from the genetically closely related population of the Central Valley of Costa Rica[178,244]. While follow-up studies in pedigree and trio samples from Antioquia and Costa Rica continue to support the existence of a susceptibility locus for severe bipolar disorder on chromosome 5q in both populations[184,245], the most important contribution to the linkage signal is made by the Costa Rican pedigrees, indicating that that there might be other predisposing loci of greater relevance in the Antioquian population (see also the discussion of the previous chapter, section 3.4).

We wanted to explore this possibility further by performing a linkage scan in an additional nine BPI families from the *paisa* community that had become available for study. Of these, seven families stem from the Oriente region in Antioquia, while two families originate in the village of Aranzazu in the Colombian province of Caldas. As described in chapter 1.3.2, the population of Caldas forms part of the *paisa* community through historical connections. Furthermore, a genealogical study of the families from Aranzazu conducted by our collaborators in Medellín has shown that the founder of these families immigrated from the village of Marinilla in the Oriente region of Antioquia. This places all fifteen *paisa* pedigrees (six families from the original linkage scan and nine newly ascertained families) in the same circumscribed and genetically homogeneous sub-isolate of Oriente, making them an ideal pedigree collection for the study of a complex disease such as BP. We therefore decided to genotype the same set of microsatellite markers used for the original scan in the nine

new families and carry out a joint analysis of the two data sets with the aim of identifying chromosomal regions harbouring potential susceptibility variants for bipolar disorder in the population of Antioquia.

4.2. Study Design

Ascertainment and diagnostic procedures for all fifteen pedigrees are described in detail in chapter 2.1. Genotype data were obtained for 382 evenly spaced autosomal microsatellite markers from the ABI Linkage Mapping Set v2.5 (average distance ~10cM) were analysed. Data for nine extended pedigrees (Figure 2.2) were obtained for this thesis and combined with data from the earlier genome-wide linkage scan of six BPI families (Figure 2.1). All analyses were carried out on the combined data set. Because the patterns of disease transmission did not support an X-chromosomal mode of inheritance in the Antioquian pedigrees, the X-chromosome was not analysed. The present study therefore represents an autosome-wide linkage scan.

The original design of this study was focused on the phenotype of bipolar disorder type I, and before embarking on the data collection, a simulation analysis based on this phenotype was carried out to assess the power of the pedigrees for linkage analysis (see section 4.3). However, because of the uncertainties attached to the nosological classification of mood disorders, including bipolar disorder, and the difficulties in choosing an adequate definition of the phenotype to study, it was decided to analyse the data under three different models as follows:

1. "Narrow model": As discussed previously, BP is a complex disorder, whose considerable genetic heterogeneity is reflected in a broad range of clinical phenotypes. One strategy to reduce the genetic heterogeneity within a linkage study lies in focussing on a particular, well-defined subtype of the disorder. Furthermore, severe forms of the disorder might have a greater genetic component than milder phenotypes. In this model, only patients diagnosed with BPI are considered as affected. All other family members were considered of unknown phenotype.
2. "Broad model": All pedigrees were ascertained through probands with a diagnosis of BPI as described in chapter 2.1; however, several other

psychiatric diagnoses occurred in the relatives of the BPI probands, most notably major unipolar depression (see Table 2.1). This is consistent with epidemiological findings of an increased rate of unipolar depression in the first-degree relatives of patients with BP[307] and lends support to the notion of a genetic relatedness between bipolar and unipolar affective disorders. To explore the possibility of a shared genetic susceptibility between the major mood disorders in the pedigrees from Antioquia, all family members diagnosed with BPI, BPII and unipolar depression were considered as affected under this broad model; the phenotype of all other family members was considered unknown.

3. "Psychosis model": This last model is concerned with the phenotype of psychosis, the experience of perceptions that are not grounded in reality. Psychosis does not occur in all BP patients and might therefore characterise a distinct subtype of the disorder. Selecting patients for psychosis might further reduce the genetic heterogeneity within the sample. On the other hand, as discussed in chapter 3, there is a growing amount of evidence for an at least partially shared aetiology between BP and schizophrenia, and there might be genetic variants predisposing to psychosis rather than to the phenotypes of schizophrenia or BP as such. The "psychosis model" therefore considers those pedigree members as affected that have experienced psychosis at some point of their illness. This includes many of the BPI patients, but also one schizophrenic and two individuals with a psychotic mood disorder that did not meet the full criteria for BP (these individuals are from family ANT24 and families ANT14 and ANT15, respectively; see Figures 2.1 and 2.2). A similar approach has been taken by Kerner and colleagues in analysing a collection of BP pedigrees from the U.S. National Institute of Mental Health[283]. As for the other two models, all other family members were considered as of an unknown phenotype. Importantly, not all families included two or more members affected by psychosis, so that the analysed data set for this model comprised only eleven extended pedigrees (see Figures 2.1 and 2.2).

The pedigree data from Antioquia were analysed using both parametric and non-parametric linkage approaches. Because a genetic model based on epidemiological

data was only available for the narrow model of disease classification (see chapter 2.3.7), parametric linkage analysis was only performed for this model. Non-parametric linkage analysis was carried out for all three phenotype models.

The exploration of three different models of disease classification introduces a problem of multiple testing into the analyses carried out on the Antioquian pedigree data (further to the one caused by the analysis of a large amount of markers, which is inherently corrected for by the use of the recommended significance thresholds as discussed in chapter 1.2.3). Formally, this might be corrected for by applying a simple Bonferroni correction, taking the form of

$$\alpha' = \alpha/t, \qquad (14)$$

where α is the nominal level of significance, α' is the corrected level of significance, and t is the number of models tested[308]. Likewise, the use of both parametric and non-parametric linkage approaches in the analysis under the narrow model must formally be seen as multiple testing and should be corrected for in order to control the false positive rate. LOD scores should be adjusted using the following equation:

$$z' = z + \log_{10}(t), \qquad (15)$$

where z is the LOD score threshold for genome wide significance (i.e., 3), and z' is the LOD score to be achieved for significance after correction for multiple testing; t is the number of models tested[308]. Here, t = 4; the formal significance levels for this study are therefore LOD $\geq$ 3.60 and $NPL_{PAIRS} \geq 4.49$.

However, this study is a hypothesis-generating one and, considering the genetic heterogeneity of the disorder under study, even linkage peaks that do not fulfil the formal criteria for genome-wide linkage might contain susceptibility variants. In the interpretation of results, failure to achieve a formally significant LOD score or NPL statistic should therefore not lead to completely disregarding a chromosomal region as potentially harbouring a predisposing variant. While any follow-up studies of the present linkage scan should naturally be informed by the LOD score or NPL statistic of a potential candidate region, other important factors also need to be considered. These include consistencies between the analyses performed within this study and with previous findings for BP, as well as the presence of strong candidate genes in the chromosomal region.

4.3. Power Analysis

The power of a study is defined as the probability of rejecting a false null hypothesis (H_0) based on the data collected. In linkage analysis, the H_0 corresponds to the absence of linkage. A straightforward way to assess whether the available pedigrees will have sufficient power to reject this H_0, and thus provide evidence for genetic linkage, is by using a simulation-based approach. In such power simulation, genotype data are generated for pedigrees of a given structure, corresponding to the real pedigrees available for study. Simulations are carried out under the assumption of linkage and, for parametric analysis, based on a specific genetic model, which is thought to best describe the inheritance of the disease and which will also be used in the subsequent linkage analysis. The process of generating genotypes for the complete set of pedigrees available for study is repeated many times, until a large number of simulated data sets are available, each of them representing a study equivalent to the one to be carried out. Linkage analysis is then performed on each of these replicates, resulting in a set of pedigree and study-wide linkage statistics for each replicate (i.e., LOD scores for parametric analysis, NPL scores for non-parametric analysis). Because all marker data are simulated assuming linkage, if a sufficient number of replicates is generated, the distribution of the resulting statistics indicates whether the pedigrees are powerful enough to allow a rejection of the null hypothesis of no linkage.

This approach was first implemented for parametric linkage analysis in the programme SLINK[309,310]. In this study, for the majority of analyses, its faster (but otherwise unchanged) successor FastSLINK v2.51 was employed (distributed by Dr Daniel Weeks from the University of Pittsburgh; http://watson.hgen.pitt.edu/register/soft_doc.html). One exception, where the SLINK programme v2.60 was used, is discussed below. The FastSLINK analysis was performed within the frame of the easyLINKAGE Plus package v5.05, a tool for the easy execution of a variety of linkage programmes, which was chosen because of its comprehensive visual output[311,312].

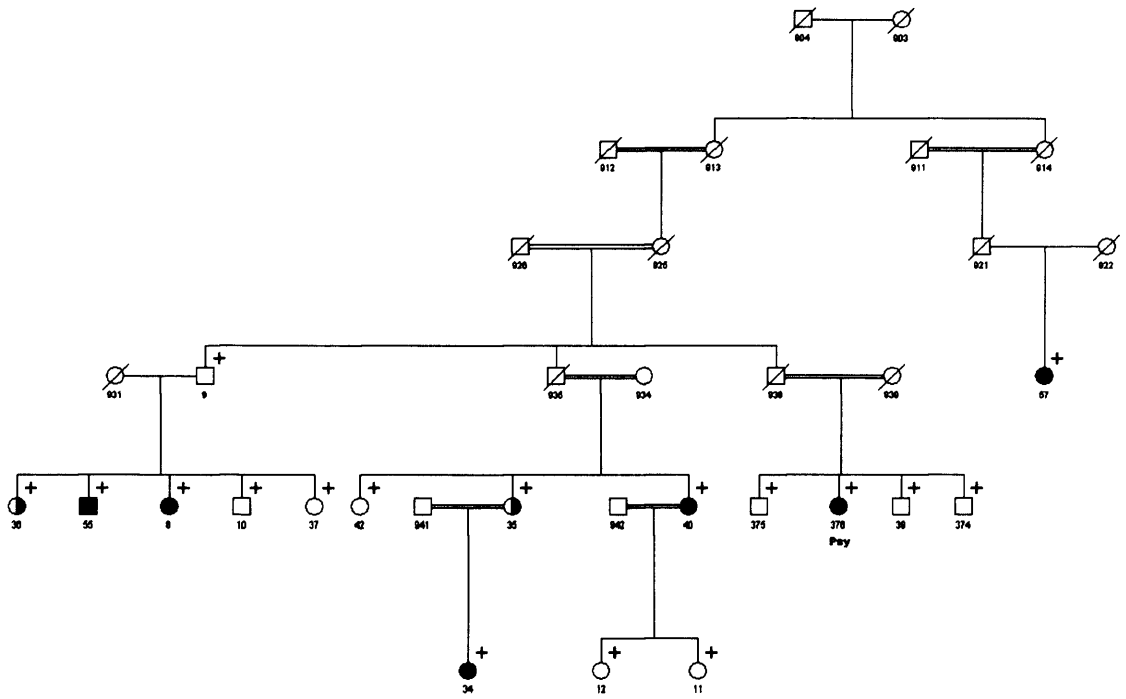
SLINK and FastSLINK perform simulations of parametric linkage (LOD score) analysis only. As discussed in the previous section, a genetic model of inheritance based on epidemiological data was only available for the narrow model where BPI

was considered as the phenotype. Parametric linkage analysis, and hence simulation-based power analysis for parametric linkage analysis, were therefore only carried out for the narrow model. It would have been of interest to assess the power of the Antioquian pedigree collection for non-parametric linkage analysis. However, the only programme that implements this type of analysis, Allegro[262], cannot handle pedigrees as complex as the ones used in this study. The power for non-parametric linkage analysis could therefore not be assessed.

For the power analysis under the narrow model, pedigree data were simulated using the parameters specified in chapter 2.3.7: frequency of the mutant allele = 0.003; penetrance of the homozygous mutant genotype = 0.9; penetrance of the heterozygous genotype = 0.81; and phenocopy rate = 0.01. It was furthermore assumed that the disease locus was 5 cM away from the nearest marker locus. Given the average marker distance of ~10 cM, this is a reasonable assumption: the maximal possible distance between a marker and the disease locus, reached if the disease locus lies right in the middle between two markers, is ~5 cM. Genotype data was generated for a marker with four equifrequent alleles, resulting in an average marker heterozygosity of 0.75, a typical value for microsatellites. The analysis was based on 1000 replicates.

Both SLINK and FastSLINK cannot handle inbreeding loops such as they occur in family FAZU01 (see Figure 2.2), and a cut-down version of this pedigree, in which all loops have been eliminated, had to be used for the simulation study (see Figure 4.1).

Figure 4.1: Cut-down version of pedigree FAZU01 used for the power simulations.



Inbreeding loops have been eliminated from the pedigree for power analysis. Double mating lines from the original pedigree have been preserved in this figure so as to indicate where the inbreeding loops are located. Filled symbols indicate a diagnosis of BPI; only those individuals were considered as affected in the power analysis. Symbols with a filled right half indicate a diagnosis of major unipolar depression; these patients were considered as of an unknown phenotype for the analyses. A cross next to a symbol indicates the availability of a DNA sample.

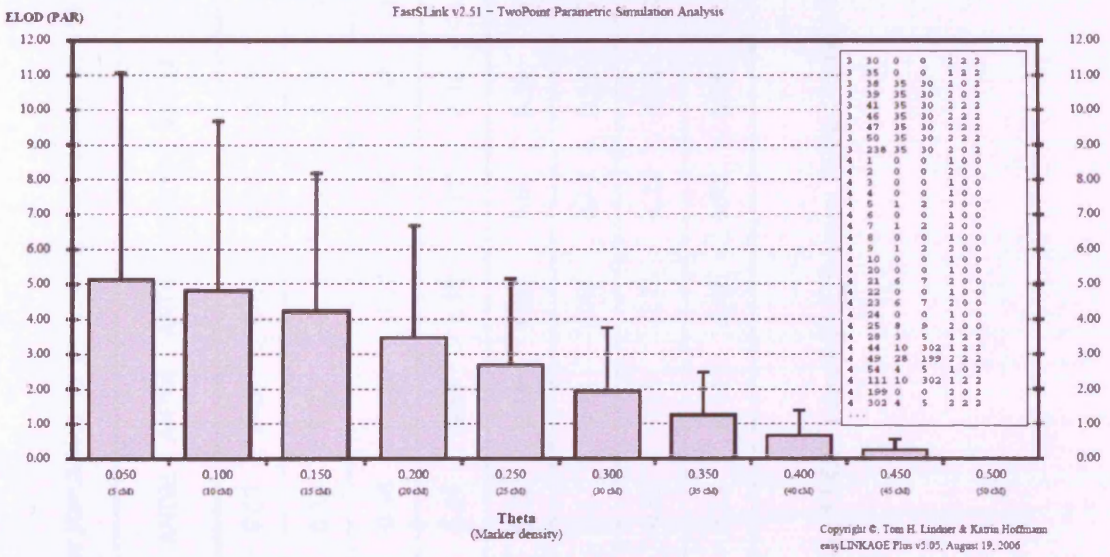
The results of the simulation-based power analysis are presented in Figure 4.2 and Tables 4.3 and 4.4. Average, minimum and maximum expected LOD scores (ELODS) were obtained for the complete study, as well as for individual pedigrees.

Table 4.3: Study-wide results of the power simulation for parametric linkage analysis under the narrow model.

Theta	Average ELOD	Standard deviation	Minimum ELOD	Maximum ELOD
0.05	5.12	1.99	-1.66	11.06
0.10	4.82	1.56	-0.38	9.67
0.15	4.21	1.24	0.20	8.19
0.20	3.48	0.93	0.24	6.67
0.25	2.70	0.74	0.19	5.16

ELOD, expected LOD score.

Figure 4.2: Results of the power simulation for parametric linkage analysis under the narrow model.



Average study-wide expected parametric LOD (ELOD) scores are shown for θ between 0.05 and 0.5 (filled grey bars). Maximum LOD scores are indicated by the black lines. The exact values are presented in Table 4.3. The box on the right side of the graph shows the first lines of the pedigree input file used in the simulation.

Table 4.4: Pedigree-wise results of the power simulation for parametric linkage analysis under the narrow model.

Theta		FAZU01	FAZU28	ANT03	ANT04	ANT07	ANT10	ANT14	ANT15	ANT18	ANT19	ANT21	ANT23	ANT24	ANT26	ANT27
0.05	Av ELOD	0.08	0.20	0.03	0.15	0.25	0.70	0.63	0.46	0.59	0.76	0.49	0.14	0.13	0.19	0.31
	St Dev	0.26	0.39	0.16	0.32	0.53	0.71	0.71	0.58	0.68	0.79	0.63	0.32	0.29	0.32	0.50
	Min ELOD	-0.68	-0.77	-0.56	-1.03	-0.97	-1.80	-1.52	-1.44	-1.74	-1.44	-1.42	-1.19	-0.61	-1.20	-1.08
	Max ELOD	**0.59**	**0.78**	**0.24**	**0.54**	**1.61**	**2.07**	**2.31**	**1.29**	**2.01**	**2.64**	**1.82**	**0.67**	**0.51**	**0.42**	**0.78**
0.10	Av ELOD	0.08	0.19	0.03	0.14	0.24	0.66	0.59	0.44	0.55	0.71	0.46	0.13	0.12	0.19	0.30
	St Dev	0.21	0.30	0.12	0.24	0.44	0.55	0.54	0.47	0.54	0.64	0.49	0.24	0.22	0.22	0.40
	Min ELOD	-0.44	-0.53	-0.37	-0.68	-0.76	-1.22	-1.01	-1.08	-1.27	-1.02	-1.00	-0.77	-0.40	-0.76	-0.73
	Max ELOD	**0.50**	**0.67**	**0.19**	**0.44**	**1.42**	**1.81**	**1.96**	**1.14**	**1.79**	**2.34**	**1.58**	**0.57**	**0.45**	**0.34**	**0.68**

Av, average; Min, minimum; Max, maximum; ELOD, expected LOD score; St Dev, standard deviation.

Although none of the pedigrees on its own reaches the significance thresholds in any instance (see Table 4.4), Table 4.3 shows that for a θ up to 0.15, the average study-wide expected LOD score is well above the widely accepted genome-wide LOD score threshold of 3.00, and even above the LOD score threshold for significance after correction for multiple models (LOD = 3.60, as discussed in chapter 4.1). However, the standard deviation for the average expected LOD scores are relatively large, and it is difficult to make inferences about the power of the Antioquian pedigrees to detect linkage from these figures alone. A direct power estimate is therefore given in the FastSLINK output by listing the percentage of simulated studies that achieved a study-wide LOD score above a certain threshold for any θ. When running FastSLINK within the easyLINKAGE package, these thresholds are fixed at LOD = 1.0, LOD = 2.0 and LOD = 3.0. In the present power simulation, 99.6% of all simulated studies achieved a LOD score $\geq$ 1.0; 96.7% resulted in a LOD score $\geq$ 2.0; and 87.6% gave a LOD score $\geq$ 3.0. The power of the present study to detect linkage at a genome-wide significance level of LOD = 3.0 using a parametric approach under the narrow model as defined in chapter 4.1 is therefore estimated to be 87.6%. Because the design of the study entailed the analysis of multiple disease models, increasing the formal threshold for significance of linkage to 3.60, it was of interest to gauge the power of the study to reach this level of significance. Changing the thresholds for significance was not possible within the easyLINKAGE package; this last analysis was therefore performed using SLINK v2.60. The same parameters were used as for the FastSLINK analysis performed with the easyLINKAGE package. 79.0% of the studies reached a LOD score $\geq$ 3.60, translating into an estimated study-wide power of 79% to detect linkage even in the context of testing multiple models.

It is widely accepted that the power of a study to reject the H_0 should be at least 80%. The power of the present study to result in a LOD score $\geq$ 3.0, and thus to detect genome-wide linkage, lies above this threshold. Even in the context of testing multiple phenotype models, the power of the Antioquian pedigree collection to reach significance is very close to 80%. Under the given models (i.e., both the genetic and disease classification models), the present study has therefore a high chance in succeeding to reject the null hypothesis of no linkage.

The main limitation of the simulation-based power analysis presented here lies in its being restricted to parametric analysis. On the one hand, this allowed only the assessment of power for the narrow model of phenotype classification, because a genetic model was not available for broad and psychosis models. On the other hand, although based on epidemiological data, it is not certain whether the assumed model is correct. Indeed, in view of the fact that the model specifies a near-dominant pattern of inheritance, for which a gene should be straightforward to localise, yet no single major locus has been identified for bipolar disorder, there is the distinct possibility of it not being correct. It would therefore have been of great interest to perform a power analysis for non-parametric linkage, which, due to computational restrictions of the Allegro programme, could not be accomplished. However, this is part of the difficulties inherent to the analysis of complex disorders and should not deter us from the study of a collection of pedigrees comprising as many heavily BP-loaded pedigrees as that from Antioquia.

4.4. Data Collection

This section deals with the process of obtaining the joint data set for linkage analysis of the 15 extended BP families available. First, a description of the data collection for the nine extended pedigrees genotyped as part of this thesis is given in section 4.4.1. Section 4.4.2 then explains how the final data set for analysis was constructed by adding the genotype data that had previously been obtained by Herzberg and colleagues[184] to those obtained here.

4.4.1. Genotype Collection for Nine Extended Bipolar Pedigrees from the *Paisa* Community

PCRs for all 382 autosomal microsatellite markers from the ABI Linkage Mapping Set v2.5 were performed for all samples from the nine Antioquian BPI families shown in Figure 2.2. Every run included a negative and a positive control (dH_2O and the CEPH sample 1347-02, respectively). The CEPH sample 1347-02, for which ABI provides genotype information for each of the markers that are part of the Linkage Mapping Set, was used for genotype calibration within and between runs.

The aim was to collect at least 90% of the data for each marker, and 95% of the data for each chromosome. A maximum of three repeat rounds were performed for markers with a data completeness under 90%, unless it seemed sensible to perform more, e.g. because a new pair of primers had been ordered that was expected to yield better results.

PCRs for all markers were initially carried out using the primers provided in the ABI Linkage Mapping Set. However, whenever it was necessary to re-order primers for a marker, primer sequences were obtained from the Genome Database (http://www.gdb.org), and primers ordered from Sigma Aldrich. To reduce genotyping costs, marker-specific primers were not fluorescently labelled; instead, each forward primer was designed to contain a universal 5' tail, whose sequence stemmed from the M13 virus, so as not to interfere with PCR in humans. This tail matched a third, fluorescently labelled, primer (see also chapter 3.2). The universal primer was available with different fluorescent labels, enabling a flexible approach to

setting up panels for fragment length analysis[313]. Markers for which repeats were performed using the M13 system are listed in Table 4.5.

Table 4.5: Microsatellite markers for which repeats were performed using M13-tailed primers.

Chromosome #	Marker name	Fragment length range (bp)
7	D7S2465	172-196
14	D14S68	160-172
15	D15S994	209-233
20	D20S117	170-196
	D20S195	262-280
	D20S889	282-310
21	D21S263	190-220

All PCRs using ABI primers were set up according to the manufacturer's instructions. PCRs using M13-tailed primers were set up according to the protocol shown in Table 4.6, and cycling conditions for the M13-PCRs are listed in Table 4.7. Amplification using the M13-tailed primers resulted in fragment sizes that were different from the ones obtained using the original ABI primers, and consistency between runs was achieved by calibration to the CEPH sample 1347-02.

Table 4.6: Set-up for PCRs using the M13-tailed primers (per reaction).

PCR component	Volume (µl)	Final concentration
HotStar Mastermix (Qiagen)	5	?
M13-tailed forward primer	0.25	0.25 µM
reverse primer	1	1.0 µM
M13 primer	1	1.0 µM
DNA	2	1.6 ng/µl
dH_2O	0.75	-
total	10	-

Table 4.7: Cycling conditions for the PCRs with M13-tailed primers.

PCR step	Temperature	Time	Cycles
Initiation	94°C	15 min	
Denaturing	94°C	30 sec	30
Primer Annealing	56°C	45 sec	
Elongation	72°C	45 sec	
Denaturing	94°C	30 sec	8
Primer Annealing	53°C	45 sec	
Elongation	72°C	45 sec	
Final Elongation	72°C	5 min	
Cool-Down	4°C	15 min	

In five cases, M13-based repeats had to be performed for the entire set of samples. PCR products for four of these markers were pooled together in a panel prior to fragment length analysis as shown in Table 4.8. The fifth marker, D7S2465, was not included in this panel because the repeats were performed at a later time than those for the other markers. PCRs for the remaining M13-markers (D14S68 and D20S889) were only used to perform repeats of few samples at a time. For these markers, the data obtained from the M13-PCR repeats were integrated with the data obtained using the ABI-primers. In these cases, genotype calibration based on the CEPH sample was of particular importance.

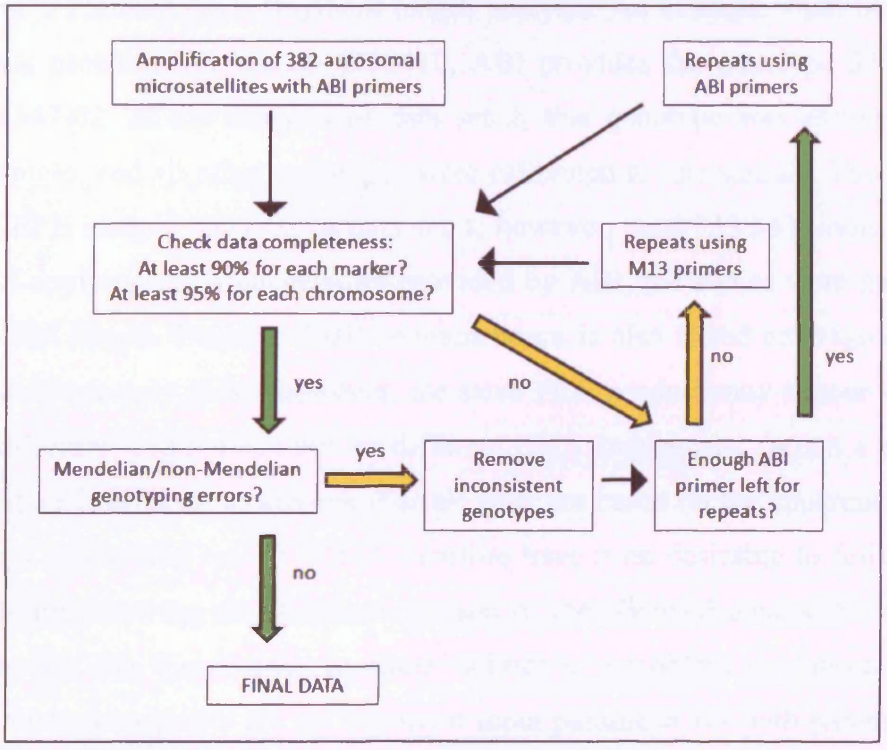
Table 4.8: Pooling panel comprising four of the M13-PCR products.

Marker name	Fragment length range (bp)	Fluorescent label
D20S117	170-196	FAM (blue)
D15S994	209-233	FAM (blue)
D21S263	190-220	HEX (green)
D20S195	262-280	HEX (green)

Fragment length analysis, genotyping and double scoring were done as described in chapter 2.2.5.2. When data completeness for a chromosome had reached 95%, and at least 90% of the data for each marker on that chromosome were available, the PedCheck programme was used to test the genotype data for consistency with Mendelian transmission as described in chapter 2.3.4. The data were also checked for non-Mendelian errors using SimWalk2 (see chapter 2.3.5). Any inconsistent genotypes were removed and, if as a consequence the genotyping completeness for a marker or the whole chromosome dropped under 90% or 95%, respectively, the samples in question were repeated. As mentioned above, however, generally no more than three repeat rounds were performed for any one marker. The data set resulting from this procedure was then merged with the data collected in the earlier BPI linkage scan performed in Antioquia[184] as described in section 4.4.2.

A schematic overview of the data collection procedure is shown in Figure 4.3.

Figure 4.3: Schematic representation of the data collection procedure.



See text for details.

4.4.2. Building the Joint Data Set

The genotype data that formed the basis of the linkage study carried out by Herzberg and colleagues[184] were available as final clean data. The original samples, as well as most of the raw data, were also available. The data from Herzberg and colleagues will from now on be referred to as data set 1, while the data obtained as part of this thesis will be called data set 2.

To enable a straightforward joint analysis of both data sets, all genotypes have been obtained using the ABI Linkage Mapping Set v2.5. In theory, because all genotypes in a run are calibrated to the same CEPH sample – 1347-02 –, it should have been possible to simply join both data sets, which had separately undergone strict genotyping quality controls including double scoring and checks for genotyping errors. However, when attempting to join the two data sets, it became evident that although the genotypes in data set 1 had been calibrated to the CEPH sample 1347-02, this sample had not been assigned its genotype as provided by ABI in the analysis of data set 1. Instead, the alleles were named after the size of the PCR product as it resulted from fragment length analysis. An example shall be given to clarify this problem: for marker D5S410, ABI provides the genotype 331/341 for sample 1347-02. In the analysis of data set 2, this genotype was assigned to the CEPH sample, and all other genotypes were calibrated to this sample. The genotype for the CEPH sample 1347-02 in data set 1, however, read 333/343, indicating that instead of applying the nomenclature provided by ABI, the alleles were called after the fragment length. Because ABI's nomenclature is also based on fragment sizes, the two calls are very close; however, the same PCR product may appear to be of a slightly different length when run on different DNA sequencers, so that a consistent nomenclature is difficult to achieve if allele calls are based on the apparent fragment length in a genotyping run. It would therefore have been desirable to follow ABI's nomenclature, allowing an easier comparison of the obtained data with future data sets. Note that this discrepancy in allele callings is a problem for linkage analysis because allele frequencies are an important input parameter for both parametric and non-parametric analyses. If the alleles are not called in a consistent manner, the allele frequencies will be skewed, and any results obtained from joint analyses are likely to be inaccurate, or even wrong. In haplotype analysis, a consistent nomenclature is

essential in order to be able to detect similarities in potential disease-carrying haplotypes between families.

Before being able to carry out any analysis on the joint data, the genotypes from both data sets therefore had to be adjusted to each other. In order to achieve this, the difference between the CEPH genotype calls in both data sets was noted, and all genotypes from data set 1 were corrected by this difference, thereby resulting in an adjusted data set which was compatible with the genotypes provided by ABI for sample 1347-02, and therefore with the genotypes in data set 2. In some instances, calibration was not possible based on the original data because the CEPH genotype had not successfully been obtained in one of the data sets. In these cases, a PCR involving two samples from each data set and the CEPH sample 1347-02 was carried out for calibration purposes. After this additional experiment, the great majority of marker genotypes were successfully calibrated. There were only four markers for which the calibration process was unsuccessful because the genotypes of the samples from data set 1 obtained in the calibration experiment differed from the genotypes that were originally included in data set 1. Since it was impossible to decide which genotypes were wrong, the genotypes from data set 1 for these makers were excluded from the joint analysis. These markers were D13S159, D17S798, D19S221, and D21S263.

A further problem was the presence of "intermediate" alleles in the data. In contrast to the usual microsatellite variation, where alleles differ from each other by complete repeat motifs, "intermediate" or "odd-sized" alleles fall outside the normal allele ladder, i.e., in between regular alleles. This phenomenon seems to be particularly common in compound microsatellites of the form $(CA)_m(CG)_n$, possibly because of the tendency of CG-rich repeats to form secondary hair-pin structures that might alter the motility of the PCR product during gel electrophoresis[314]. Some of the markers for which intermediate alleles were observed in this study are of this structure (e.g. D6S434); other markers have a different complex repeat structure, such as $(TA)_m(CA)_n$ (e.g., D7S486). Alternative sources of intermediate alleles can lie in deletions/insertions interrupting the homogeneous repeat stretch[315], or variation in the flanking non-repeat sequence[316].

While genotyping the ABI microsatellite markers for data set 2, intermediate alleles were identified at 21 markers. After cross-checking with data set 1, it was found that

the occurrence of intermediate alleles had not been taken into account for ten of these markers in that data set. It was therefore attempted to re-analyse available raw data for data set 1 at these markers. These raw data were in the format of GeneMapper's precursor programme, the Genotyper® software v3.6 (Applied Biosystems); this programme was therefore used for their re-analysis.

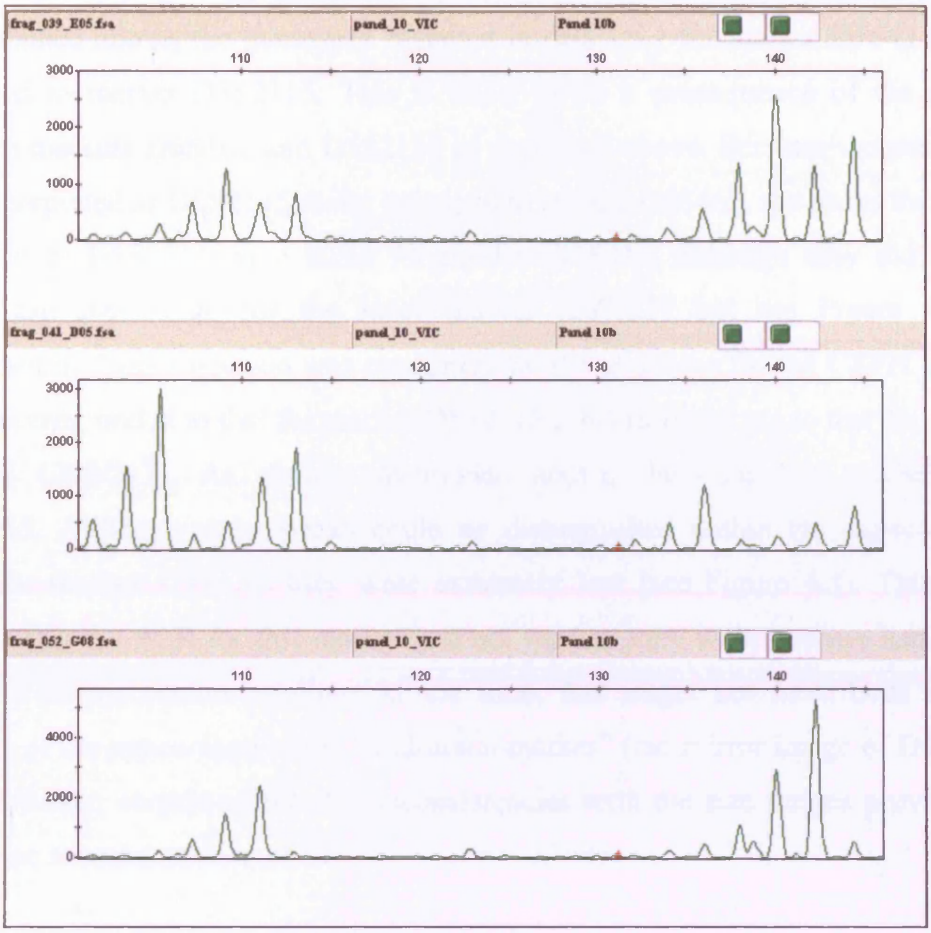
Genotypes could successfully be obtained for five markers (D6S434, D7S486, D11S925, D15S127, and D18S70). Re-analysis of markers D1S498, D1S2836, D6S262, and D15S994 was not possible because the correspondent raw data were not available. For these markers, genotypes from data set 1 were therefore excluded from the joint analyses.

Additional problems were encountered with the following markers:

D5S2115

In data set 1, the genotypes labelled as D5S2115 really belonged to marker D6S462. This was discovered because the genotype patterns seemed exactly the same for both markers; i.e., all heterozygotes for one marker were also heterozygous for the other, and the distances in bp between the two alleles were always the same. In the same manner, all homozygotes for one marker were homozygous for the other, and allele differences between two individuals were the same for both markers. Since it is highly improbable for such a situation to occur naturally, the raw data for both markers were checked. It was discovered that the marker D6S462, which belongs to the same genotyping panel as D5S2115 and occupies an adjacent size range in the same colour lane (green), produced a "mirror image", where there are two PCR products for each allele, which differ by a constant number of bp (see Figure 4.4).

Figure 4.4: "Mirror image" for marker D6S462.



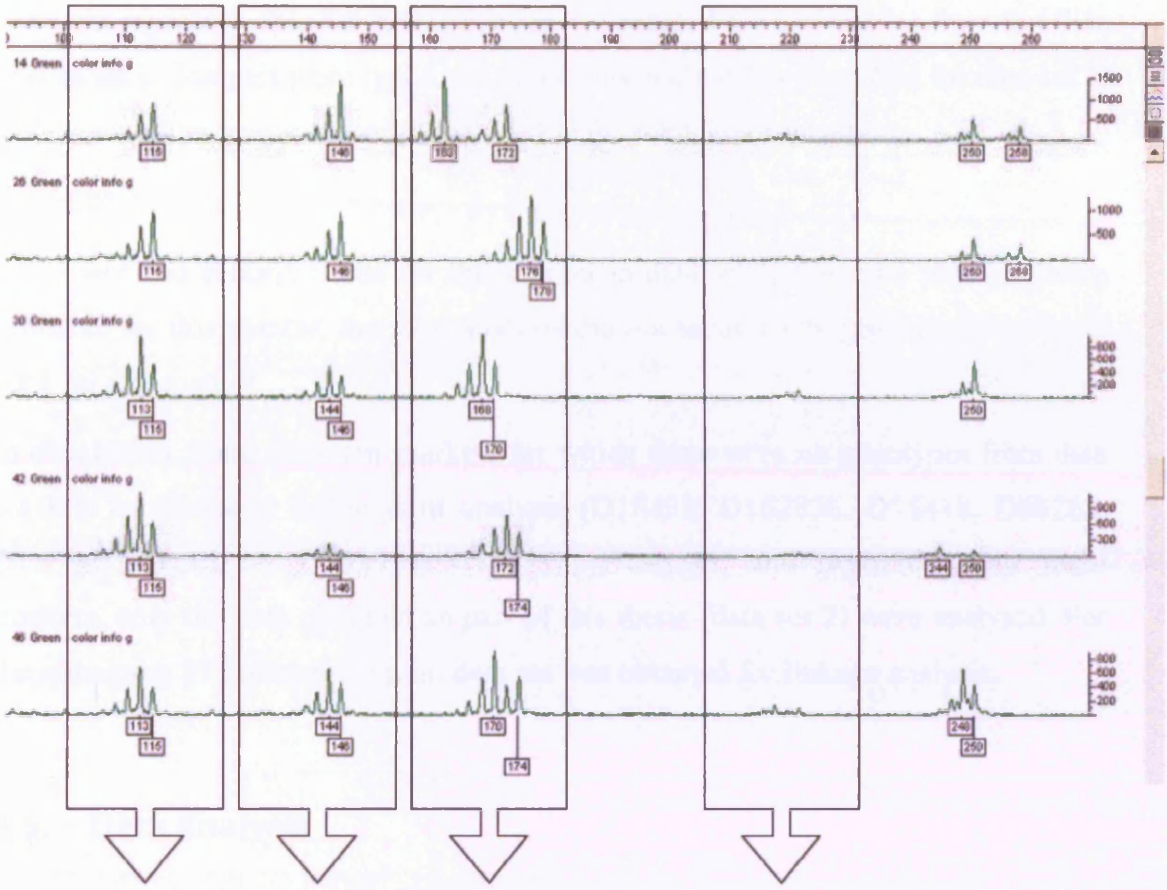
Electropherograms for three samples (from GeneMapper®). What initially looks like the product of two different markers could be identified as a "mirror image" phenomenon. The group of peaks at the left lie within the predicted size range for marker D6S462 (103-120 bp). The peaks at the right lie close to, or even within, the predicted size range for marker D5S2115 (142-170 bp). In data set 1, they were therefore mislabelled as marker D5S2115. In reality, this is a mirror image of marker D6S462, as can be seen from the equal patterns of the peaks in both groups. Although this pattern is only shown for three samples here, it could be observed throughout all samples.

The biological explanation for this phenomenon might be that the binding site for one of the primers lies in a duplicated segment. Alternatively, this pattern might be due to primer degeneration. Degeneration might reduce primer specificity, thereby allowing it to anneal at a close-by site which might be similar, but not identical, to the targeted primer binding site. Upon discovering this issue, it was also found out that the genotypes that really belonged to marker D5S2115 were labelled as D5S418, another marker from panel 10 (see below). These genotype data were correctly re-labelled as D5S2115 and could subsequently be analysed as part of the joint data set.

D5S418

As explained above, the genotypes included in data set 1 for marker D5S418 really belonged to marker D5S2115. This is likely to be a consequence of the mix-up between markers D6S462 and D5S2115 as described above. Because the peaks that were interpreted as D5S2115 really belonged to marker D6S462, the peaks that really belonged to D5S2115 were taken for marker D5S418, although they did not lie within the size range for the latter marker (207-227 bp; see Figure 4.5 for clarification). This suspicion was confirmed by the genotype for the CEPH sample, which corresponded to that for marker D5S2115 (168/168) and not to that for marker D5S418 (209/211). As already mentioned above, the data was relabelled as D5S2115. Although some peaks could be distinguished within the expected size range for marker D5S418, they were extremely low (see Figure 4.5). This might indicate that the PCR for this marker had not worked very well, or there had been a problem during marker pooling. At the time, this might not have been noticed because of the appearance of the "additional marker" (the mirror image of D6S462). It is, however, surprising that the inconsistencies with the size ranges provided by ABI were not taken notice of.

Figure 4.5: Mix-up between genotypes of panel 10 in data set 1.



Electropherogrammes showing the green lane of panel 10 from the ABI Linkage Mapping Set for samples from data set 1 (from Genotyper® v3.6). To clarify the mix-up in genotype calling, the labels at the bottom of the figure describe which marker truly lies within each marked size range, and for which marker the genotypes might have been mistaken. The presence of very low PCR products within the size range of marker D5S418 indicates that the PCR for this marker might not have worked very well, or that there might have been problems with the pooling procedure.

D7S2465

This marker contained intermediate alleles but was very difficult to retype for data set 1 based on the available raw data. Because a new primer had to be ordered to carry out repeats in data set 2, this marker was retyped for all samples from families of data set 1. Samples were typed and genotypes analysed as described for data set 2, and the resulting genotypes were included in the joint data analysis.

D11S905

There were no genotype data for this marker in data set 1. Since no raw data were available for this marker, the joint analysis did not include any genotypes from data set 1 for this marker.

In conclusion, there were ten markers for which there were no genotypes from data set 1 to be included in the joint analysis (D1S498, D1S2836, D5S418, D6S262, D11S905, D13S159, D15S994, D17S798, D19S221, and D21S263). For these markers, only the data obtained as part of this thesis (data set 2) were analysed. For the remaining 372 markers, a joint data set was obtained for linkage analysis.

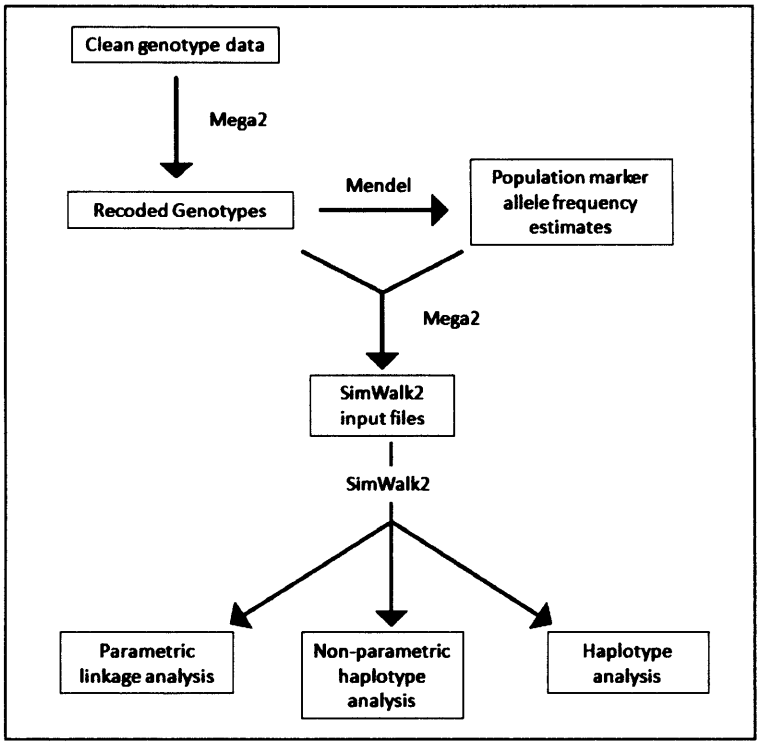
4.5. Data Analysis

The aim of this section is to provide an outline of the analysis procedure. For details of the methods employed, please refer to chapter 2.3.

As a first step, the programme Mega2 was used to recode allele labels from the 2- or 3-digit fragment size-based nomenclature used in data collection, to a consecutive numeric nomenclature running from 1 to n, where n is the number of alleles observed at each locus. After allele recoding, Mega2 was used to convert the linkage format files, in which the genotype data were stored, to the input format for Mendel v8.0.1. Mendel was then used to estimate population allele frequencies from the pedigree data as described in chapter 2.3.6. After completing these preparatory analyses, Mega2 was used to create the input files for the SimWalk2 programme based on the recoded genotypes and incorporating the population allele frequency estimates obtained from the Mendel programme. Finally, SimWalk2 was used to carry out the analyses that are at the core of this chapter: parametric and non-parametric linkage analyses, as well as haplotype analysis.

A flow chart summing up the analysis procedure is presented in Figure 4.6.

Figure 4.6: Flow chart of the data analysis procedure.



4.6. Results

In this section, I present a description of the results obtained in the genome-wide scan of 15 extended pedigrees from the *paisa* community segregating severe bipolar disorder. First, an overview on data completeness shall be given. Subsequently, the results of the linkage scan for each phenotype model will be summarised. Haplotype analyses have been conducted to facilitate the identification of potential common disease-carrying haplotypes in regions selected for a high LOD or NPL score; therefore, results from haplotype analyses will be presented together with the results of the linkage analyses.

4.6.1. Data Completeness

Genotyping of 382 autosomal microsatellite markers was attempted in 75 samples from nine pedigrees (data set 2). Genotype data for a further 91 samples from six pedigrees that had been subject of a previous linkage study were available for the same set of markers (data set 1).

In collecting the genotypes for data set 2, it was aimed at obtaining at least 90% of the genotypes for any one marker, and at least 95% of all genotypes for each chromosome. It was generally not attempted to obtain missing genotypes from data set 1; changes that were made to data set 1 in order to prepare a merged data set for joint analysis were described in chapter 4.4.2. The most important reasons for excluding genotypes from data set 1 from the joint analyses were the presence of intermediate alleles in the data which had not been accounted for in the genotype scoring of data set 1, and problems in calibrating genotype data from both data sets to the CEPH genotypes. Genotypes from data set 1 had to be excluded for ten markers (D1S498, D1S2836, D5S418, D6S262, D11S905, D13S159, D15S994, D17S798, D19S221, and D21S263).

Overall and chromosome-wise data completeness

The overall completeness of the data collected here (data set 2) was 97%, with chromosome averages ranging from 94% (for chromosome 16) to 98% (for chromosomes 5, 6, 9, and 11; see Table 4.9 on page 164). The overall completeness of data set 1 was 93%. Chromosome averages for data set 1 ranged from 72%

(chromosome 21) to 97% (chromosome 10; see Table 4.9). The low value for chromosome 21 results from there only being five markers on that chromosome, of which one (D21S263) had to be excluded because the genotypes of both data sets could not be calibrated to each other.

Data completeness by marker

For data set 2, data completeness for single markers ranged from 83% for marker D6S516, to 100% for 72 out of 382 markers (average 97 ± 3%). Overall, there were 15 markers for which less than the targeted 90% of genotypes could be collected in data set 2. In all cases, the maximum number of repeat rounds had already been completed, and in all but one case (marker D16S516 on chromosome 16), chromosome averages of at least 95% were achieved nonetheless. For data set 1, the lowest percentage of completeness of all markers for which genotypes were available (i.e., disregarding the 10 markers for which genotypes from data set 1 could not be used for joint analysis) was 77% (D8S1784). For 51 of the 382 markers, 100% of genotypes were available. The average single marker data completeness for data set 1 was 93 ± 16%. This high variability is due to the markers for which genotypes could not be used in the joint analyses; without these markers, the average data completeness for single markers was 95 ± 4%.

For details of marker data completeness by chromosome, see Tables 8.1 to 8.22 in the Appendix.

Data completeness by sample

Data completeness for individual samples lied between 57% and 97% for data set 1 (average 93 ± 6%), and between 74% and 100% for data set 2 (average 97 ± 4%) (data not shown).

In conclusion, the genotyping success for both data sets was very high (93% and 97% for data sets 1 and 2, respectively), and variation in genotyping success was small for both markers and samples. Also, the standard deviations of the averages of data completeness for markers and samples are very similar, both within and between data sets (when excluding the markers for which data set 1 genotypes were not used in the joint analyses); thereby indicating that neither DNA quality nor PCR efficiency represented a major source of variation in genotyping success. High genotyping success was of particular importance for the families from data set 2, for

which linkage analysis was performed here for the first time. With an overall data completeness of 97%, it can be said that this has been achieved. Furthermore, the genotypes included in the final data set were subject to strict quality checks, and the resulting data therefore represent a solid basis for all analyses to be performed in the frame of this thesis.

Table 4.9: Completeness of the data obtained for the genome-wide linkage scan in fifteen extended pedigrees from Antioquia (by chromosome).

Chromosome	Data Completeness (%)		
	Data Set 1	Data Set 2	Weighted Average[1]
1	0.87	0.97	0.92
2	0.97	0.97	0.97
3	0.94	0.96	0.95
4	0.93	0.97	0.95
5	0.93	0.98	0.95
6	0.90	0.98	0.93
7	0.96	0.97	0.96
8	0.96	0.97	0.96
9	0.96	0.98	0.97
10	0.97	0.97	0.97
11	0.89	0.98	0.93
12	0.94	0.95	0.95
13	0.88	0.96	0.92
14	0.95	0.96	0.95
15	0.90	0.97	0.93
16	0.94	0.94	0.94
17	0.89	0.96	0.92
18	0.95	0.96	0.95
19	0.87	0.96	0.91
20	0.95	0.96	0.95
21	0.72	0.95	0.82
22	0.94	0.97	0.95
Weighted Average[2]	**0.93**	**0.97**	**0.94**

[1]Weighted by the number of individuals in each sample. [2]Weighted by the number of markers per chromosome.

4.6.2. Linkage Analysis: Narrow Model

4.6.2.1. Parametric Analysis

Parametric linkage analysis of the fifteen extended *Paisa* pedigrees under the narrow model identified three genomic regions with study-wide multipoint heterogeneity LOD scores ≥ 1.3 (see Table 4.10). The threshold of 1.3 is arbitrary; however, the same cut-off has been chosen by Herzberg and colleagues to indicate the most important results of their genome scan for bipolar disorder[184]. The highest HLOD score (2.14) was obtained for chromosome 21q21.1-q22.13 at marker D21S1914. The proportion of families linked to this locus (α) was 0.15. The two other regions identified are located on chromosomes 13q33 and 1p22-31. No LOD scores above the previously defined thresholds for genome-wide significance were obtained (LOD = 3.0, or LOD = 3.6 after correction for multiple models; see chapter 4.1); and for α = 1.0, i.e., under the assumption of locus homogeneity, no LOD scores ≥ 1.3 occurred anywhere in the genome (data not shown).

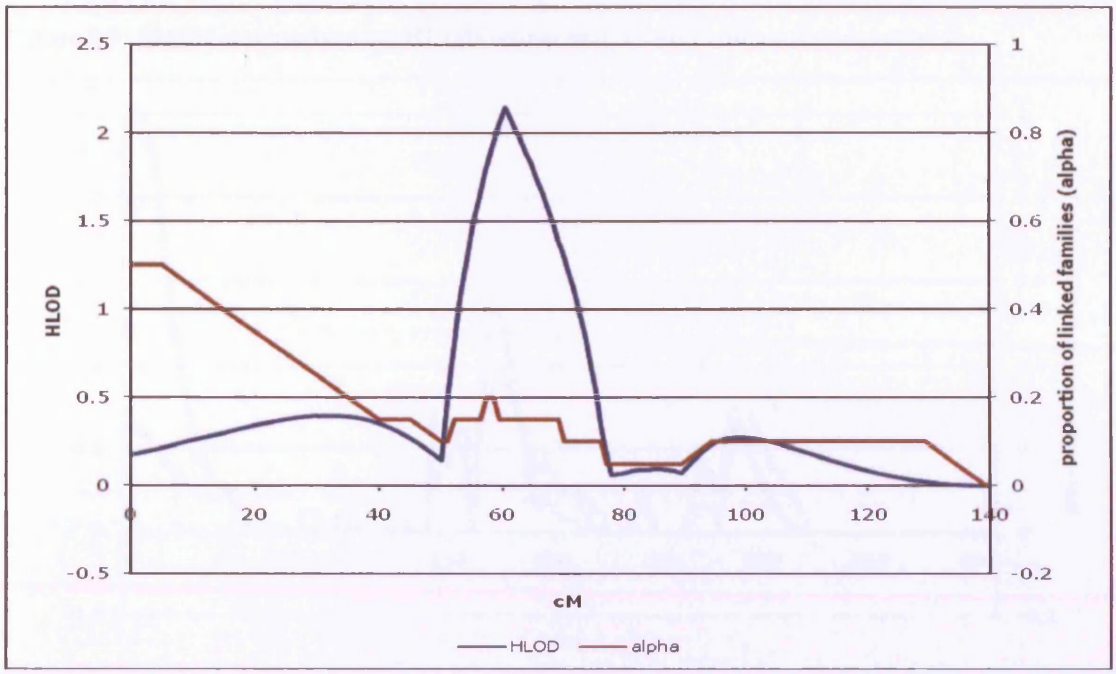
Plots showing multipoint HLOD scores and α-values along each of the chromosomes for which HLOD scores ≥ 1.3 were found, are displayed in Figures 4.7 to 4.9.

Table 4.10: Regions with study-wide HLOD scores ≥ 1.3.

Chromosome region	Centromeric marker	Telomeric marker	highest HLOD	closest marker[1]	α
21q21.1-q22.13	D21S1256	D21S1252	2.14	D21S1914	0.15
13q33.1-q33.3	D13S158	D13S173	1.46	D13S158	0.3
1p22.1-p31.1	D1S2868	D1S2841	1.32	D1S207	0.35

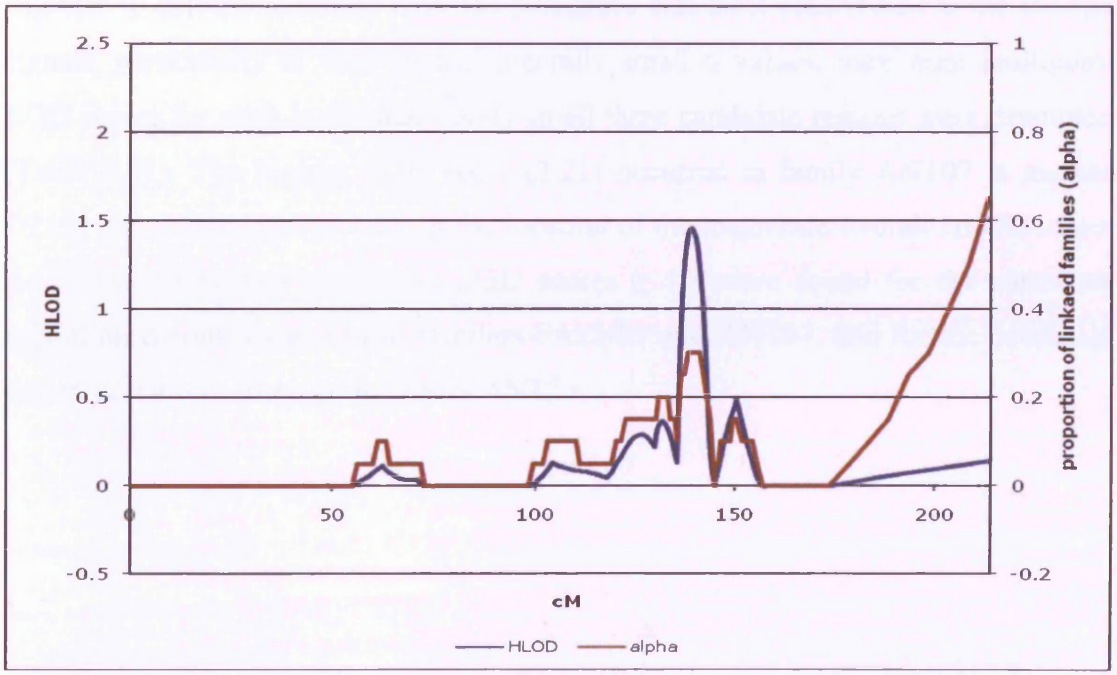
[1]Marker at the shortest genetic distance from the position at which the highest HLOD score has been obtained.

Figure 4.7: Multipoint study-wide HLOD scores along chromosome 21.



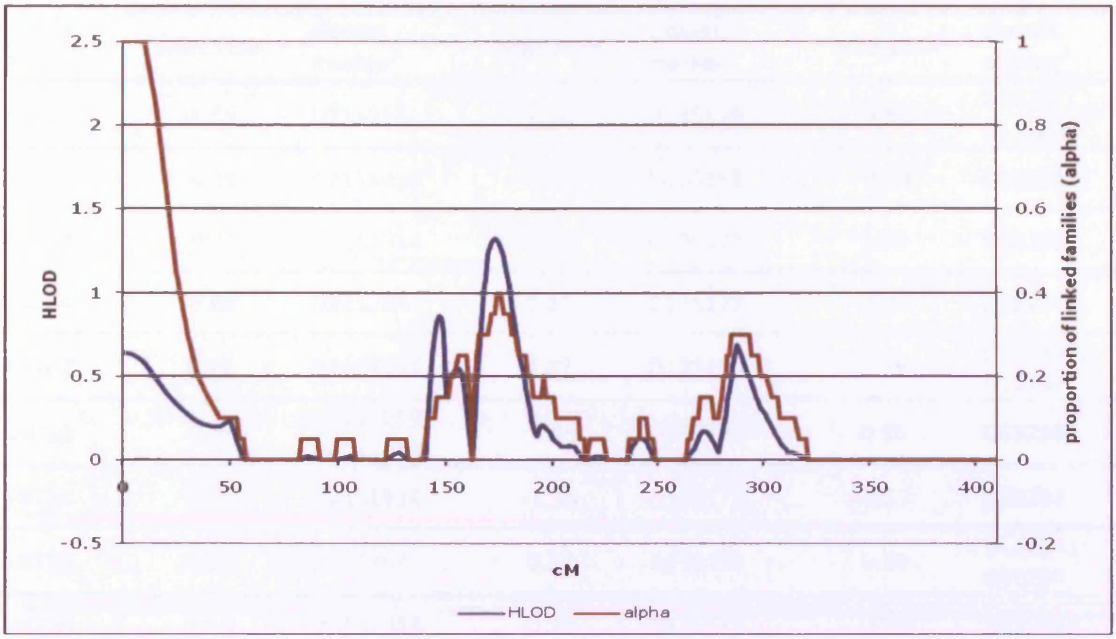
Multipoint study-wide HLOD scores for chromosome 21 are plotted against the genetic distance on the chromosome in cM (blue line). Also shown are the estimates of the proportion of families showing linkage to each point along the map (α; red line).

Figure 4.8: Multipoint study-wide HLOD scores and α-values along chromosome 13.



Multipoint study-wide HLOD scores for chromosome 13 are plotted against the genetic distance on the chromosome in cM (blue line). Also shown are the estimates of the proportion of families showing linkage to each point along the map (α; red line).

Figure 4.9: Multipoint study-wide HLOD scores and α-values along chromosome 1.



Multipoint study-wide HLOD scores for chromosome 1 are plotted against the genetic distance on the chromosome in cM (blue line). Also shown are the estimates of the proportion of families showing linkage to each point along the map (α; red line).

In order to determine which were the pedigrees that most contributed to the linkage signals, particularly in view of the generally small α-values, maximum multipoint LOD scores for each individual family in all three candidate regions were examined (Table 4.11). The highest LOD score (3.21) occurred in family ANT07 at marker D21S1914 on chromosome 21, at the location of the maximum overall HLOD score. Additional individual multipoint LOD scores ≥ 1.3 were found for the candidate region on chromosome 13q in families FAZU01 and ANT07, and for the candidate region on chromosome 1p in family ANT27.

Table 4.11: Maximum multipoint LOD scores for each of the 15 extended pedigrees in the three chromosomal regions with a study-wide HLOD score ≥ 1.3.

Family	21q21.1-q22.13		13q33.1-q33.3		1p31.1-p22.1	
	Max LOD	closest marker[1]	Max LOD	closest marker[1]	Max LOD	closest marker[1]
FAZU01	-1.15	D21S263	1.38	D13S158	-0.89	D1S207/ D1S2868
FAZU28	-0.39	D21S1256	-0.54	D13S158	-0.18	D1S2841
ANT03	-0.11	D21S1914	0.27	D13S173	0.55	D1S2868
ANT04	-3.08	D21S263	1.11	D13S173	-1.47	D1S207
ANT07	**3.21**	D21S1914	1.47	D13S158	0.19	D1S2841/ D1S207
ANT10	-0.19	D21S1256/ D21S1914	-0.45	D13S173	0.85	D1S2868
ANT14	0.80	D21S1914	-1.16	D13S173	-0.53	D1S207
ANT15	0.38	D21S263	0.30	D13S158	0.29	D1S2841/ D1S207
ANT18	0.13	D21S1914	-1.29	D13S173	-1.35	D1S2868
ANT19	-0.01	D21S263	-0.97	D13S173	0.82	D1S207
ANT21	-0.95	D21S1252	-0.82	D13S173/ D13S158	-0.04	D1S2868
ANT23	-0.59	D21S1256	0.37	D13S158	0.37	D1S207
ANT24	0.28	D21S1256	0.00	whole interval	0.28	D1S207
ANT26	-1.26	D21S1252	-0.61	D13S158	0.86	D1S207
ANT27	-0.81	D21S263	-2.29	D13S158	1.52	D1S207
All	**2.14**	**D21S1914**	**1.46**	**D13S158**	**1.32**	**D1S207**

[1]Marker at the shortest genetic distance from the position at which the highest LOD score (HLOD score for "All families") has been obtained. If the highest LOD score occurred right in between two markers, both have been named. Instances where the LOD score was constant for the whole interval have been indicated. Individual multipoint LOD scores ≥ 1.3 are underlined; LOD scores ≥ 2.0 are printed in bold.

In addition to considering regions identified based on study-wide heterogeneity LOD scores ≥ 1.3, single-family results were scanned for the occurrence of individual LOD scores ≥ 2.0 anywhere along the genome (Table 4.12). Of the five chromosomal regions for which individual LOD scores ≥ 2.0 were obtained, one showed a study-wide HLOD score suggestive of linkage (chromosome 21q). In addition, a LOD score of 2.06 in family ANT27 occurs close to the candidate region

on chromosome 1p. For the other three regions on chromosomes 3q, 15q and 11q, no study-wide signal was picked up.

Table 4.12: Chromosome regions with single-family LOD scores ≥ 2.0.

Family	Chromosome region	Max LOD[1]	closest marker[2]
ANT07	21q21.2	3.21	D21S1914
ANT07	3q27.3	2.57	D3S1262
ANT18	15q26.3	2.46	D15S120
FAZU01	11q12.2	2.19	D11S4191
ANT27	1p31.3	2.06	D1S230

[1]For each region with LOD scores ≥ 2.0, the highest LOD score is given.
[2]Marker at the shortest genetic distance from the position at which the highest LOD score occurred.

Haplotype analysis

In determining regions of potential interest in individual families, the threshold of LOD = 2.0 was chosen because it is commonly considered to indicate suggestive evidence for linkage (see chapter 1.2.3). To explore the basis for the linkage signals detected, haplotypes at each of the regions with LOD scores ≥ 2.0 were visually inspected in each family. These haplotypes are shown below (Figures 4.10 – 4.14). Haplotypes are displayed for the candidate regions listed in Table 4.12. For chromosome 21, on which only five markers have been typed, the whole chromosome is shown. For all other chromosomes, haplotypes are limited by markers with LOD scores ≤ -2.0 (i.e. where there is significant evidence for exclusion of linkage).

Despite the high LOD score for family ANT07 on chromosome 21q, there is no co-segregation of one haplotype with the disease phenotype. There is, however, co-segregation of a haplotype in the leftmost branch of the pedigree with BPI (light blue haplotype; see Figure 4.10), and partial co-segregation of another haplotype in the other branches of the pedigree: the majority of all individuals that do not share the light blue haplotype, do share a haplotype comprising alleles at markers D21S1814 to D21S266 (dark blue haplotype in Figure 4.10; the allele at marker D21S1914 is

represented in red in some individuals, indicating that it is assumed to be part of a different haplotype; however, it cannot be distinguished with certainty where the crossovers in individuals 8 and 13 have happened, and there is a possibility of identity by descent for all three markers).

On chromosome 3q – again for family ANT07 –, there is co-segregation of a haplotype ranging from marker D3S1614 to marker D3S1580 (inclusive) with BPI within the left branch of the pedigree (dark red haplotype; see Figure 4.11). As is the case for chromosome 21, there seems to be no co-segregation within the right branch of the pedigree.

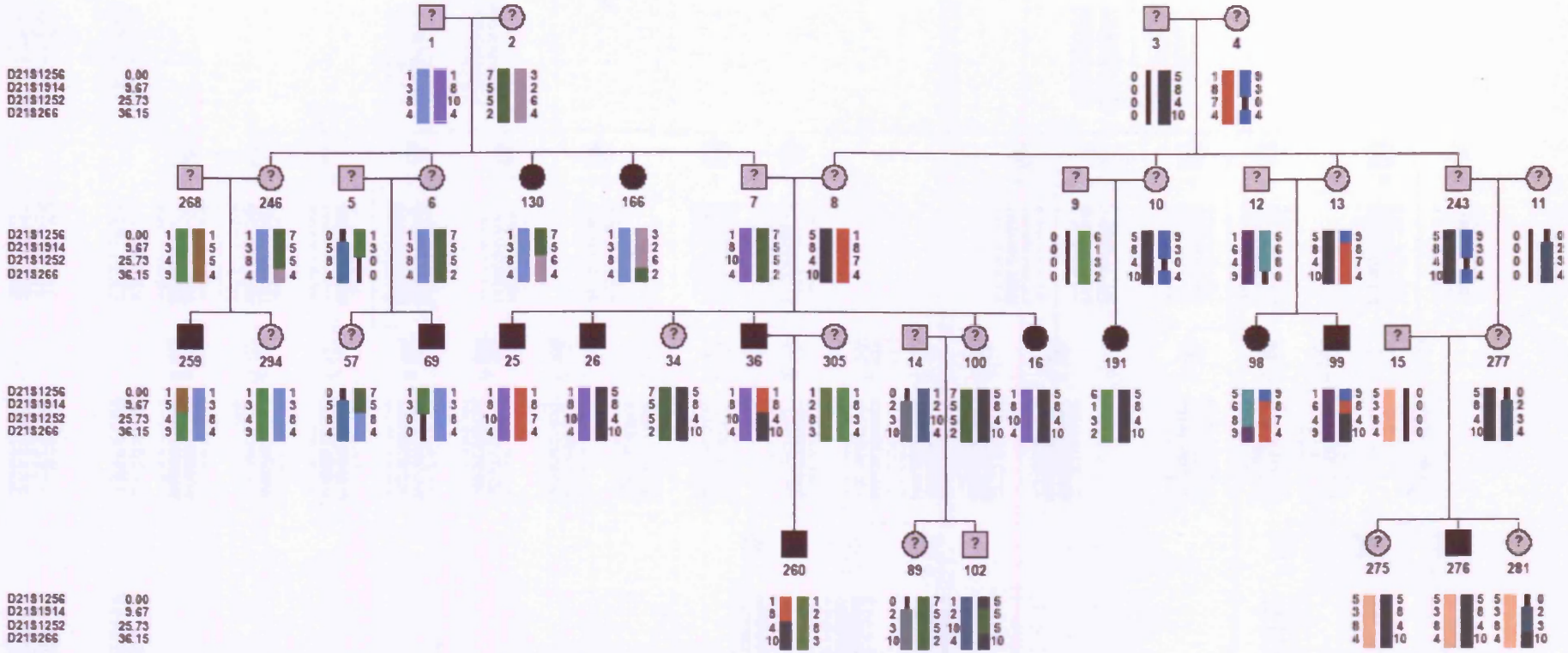
For family ANT18, no co-segregation of a chromosome 15q haplotype and the disease phenotype could be identified (see Figure 4.12): although some alleles are identical by state in affecteds, they do not seem to be identical by descent.

In family FAZU01, for the candidate region on chromosome 11q, there is a haplotype showing near perfect co-segregation with BPI (because BP is a complex disease, in this context, "near perfect co-segregation" only refers to affecteds sharing a haplotype, while it is not taken into account whether family members of an unknown phenotype status share this haplotype or not). In Figure 4.13, a haplotype comprising markers D11S904 to D11S4191 occurs in all but one (individual 55) of the affecteds. In most individuals, this haplotype is represented in light red; however, in individuals 40 and 376, parts of the haplotype are shown in blue; this is probably due to the fact that the family is inbred, which might cause the SimWalk2 programme difficulties in assigning the most probable haplotypes. However, inspection of the alleles shows that there is co-segregation between disease and haplotype. Similarly, for individual 34, part of the haplotype is shown in green. This means that SimWalk2 inferred the occurrence of a crossover between markers D11S905 and D11S935 (e.g. because it is likely that at least one crossover occurs on this chromosome), but this inference, although based on a maximum likelihood approach, might be wrong, and the haplotype shown in green in individual 34 might indeed be identical by descent to the light red and the blue haplotypes in the remaining affecteds.

Inspection of the haplotypes on chromosome 1p in family ANT27 shows co-segregation of a putative disease-causing haplotype comprising markers D1S207 and

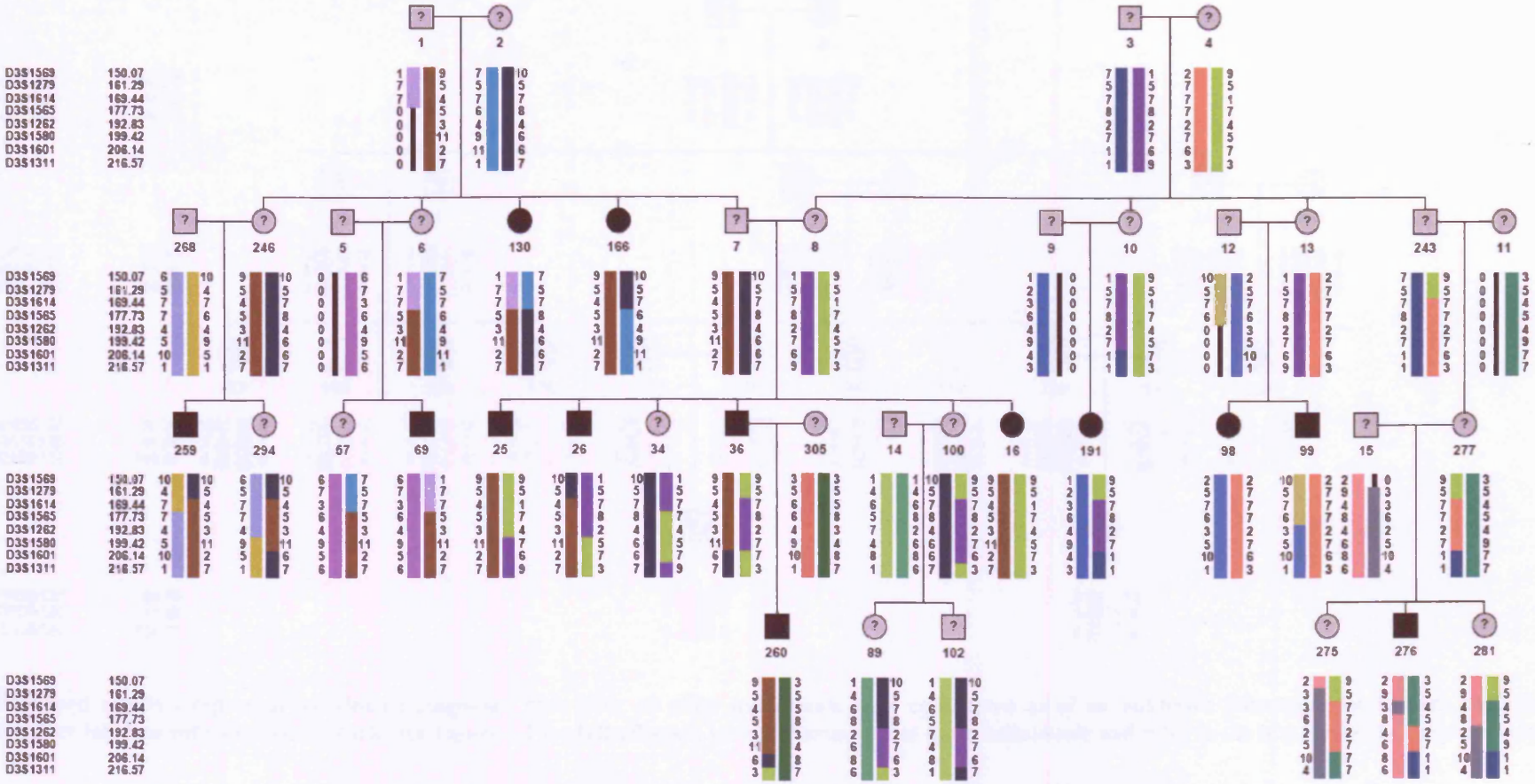
D1S2868 with the phenotype (see Figure 4.14). This haplotype is represented in blue for some individuals, and in pale green for others because the maximum likelihood approach employed by SimWalk2 did not result in an IBD-inference for these haplotypes. It has to be stressed that this is a statistical inference, which does not preclude that in reality, these haplotypes might be identical by descent.

Figure 4.10: Haplotype reconstruction for chromosome 21 in family ANT07. Marker D21S263, for which no genotype data were available in data set 1, is not shown.



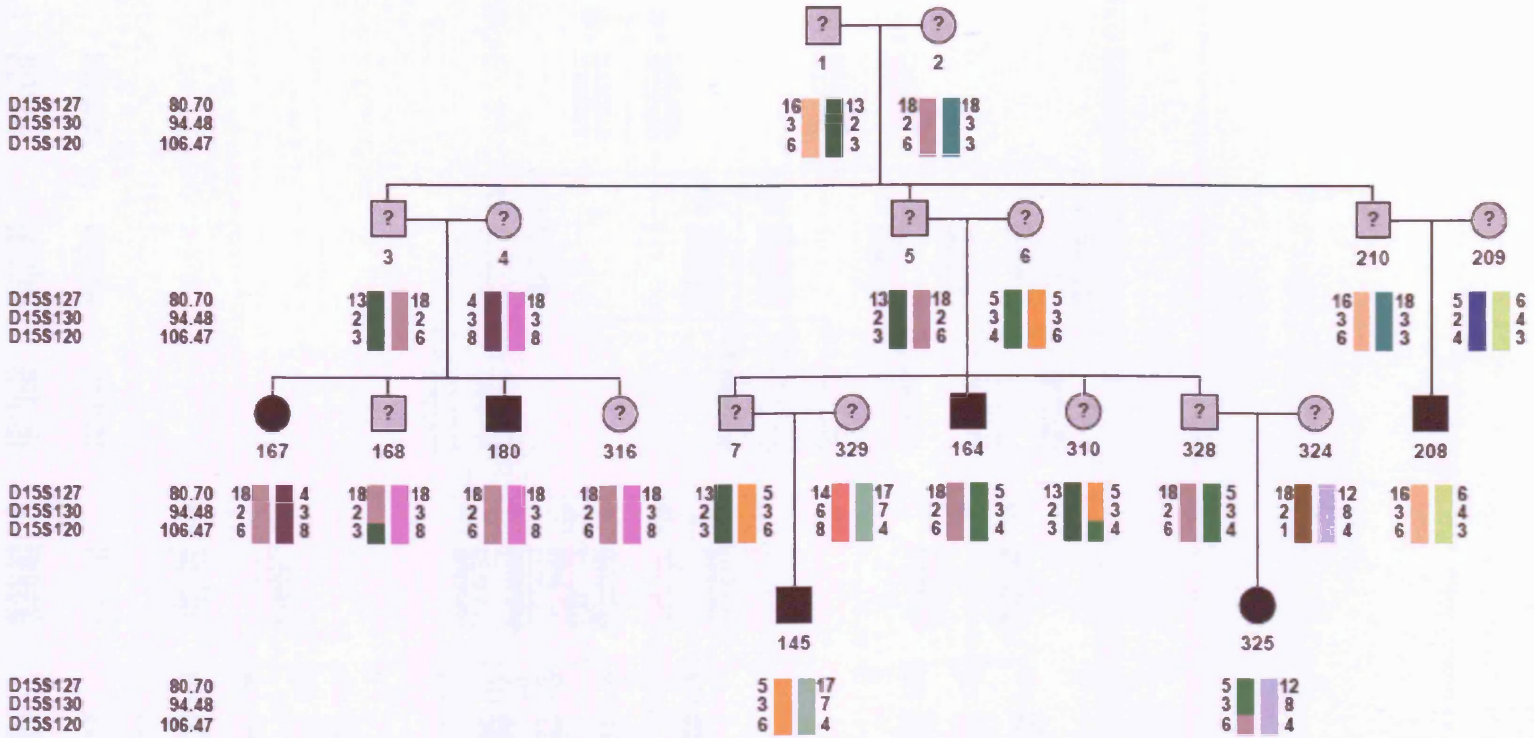
Blackened symbols represent individuals diagnosed with BPI; all other individuals were considered as of an unknown phenotype for linkage analysis and are therefore labelled with a question mark. See Figure 2.1 for full affection status information of these individuals; refer to the text for further explanations.

Figure 4.11: Haplotype reconstruction for a candidate region on chromosome 3 in family ANT07.



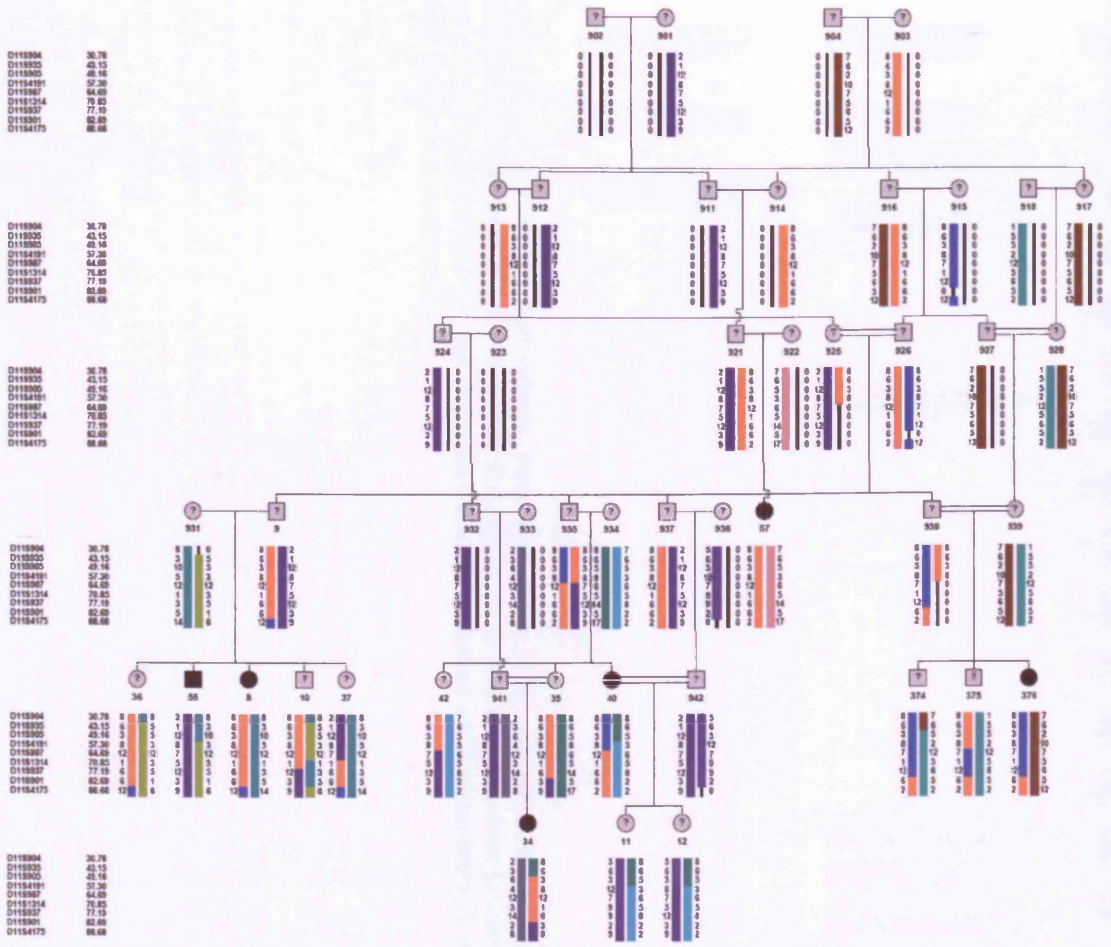
Blackened symbols represent individuals diagnosed with BPI; all other individuals were considered as of an unknown phenotype for linkage analysis and are therefore labelled with a question mark. See Figure 2.1 for full affection status information of these individuals; refer to the text for further explanations.

Figure 4.12: Haplotype reconstruction for a candidate region on chromosome 15 in family ANT18.



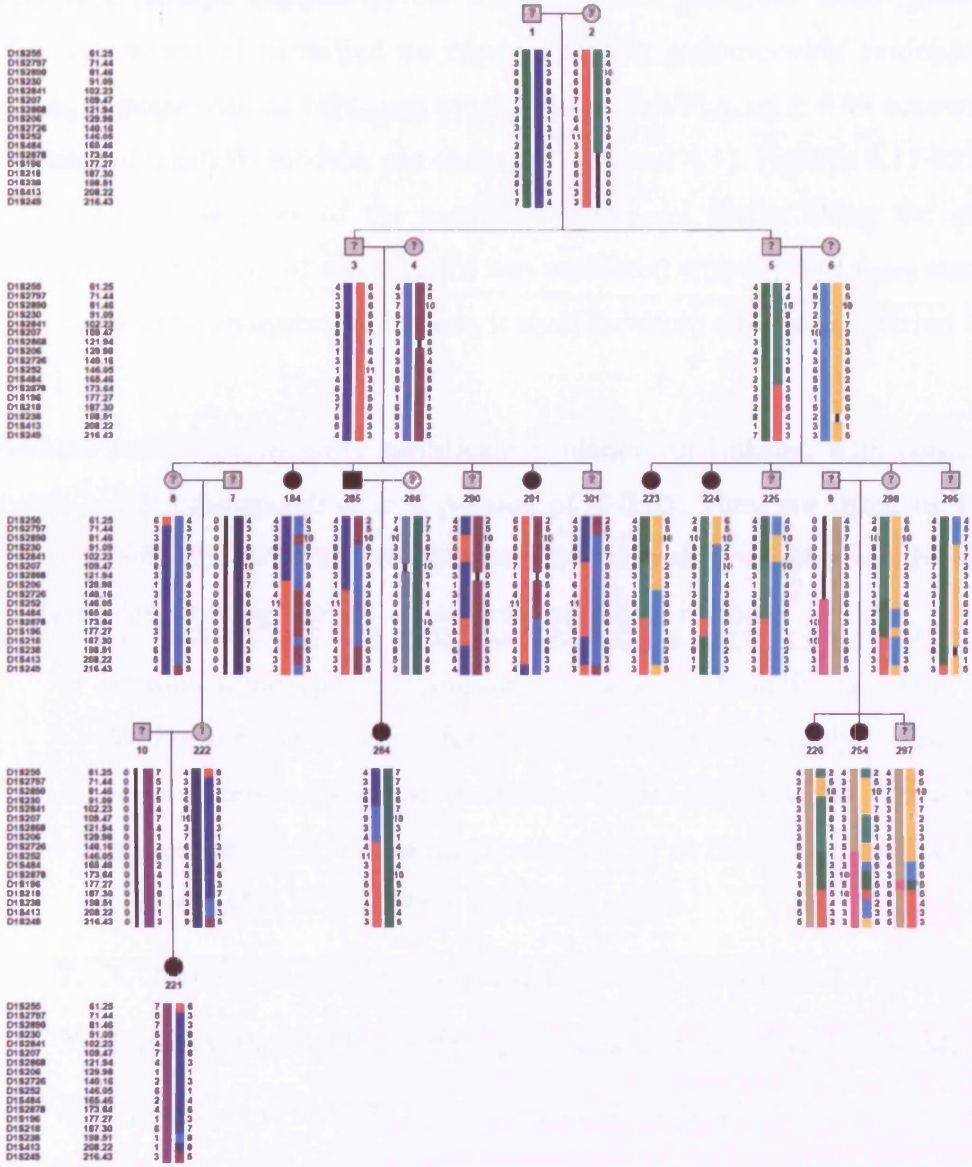
Blackened symbols represent individuals diagnosed with BPI; all other individuals were considered as of an unknown phenotype for linkage analysis and are therefore labelled with a question mark. See Figure 2.1 for full affection status information of these individuals and refer to the text for further explanations.

Figure 4.13: Haplotype reconstruction for a candidate region on chromosome 11 in family FAZU01.



Blackened symbols represent individuals diagnosed with BPI; all other individuals were considered as of an unknown phenotype for linkage analysis and are therefore labelled with a question mark. See Figure 2.2 for full affection status information of these individuals and refer to the text for further explanations.

Figure 4.14: Haplotype reconstruction for a candidate region on chromosome 1 in family ANT27.



Blackened symbols represent individuals diagnosed with BPI; all other individuals were considered as of an unknown phenotype for linkage analysis and are therefore labelled with a question mark. See Figure 2.1 for full affection status information of these individuals and refer to the text for further explanations.

4.6.2.2. Non-Parametric Analysis

Non-parametric linkage analysis of the fifteen bipolar pedigrees from Antioquia under the narrow model identified no region showing genome-wide evidence for linkage (i.e., regions with an NPL_{PAIRS} score ≥ 3.88, or $NPL_{PAIRS} \geq 4.49$ accounting for the testing of multiple models; see chapters 1.2.3 and 4.1). Figures 4.15 to 4.17 (pages 179-181) show plots of the combined NPL_{PAIRS} scores along the entire genome (note that the level of significance was measured with the NPL_{PAIRS} statistic for all non-parametric analyses; in the text, it shall therefore simply be referred to as NPL score).

Nine markers provided nominally significant evidence for linkage, with combined NPL scores ≥ 1.3, corresponding to a p-value of ≤ 0.05. They are listed in Table 4.13, together with the NPL scores obtained for individual families at the same markers. These nine markers define six separate candidate regions:

1. Chromosome 1p13-31 (limited by markers D1S230 and D1S2726). While the NPL score for marker D1S2841 is only 1.26, it is immediately adjacent to markers D1S207-D1S206, for which NPL scores are ≥ 1.3, with a maximum of 2.13 at D1S2868; it is therefore considered to be part of the candidate region.
2. Chromosome 1q25-31 (limited by markers D1S218 and D1S413);
3. Chromosome 5q11-12 (limited by markers D5S407 and D5S424);
4. Chromosome 9p13-21 (limited by markers D9S171 and D9S1817);
5. Chromosome 12p12-12ct (telomeric limit: marker D12S1617);
6. Chromosome 12ct-12q14 (telomeric limit: marker D12S83).

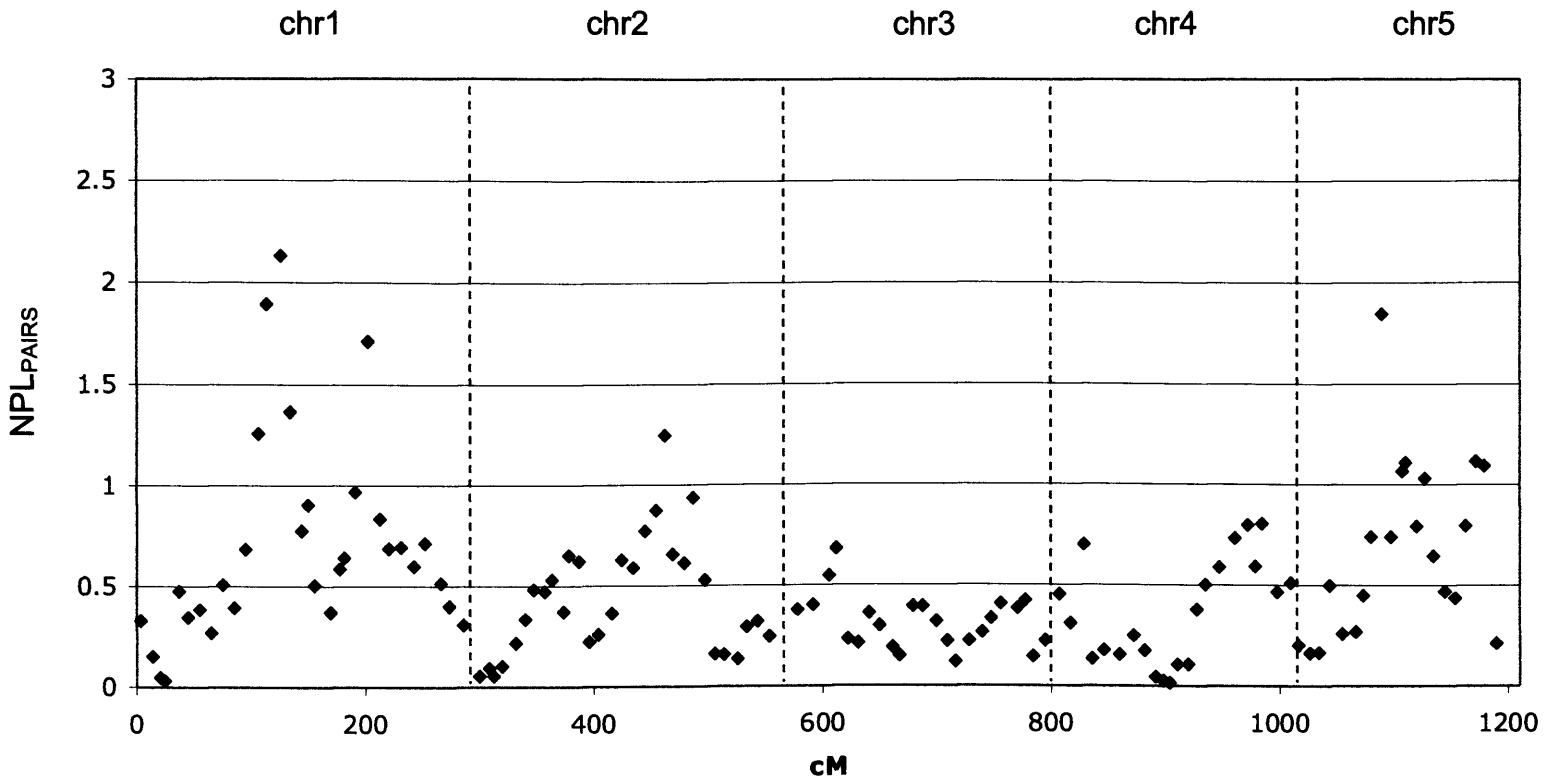
The highest combined NPL scores were observed on chromosomes 12q and 1p (2.55 and 2.13, respectively). Several of the pedigrees show individual NPL scores ≥ 1.3 within these candidate regions (see Table 4.13). The highest individual statistic in any of these candidate regions was obtained for family ANT27 on chromosome 1p31.1 (marker D1S207; NPL = 2.0).

Table 4.13: Markers with combined NPL_{PAIRS} scores ≥ 1.3 (equivalent to p ≤ 0.05) in the genome scan of fifteen BPI pedigrees (narrow model).

Marker	Chromosome region	Position on chromosome	NPL_{PAIRS}														
			all	FAZU01	FAZU28	ANT03	ANT04	ANT07	ANT10	ANT14	ANT15	ANT18	ANT19	ANT21	ANT23	ANT24	ANT26	ANT27
D12S85	12q13.11	68.90	**2.55**	0.54	0.84	0.47	1.57	0.17	0.23	0.17	0.36	0.90	0.62	0.76	0.23	0.12	1.37	0.51
D1S2868	1p22.1	136.57	**2.13**	0.11	0.28	1.10	0.26	0.84	0.75	0.47	0.08	0.65	1.21	0.24	0.03	0.13	0.38	1.58
D12S368	12q13.3	74.12	**1.93**	0.56	0.84	0.41	1.69	0.01	0.21	0.15	0.30	0.86	0.59	0.86	0.25	0.10	1.23	0.24
D1S207	1p31.1	122.43	**1.90**	0.14	0.02	0.07	0.31	1.01	0.26	0.57	0.35	0.08	1.33	0.32	0.24	0.52	0.73	**2.00**
D5S647	5q12.3	81.62	**1.85**	0.26	0.85	0.24	1.35	0.32	0.59	0.16	0.15	0.60	0.14	0.00	1.14	0.08	0.06	1.77
D12S345	12p11.21	56.90	**1.77**	0.60	0.71	0.62	0.18	0.44	0.22	0.12	0.51	0.87	0.52	0.76	0.24	0.12	1.42	0.35
D1S238	1q31.1	221.17	**1.71**	0.49	1.48	0.33	0.48	0.59	0.23	0.24	0.35	0.09	0.28	0.07	1.26	0.00	0.44	1.10
D9S161	9p21.2	46.90	**1.61**	0.53	0.82	0.31	0.74	0.38	0.24	0.13	0.35	1.20	0.15	0.13	0.21	0.60	0.27	1.64
D1S206	1p21.2	145.27	**1.37**	0.13	0.44	1.05	0.33	0.68	0.65	0.10	0.08	0.42	1.15	0.25	0.07	0.13	0.23	1.06

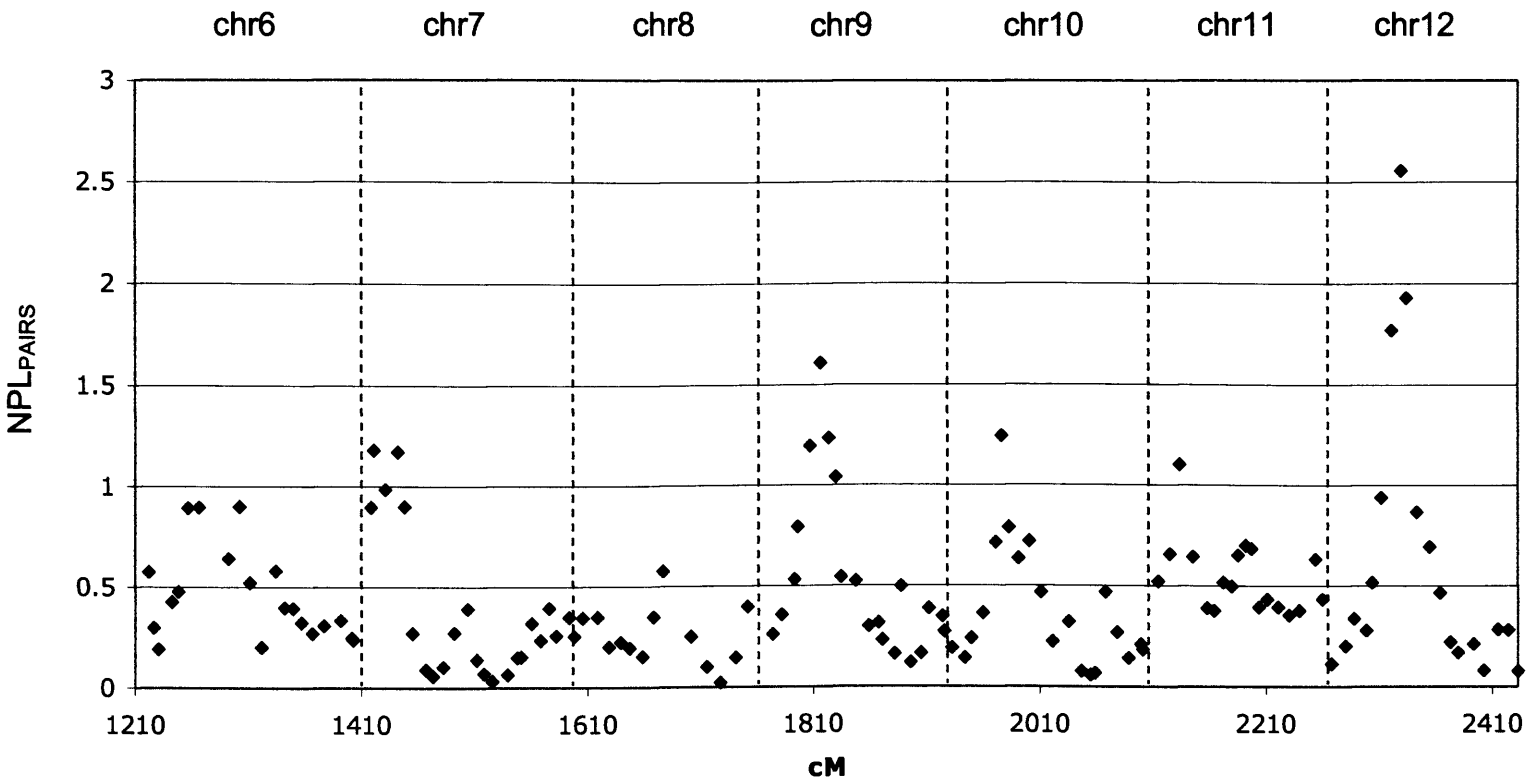
Family-specific NPL scores ≥ 1.3 are underlined (scores ≥ 2.0 are printed in bold).

Figure 4.15: Combined NPL_{PAIRS} scores for chromosomes 1-5 (narrow model).



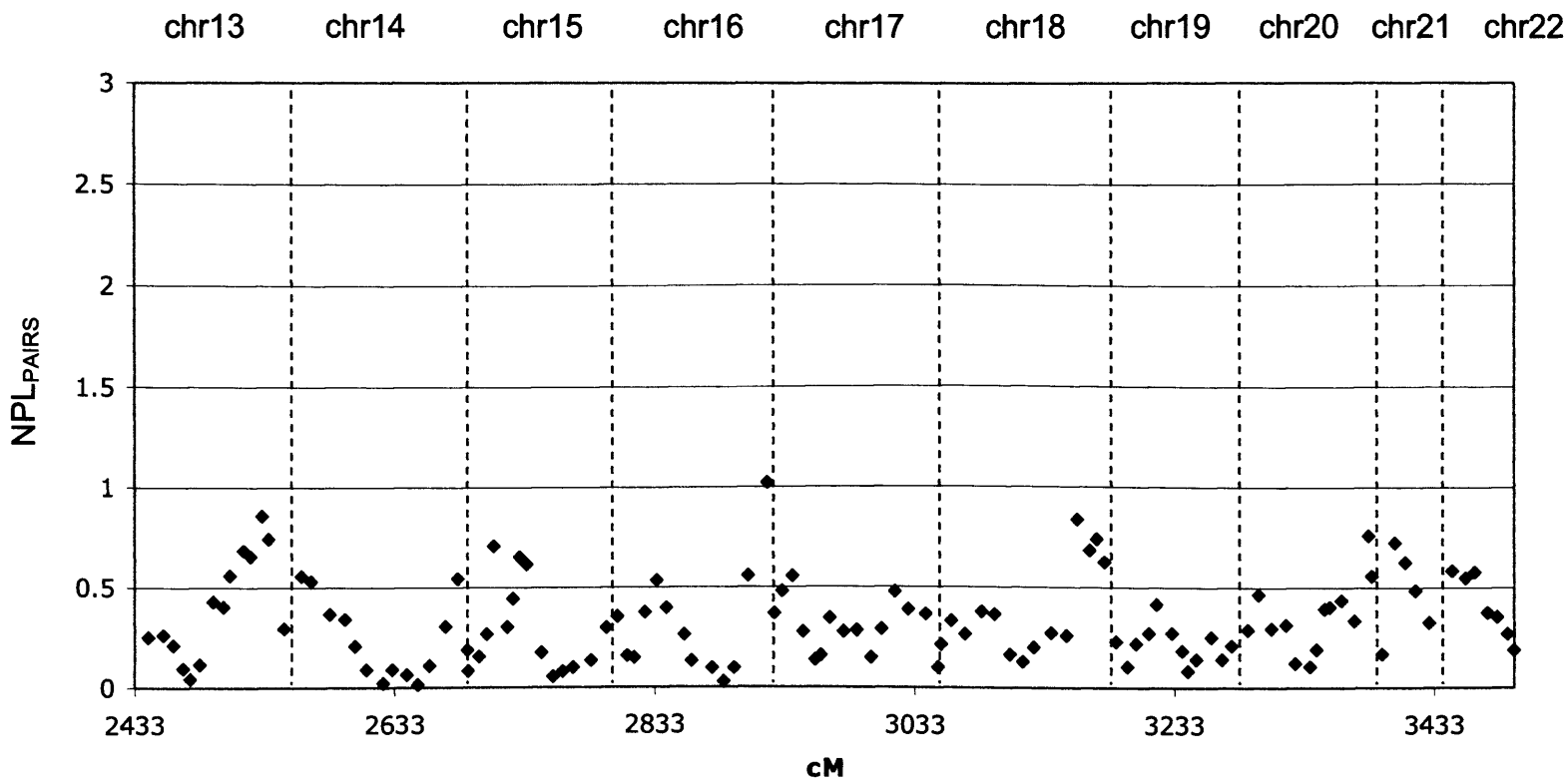
Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Figure 4.16: Combined NPL_{PAIRS} scores for chromosomes 6-12 (narrow model).



Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Figure 4.17: Combined NPL_{PAIRS} scores for chromosomes 13-22 (narrow model).



Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Markers, for which single-family NPL scores ≥ 2.0 were obtained under the narrow model, are listed in Table 4.14. Haplotype reconstructions for these regions are shown in Figure 4.18 (chromosome 16, family ANT07) and Figure 4.19 (chromosome 21, family ANT27). Haplotypes for chromosome 3q in family ANT07 have already been examined in the previous section, in the frame of the results from the parametric linkage analysis (see also Figure 4.11). Haplotypes for chromosome 21 are again shown for the entire chromosome, whereas haplotypes for chromosome 16 in family ANT07 are displayed for all markers with NPL ≥ 1.3, as well as for the adjacent markers on both sides.

Table 4.14: Markers with single-family NPL_{PAIRS} scores ≥ 2.0 (narrow model).

Family	Chromosome region	NPL_{PAIRS}	Marker
ANT07	16p12.2	2.49	D16S3046
ANT07	3q26	2.32	D3S1580
ANT14	21q21.2	2.32	D21S1914
ANT07	16p12.1	2.07	D16S3068
ANT27	1p31.1	2.00	D1S207

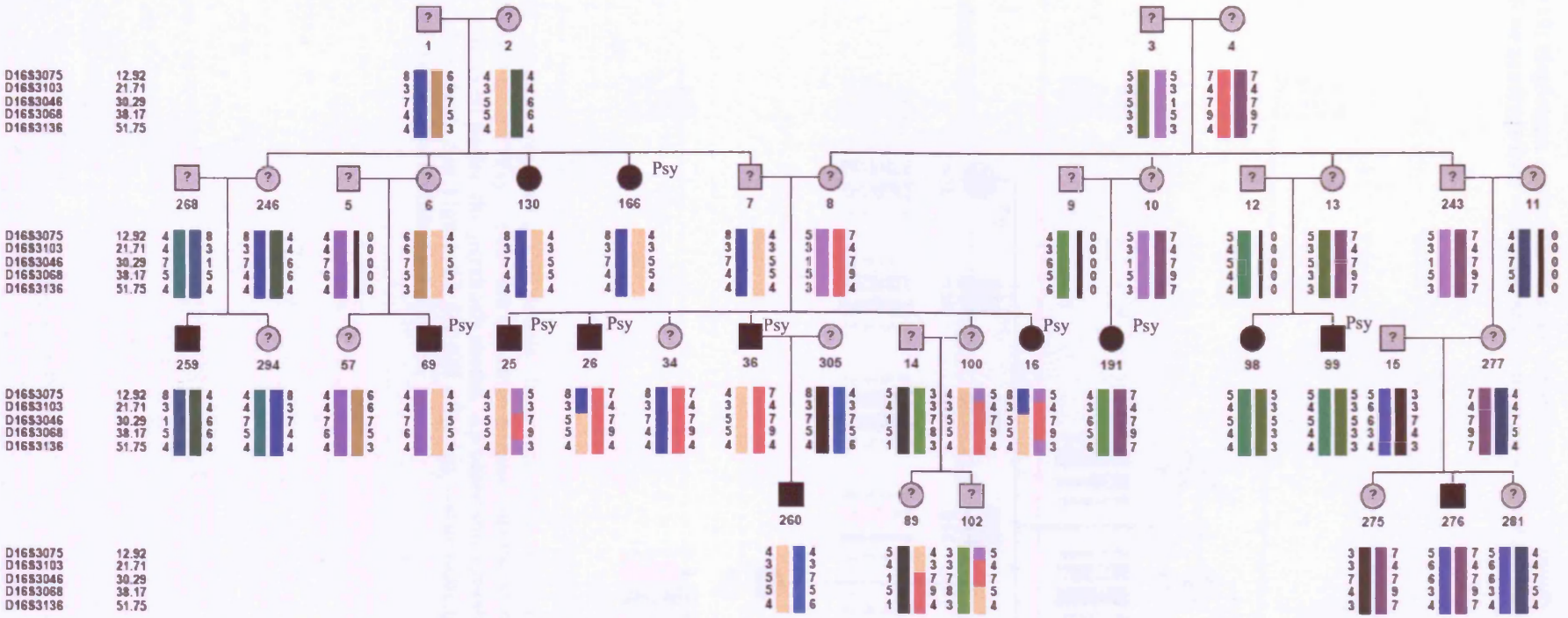
Haplotype analysis

The haplotype reconstruction for chromosome 16p in family ANT07 shows the occurrence of a particular haplotype (shown in light brown in Figure 4.18) in the majority of the affecteds. Haplotype patterns in the remaining affecteds, however, provide a less clear picture.

On chromosome 21, haplotype reconstruction for pedigree ANT14 shows near-perfect co-segregation of a haplotype comprising markers D21S1256 and D21S1914 with BPI (the blue haplotype in Figure 4.19). Individual 172, the only one not to display the "blue" haplotype, nonetheless shares the same alleles at the markers in question. Since the mother of individual 172, individual 188, is homozygous for both markers, the haplotype for individual 172 cannot be confidently reconstructed, and it is indeed possible that all affecteds share a haplotype that is identical by descent.

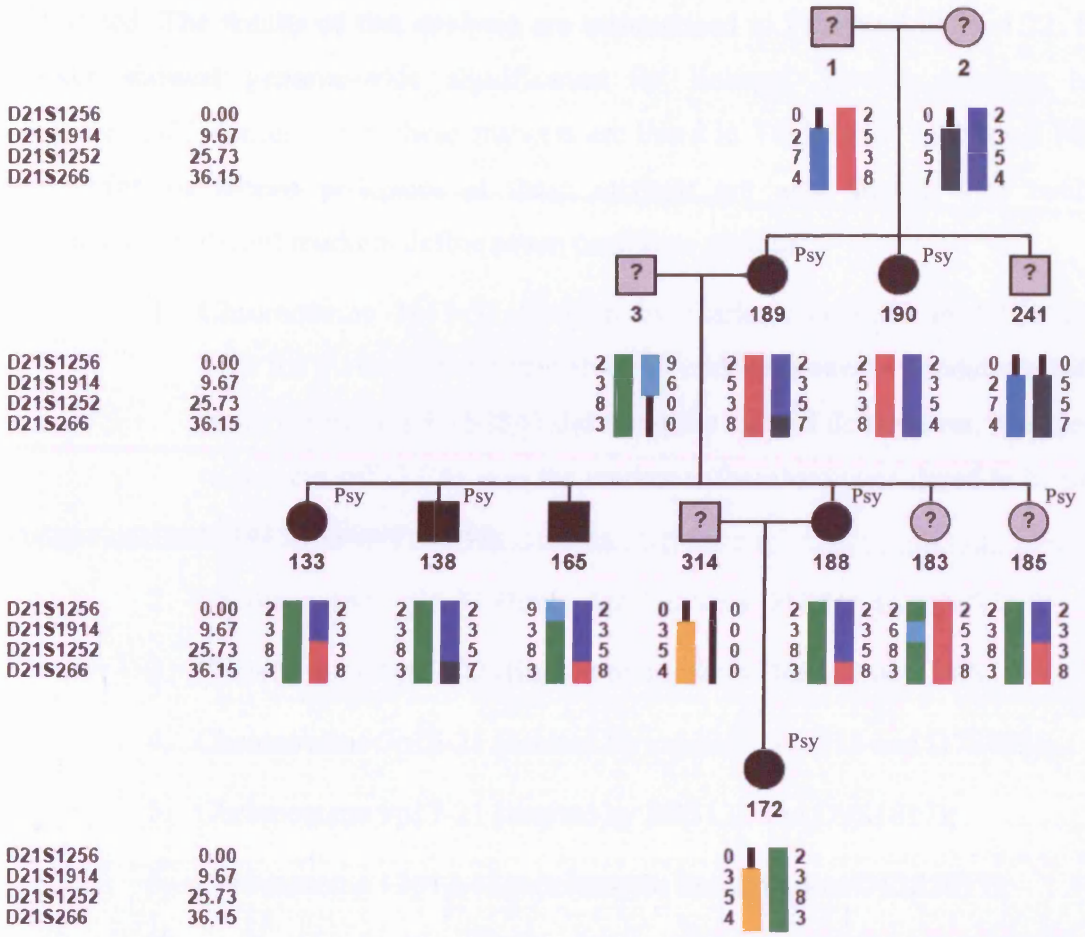
The reconstruction of haplotypes on chromosome 1p in family ANT27 has already been discussed in the context of parametric linkage analysis in the previous section (chapter 4.6.2.1; see also Figure 4.14).

Figure 4.18: Haplotype reconstruction for a candidate region on chromosome 16 in family ANT07.



Blackened symbols represent individuals diagnosed with BPI; individuals 57, 100, 89, 275 and 281 are diagnosed with MD, and individual 102 has a diagnosis of BPII. Individuals suffering from psychosis are labelled "Psy". Diagnoses differing from BPI are indicated in this pedigree because the region on chromosome 16p was picked up under all three analysis models for family ANT07. All other pedigree members are of an unknown phenotype status. See text for further explanations.

Figure 4.19: Haplotype reconstruction for chromosome 21 in family ANT14. Marker D21S263, for which no genotype data were available in data set 1, is not shown.



Blackened symbols represent individuals diagnosed with BPI; individuals suffering from psychosis are labelled "Psy". They are indicated because chromosome 21 was also picked up as a region of interest under the psychosis model. All other individuals were considered as of an unknown phenotype. See Figure 2.1 for full affection status information of these individuals, and refer to the text for further explanations.

4.6.3. Linkage Analysis: Broad Model

Under the broad model, where all family members diagnosed with BPI, BPII or major depression were considered affected, only non-parametric analysis was performed. The results of this analysis are summarised in Figures 4.20 to 4.22. No marker showed genome-wide significance for linkage. Twelve markers had combined NPL scores ≥ 1.3; these markers are listed in Table 4.15. Individual NPL scores for all fifteen pedigrees at these markers are also shown. The twelve nominally significant markers define seven candidate regions:

1. Chromosome 1p13-31 (limited by markers D1S230 to D1S2726). Like for the non-parametric analysis under the narrow model, the NPL score for marker D1S2841 did not quite reach 1.3; however, it is close to this cut-off (1.28), and the marker is therefore considered to be part of the candidate region.
2. Chromosome 1q25-31 (limited by markers D1S218 and D1S413);
3. Chromosome 6p12-22 (limited by markers D6S422 and D6S257);
4. Chromosome 7p15-21 (limited by markers D7S513 and D7S493);
5. Chromosome 9p13-21 (limited by D9S171 and D9S1817);
6. Chromosome 12p12-12ct (telomeric limit: marker D12S1617);
7. Chromosome 12ct-12q21 (telomeric limit: D12S351; marker D12S326, which is directly adjacent to the markers with NPL ≥ 1.3, has an NPL score of 1.24 and might therefore still considered to be part of the candidate region).

The candidate regions with the highest combined NPL scores (≥ 2.0) were chromosome 12p and q, and 1p and q (NPL scores between 2.07 and 2.35).

Several of the families show individual NPL scores ≥ 1.3 within these candidate regions (see Table 4.15). Again, the highest individual statistic in any of these candidate regions was obtained for family ANT27 on chromosome 1p31.1 (marker D1S207; NPL = 2.0). This NPL score is the same as for the analysis under the narrow model because all affected pedigree members in family ANT27 are diagnosed with BPI, rendering the analyses under the two different models identical (see Figure 2.1).

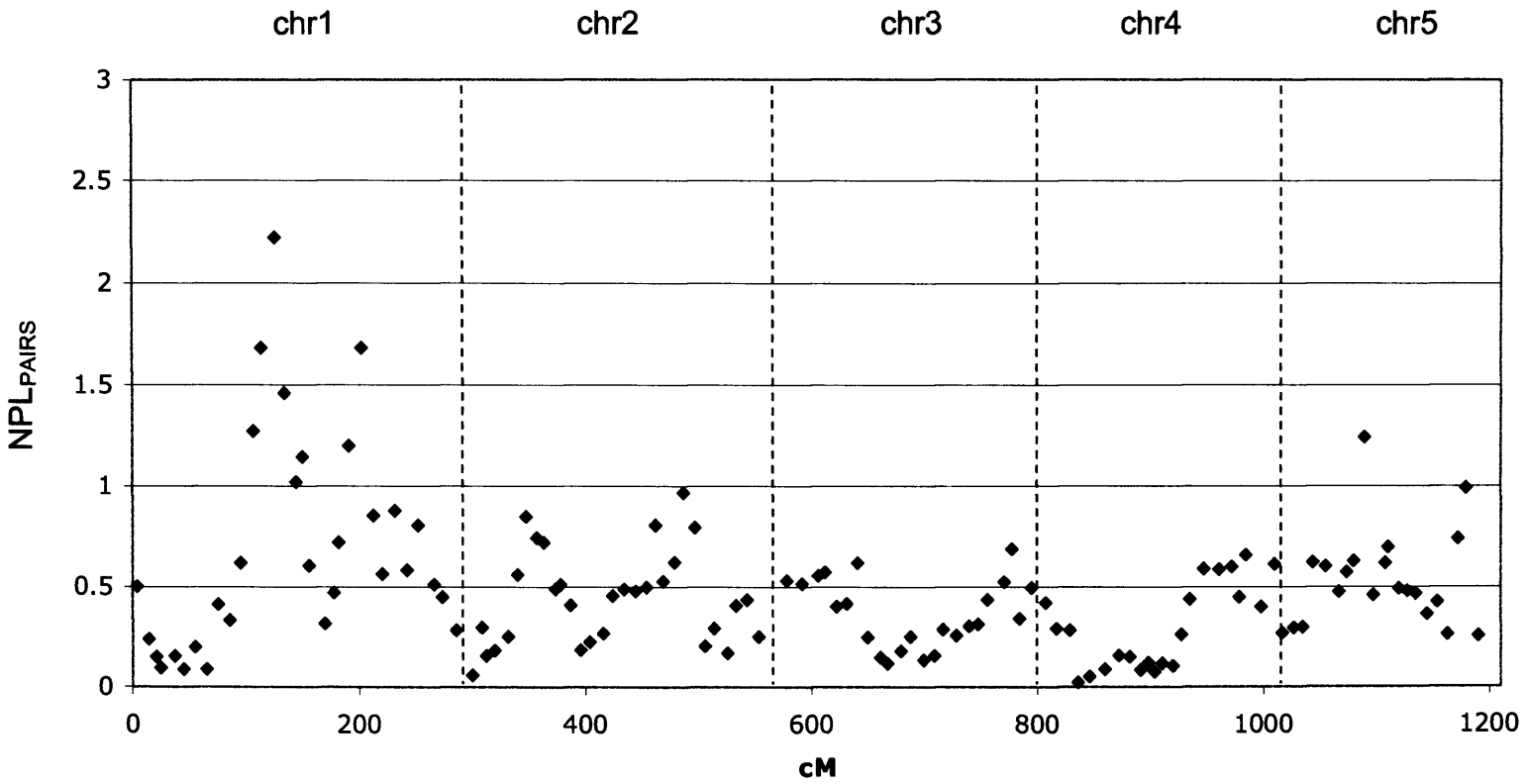
Of the seven candidate regions identified in non-parametric analysis under the broad model, five overlap partly or completely with the ones identified in non-parametric analysis under the narrow model; these are the regions on chromosome 1p, 1q, 9p, 12p, and 12q. The candidate region on chromosome 1p was also identified in parametric linkage analysis.

Table 4.15: Markers with combined NPL_{PAIRS} scores ≥ 1.3 (equivalent to $p \leq 0.05$) in the genome scan of fifteen extended *paisa* pedigrees (broad model).

Marker	Chromosome region	Position on chromosome	NPL_{PAIRS}															
			all	FAZU01	FAZU28	ANT03	ANT04	ANT07	ANT10	ANT14	ANT15	ANT18	ANT19	ANT21	ANT23	ANT24	ANT26	ANT27
D12S85	12q13.11	68.90	**2.35**	0.38	0.84	0.36	0.73	0.51	0.71	0.31	0.36	0.90	0.62	0.76	0.28	0.35	1.37	0.51
D1S2868	1p22.1	136.57	**2.22**	0.19	0.28	1.06	0.10	1.39	0.57	0.77	0.08	0.65	1.21	0.24	0.09	0.00	0.38	1.57
D12S345	12p11.21	56.90	**2.10**	0.58	0.71	0.43	0.26	0.91	0.65	0.12	0.51	0.87	0.52	0.76	0.29	0.35	1.42	0.35
D12S368	12q13.3	74.12	**2.07**	0.31	0.84	0.34	1.19	0.33	0.64	0.29	0.30	0.86	0.59	0.86	0.32	0.28	1.23	0.25
D1S207	1p31.1	122.43	**1.68**	0.10	0.02	0.16	0.23	0.80	0.66	0.70	0.35	0.08	1.33	0.32	0.23	0.31	0.73	**2.00**
D1S238	1q31.1	221.17	**1.68**	0.50	1.47	0.18	0.49	0.36	0.33	0.23	0.35	0.09	0.28	0.07	1.87	0.00	0.44	1.10
D7S507	7p21.1	26.00	**1.67**	0.40	0.01	0.94	0.15	1.13	0.62	0.54	0.35	0.01	0.59	0.07	0.78	0.78	0.87	0.68
D6S1610	6p21.2	48.86	**1.54**	0.21	0.04	0.13	0.49	0.19	1.29	1.73	0.00	0.47	0.78	0.84	0.27	1.21	0.22	0.54
D9S161	9p21.2	46.90	**1.50**	0.75	0.82	0.62	0.61	0.47	0.36	0.10	0.35	1.20	0.15	0.13	0.25	0.00	0.27	1.64
D1S206	1p21.2	145.27	**1.46**	0.25	0.44	1.05	0.17	1.24	0.65	0.16	0.08	0.42	1.15	0.24	0.05	0.00	0.23	1.06
D6S276	6p22.2	38.44	**1.37**	0.19	0.20	0.15	0.53	0.11	1.33	1.57	0.00	0.37	0.71	0.72	0.30	1.23	0.17	0.57
D12S83	12q14.1	84.54	**1.32**	0.25	0.54	0.23	0.52	0.92	0.28	0.35	0.18	0.47	0.52	0.84	0.32	0.22	1.09	0.12

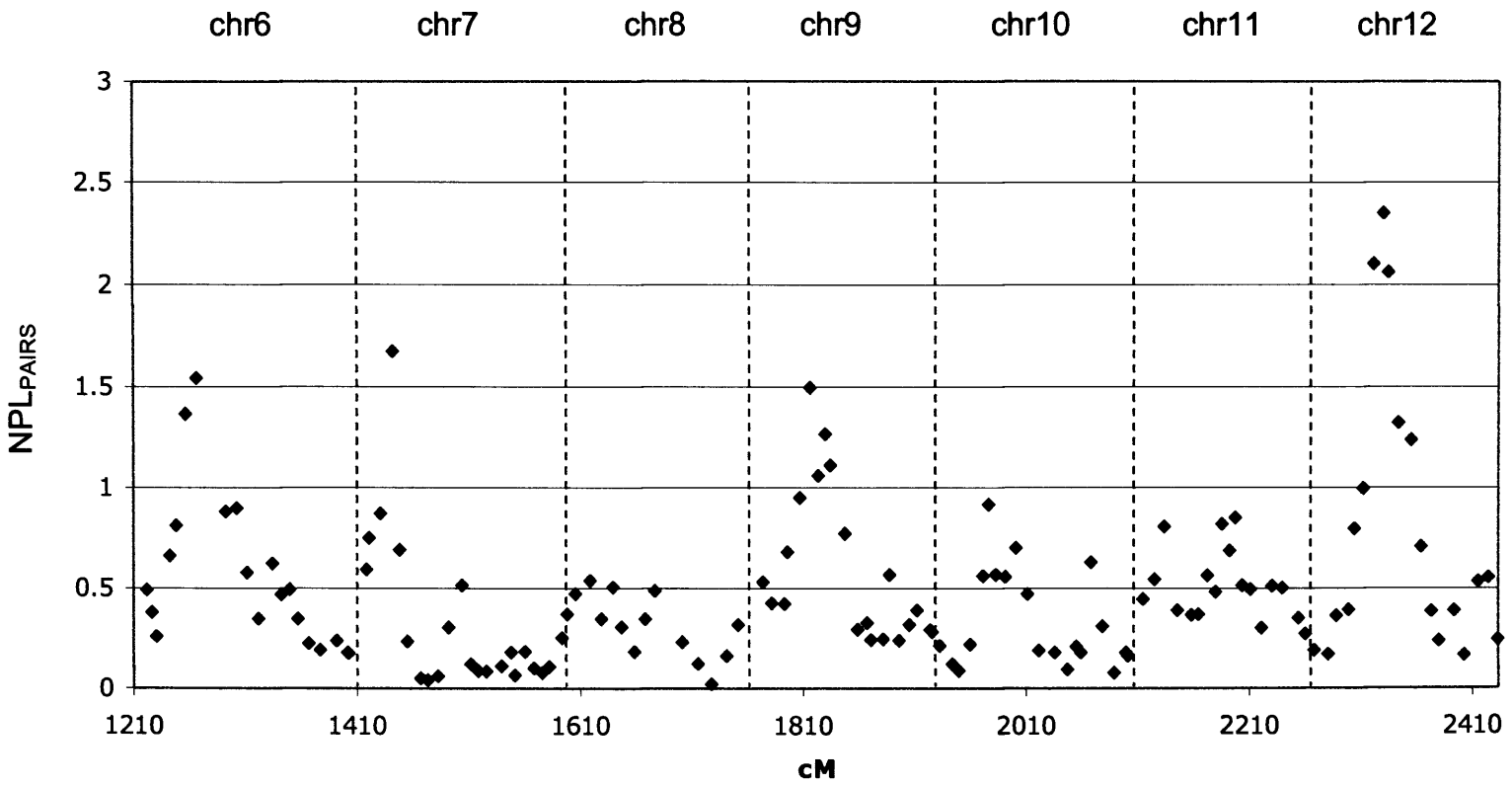
Individual NPL scores ≥ 1.3 are underlined; NPL_{PAIRS} scores ≥ 2.0 are additionally printed in bold.

Figure 4.20: Combined NPL_{PAIRS} scores for chromosomes 1-5 (broad model).



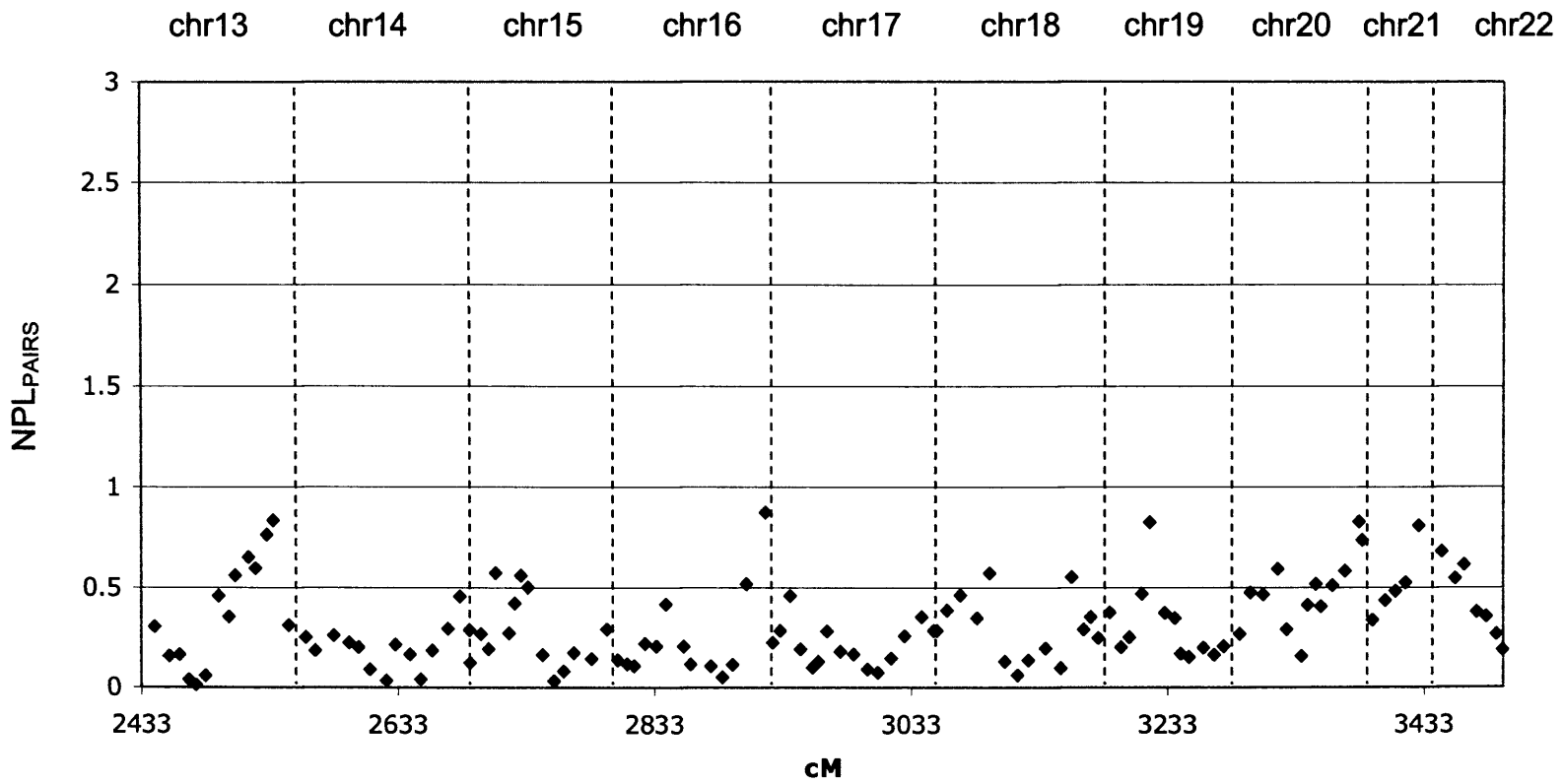
Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Figure 4.21: Combined NPL_{PAIRS} scores for chromosomes 6-12 (broad model).



Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Figure 4.22: Combined NPL_{PAIRS} scores for chromosomes 13-22 (broad model).



Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Single-family NPL scores ≥ 2.0 were found in three pedigrees (see Table 4.16). Haplotype reconstructions for these families are discussed below; as in section 4.6.2.2, haplotypes are displayed for all markers with NPL ≥ 1.3, as well as for the adjacent markers on both sides.

Table 4.16: Markers with single-family NPL_{PAIRS} scores ≥ 2.0 (broad) model).

Family	Chromosome region	NPL_{PAIRS}	Marker
ANT23	2q33.3	2.23	D2S325
ANT23	2q32.3	2.19	D2S117
ANT07	16p12.1	2.12	D16S3068
ANT27	1p31.1	2.00	D1S207

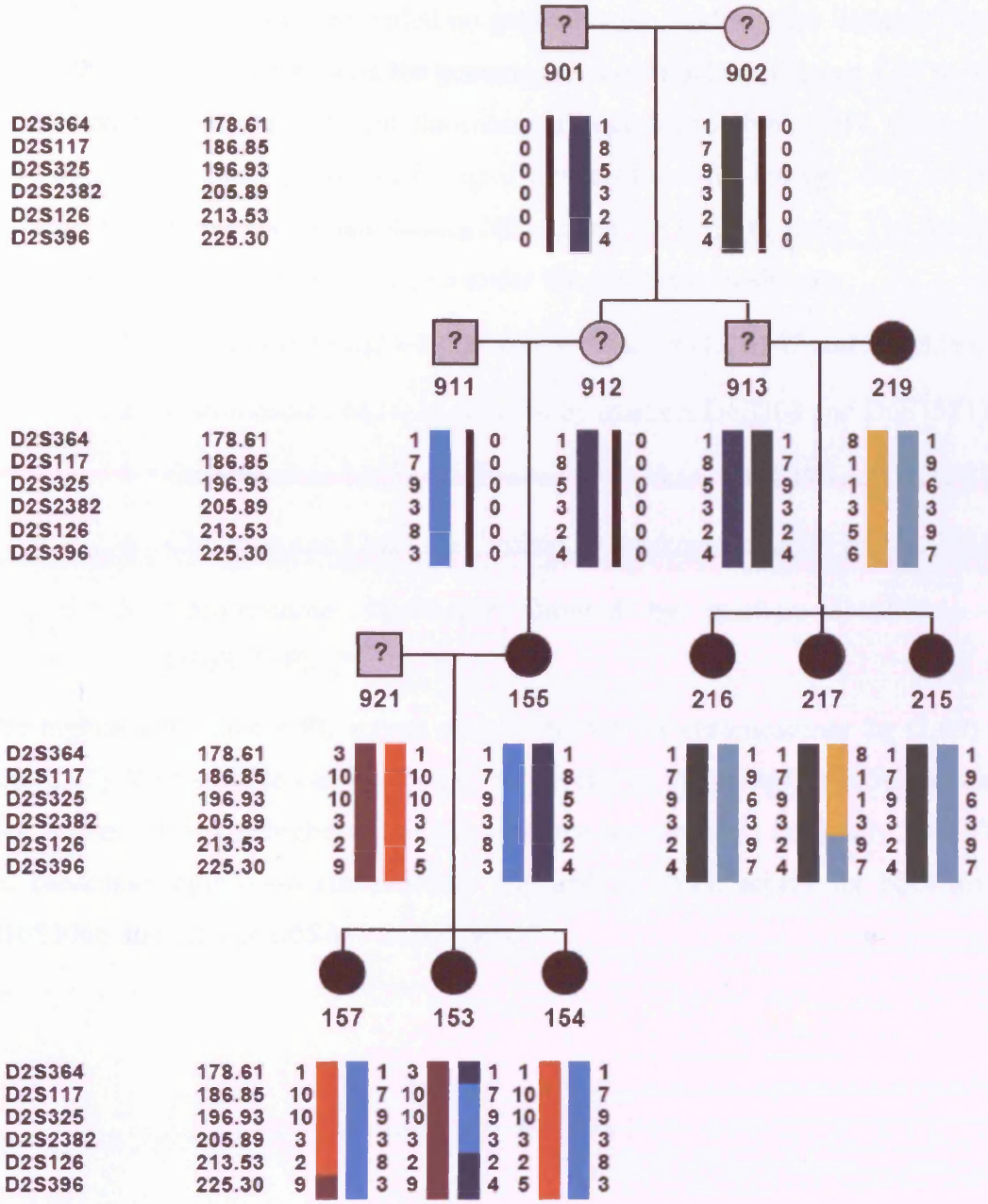
Visual inspection of the results of the haplotype analysis suggests that in family ANT23, a 3-marker haplotype comprising markers D2S117 to D2S2382 on chromosome 2 is co-segregating with the broadly defined phenotype (see Figure 4.23). Although according to the reconstruction by SimWalk2, the haplotypes do not seem to be identical by descent in all affecteds, it has to be remembered that SimWalk2 uses a maximum likelihood approach, and it is possible that there is another, equally likely haplotype configuration (or even several), which allows for the two haplotypes that are identical by state (dark green and light blue in Figure 4.23) to be also identical by descent. Note that at first sight, the haplotype reconstruction as shown in Figure 4.23 seems less likely than an alternative where the potential disease-carrying haplotype is identical by descent in all affecteds; however, this has to do with the fact that only a limited region on chromosome 2 is shown in the figure, whereas the haplotypes were reconstructed based on information for the whole chromosome.

The haplotypes on chromosome 16p in pedigree ANT07 have been previously discussed in section 4.6.2.2 (see Figure 4.18); however, in the present analyses, individuals diagnosed with BPII and MD were also considered affected. This makes

the co-segregation patterns between haplotypes and phenotypes less clear; however, the light brown haplotype (as shown in Figure 4.18) still shows co-segregation with the phenotype in part of the pedigree.

Co-segregation patterns between haplotypes in a candidate region on chromosome 1p and the phenotype in family ANT27 have equally been previously discussed (see section 4.6.2.1 and Figure 4.14). Because there are no individuals affected with either BPII or MD in this family, the conclusions remain the same: there is co-segregation of a putative disease-carrying haplotype comprising markers D1S207 and D1S2868 with BPI, the only affecteds without the haplotype under consideration being individuals 284 and 291.

Figure 4.23: Haplotype reconstruction for a candidate region on chromosome 2 in family ANT23.



Blackened symbols represent individuals considered affected in linkage analysis; all other individuals were considered as of an unknown phenotype and are therefore labelled with a question mark. See Figure 2.2 for full affection status information of these individuals. See text for further explanations.

4.6.4. Linkage Analysis: Psychosis Model

Non-parametric linkage analysis of the fifteen bipolar pedigrees from Antioquia under the psychosis model provided no genome-wide evidence for linkage. Plots of the combined NPL scores along the genome are shown below (Figures 4.24 to 4.26). For five markers in five different chromosomal regions, combined NPL scores ≥ 1.3 were obtained, indicating nominally significant evidence for linkage; they are listed in Table 4.17, along with single-family NPL scores for these markers. The candidate regions identified through the analysis under the psychosis model are:

1. Chromosome 2q24-31 (limited by markers D2S142 and D2S335);
2. Chromosome 6q24-25 (limited by markers D6S308 and D6S1581);
3. Chromosome 10q11-22 (limited by markers D10S196 and D10S537);
4. Chromosome 12q21-23 (limited by markers D12S326 and D12S346);
5. Chromosome 16p12-q12 (limited by markers D16S3046 and D16S3136).

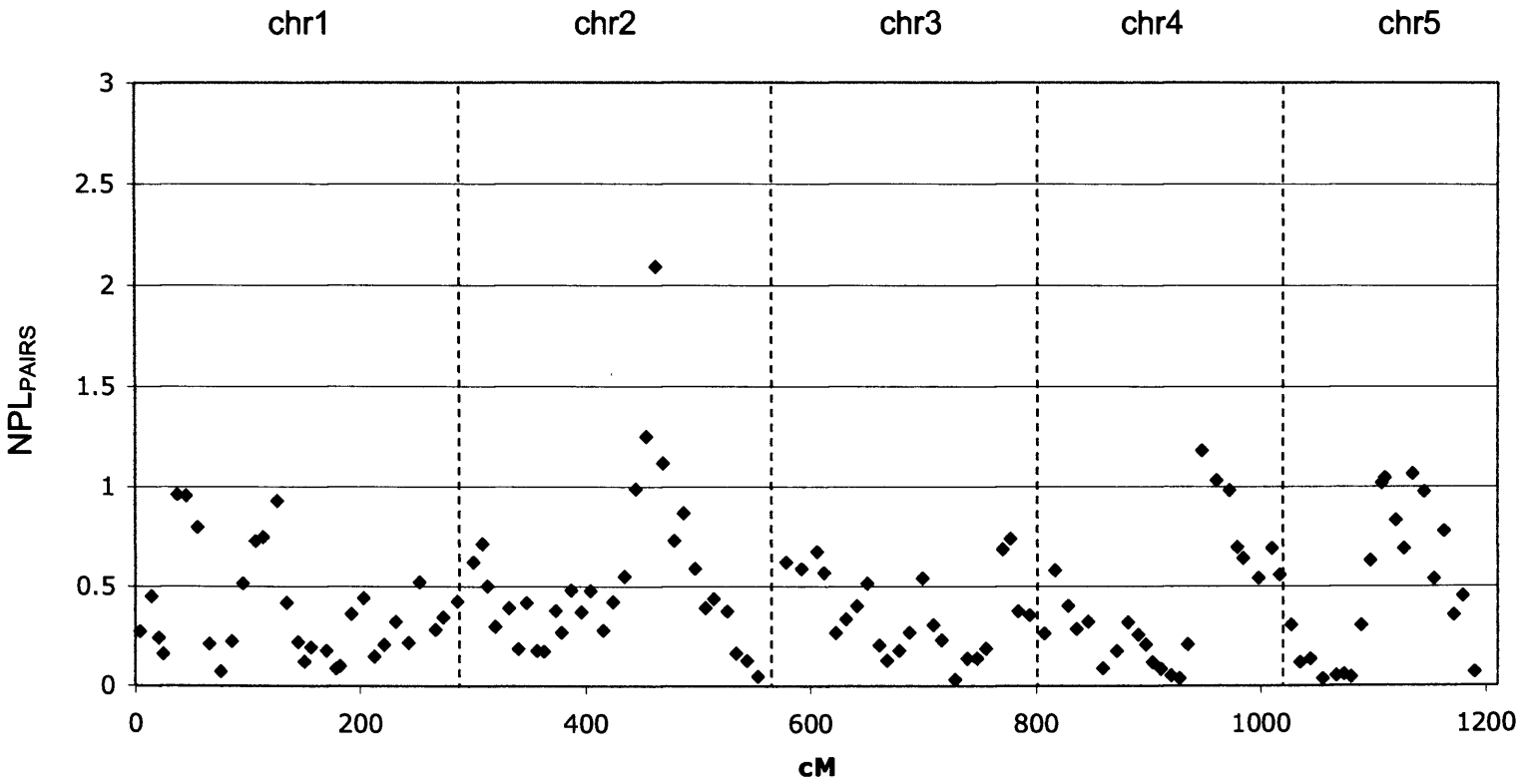
The highest combined NPL scores were achieved for chromosomes 2q (2.09) and 16p (2.05). Within these candidate regions, several of the pedigrees show individual NPL scores ≥ 1.3. The highest individual statistic was obtained for family ANT07 for the candidate regions on chromosome 16p and 6q (NPL scores for both marker D16S3068 and marker D6S441 were 1.90).

Table 4.17: Markers with combined NPL_{PAIRS} scores ≥ 1.3 (equivalent to p ≤ 0.05) in the genome scan of fifteen extended *paisa* pedigrees (psychosis model).

Marker	Chromosome region	Position on chromosome	NPL_{PAIRS}										
			all	FAZU28	ANT03	ANT04	ANT07	ANT14	ANT15	ANT18	ANT21	ANT23	ANT24	ANT27
D2S2330	2q24.3	178.85	**2.09**	0.60	0.37	0.02	0.98	0.80	0.60	0.29	0.36	0.60	0.13	0.26
D16S3068	16p12.1	41.82	**2.05**	0.60	0.00	0.00	<u>1.90</u>	0.53	0.62	0.26	1.09	0.12	0.61	0.09
D6S441	6q25.2	166.61	**1.44**	0.13	1.20	0.06	<u>1.90</u>	0.05	0.58	0.11	0.00	0.60	0.05	0.33
D12S351	12q21.33	106.94	**1.39**	0.12	0.01	0.62	0.13	<u>1.44</u>	0.13	0.17	0.33	0.61	0.41	0.33
D10S1652	10q21.2	87.87	**1.36**	0.23	0.00	0.00	<u>1.68</u>	0.42	0.37	0.17	0.36	0.13	0.13	0.57

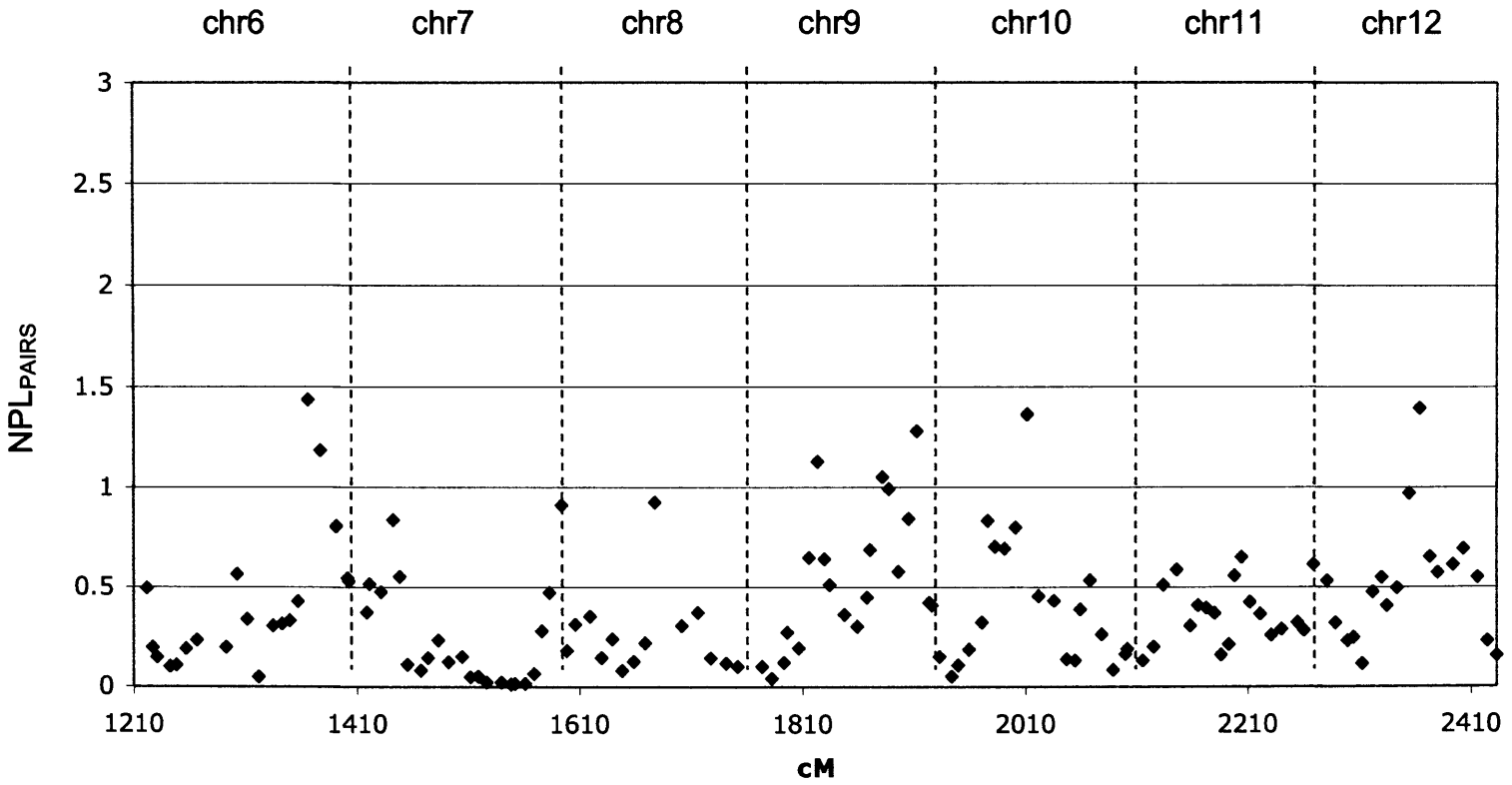
Individual NPL scores ≥ 1.3 are underlined; NPL_{PAIRS} scores ≥ 2.0 are additionally printed in bold.

Figure 4.24: Combined NPL_{PAIRS} scores for chromosomes 1-5 (psychosis model).



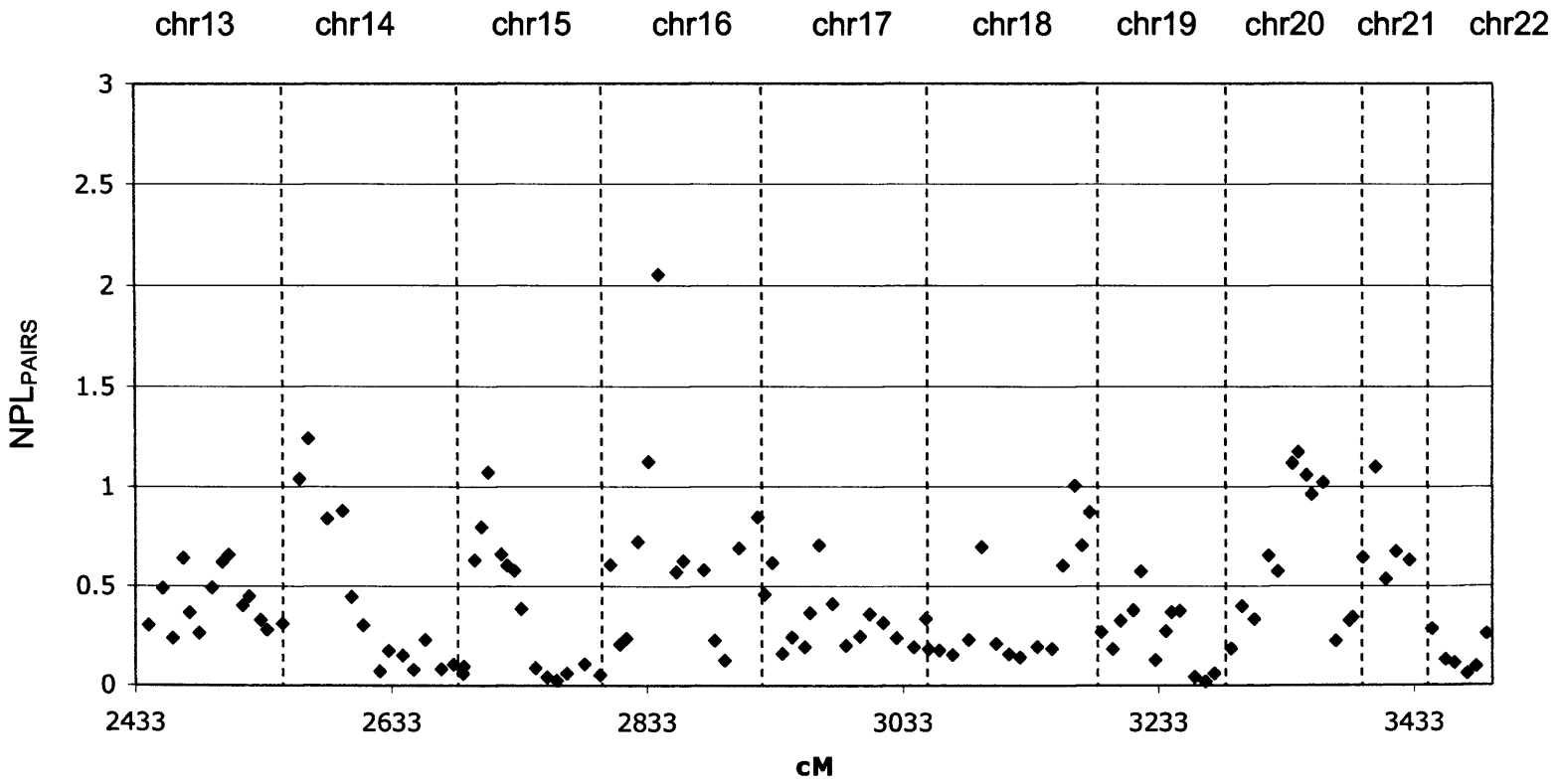
Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Figure 4.25: Combined NPL_{PAIRS} scores for chromosomes 6-12 (psychosis model).



Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Figure 4.26: Combined NPL_{PAIRS} scores for chromosomes 13-22 (psychosis model).



Dotted lines separate individual chromosomes. The X-axis represents the cumulative genetic distance from chromosome 1pter.

Individual NPL scores ≥ 2.0 were found in two families for three markers, as shown in Table 4.18.

Table 4.18: Markers with single-family NPL_{PAIRS} scores ≥ 2.0 (psychosis model).

Family	Chromosome region	NPL_{PAIRS}	Marker
ANT07	16p12.2	2.40	D16S3046
ANT14	21q21.2	2.33	D21S1914
ANT14	4q31.21	2.30	D4S424

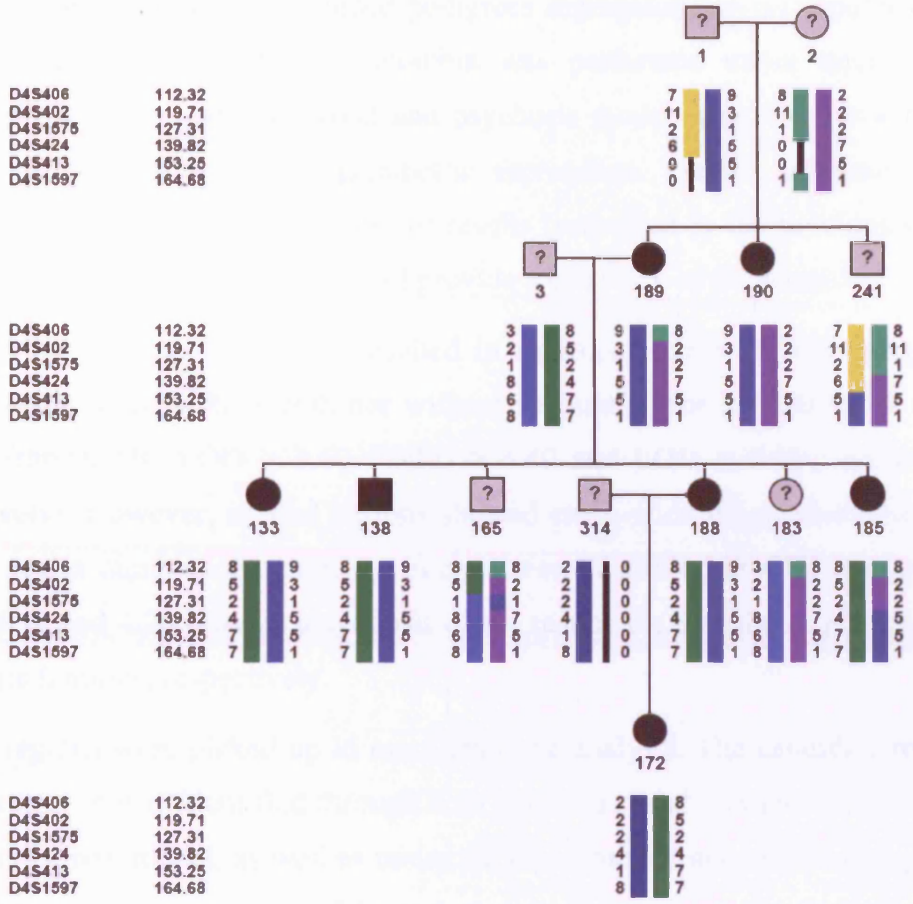
Haplotype analysis

In family ANT07, chromosome 16p was picked up as a region of interest in non-parametric analysis under all three models (see sections 4.6.2.2 and 4.6.3). The haplotype reconstruction is shown in Figure 4.18; visual inspection of the haplotypes suggests co-segregation of the light brown haplotype with psychosis in the left branch of the pedigree.

Haplotype reconstructions of chromosome 21 in pedigree ANT14 are shown in Figure 4.19 and have been previously discussed in section 4.6.2.2. The only individual that was not considered affected in the analysis under the narrow model, individual 185, a woman with an affective disorder that did not meet the criteria for BP but who is affected by psychosis, displays the same haplotype comprising markers D21S1256 and D21S1914 as the other affecteds in that family, making chromosome 21 an interesting candidate region in that family.

A haplotype reconstruction for chromosome 4q in family ANT14 is shown in Figure 4.27. Shown are all markers with NPL scores ≥ 1.0, as well as one adjacent marker on each side. Co-segregation of a haplotype comprising the three bottom markers with psychosis is nearly perfect; individual 172 alone does not share this haplotype with the remaining affecteds.

Figure 4.27: Haplotype reconstruction for a candidate region on chromosome 4 in family ANT14.



Blackened symbols represent individuals with psychosis; all other individuals were considered as of an unknown phenotype in linkage analysis and are therefore labelled with a question mark. See Figure 2.2 for full affection status information of these individuals, and refer to the text for further explanations.

4.6.5. Summary of the Linkage Results

Linkage analysis of fifteen extended pedigrees segregating severe bipolar disorder from the *paisa* community in Colombia was performed under three different phenotype models – narrow, broad and psychosis models (see chapter 4.1) – and using both parametric and non-parametric approaches. The use of these different approximations has yielded a wealth of results (presented in the previous sections, 4.6.2 - 4.6.4), and this subchapter shall provide a summary of these results.

None of the analyses performed resulted in significant evidence for linkage on a genome-wide level, neither with nor without accounting for the testing of multiple models (thresholds: LOD $\geq$ 3.60 / NPL $\geq$ 4.49 and LOD $\geq$ 3.00 / NPL $\geq$ 3.88, respectively). However, several regions showed study-wide suggestive evidence for linkage, and a number of regions of interest were identified in different pedigrees. Tables 4.19 and 4.20 provide a synopsis of the results for the whole set of pedigrees and single families, respectively.

Several regions were picked up in more than one analysis. The candidate region on chromosome 1p was identified through both parametric and non-parametric analyses under the narrow model, as well as under the broad model, and candidate regions on chromosome 1q, chromosome 12p and q and chromosome 9p showed nominal significance in non-parametric analysis under both narrow and broad models. The region on chromosome 1p produced consistently high LOD/NPL scores under narrow and broad models in family ANT27. On chromosome 21q, parametric linkage analysis produced high LOD scores both study-wide and for family ANT07, and non-parametric analyses under both narrow and psychosis models resulted in NPL scores $\geq$ 2.0 for family ANT14 in the same chromosomal region. Finally, for family ANT07, the candidate region on chromosome 16p was identified through non-parametric analysis under all three models.

Table 4.19: Synopsis of the chromosomal regions for which a HLOD score (in parametric analysis) or a combined NPL_{PAIRS} score (in non-parametric analysis) ≥ 1.3 were obtained (across all diagnostic models).

Narrow Model		Broad Model (Non-parametric LA)	Psychosis Model (Non-parametric LA)
Parametric LA	Non-parametric LA		
chr 1p22-31 (1.32)	chr 1p13-31 (2.13)	chr 1p13-31 (2.22)	chr 2q24-31 (2.09)
chr 21q21-22 (2.14)	chr 1q25-31 (1.71)	chr 1q25-31 (1.68)	chr 16p12-q12 (2.05)
chr 13q33 (1.46)	chr 12p12-ct (1.77)	chr 12p12-ct (2.10)	chr 6q24-25 (1.44)
	chr 12ct-12q14 (2.55)	chr 12ct-12q21 (2.35)	chr 12q21-23 (1.39)
	chr 9p13-21 (1.37)	chr 9p13-21 (1.50)	chr 10q11-22 (1.36)
	chr 5q11-12 (1.85)	chr 7p15-21 (1.67)	
		chr 6p12-22 (1.54)	

LA, Linkage analysis. Maximum HLOD (for parametric analysis) or combined NPL_{PAIRS} (for non-parametric analyses) scores for each region are shown in brackets.

Table 4.20: Synopsis of the chromosomal regions for which a LOD score (in parametric analysis) or an individual NPL_{PAIRS} score (in non-parametric analysis) ≥ 2.0 were obtained for in a single pedigree (all diagnostic models).

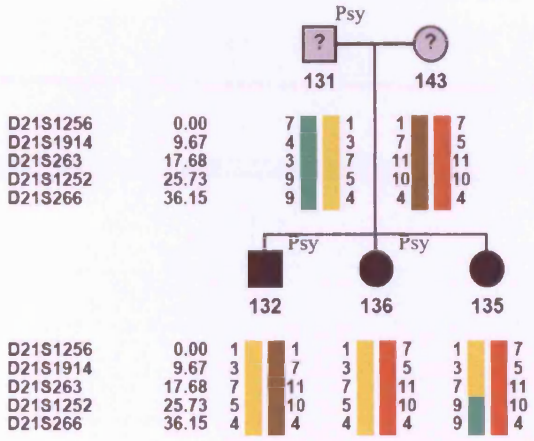
Narrow Model		Broad Model (Non-parametric LA)	Psychosis Model (Non-parametric LA)
Parametric LA	Non-parametric LA		
ANT07: chr 21q21 (3.21)	ANT14: chr 21q21 (2.32)	ANT23: chr2q32-33 (2.23)	ANT14: chr 21q21 (2.33)
ANT07: chr 3q27 (2.57)	ANT07: chr 16p12 (2.49)	ANT07: chr 16p12 (2.12)	ANT07: chr 16p12 (2.40)
ANT18: chr 15q26 (2.46)	ANT27: chr 1p31 (2.00)	ANT27: chr 1p31 (2.00)	ANT14: chr 4q31 (2.30)
FAZU01: chr 11q12 (2.19)	ANT07: chr 3q26 (2.32)		
ANT27: chr 1p31 (2.06)			

LA, Linkage analysis. Maximum LOD (for parametric analysis) or NPL_{PAIRS} (for non-parametric analyses) scores for each region are shown in brackets.

Haplotype analyses showed co-segregation of specific haplotypes with the disease phenotype in several cases. The most consistent pattern of co-segregation was seen between BPI and a haplotype comprising markers D21S1252 and D21S1914 on chromosome 21 in family ANT14 (Figure 4.19). This co-segregation is also seen when psychosis is considered as the phenotype. A comparison between chromosome 21 haplotypes in families ANT14 and ANT07 shows that the former pedigree and the

left branch of the latter share an allele at marker D21S1914 (allele 3, see Figure 4.10 for haplotypes in family ANT07). Whether it is identical by state or identical by descent, however, cannot be decided based on the available information. Inspection of haplotypes for chromosome 21 in the remaining pedigrees showed that the same allele was present in a haplotype co-segregating with BPI and psychosis in family ANT15 (see Figure 4.28). The potential susceptibility haplotype additionally shows the same allele at marker D21S1256 in family ANT15 and the left branch of family ANT07 (allele 1).

Figure 4.28: Haplotype reconstruction for chromosome 21 in family ANT15.



Blackened symbols represent individuals diagnosed with BPI; individuals suffering from psychosis are labelled "Psy". Individual 143 was considered as of an unknown phenotype in linkage analysis. See text for further explanations.

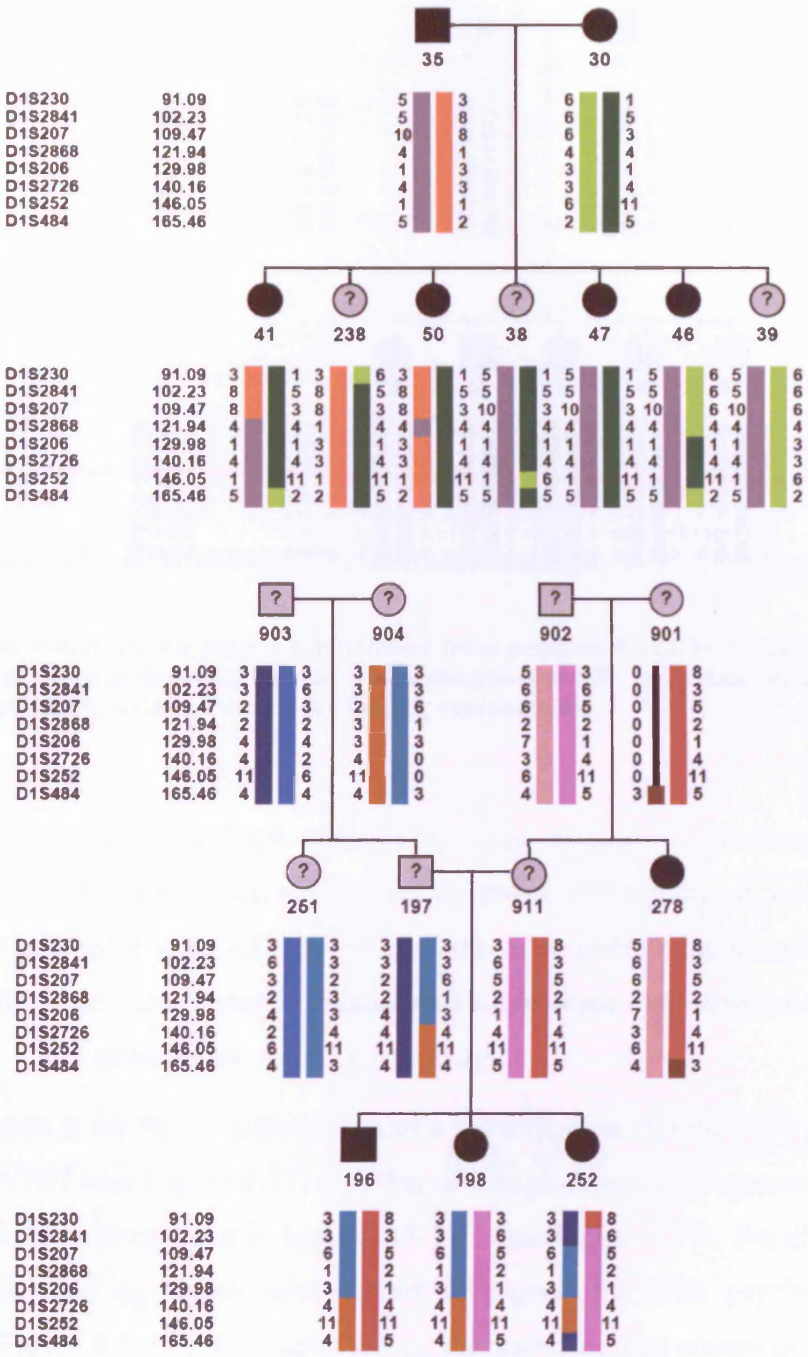
Haplotype comparison between pedigrees ANT07 and ANT15 furthermore reveals the occurrence of a shared haplotype comprising markers D21S1525 and D21S266 (different from the potential susceptibility haplotype discussed so far), possibly indicating that members of these two pedigrees are related.

A haplotype comprising markers D1S207 to D1S206 co-segregates with the phenotype of BPI in family ANT27 (see Figure 4.14). For two other families, individual NPL scores ≥ 1.0 were obtained for the same region under both narrow and broad models: ANT03 and ANT19 (see Table 4.14 and Table 4.16 for narrow and broad models, respectively). Haplotype analysis of these two families showed that, although they do not share any alleles with family ANT27, they do share a

common haplotype between them, which comprises markers D1S206 to D1S252 (see Figure 4.29 on page 206). Visual inspection of the haplotypes for this region in the remaining pedigrees revealed that BPI affecteds in one nuclear family (forming part of pedigree FAZU01) also shared the same haplotype (see Figure 4.30 on page 207).

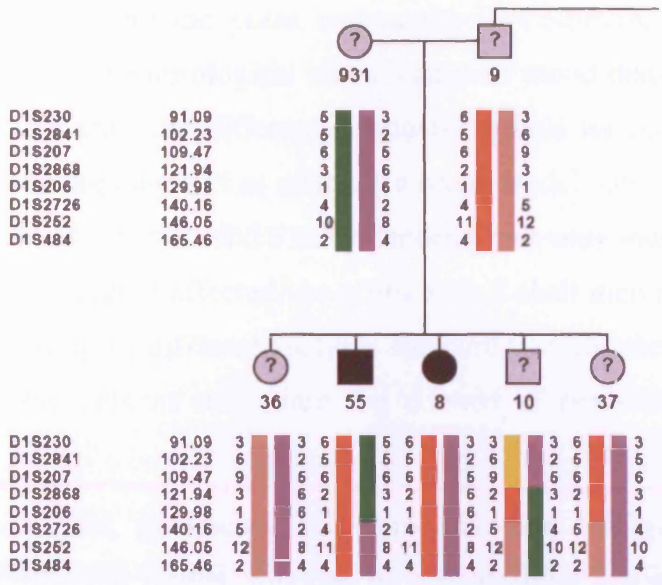
The common region of co-segregation with BPI (but not of haplotype sharing between families) in all three families is around marker D1S206 on chromosome 1p21.2. Co-segregation between chromosome 1p and the broadly defined phenotype is far less consistent, with two individuals diagnosed with unipolar depression in families ANT03 and FAZU01 not sharing the haplotype.

Figure 4.29: Haplotype reconstruction for a candidate region on chromosome 1p in pedigrees ANT03 (top) and ANT19 (bottom).



Blackened symbols represent individuals diagnosed with BPI. Individuals 238, 38 and 39 in family ANT03 (top), and individual 197 in family ANT19 (bottom) have a diagnosis of major unipolar depression. See text for further explanations.

Figure 4.30: Haplotype reconstruction for a candidate region on chromosome 1p in a nuclear family forming part of pedigree FAZU01.



The nuclear family shown here is the leftmost from pedigree FAZU01 as shown in Figure 2.2; blackened symbols indicate individuals with a diagnosis of BPI. Individual 36 has a diagnosis of major unipolar depression. See text for further explanations.

In family FAZU01, a haplotype comprising alleles at markers D11S904 to D11S4191 is shared between all but one affecteds, making this an interesting region in this family (see Figure 4.13). This is all the more interesting because the affecteds in this family are quite distantly related and a haplotype shared between all affecteds should be a rare event in the absence of linkage.

Also of interest are the co-segregation of a haplotype on chromosome 3q with BPI in family ANT07 (see Figure 4.11) and that of a haplotype on chromosome 2q with the broadly defined phenotype in family ANT23 (see Figure 4.23). Finally, a haplotype on chromosome 4q shows near-perfect co-segregation with psychosis in family ANT14 (Figure 4.27), and a haplotype on chromosome 16p occurs in most affecteds under any phenotype definition in family ANT07 (Figure 4.18).

4.7. Discussion

I performed linkage analysis in a collection of fifteen extended pedigrees segregating severe bipolar disorder from the *paisa* community in Colombia. In view of the uncertainties attached to the nosological classification of mood disorders in general, and BP in particular, I explored different diagnostic models for linkage analysis: a narrow model considering only BPI as affected, a broad model considering BPI, BPII and major depression as affected, and a model under which only individuals affected by psychosis were considered affected. As a first step, I shall therefore discuss how the results obtained from the different analyses compare to each other, before putting the findings from the present study into the context of previous reports in the literature.

Under the narrow model, parametric and non-parametric linkage analyses have yielded somewhat differing results. Regions on chromosome 21q and chromosome 13q have been highlighted by parametric linkage analysis only, while regions on chromosomes 12p and q, 1q, 5q and 9p have provided suggestive evidence for linkage in non-parametric, but not in parametric analysis. The only region supported by both analyses is that on chromosome 1p. These discrepancies are most likely due to the different characteristics of parametric and non-parametric analyses: while LOD score analysis is more powerful upon specification of an (at least approximately) correct genetic model[112], non-parametric analysis is more suitable to detecting signals when the mode of inheritance is unknown[115]. In the context of the present study, a possible interpretation is that co-segregation of loci on chromosomes 21q and 13q with the disease phenotype, BPI, in the Antioquian families is compatible with the specified, near-dominant genetic model, while segregation patterns of the loci identified in non-parametric analysis were more complex. On the other hand, the regions on chromosome 21q and 13q might not have been picked up by the non-parametric analysis because of lack of power.

When comparing the non-parametric analyses under the three different models, it can be seen that the analyses under the narrow and the broad models yielded similar results, i.e., several chromosomal regions were highlighted by both analyses, while none of the regions identified in the study-wide analysis under the psychosis model overlapped with any of the regions of highest significance under narrow and broad

models. This might be due to real differences between susceptibility factors implicated in psychosis on the one hand, and affective disorder as such on the other. Alternatively, the differences between findings might reflect differences between the samples on which the analyses are based: only eleven out of the fifteen families available for analysis under the narrow and broad models could be analysed under the psychosis model. Families FAZU01, ANT10, ANT19 and ANT26 comprised less than two cases of psychosis and were therefore not included in the analysis under the psychosis model. Furthermore, most other families had less affecteds under the psychosis model than under the other two models, most notably families FAZU28, ANT03, ANT04 and ANT23. However, these two explanations (based on real aetiological differences and power issues, respectively) are not mutually exclusive. Some of the regions highlighted under narrow and broad models could also be implicated in the aetiology of psychosis, but might have a weak effect on susceptibility, thereby being more difficult to detect with a smaller sample. On the other hand, the fact that some regions, such as those on chromosomes 2q and 16p, reach suggestive linkage only in the analysis under the psychosis model, might imply that there are indeed real differences in predisposition to psychosis and to mood disorders as such. There are very few examples in the literature of the analysis of psychosis as a phenotype in extended bipolar pedigrees; however, the available results are consistent with findings from the present study in that they implicate a number of regions in the predisposition to psychosis, which have not been found to be linked to the phenotype of bipolar disorder[317,318], thereby strengthening the hypothesis that there are genetic loci conferring susceptibility to psychosis rather than affective disorders *per se*. This is also in line with the growing body of evidence supporting shared genetic susceptibility between bipolar disorder and schizophrenia[16,193] (see also chapter 3).

As mentioned above, the results obtained in the analyses under narrow and broad models show many overlaps. Most notably, both analyses provide study-wide suggestive evidence for the involvement of chromosomal regions 1p13-31, 1q25-31, 12ct-12q14 and 12p12-12ct in disease susceptibility. A possible explanation is that some of the factors causing susceptibility to narrowly defined bipolar disorder type I, might also predispose to affective disorders in general, including major unipolar depression. This is consistent with reports that unipolar depression occurs more often

in the first-degree relatives of BP patients than in the general population[307]. There are, however, some regions that are only supported by one of the two analyses, such as chromosome 5q11-12, which has only been detected under the narrow model, and chromosomes 6p12-22 and 7p15-21, for which suggestive evidence for linkage was provided under the broad, but not under the narrow model. This might again either be due to changes in sample composition (although the same families were analysed, the analysis under the broad model included more affecteds and should therefore have had more power to detect susceptibility regions), or it might reflect real differences in genetic predisposition to the two phenotypes. Analysis of the same linkage data under both narrow and broad models is common in the BP gene mapping literature; the most distinctive difference between the two models is usually the addition of recurrent unipolar depression as a phenotype under the broad model, while the narrow phenotype definition mostly considers only BPI, or BPI and II as affected. The results of these studies are consistent with findings from this thesis in that they show important overlaps between the analyses under the different models, while at the same time, some regions are only highlighted under one of the models[319-324]. It seems therefore likely that there are loci which confer susceptibility to developing a mood disorder in general, while others predispose specifically to bipolar disorder. This might help explain why, although unipolar depression is more common in the relatives of bipolar probands, the opposite is not true[307]: families segregating bipolar disorder might carry susceptibility loci for both mood disorders in general and bipolar disorder in particular, while the risk of carrying a predisposing variant for bipolar disorder is not elevated in relatives of unipolar patients, in whom "only" genetic variants predisposing to mood disorders in general are enriched.

The most important findings of the present study will now be discussed individually.

Chromosome 1p

Non-parametric analysis under both narrow and broad models provided suggestive study-wide evidence for linkage to a region on chromosome 1p13.3-31.1, a region that was also found to be of interest in our previous linkage scan, where markers D1S2868 and D1S207 produced NPL scores of 1.53 and 1.33, respectively. The evidence of linkage has increased in the present genome scan, where we obtained a

maximum NPL score of 2.13 for marker D1S2868 under the narrow model. For the broad model, the NPL score for this marker is even higher (2.22). The addition of a further nine families has therefore strengthened the evidence for the presence of a susceptibility locus for severe mood disorder on chromosome 1q in Antioquia.

Chromosome 1p22-31 is also the only region picked up by both parametric and non-parametric analyses under the narrow model. One interpretation of this consistency between parametric and non-parametric analyses might lie in the possibility that inheritance patterns in the region are consistent with the mode of inheritance specified for the parametric analysis, while at the same time, the signal is strong enough across families to be detected in the non-parametric analysis of lesser power.

This region is also supported by previously published findings. Ewald and colleagues obtained a two-point LOD score of 2.75 for marker D1S216 on chromosome 1p31.1 in a sample of Danish BP pedigrees, using a broad affection model similar to the one used here[325]. The NIMH Genetics Initiative also found significant evidence for linkage to chromosome 1p31.1 using affection models very similar to the ones used in the present study including BPI, BPII and unipolar depression (broad model), or only BPI/II (narrow model)[326]. Interestingly, the bipolar genome scan meta-analysis for linkage by Segurado and colleagues ranked a 30 cM-bin on chromosome 1p, delimitated by markers D1S2742 and D1S226, third (out of 120 bins covering the entire genome) for significance under a narrow model, where only BPI and schizoaffective disorder are considered as affected[205]. Under a model that additionally included BPII, the same bin was still ranked 10th. The most significant marker identified here, D1S2868, lies outside this bin; however, there is an important overlap between the region identified in the meta-analysis and the candidate region found here. The fact that the region on chromosome 1p31.1 has been implicated in bipolar disorder in a number of other studies, as well as in a comprehensive genome scan meta-analysis, adds to the interest of this finding.

Haplotype analyses of the three families that contribute most to the linkage signal in this region – families ANT03, ANT19 and ANT27 – revealed co-segregation of marker D1S206 with BPI in all three pedigrees. At the same time, families ANT03 and ANT19 share a haplotype comprising marker D1S206 and two additional makers centromeric of the latter. The same haplotype is additionally shared by the affecteds of a nuclear family belonging to pedigree FAZU01. Although the alleles forming this

haplotype are common, with frequency estimates between 11.6% for allele 11 at marker D1S252 and 41.4% for allele 4 at marker D1S2726, the fact that these families stem from the same population isolate allows for the possibility of identity by descent-sharing of this haplotype. Co-segregation of a possible IBD-haplotype with the disease phenotype means that the shared haplotype might carry a founder mutation conferring susceptibility to BPI in these three pedigrees.

In conclusion, parametric and non-parametric analyses under different models, as well as haplotype analyses, suggest the presence of a susceptibility locus for bipolar disorder on chromosome 1p21-31 in the sample from Antioquia. The region identified in this study overlaps with previously reported candidate regions, although there is some evidence from both linkage and haplotype analyses pointing towards a slightly more centromeric location of the locus identified here. In any case, there is accumulating evidence for an important susceptibility locus for BPI on chromosome 1p, which has consistently been replicated in a number of different populations.

Chromosome 1q

Non-parametric analysis under both narrow and broad models has yielded suggestive evidence of linkage to a region on chromosome 1q25-31. This initially suggests a possible involvement of a locus in this region in genetic susceptibility to mood disorders, rather than bipolar disorder as such. However, in the two families that contributed the highest individual NPL scores to the combined score, ANT27 and FAZU28, all affecteds have a diagnosis of BPI, and it remains therefore possible that the locus on 1q is involved in susceptibility to BPI, and not so much to mood disorders in general.

A number of studies have reported linkage of BP to loci on chromosome 1q[319,327-330], and this region has also repeatedly been implicated in genetic susceptibility to schizophrenia[228,331-333]. Most notably, the genes encoding the regulator of G signalling protein, *RGS4*, and the nitric oxide synthase 1 (neuronal) adaptor protein, *NOS1AP*, which have both been found to be associated to schizophrenia[146,229,334,335] (see also chapter 5), are located on the long arm of chromosome 1 (1q23.3). In view of the possible shared genetic susceptibility to schizophrenia and bipolar disorder, there have been attempts to replicate the association results obtained for

schizophrenia in bipolar disorder, and a study carried out in a Brazilian population has indeed found *RGS4* to be associated to BP[336]. Later studies in Scottish and Ashkenazi Jewish samples, however, failed to replicate this result[337,338]. There are no published genetic association studies between *NOS1AP* and BP, but Xu and colleagues reported increased expression of the NOS1AP protein in the prefrontal cortex of bipolar subjects[339].

Together, these results indicate that there might be at least one locus predisposing to both schizophrenia and BP on the long arm of chromosome 1; however, the exact location of that locus, and whether it is indeed the same locus that confers susceptibility to both disorders, is not clear. Results from this thesis place the candidate region for BPI on chromosome 1q25-31, while previously published results point at slightly different locations including chromosome 1q23 and 1q32. More work remains to be done in order to establish how many susceptibility loci for BP there are on the long arm of chromosome 1, and what their role might be in predisposition to affective disorders in general, to BP in particular, and to schizophrenia.

Chromosome 2q

A candidate region on chromosome 2q24-31 provided suggestive evidence for linkage to psychosis in the pedigree sample from Antioquia. The same region showed suggestive linkage to bipolar disorder in a study including families of German, Israeli and Italian origin; however, that study used a broad phenotype definition, which included BPI and II, schizoaffective disorder and recurrent unipolar depression[340]. A recent linkage study by Zandi and colleagues, found – again suggestive – evidence for linkage to chromosome 2q24 in a collection of North American BP pedigrees using both narrow (including BP and schizoaffective disorder, manic type) and broad (additionally including major depression) phenotype definitions[341]. There seem to be no published studies reporting linkage to chromosome 2q24-31 using psychosis as a phenotype, making this the first study to implicate that region in genetic susceptibility to psychosis rather than mood disorders in general, or bipolar disorder in particular. It is, however, of interest that a meta-analysis of linkage studies in schizophrenia ranked a 30cM-bin adjacent to the locus

reported here 5th for significance (out of 120 bins covering the whole genome), thereby corroborating the hypothesis that there might be a locus predisposing to psychosis on chromosome 2q[228]. Further work needs to be carried out in order to evaluate the possible role of the region identified here in BP and psychosis.

Chromosome 12q

In the present study, the most significant evidence for linkage to BP was found for a region on chromosome 12q, stretching from the centromere to 12q14/12q21 (for narrow/broad models, respectively). While a locus on chromosome 12q23-24 has consistently been implicated in susceptibility to bipolar disorder in a number of studies[324,325,328,342], so far, there has only been one report of linkage for a locus centromeric of that region: a French Canadian study reported suggestive LOD scores for chromosome 12q13.12-q14.1, under a recessive model of inheritance, and using a broad affection model including BPI, BPII, schizoaffective disorder and recurrent unipolar depression as affected[343]. However, the authors of that study conclude that the main susceptibility locus in their sample coincides with the previously reported locus on chromosome 12q23-24, where they identified two shared, possibly disease-carrying haplotypes in two branches of the largest pedigree analysed in their study; thereby making this the first study to identify the region on chromosome 12ct-12q14 as the most significant finding in a genome-wide linkage scan. Also, to the best of my knowledge, there are no reports on candidate gene association studies for the region reported here. It is therefore possible that we have identified a novel locus, possibly one that might be of major importance for BP susceptibility in the population of Antioquia. Because this locus was supported by both narrow and broad analyses, it might represent a predisposing locus for mood disorders including both BP and major unipolar depression.

Chromosome 12p

Like the candidate region on the long arm of chromosome 12, the candidate region on chromosome 12p was supported by suggestive evidence for linkage in non-parametric analysis under both narrow and broad models. There are no previous reports in the literature explicitly implicating this region in the genetic susceptibility

to bipolar disorder, neither from linkage nor from association studies, although the Canadian linkage study that also found linkage on chromosome 12q, reported a near-suggestive LOD score of 1.61 for marker D12S87 on chromosome 12p11.22, under a recessive model[343]. However, the authors of that study have not discussed their finding any further, and it is not clear what importance they attribute to it.

Like the region on chromosome 12q, the candidate region on the short arm of chromosome 12 is a novel finding, and because of it being identified under both narrow and broad models, it might contain a susceptibility locus for mood disorders in general. On the other hand, however, it is noteworthy that the region on chromosome 12p is supported by linkage of a single marker, D12S345, to the disease phenotype. This marker is immediately adjacent to the first marker analysed on the long arm of chromosome 12, D12S85, which is part of the candidate region on chromosome 12q. Recombination maps of single chromosomes[344], as well as of the entire human genome[345,346], show a suppression of recombination events in centromeres, with cross-overs concentrated towards the telomeres in males. While in females, recombination events near the centromeres seem to be more common[344,345], it is nevertheless possible that a reduced recombination rate around the centromere leads to the chromosome 12q signal stretching into the short arm of the chromosome. One possible interpretation of our findings is therefore that the region on chromosome 12p does not represent an independent candidate region; instead, the "real" candidate region might be that on chromosome 12q. It remains, of course, possible that there is an additional susceptibility locus for mood disorders on chromosome 12p12-ct, and further work is needed to establish the role of chromosome 12p in BP.

Chromosome 13q

Parametric linkage analysis under the narrow model yielded some evidence for linkage of chromosome 13q33 to severe BP. This region was not identified in the non-parametric analysis, possibly because of the reduced power of that approach. Chromosome 13q33 has been implicated in BP through linkage analysis of a series of North American pedigrees[347], and this finding was later confirmed in an expanded sample from the same North American population; although the linkage peak in that

second study occurred on chromosome 13q32.3 rather than 13q33, it is still located very close to the region identified here[348]. Chromosome 13q is also the region that obtained strongest support in a meta-analysis of BP linkage scans performed by Badner and Gershon (2002)[204]. It is of interest that the *DAOA* gene, which encodes the D-Amino Acid Oxydase Activator, a protein involved in glutamatergic neurotransmission and which has been found to be associated with both BP and schizophrenia, is located in the same region[172,349-352]. In fact, this gene is only one of nine in the candidate region identified here. It would be extremely interesting to investigate whether association to the *DAOA* locus can be detected in a sample of unrelated bipolar patients from Antioquia.

Chromosome 16p

Marker D16S3068 on chromosome 16p12.1 provided suggestive study-wide evidence for linkage to psychosis, and family ANT07 yielded high individual NPL scores for markers in that region (including D16S3068) under all three phenotypic models (see Table 4.20). When comparing the individual NPL scores for pedigree ANT07 under the three models, an interesting observation is that, in spite of the lower power of analyses under narrow and psychosis models compared to that under the broad model (due to the reduced number of affecteds), it was under these more stringently defined phenotypic models that higher NPL scores for chromosome 16p in family ANT07 were reached. Together with the study-wide evidence for linkage in the psychosis analysis, this might indicate that chromosome 16p carries a predisposing locus for severe BPI with occurrence of psychosis.

There are numerous reports of linkage and association of bipolar disorder to chromosome 16p12-13 in the literature. While some studies have identified a locus telomeric of the region found to be linked to the disease here[321,324,325,330], there have also been reports of linkage to chromosome 16p12, the same region that was highlighted by analyses in the present study. A sample of BP pedigrees from an isolated Finnish population showed linkage to that region under a broad phenotype definition including bipolar spectrum disorders and major unipolar depression[320], and pedigrees from the NIMH Genetics Initiative were found to be linked to 16p12 using a range of diagnostic models including one that considered only cases of BPI that

had also experienced psychosis as affected[318]. Interestingly, chromosome 16p12 also contains the SNP that shows the most significant association to BP in a whole-genome association scan carried out as part of the Wellcome Trust Case Control Consortium initiative, rs420259, located in the gene encoding the partner and localiser of BRCA2, *PALB2*[49]. Taken together, there is accumulating evidence for an involvement of a candidate region on chromosome 16p12 in susceptibility to BP, to which the findings from the present study add.

Chromosome 21q

Parametric linkage analysis under the narrow model produced suggestive evidence for linkage of BPI to a candidate locus on chromosome 21q21-22. Non-parametric analyses failed to provide study-wide evidence for this locus; as discussed above, this might reflect the greater power to detect linkage using a parametric approach when an approximately correct model of genetic inheritance is specified. The genetic model used in the parametric analysis specifies a near-dominant mode of inheritance (see chapter 2.3.7), and, as discussed in section 4.3, it is far from certain, even unlikely, that this model holds for the inheritance of bipolar disorder *per se*. However, it has been shown that the crucial condition for parametric linkage analysis consists in that the specified mode of inheritance should hold at the locus under consideration, even though it might not be true for the disease as such[121]. It seems therefore possible that a locus on chromosome 21q21-22 acts as a major susceptibility locus in the present sample, most notably so in pedigree ANT07, for which the LOD score at this locus was 3.21. On inspecting the reconstructed haplotypes for this family, however, it becomes evident that there are at least two haplotypes co-segregating with the phenotype; one in the left branch of the pedigree (shown in light blue in Figure 4.10 on page 172), and a different one in the remaining branches of the pedigree (shown in dark blue/red; see also section 4.6.2.1). In view of the complex mode of inheritance of bipolar disorder, it would seem likely that, if there were one locus of major impact on the genetic susceptibility to the disease, it should be a rare event, accounting only for a minute fraction of affecteds, and all affecteds in a family with a high LOD score should share one haplotype. In other words, in the – perhaps unlikely, but possible – event of there being one major locus responsible for the susceptibility to disease in one family, it would seem unlikely for

the different branches of this family to show allelic heterogeneity. This, however, is the case in family ANT07, thereby somewhat weakening the evidence for linkage in that pedigree.

On the other hand, family ANT14 produced NPL scores suggestive of linkage under both narrow and psychosis models for marker D21S1914, thereby implicating the same locus on chromosome 21q in disease susceptibility in that family. Additionally, haplotype analysis of families ANT14 and ANT15 revealed that these two families share an allele at locus D21S1914 with the left branch of family ANT07 (allele 3). Family ANT14 and the left branch of family ANT07 furthermore share an allele at the telomeric neighbouring marker D21S1252 (allele 8), while the latter and ANT15 share an additional allele at the centromeric adjacent marker D21S1256 (allele 1). Although all of these alleles are relatively common, with population allele frequency estimates ranging from 10.3% for allele 8 at locus D21S1252 and 11.5% for allele 3 at marker D21S1914, to 36.5% for allele 1 at marker D21S1256, the sharing of a haplotype that is identical by state and that additionally co-segregates with the disease in three different pedigrees might indicate the presence of a common disease-predisposing founder mutation at a locus on chromosome 21q in these three families. None of the other families have produced evidence for linkage at markers on chromosome 21q, and the α-value of 0.15 corresponding to the study-wide HLOD score of 2.14 at this locus further corroborates the notion that the linkage signal is mainly driven by the three families discussed here: ANT07, ANT14 and ANT15. This heterogeneity between pedigrees, however, is to be expected in the genetic analysis of a complex disease; it can indeed be seen for all other candidate regions discussed here.

Not surprisingly, the candidate region on chromosome 21 was also implicated in our previous linkage scan, which included both families ANT07 and ANT14. Unlike the present genome scan, our previous study also implicated this region in BPI through non-parametric linkage analysis, indicating that the addition of a further nine pedigrees might have introduced a somewhat increased heterogeneity with regards to this locus. This is also supported by the difference in α-values in parametric linkage analysis between previous and present linkage scan: while in our first study, we found an α-value of 0.51, implying that half of the families studied were linked to chromosome 21q21 (see Table 4.1), as mentioned above, α is only 0.15 for the

highest HLOD score identified here. Nevertheless, there continues to be very interesting evidence for the presence of a BPI susceptibility locus on chromosome 21q21 in our Antioquian pedigrees.

Evidence for linkage of chromosome 21q21-22 to bipolar disorder has also repeatedly been reported in the literature. Detera-Wadleigh and colleagues found a LOD score of 1.85 for a region on chromosome 21q22.11-q22.12 in a collection of extended pedigrees of European ancestry[319], and a linkage study of two BP pedigrees from Québec, one of them very large, resulted in LOD scores of > 1.0 on chromosome 21q21.3-q22.13 (this is the same study that also provided evidence for linkage to chromosome 12q)[343]. A family study from Bulgaria yielded suggestive evidence for linkage around marker D21S1252[353], and finally, a recent Irish study of affected sib pair families detected linkage to chromosome 21q21[324]. Interestingly, except for the Canadian study, the analyses yielding evidence for a locus on chromosome 21q had been performed using a narrow definition of the disease phenotype in all of these studies, including only BPI, or BPI and II as well as schizoaffective disorder, but excluding unipolar depression. Where parallel analyses on the same data were conducted under a broader phenotype model including recurrent unipolar depression, evidence for linkage of chromosome 21q to the phenotype was either not found[324], or it was weaker than under the narrow model[319]. This is in line with the results from the present study, where the strongest evidence for chromosome 21q was found under the narrow model. For family ANT14, suggestive evidence for linkage was also obtained under the psychosis model, but the locus was not supported by the analysis under the broad model, neither for the complete study, nor for any individual pedigree. It therefore seems possible that a locus on chromosome 21q21-22 acts as a risk factor for severe bipolar disorder. This hypothesis is further strengthened by a finding by Lin and colleagues, who reported significant evidence for a locus on chromosome 21q22.13 as a susceptibility factor for early-onset BP[354], a sub-type of the disorder associated with increased severity of clinical symptoms, including higher incidence of psychosis[197].

Other authors have reported evidence for the presence of a susceptibility factor on chromosome 21q22.3, telomeric of the region reported here and close to the gene encoding the liver phosphofructokinase *PFKL*[355-359], although not all results for that region are conclusive[360,361]. Further work is needed to determine whether there are

indeed several distinct candidate regions on chromosome 21q, and what their respective role is in the aetiology of BP.

Additional candidate regions

Linkage and haplotype analyses have identified a number of additional regions that could be of importance for genetic susceptibility to mood disorders in general, BP in particular, or psychosis. These include regions on chromosomes 2q32-33, 3q26-28, 4q31, 5q11-12, 6p12-22, 6q24-25, 7p15-21, 9p13-21, 10q11-22, 11q12, and 12q21-23. Some of these loci, such as the regions on chromosomes 2q32-33, 3q26-27, 4q31 and 11q12, were of importance in individual pedigrees only. Chromosomes 4q31 and 3q28 had produced study-wide two-point heterogeneity LOD scores of 1.35 and 1.61, respectively, in our previous linkage scan of six families. For chromosome 3q28, we had additionally found a NPL score of 1.96 at marker D3S1580. These signals are lost in the present genome scan. This probably reflects the fact that the study-wide signals found on those chromosomes in the first scan were mainly driven by individual families (ANT27 and ANT07, respectively), and that the addition of further families might have introduced more heterogeneity with regards to these loci.

Regions that are implicated in individual families only might either contain susceptibility variants that are unique to single families, or else susceptibility variants that interact with other genetic factors present in these families, leading to the clinical phenotype only on the background of the genetic makeup of a specific family. A further possibility that always has to be accounted for, especially in the context of the genetic dissection of complex disease, is the occurrence of false positive results. Where many families are involved in a linkage study, the probability of obtaining high individual NPL or LOD scores is substantial even in the absence of linkage, making a comparison to findings from other studies all the more important.

The majority of the regions reported above have previously been implicated in the genetic susceptibility to BP through linkage, and, in some cases, association analyses (reviewed in ref. [207]). Exceptions are the regions on chromosomes 5q11-12, 6q24-25, and 9p13-21, for which no previous reports of linkage are available to the best of my knowledge. However, a case-control study from Taiwan reported an association between BP and the gene encoding the serotonin receptor 1A (*HTR1A*) on

chromosome 5q12.2[362], and although other authors have found less conclusive results[363], it might be of interest to study this gene in the Antioquian population.

The present study has implicated several chromosomal regions in the genetic susceptibility to bipolar disorder that were not identified in our initial linkage scan. Most notably, regions on chromosomes 12q, 13q and 1q have provided suggestive evidence for linkage in the analysis of the joint data set under the narrow model, which corresponds to the phenotype model used in our previous scan. These new findings most likely reflect the increased power to detect linkage of the expanded sample used in this thesis. Moreover, genetic heterogeneity between families remains an issue, even within a population isolate, and the addition of new families might indeed have introduced novel signals.

On the other hand, our first linkage scan had identified several regions which are not supported by the analysis of the expanded sample. This includes regions on chromosome 2q33, 6q22, and 18q23, as well as the region on chromosome 5q33 on whose follow-up we had concentrated because of the agreement with linkage signals from the Central Valley of Costa Rica. The signals for chromosomes 2q33 and 18q23, identified in parametric and non-parametric analysis, respectively, were only of modest strength, with a HLOD score of 1.33 for chromosome 2q33 and an NPL score of 1.34 for chromosome 18q (see Tables 4.1 and 4.2). These signals could either have been of only minor significance in the original sample, or they might even have represented false positive findings. It is surprising that the signal on chromosome 6q22, which was relatively strong, with a parametric HLOD score of 2.01, disappeared in the joint analysis. One reason for this might also lie in an increased genetic heterogeneity at this locus. However, because the signal was previously so strong, this explanation alone seems unlikely to account for the discrepancy between the two studies. An additional factor might have been the re-analysis of the marker D6S434, located adjacent to the microsatellite which provided the strongest evidence for linkage in our first study, D6S287 (see Table 4.1). In our first study, the occurrence of intermediate alleles had not been taken into account for marker D6S434. Analysis of the corrected genotypes shows evidence *against* linkage at that marker in family ANT04 (LOD score -2.45), formerly the family providing strongest support for linkage to chromosome 6q22. Because we performed multipoint

analysis in the present study, the low LOD score for D6S434 might have pulled down the signal for D6S287.

An important observation is that the present study has not confirmed our previous findings of a locus predisposing to severe BP on chromosome 5q31-34[184]. A likely explanation for this discrepancy lies in that in our previous linkage scan, the region on chromosome 5q received only moderate support from the analysis of the six Antioquian pedigrees alone. The maximum NPL_{PAIRS} score in the region was 1.92 for marker D5S410, lower than the most interesting NPL scores reported here. It was through the addition of a further eight Antioquian pedigrees, and most importantly, three pedigrees from the Central Valley of Costa Rica, that a much higher maximum NPL_{PAIRS} score of 4.40 was obtained for 5q31-34. Genome-wide analysis of the largest of the Costa Rican pedigrees, followed by fine mapping, had previously yielded suggestive evidence for the same region on chromosome 5q[178,244], and without this pedigree, the evidence for linkage does probably not reach significance. This does not invalidate our earlier results for chromosome 5q; however, it suggests that it is not a major candidate locus in the population of Antioquia, and that there are other loci that contribute more to the genetic susceptibility to BP in our sample.

Bipolar disorder is a complex disorder, which is characterised by substantial genetic heterogeneity. By studying pedigrees from a population isolate, we have endeavoured to minimise this heterogeneity. Nevertheless, none of the analyses have identified a region for which there is significant evidence of linkage on a genome-wide level. This indicates that, even within a population isolate – and in fact, even within sub-isolates such as the Oriente region of Antioquia and the village of Aranzazu –, the level of genetic heterogeneity is substantial enough to complicate the use of linkage approaches in the discovery of genetic susceptibility factors to bipolar disorder. This is consistent with findings from other population isolates such as the Amish, the Ashkenazim, and the populations of the Central Valley of Costa Rica and the Canadian province of Québec, where linkage approaches have apparently failed to yield the successes promised by the increased genetic homogeneity of these populations, either because no significant evidence of linkage was found[329,364-366], or because follow-up analyses produced conflicting results[343,367]. In other cases, follow-up studies have supported initial findings, yet any interesting signals have not led to the identification of a concrete susceptibility locus[174,177]. Although disappointing, the

lack of obvious linkage signals in the study of bipolar disorder need not be interpreted as a failure of linkage approaches to yield interesting and important results. As discussed in the introduction to this thesis (see chapter 1.4.3), meta-analyses of genome-wide linkage scans have found several genomic regions to be consistently implicated in the genetic susceptibility to bipolar disorder[204-206]. Importantly, the regions highlighted in these genome scan meta-analyses did not always coincide with those observed to be the most significant in the individual studies included. This finding might be interpreted in several ways. First, the most significant results of a genome scan might be of importance in a specific family or population only, while less prominent signals could reflect loci of lesser impact but possibly of greater consistency across studies. An alternative explanation lies in that any of the signals in a linkage scan might represent false positives, especially if they do not reach genome-wide significance. It is very common for genome-wide linkage scans in psychiatric disease to produce suggestive rather than significant evidence for linkage, and it might indeed require the comparison and meta-analysis of such linkage scans to decide which of the original signals are real. It has to be stressed, however, that this does not invalidate the use of linkage approaches in the context of complex disease; instead, it highlights the need to re-adjust how results from these studies should be interpreted. In the light of complex inheritance patterns, most individual linkage studies might not have sufficient power to detect susceptibility loci for psychiatric disease on their own. Nevertheless, they can – and do, as the recent meta-analyses have shown – contribute important information to the emerging picture of genetic predisposition to bipolar disorder.

The results obtained in the present study are typical of a linkage study of an aetiologically complex and genetically heterogeneous disorder. While no significant evidence for linkage has been found at any analysed marker, there are a number of chromosomal regions for which suggestive evidence of linkage has been detected under different models, and interesting patterns of marker genotype-disease phenotype co-segregation have been observed in several pedigrees. The results reported here are therefore encouraging and will certainly contribute to our growing knowledge of genetic susceptibility to BP. Nevertheless, a few limitations to the present study shall also be discussed. First, it is possible that our study lacked power to detect loci of minor impact, or loci showing substantial heterogeneity between and

within families, such as they are typical of genetically complex disease. Because of the complexity of some of the pedigrees included in the study, we were only able to perform a power analysis for the parametric approach, leaving the (probably lower) power of the present collection of pedigrees for the non-parametric approach unexplored. This makes it difficult to judge the false-negative rate of this study. In the same manner, some of the loci that we did identify might have reached genome-wide levels of significance, had our pedigree sample been bigger. On the other hand, it was the aim of this study to generate hypotheses for further study in the population of Antioquia and elsewhere, and we did identify some promising candidate regions. Also, as has been pointed out above, it might not be possible at all to reliably identify loci involved in complex disease in a single linkage screen. Our results add significance to some of the findings reported by other groups, and we might also have identified some novel loci, especially on chromosome 12, which now await replication in additional samples.

Replication, in fact, is required for all findings from the present study: an important implication of the fact that none of our results reached statistical significance on a genome-wide level is that some of our findings, or, in the worst case, all of them, could be false positives, which have occurred by chance in the absence of linkage. Although the consistency of our results with those from previous studies is very encouraging because it indicates that our findings are not mere products of chance, it is still essential to seek replication of the results from the present study in additional samples from Antioquia and other populations.

A further limitation of the present work lies in the fact that the X-chromosome has not been analysed. It was decided to leave this chromosome out because there was no indication of sex-linked segregation of the phenotype in the pedigrees under study. Also, non-parametric analysis of X-chromosome data is not straightforward and cannot be performed using SimWalk2. Nevertheless, complex inheritance patterns mean that there might be susceptibility genes of minor impact or modifier genes on the X-chromosomes without reflecting into a clear sex-linked transmission pattern of the disease. Linkage studies have found some evidence for the implication of X-linked loci in BP, and there has been support for genetic association between several genes, such as the gene encoding the monoamine oxidase A, *MAOA*, and BP[207]. It

might therefore be of interest to perform linkage analysis on the X-chromosome in the Antioquian sample studied here.

4.8. Conclusion and Future Work

Linkage analysis of a collection of fifteen extended *paisa* pedigrees segregating severe bipolar disorder has yielded suggestive evidence for the implication of a range of loci in genetic susceptibility to bipolar disorder in particular, mood disorders in general, and the experience of psychosis. The most interesting loci specific to bipolar disorder are chromosomes 21q21-22 and 13q33. We have also found loci that might predispose to mood disorders in general, including unipolar depression, rather than BP in particular, in our sample; the most interesting results are candidate regions on chromosomes 1p13-31, 1q25-31 and 12ct-12q14. Linkage analysis using psychosis as the disease phenotype identified candidate regions on chromosomes 2q24-31 and 16p12. The fact that many of these loci had been previously identified as susceptibility loci for BP in other samples, often even in several independent studies, is very encouraging and fosters the hope that a clearer picture of the genetic aetiology of BP will emerge. On the other hand, we have also identified a novel locus on chromosome 12q and, possibly, 12p.

Most of the candidate regions supported by the present study are several tens of cM long and harbour hundreds of genes, thereby complicating a follow-up of this linkage study by candidate gene analysis. The only exception is the candidate region on chromosome 13q33, which contains no more than nine genes (www.ensembl.org; last accessed on 13/06/2008). One of them is the *DAOA* gene, which has repeatedly been found to be associated to both schizophrenia and bipolar disorder. It will be of great interest to follow up this finding by sequencing the *DAOA* gene in the families analysed here, especially in families FAZU01 and ANT07, in which the LOD scores for this region were highest. Additionally, an association study of that gene should be performed in the Antioquian population using established genetic variants as well as possible variation identified through sequencing in the families, to try and further define the role of the *DAOA* gene in the genetic susceptibility to BP.

In a similar manner, further candidate genes implicated in previously published studies and located within the candidate regions found here, such as the *HTR1A* gene

on chromosome 5q12.2, could be sequenced in the pedigrees and/or studied by association analysis in the Antioquian population. Association analysis of candidate genes can be carried out immediately, using the well-characterised sample of sporadic cases that has already been collected in Antioquia and that has also been used in the context of this thesis (see chapter 3).

Additionally, in an effort ultimately aimed at finding new candidate genes for BP, fine mapping should be undertaken in all of the promising candidate regions found in this study by typing additional markers in those regions, and even more importantly, by typing additional individuals from the same pedigrees. For a number of samples from the pedigrees analysed here, not enough DNA was available to carry out a whole-genome linkage scan. However, enough DNA is left for most of these samples to allow including them in a follow-up study involving few markers. The addition of further affected individuals increases the power of follow-up fine mapping efforts, and while some regions might not gain further support, others might show more significant evidence for linkage, thereby guiding further attempts to identify suitable candidate loci. Fine mapping of the regions on chromosomes 1p and q and 12q is already underway.

Because none of our findings reached statistical significance on a genome-wide level, it is essential to confirm the results from the present study through the analysis of additional pedigree, trio or case-control samples from Antioquia, as well as from other populations. As a first step, an association analysis of genetic variants in the candidate regions with BPI should be performed in the available BP trio sample; however, further replication will be required. Moreover, it is of special interest to further investigate the role of specific loci in the causation of the different phenotypes studied here. This might involve the collection of a large sample of affecteds and either their parents or matched controls, allowing enough power to study BP with and without psychosis separately in an association approach (the latter possibly combined with schizophrenia), in order to test the hypothesis that at least partly different loci are responsible for the causation of these phenotypes. It would naturally also be of great interest to study further family samples, but it might not be realistic to hope for the identification of an additional collection of large and equally heavily BPI- and psychosis-loaded pedigrees in the *paisa* population.

While it could be argued that our findings should be replicated before attempting to fine map specific regions and identify candidate genes, replication requires the collection of additional samples and is therefore a task for the medium to long term. Other follow-up work, on the other hand, can be carried out immediately using the available pedigree and trio samples: fine mapping and sequencing of previously reported candidate genes, as well as testing previously reported genes for association in our trio sample will therefore represent the short-term goals to aim for, while long-term goals lie in the replication of our findings in additional samples, as well as the identification of susceptibility genes within the candidate regions identified here.

A further approach to be pursued in our collection of *paisa* pedigrees segregating BP is the analysis of linkage to endophenotypes of the disorder. The endophenotype approach is based on the concept that, while the behavioural outcome of psychiatric disease is extremely complex, there are simpler phenotypes on a lower physiological level that are associated with the disease. Such internal phenotypes, or *endo*phenotypes, are thought to be closer to the genetic basis of the disease, and linkage analysis of these traits holds the promise of being more straightforward because of a simpler genotype-(endo)phenotype relationship[368,369]. While endophenotypes are only starting to be explored in BP, they have already been studied for a long time in schizophrenia. An example is eyetracking dysfunction, where affected individuals have difficulties in the smooth visual pursuit of moving objects. As is characteristic for an endophenotype, this trait is not only associated with the disorder, it is also significantly more common in unaffected relatives of schizophrenia patients than in the general population[369].

In collaboration with UCLA and Universidad de Antioquia, we have recently started a project aiming at establishing endophenotypes in bipolar disorder and subsequently conducting linkage analysis to these traits in bipolar families from Antioquia and the Central Valley of Costa Rica. The families investigated as part of this new project are extended versions of some of the pedigrees involved in this study (including ANT04, ANT07 and ANT10), and it shall be of great interest to see whether the results will be consistent with the ones obtained here.

CHAPTER FIVE

TRANSMISSION DISTORTION ANALYSIS OF A SCHIZOPHRENIA TRIO SAMPLE FROM ANTIOQUIA AT THE *NOS1AP* LOCUS

5. Transmission distortion analysis of a schizophrenia trio sample from Antioquia at the *NOS1AP* locus

5.1. *NOS1AP* as a Candidate Gene for Schizophrenia

Linkage studies of families with schizophrenia have identified several potential susceptibility regions throughout the genome[172,228,230,331,370-372]. One of the most promising linkage findings in schizophrenia to date was reported by Brzustowicz and colleagues, who carried out a whole genome scan in a collection of 22 extended Canadian pedigrees segregating schizophrenia and schizoaffective disorder and found a maximum parametric multipoint LOD score of 6.5 for a region on chromosome 1q21-23[331]. This finding is supported by several other studies involving independent pedigree collections of Caucasian origin. Shaw and colleagues reported a heterogeneity LOD score of 2.4 for marker D1S196 on chromosome 1q24 for 70 families from the U.S.A. and Europe[373], and interestingly, the same marker produced a HLOD score of 3.2 in a sample of British and Icelandic schizophrenia pedigrees[332]. Finally, a Taiwanese study of 45 schizophrenia sib pairs found an estimated proportion of IBD sharing of 57% (p-value 0.03) at marker D1S1679, the same marker which showed the most significant evidence of linkage in the Canadian study[374]. The consistency of linkage findings for chromosome 1q21-23 is further stressed by the results of a recent comprehensive meta-analysis including 20 genome-wide linkage scans of schizophrenia families, which found evidence for the presence of a schizophrenia susceptibility locus in this region[228].

Chromosome 1q23 harbours several potential susceptibility genes for schizophrenia, including the Regulator of G Protein Signalling 4 (*RGS4*), the U2AF Homology Motif Kinase 1 (*UHMK1*), and the Nitric Oxide Synthase 1 (neuronal) Adaptor Protein (*NOS1AP*; also called *CAPON*). Fine mapping of a 15 cM interval on chromosome 1q23 in the Canadian sample confirmed the previously obtained LOD score of 6.5 and narrowed the candidate region down to 3 cM (~1 Mb)[375]. Both *UHMK1* and *RGS4* lie outside this narrow region, and LD analysis of genetic markers across the original linkage peak in the Canadian family sample yielded

significant evidence of association between SNPs within the *NOS1AP* gene and schizophrenia (minimum p-value 0.0016)[334].

The *NOS1AP* gene is not only a prime positional candidate gene for schizophrenia; it is also an interesting functional candidate due to its role in the glutamatergic neurotransmission system. Glutamatergic neurotransmission was first implicated in the aetiology of schizophrenia when it was discovered that subanaesthetic doses of dissociative anaesthetics, such as phencyclidine and ketamine, induce negative symptoms and cognitive deficits in healthy volunteers that are clinically indistinguishable from schizophrenia[376]. Phencyclidine and ketamine were known to act as non-competitive antagonists of N-methyl-D-aspartate sensitive glutamate receptors (NMDARs); the observation that this antagonistic action could lead to schizophrenia-like symptoms laid the foundation for the NMDAR hypofunction hypothesis of schizophrenia. This hypothesis postulates that a reduced function of the NMDAR on corticolimbic GABAergic neurons is at the root of negative, cognitive and, as a downstream effect, positive symptoms, and that NMDAR hypofunction also triggers the cortical atrophy that is characteristic of schizophrenia[376]. The NMDAR hypofunction hypothesis has gained further support through pharmacological studies, which found that administration of NMDAR agonists, such as glycine and D-serine, to schizophrenic patients led to a significant improvement of negative and cognitive symptoms[377].

The protein encoded by the *NOS1AP* gene has been shown to act within the pathway of NMDAR mediated neurotransmission. Intracellular NMDAR induced signal transmission relies on the interaction of the receptor molecule with the neuronal Nitric Oxide Synthase (nNOS) through a mediator protein, PSD95. The NOS1AP protein competes with PSD95 for interaction with nNOS and is thought to be involved in the regulation of nNOS activity in the neuron[378]. Dysregulation of NOS1AP availability might lead to a disruption of signalling processes following glutamatergic neurotransmission downstream of the NMDAR, thereby possibly eliciting a similar effect to that of NMDAR hypofunction. In line with a possible role for abnormal NOS1AP expression in schizophrenia, Xu and colleagues have recently identified a short isoform of the NOS1AP protein and shown its increased expression in the schizophrenic brain in comparison to healthy subjects in a post-mortem brain study[339].

In an effort to further characterise the role of *NOS1AP* in schizophrenia, several studies have evaluated association between markers across the gene region and the disorder. A study of Han Chinese detected significant association between a synonymous SNP located in exon 9 of the gene, rs348624, and the disorder ($p = 0.000017$)[379]; however, the results from Canada and China could not be replicated in a large British sample[380]. It is therefore of interest to examine a possible association in additional independent samples.

In an evaluation of candidate genes, our group analysed D1S1679, a microsatellite marker 23.5 kb downstream of the *NOS1AP* gene, in a trio sample from Antioquia and found a significant association of this marker with schizophrenia ($p = 0.019$)[381]. To further evaluate this association, I examined the same collection of 102 Antioquian schizophrenia trios with a dense set of SNPs spread throughout the *NOS1AP* gene and performed single-marker as well as haplotype-based TDT analyses. In view of the psychopathological heterogeneity of schizophrenia, I also used a dimensional approach to evaluate association between clinical features and the *NOS1AP* gene. This dimensional approach was developed together with Dr Jenny García from Universidad de Antioquia in Medellín and will be explained in greater detail in the following section.

5.2. Clinical Dimensions of Schizophrenia

As discussed earlier, schizophrenia is a clinically heterogeneous disorder. Age of onset, inter-episode recovery, and symptom constellations can vary widely between patients, and two patients with the same diagnosis of schizophrenia might in fact not share a single symptom[13,212]. It seems therefore likely that the diagnostic category of "schizophrenia" encompasses several distinct disease entities with different, yet probably related, aetiologies (see chapter 1); and the psychopathological heterogeneity of the disorder known to us as schizophrenia is likely to reflect the genetic heterogeneity underlying its aetiology. One possible way of unravelling this heterogeneity lies in the use of clinical dimensions. The concept of clinical dimensions is based on the notion that there are symptomatic complexes coexisting within the disorder, which vary across affected individuals and which can be quantified by using clinical scales measuring the prominence of each symptom

complex in affecteds[382,383]. The rationale for the use of clinical dimensions, rather than the diagnosis of schizophrenia, as a phenotype in gene mapping studies is that there should be fewer genes contributing to each dimension than to the disorder as such, thereby facilitating the identification of these genes.

There are a number of examples of studies using the dimensional approach in the literature. The His452Tyr polymorphism in the gene encoding the serotonin receptor HTR2A has been found to be associated to affective symptoms of schizophrenia[384], and negative symptoms of schizophrenia have been reported to be associated with the number of copies of a CAG repeat within the hKCa3 potassium channel gene[385,386], with a specific allele of a microsatellite within the gene encoding the brain-derived neurotrophic factor (*BDNF*)[384], and with a haplotype in the dystrobrevin binding protein 1 gene (*DTNBP1*)[387]. While these results will need to be replicated in independent samples, they represent encouraging examples of the feasibility of the dimensional approach in identifying susceptibility genes for complex disease. We therefore decided to use this approach in addition to the categorical one in assessing association between the *NOS1AP* gene and schizophrenia in the sample from Antioquia.

The clinical dimensions used in this study were obtained from the Scales of Assessment of Positive and Negative Symptoms (SAPS and SANS)[388-390] by factor analysis. The SANS and SAPS were applied to all schizophrenic patients by an experienced psychiatrist. To comply with the minimum sample size recommended for factor analysis[391,392], the scales were also applied to an additional sample of 150 schizophrenic patients diagnosed according to the same diagnostic criteria as the patients belonging to the trio sample (see chapter 2.1.1) and with similar clinical parameters. However, these additional individuals were not available for genotyping. On the other hand, the SANS and SAPS could only be applied to 98 out of the 102 patients available for genotyping, making for a total sample of 248 for factor analysis and 98 for the genetic analysis of the clinical dimensions (see below).

Dimensional scores were obtained by principal component factor analysis performed on the individuals items of the SANS and SAPS. Sampling adequacy was evaluated using the Kaiser-Meyer-Olkin measure (KMO)[393]. The resulting KMO of 0.89 indicated good variable factorability (i.e., the variables under study have a low partial correlation coefficient). Factor analysis was then performed using the programme

SPSS 13.0 (SPSS Inc.), using the Scree criterion for factor selection[394]. In order to assign items to factors, only the items with a loading of 0.40 or greater were taken into account. The factor solution was then rotated (using the VARIMAX procedure) and factor scores calculated using regression. All factors had eigenvalues > 1.0, indicating that they account for more variance than any single SANS or SAPS item. The resulting dimensions and the factor loadings are presented in Table 5.1. The dimensions are: (1) affective flattening and alogia; (2) auditory, somatic and visual hallucinations, and first rank and paranoid delusions; (3) formal thought disorders; (4) avolition and social isolation; (5) bizarre behaviour; and (6) olfactory hallucinations and other delusions.

After factor analysis, dimensional scores were calculated for each patient. These scores represented the trait, or phenotype, input for the quantitative genetic association analysis using the QTDT programme[277,395]. Trait values should follow a normal distribution for analysis with the QTDT programme (Gonçalo Abecasis, personal communication); therefore, the dimensional scores were normalised before quantitative TDT analysis. To achieve this, all 98 individuals were ranked based on their dimensional score. Ranks were converted to percentiles [rank / (N + 1), where N is the number of individuals], and z-scores were obtained using the inverse standard normal cumulative distribution. The z-scores were then used as input for the quantitative genetic association analysis (see section 5.3.2).

The factor analysis, and the normalisation of the resulting dimensional scores were performed by Dr Jenny García at Universidad de Antioquia, Medellín.

Table 5.1: Clinical dimensions obtained from SANS and SAPS by factor analysis.

		Dimension					
		1	2	3	4	5	6
Eigenvalue		12.5	7.3	3.7	2.04	1.9	1.5
% of variance		25.1	14.7	7.3	4.1	3.7	3
Items	Facial expression	**0.80**	0.05	0.003	0.37	0.03	0.004
	Spontaneous movements	**0.83**	0.01	-0.04	0.23	0.02	-0.09
	Expressive gestures	**0.84**	0.04	-0.05	0.34	0.01	0.01
	Eye contact	**0.79**	0.04	0.09	0.20	0.17	-0.10
	Affective non-responsiveness	**0.79**	0.04	0.001	0.34	0.09	0.005
	Vocal inflections	**0.84**	-0.009	0.02	0.27	0.07	0.11
	Poverty of speech	**0.74**	-0.09	0.13	0.19	0.35	-0.03
	Poverty of content	**0.64**	-0.10	0.31	0.27	0.24	0.10
	Blocking	**0.49**	0.09	0.33	-0.08	0.25	0.05
	Latency of response	**0.71**	-0.02	0.14	0.07	0.27	-0.13
	Grooming and hygiene	0.38	0.07	0.13	0.34	**0.42**	0.17
	Impersistence at work	0.23	-0.01	0.04	**0.60**	0.20	0.08
	Physical anergia	0.37	0.08	0.07	**0.64**	0.01	-0.04
	Recreational interests	0.36	0.16	0.07	**0.71**	0.073	-0.04
	Sexual interest	0.38	0.05	0.02	**0.66**	0.01	0.05
	Intimacy and closeness	0.38	0.12	0.11	**0.67**	0.15	0.07
	Relationship with friends	0.37	0.11	0.08	**0.72**	0.03	0.03
	Social inattentiveness	**0.49**	0.001	0.34	0.33	**0.42**	0.02
	Inattentiveness during testing	0.30	-0.05	0.26	0.29	**0.46**	0.03
	Auditory hallucinations	-0.13	**0.72**	0.06	0.24	0.16	0.07
	Voices commenting	-0.20	**0.75**	0.11	0.16	0.09	-0.03
	Voices conversing	-0.08	**0.75**	0.16	0.15	0.08	0.09
	Somatic hallucinations	0.03	**0.51**	0.12	0.18	0.13	0.41
	Olfactory hallucinations	0.01	0.29	0.05	0.26	0.06	**0.48**
	Visual hallucinations	-0.02	**0.50**	0.06	0.12	0.19	0.39
	Persecutory delusions	-0.07	**0.75**	0.04	0.16	0.17	0.12
	Delusions of jealously	0.08	0.05	-0.03	-0.12	0.16	**0.55**
	Delusions of guilt	-0.12	0.08	0.02	0.0001	0.12	**0.46**
	Grandiose delusions	-0.05	0.08	0.06	0.03	0.05	**0.61**
	Religious delusions	0.002	0.19	0.04	-0.02	-0.09	**0.68**
	Somatic delusions	0.002	0.37	0.17	0.15	0.07	**0.52**
	Delusions of reference	-0.07	**0.71**	-0.04	0.09	0.17	0.11
	Delusions of being controlled	0.06	**0.77**	0.19	0.01	0.06	0.16
	Delusions of mind reading	0.14	**0.78**	0.06	-0.08	-0.004	0.12
	Thought broadcasting	0.14	**0.75**	0.07	-0.13	-0.01	0.07
	Thought insertion	0.13	**0.78**	0.04	-0.03	0.004	0.13
	Thought withdrawal	0.15	**0.77**	0.06	-0.14	-0.06	0.10
	Clothing and appearance	0.24	0.21	0.23	-0.12	**0.57**	0.07
	Social and sexual behaviour	0.22	0.25	0.17	0.08	**0.59**	0.15
	Aggressive behaviour	0.09	0.26	0.02	0.13	**0.49**	0.20
	Stereotyped behaviour	0.19	0.11	0.05	0.21	**0.54**	0.29
	Derailment	0.26	0.21	**0.74**	0.08	0.01	0.22
	Tangentiality	0.27	0.16	**0.66**	0.15	0.06	0.26
	Incoherence	0.27	0.04	**0.76**	-0.01	0.08	0.11
	Illogicality	0.25	0.14	**0.71**	0.02	-0.005	0.19
	Circumstantiality	-0.19	0.14	**0.68**	-0.04	0.05	-0.07
	Pressure of speech	-0.26	0.10	**0.60**	0.09	0.04	0.04
	Distractible speech	0.16	-0.04	**0.61**	0.16	0.33	-0.06
	Clanging	-0.06	0.04	**0.54**	0.07	0.26	-0.15
	Inappropriate affect	0.17	0.09	**0.48**	-0.05	0.51	-0.06

Item loadings after VARIMAX rotation. An item contributes to a dimension if its loading value is > 0.4 (indicated by bold type). The dimensions are: 1, affective flattening and alogia; 2, auditory, somatic and visual hallucinations, and first rank and paranoid delusions; 3, formal thought disorders; 4, avolition and social isolation; 5, bizarre behaviour; 6, olfactory hallucinations and other delusions.

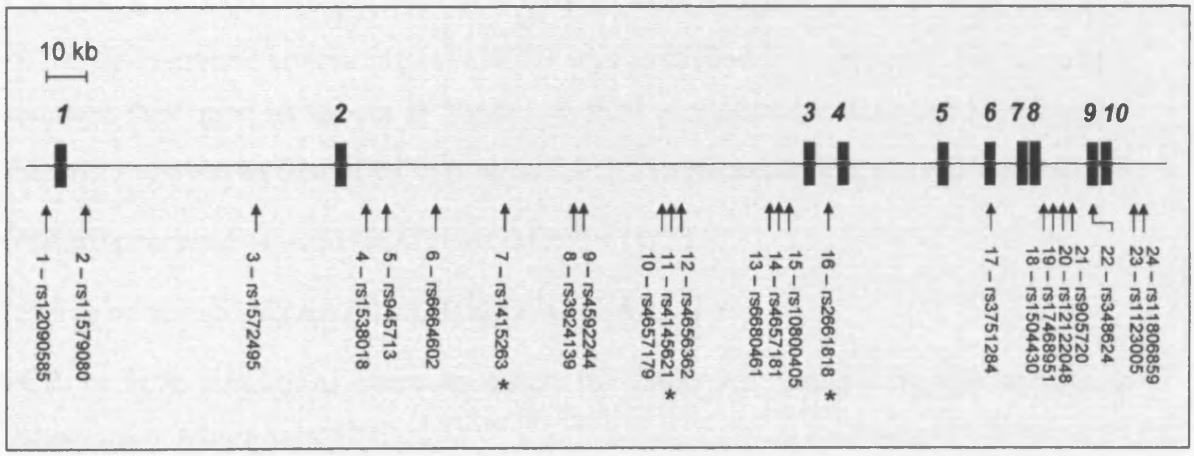
5.3. Materials and Methods

The study sample, the diagnostic procedures and the statistical analyses have been described in detail in chapter 2. It is therefore the main aim of this section to provide details on marker selection and genotyping methods, and to give a brief overview over the data analysis.

5.3.1. Marker Selection and Genotyping

24 SNP markers covering 314 kb across the *NOS1AP* gene region were genotyped in this study. A schematic overview of the markers chosen and their location with respect to NOS1AP is given in Figure 5.1. The 24 markers included 9 SNPs from the original association study of NOS1AP[334] (numbers in brackets refer to SNP numbering as in Figure 5.1): rs1572495 [#3], rs1538018 [#4], rs945713 [#5], rs1415263 [#7], rs3924139 [#8], rs4145621 [#11], rs2661818 [#16], rs3751284 [#17], and rs348624 [#22], and additional SNPs selected from evolutionary conserved regions within or close to NOS1AP[396]: rs12090585 [#1], rs11579080 [#2], rs6664602 [#6], rs4592244 [#9], rs4657179 [#10], rs4656362 [#12], rs6680461 [#13], rs4657181 [#14], rs10800405 [#15], rs1504430 [#18], rs17468951 [#19], rs12122048 [#20], rs905720 [#21], rs1123005 [#23], rs11806859 [#24].

Figure 5.1: Location of all genotyped markers along the *NOS1AP* gene region,



This figure shows all markers genotyped in the present study, including markers rs6664602 [#6] and rs4656362 [#12] that were later excluded from all analyses (see text). The locations of *NOS1AP* exons are indicated by black boxes; exon numbers are in italics. Consecutive marker numbers from 1 to 24 are included for easier comparison with the LD plot in Figure 5.2. Markers significantly associated with schizophrenia in Brzustowicz et al. (2004)[334] are labelled with an asterisk.

Genotyping for all but one marker (rs1415263) was conducted by our collaborators on this project at Rutgers University, U.S.A.; the genotyping method used by them for this project is based on a ligation detection assay[397,398]. Clean genotypes were sent to our laboratory for data analysis.

Genotyping for marker rs1415263 was performed in our laboratory using the SNaPshot® genotyping kit (Applied Biosystems). The SNaPshot® system is a primer extension-based genotyping method that allows the multiplex typing of up to 10 SNPs. This system was chosen because it enabled us to genotype the SNP rs1415263 together with four other SNPs that were used in a different project.

In SNaPshot® genotyping, the genomic DNA containing the SNP is first amplified by PCR. In a second step, an extension primer and the four different ddNTPs, each labelled with a different fluorescent dye, are added to the cleaned-up PCR product. The extension primer comprises a ~20 bp-sequence matching the sequence of the PCR product directly adjacent to the polymorphic site, and a universal tail of variable length, which can be adjusted to distinguish the different products of the multiplex assay from each other. In the primer extension reaction, the ddNTP that matches the polymorphic site is added to the 3' end of the extension primer. After denaturing, the product of the extension reaction is run on a capillary sequencer, where the detection of the different fluorescent labels enables genotyping (one peak is detected for homozygotes, two peaks of different colours are detected for heterozygotes). The position of the peak corresponds to the length of the extended SNaPshot® primer.

A 209 bp-fragment containing rs1415263 was amplified from genomic DNA using a standard PCR mix, as shown in Table 2.4. PCR primers were designed by using the Primer3 program as described in chapter 2.2.3. Primer sequences were as follows:

forward primer: 5'-CAGTGCCTCAGTGCTTTGTT-3'

reverse primer: 5'-CTAAATGGTGAGCCCCAATG-3'

PCR cycling conditions were as listed in Table 2.5, except for the annealing temperature, which was 57°C.

After checking the amplification success by agarose gel electrophoresis as described in chapter 2.2.4, the PCR product was cleaned in preparation for the SNaPshot® reaction. This clean-up step is equivalent to the one used before sequencing (see chapter 2.2.6). 2.5µl (2.5U) SAP and 0.1µl (1U) Exo-I (both USB Corporation) were

added to 10µl PCR product and topped up with dH_2O to a total volume of 20µl. The mix was then left to incubate for 1h at 37°C, followed by 15min at 72°C for enzyme deactivation.

1.6µl of the clean PCR product were carried forward to the SNaPshot® reaction, which was performed following the manufacturer's instructions. The extension primer was designed using the Jellyfish™ v1.5 program (Labvelocity) taking into account the following criteria:

- The extension primer must anneal adjacent to the polymorphic site.
- The optimal length of the extension primer is between 15 and 25bp (excluding the universal tail).
- Its GC content should be between 40 and 60%.
- The extension primer should have no more than 4bp self-complementarity.
- If mismatches are to be incorporated, the 3' bp (adjacent to the polymorphism) must not be a mismatch.

The extension primer used to type SNP rs1415263 had the following sequence:

5'-$(GACT)_{10}$TTCCCTATTCCTTTATC-3'

After the SNaPshot® reaction, the samples were sent to the Centre of Comparative Genomics, where they were run on an ABI 3730xl® genetic analyser (Applied Biosystems). The resulting raw data was sent back to our lab, and genotype analysis was performed with the GeneMapper® v3.7 software, using its inbuilt SNaPshot® analysis routine. All genotypes were visually checked by two independent researchers, including the author of this thesis ("double scoring"). Upon disagreement between the two scorers, a single attempt was made to re-genotype the sample in question. If re-genotyping was unsuccessful, the sample was excluded from the study.

5.3.2. Data Analysis

The methods used for the statistical analysis of the data collected as part of this project have been presented in detail in chapter 2.3. Briefly, the genotype data were checked for Mendelian inconsistencies using PedCheck[268]. Hardy-Weinberg equilibrium was evaluated separately in founders and cases using the Genepop program[266], and allele frequencies of non-transmitted alleles were estimated using TDTPHASE v.2.4[275].

The Transmission Disequilibrium Test (TDT)[154] for single markers was carried out as implemented in the TRANSMIT program, version 2.5.4.[155].

Linkage disequilibrium across the *NOS1AP* gene was evaluated using Haploview v3.2[276] based on parental genotypes. To compare the LD structure in the Antioquian sample to the LD structure in the European population, to which the Antioquian population is very close with 80% autosomal European ancestry[182], the CEU HapMap data for the same region was downloaded from the HapMap project website (http://www.hapmap.org), and the LD plot was also displayed in Haploview. Regions with the highest levels of linkage disequilibrium were used for haplotype-based TDT with WHAP v.2.09[274] as described in chapter 2.3. Because WHAP only accepts data from parent-offspring trios or duos and cannot accommodate other relatives, WHAP analyses included only 99 index cases: 38 complete trios and 61 duos. Two tests were performed: an omnibus haplotype test (testing the effects across all haplotypes) and a specific haplotype test (testing the effect of each haplotype against all others).

Quantitative TDT analysis was carried out on the normalised dimensional scores (z-scores; see section 5.2) using the QTDT v.2.5.1 programme[277]. As mentioned above, four of the index cases had no quantitative data available; thus reducing the sample size for these analyses to 98.

5.4. Results

5.4.1. Hardy-Weinberg Equilibrium

All markers tested were found to be in Hardy-Weinberg equilibrium, with exception of marker rs4656362 [#12] (p = 0.0197) (see Table 5.2). Marker rs6664602 [#6] was found not to be polymorphic in the Antioquian population. These two markers were therefore excluded from all further analyses.

Table 5.2: Results of the Hardy-Weinberg equilibrium test in 23 SNPs across the *NOS1AP* gene region in the schizophrenia trio sample from Antioquia.

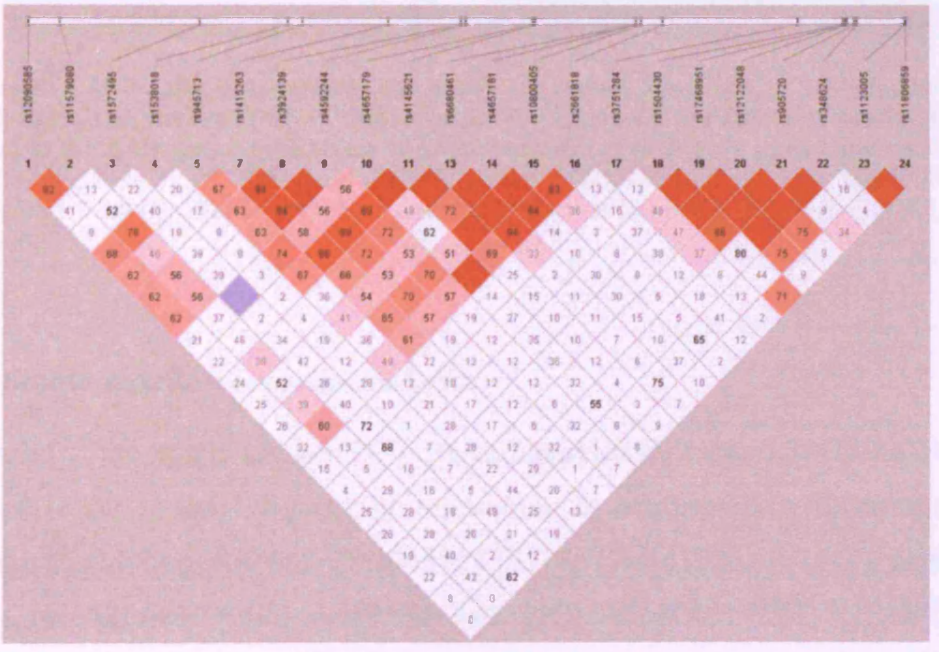
Locus	Founders (parents)		Schizophrenia patients	
	p-value	s.e.	p-value	s.e.
rs12090585	0.077	0.0029	0.841	0.0019
rs11579080	0.835	0.002	0.545	0.0043
rs1572495	0.674	0.002	1.000	0
rs1538018	0.847	0.0019	1.000	0
rs945713	1.000	0	0.364	0.0054
rs1415263	0.739	0.0031	0.227	0.0045
rs3924139	1.000	0	0.207	0.0048
rs4592244	1.000	0	0.204	0.0047
rs4657179	0.589	0.003	0.373	0.0033
rs4145621	0.712	0.0036	1.000	0
rs4656362	***0.020***	0.0015	0.821	0.002
rs6680461	0.443	0.0051	0.546	0.0039
rs4657181	0.850	0.0018	0.223	0.0044
rs10800405	1.000	0	0.411	0.0045
rs2661818	0.695	0.0033	0.140	0.0042
rs3751284	0.864	0.002	0.288	0.0051
rs1504430	0.334	0.0052	0.263	0.0054
rs17468951	0.577	0.0039	1.000	0
rs12122048	0.709	0.0033	1.000	0
rs905720	0.685	0.0031	0.137	0.0031
rs348624	1.000	0	0.367	0.0033
rs1123005	1.000	0	0.783	0.0018
rs11806859	0.494	0.0039	0.067	0.0025
Global Test[a] (44 d.f.)	0.9892	-	0.6754	-

[a]**Excludes marker rs1415263, which has been tested separately.**

5.4.2. Linkage Disequilibrium Analysis

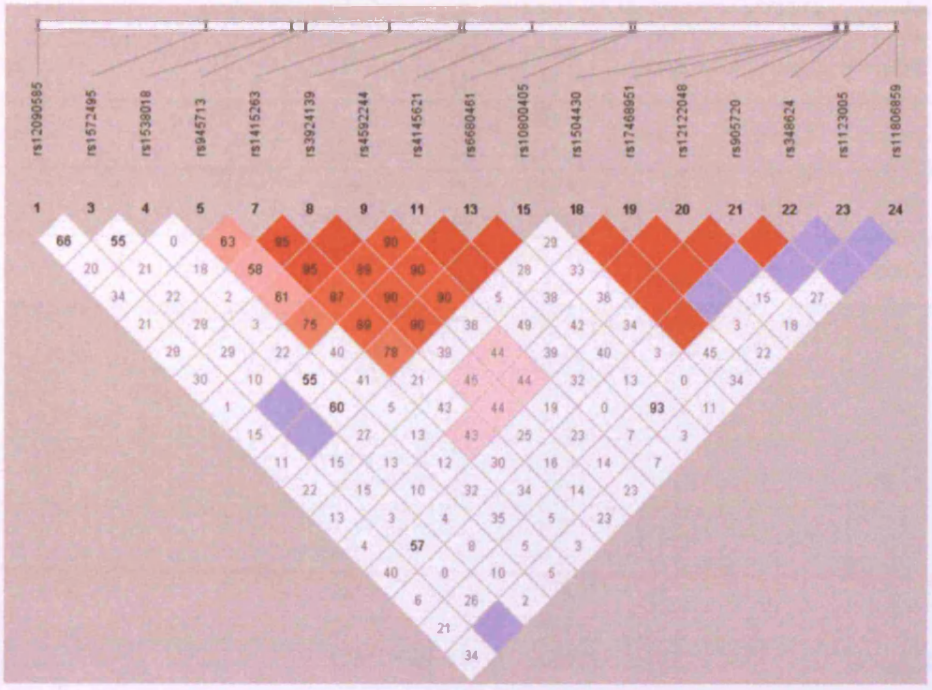
The pattern of LD between the SNPs genotyped in the Antioquian sample, as determined by Haploview, is represented in Figure 5.2. Two regions of strong LD are apparent, the first one covering markers rs945713 [#5] to rs2661818 [#16], spanning 129.1 kb and comprising introns 2 and 3 of *NOS1AP* (region 1), the second one covering markers rs1504430 [#18] to rs348624 [#22] and spanning 5.1 kb mostly within intron 8 (region 2). For comparison, the LD structure of the European HapMap population (CEU) in the same region is displayed in Figure 5.3. It shows great similarity to the pattern seen in the Antioquian sample, with two regions of high LD in the same location as observed in Antioquia.

Figure 5.2: LD structure between 22 genotyped SNPs across the *NOS1AP* gene in the Antioquian trio sample.



Marker numbers (1 to 24) are as in Figure 5.1. Values in the boxes refer to % D'. Where no number is shown, D'=1.0. Red and pink boxes indicate a LOD score of ≥ 2.0 (D' significant); blue and white boxes indicate a LOD score of < 2.0 (D' not significant).

Figure 5.3: LD structure across the *NOS1AP* gene based on data for the European HapMap population (CEU; http://www.hapmap.org).



Only HapMap SNPs also genotyped in this study are shown. Since not all SNPs included here are HapMap SNPs, the set of SNPs displayed for the European population is smaller than the one typed in the Antioquian population. Marker numbers (1 to 24) are as in Figure 5.1. Values in the boxes refer to % D'. Where no number is shown, D'=1.0. Red and pink boxes indicate a LOD score of ≥ 2.0 (D' significant); blue and white boxes indicate a LOD score of < 2.0 (D' not significant).

5.4.3. Single marker association tests

The results of the single marker TDT are summarised in Table 5.3. Of the 22 SNPs examined, eight showed significant association to schizophrenia (overtransmitted alleles are shown in parentheses): rs945713 [#5] (G), rs1415263 [#7] (T), rs3924139 [#8] (T), rs4592244 [#9] (A), rs4145621 [#11] (C), rs6680461 [#13] (G), rs4657181 [#14] (A), and rs3751284 [#17] (T). For all eight markers, both the p-value based on the chi-squared approximation and the bootstrap p-value are nominally significant. Of these eight markers, seven are in high LD with each other and are located in LD region 1, while the remaining marker, rs3751284 [#17], is not in strong LD with any other marker (Figure 5.2).

Table 5.3: Single marker TDT (TRANSMIT) for 22 SNPs within the CAPON gene.

SNP	#[a]	allele	allele frequency[b]	O	E	Var (O-E)	χ^2 (1d.f.)	p-value	bootstrap p-value[c]
rs12090585	1	G	0.667	132	132.98	19.558	0.0487	0.825	0.820
		A	0.333	68	67.024				
rs11579080	2	G	0.623	110	109.87	13.812	0.0012	0.972	0.970
		T	0.377	64	64.129				
rs1572495	3	G	0.907	180	182.74	8.5861	0.8728	0.350	0.397
		A	0.093	24	21.263				
rs1538018	4	G	0.650	146	142.31	18.396	0.7392	0.390	0.392
		C	0.350	60	63.688				
rs945713	5	G	0.470	120	109.93	18.947	***5.3500***	***0.021***	***0.011***
		A	0.530	82	92.068				
rs1415263	7	C	0.478	87	97.35	19.523	***5.4866***	***0.019***	***0.015***
		T	0.522	117	106.65				
rs3924139	8	C	0.426	95	105.48	18.506	***5.9341***	***0.015***	***0.007***
		T	0.574	107	96.521				
rs4592244	9	G	0.426	95	105.48	18.506	***5.9341***	***0.015***	***0.007***
		A	0.574	107	96.521				
rs4657179	10	G	0.802	164	165.42	13.308	0.1518	0.697	0.704
		T	0.198	40	38.579				
rs4145621	11	C	0.570	138	126.19	18.856	***7.3991***	***0.007***	***0.004***
		T	0.430	64	75.812				
rs6680461	13	T	0.740	136	144.44	15.679	***4.5391***	***0.033***	***0.028***
		G	0.260	68	59.564				
rs4657181	14	A	0.567	143	131.54	19.618	***6.6972***	***0.010***	***0.006***
		T	0.433	63	74.462				
rs10800405	15	C	0.724	132	139.15	15.149	3.3715	0.066	0.053
		G	0.276	66	58.853				
rs2661818	16	C	0.651	128	124.71	16.631	0.6498	0.420	0.391
		G	0.349	62	65.287				
rs3751284	17	C	0.638	105	114.27	20.327	***4.2276***	***0.040***	***0.019***
		T	0.362	95	85.73				
rs1504430	18	C	0.681	129	134.41	19.001	1.5430	0.214	0.254
		T	0.319	75	69.585				
rs17468951	19	A	0.639	139	136.23	18.138	0.4228	0.516	0.538
		G	0.361	65	67.769				
rs12122048	20	G	0.633	138	135.1	17.474	0.4817	0.488	0.469
		A	0.367	64	66.901				
rs905720	21	C	0.696	143	139.31	16.922	0.8057	0.369	0.389
		T	0.304	59	62.692				
rs348624	22	C	0.845	171	171.12	11.231	0.0013	0.971	0.977
		T	0.155	33	32.877				
rs1123005	23	A	0.854	169	173.3	11.254	1.6394	0.200	0.283
		A	0.718	55	56.138				
rs11806859	24	G	0.282	147	145.86	15.976	0.0810	0.776	0.801
		A	0.718	55	56.138				

[a]SNP number as in Figure 5.1. [b]in untransmitted chromosomes; [c]based on 1000 bootstrap samples; O, observed transmissions; E, expected transmissions; d.f., degree of freedom. Nominally significant χ^2- and p-values are italicised and bold. Markers belonging to LD regions 1 (above) and 2 (below) are shaded.

5.4.4. Haplotype association

Haplotype analyses were carried out based on the two LD regions shown in Table 5.3 and Figure 5.2. The results of association tests for LD region 1 (markers rs945713 [#5] to rs2661818 [#16]) are shown in Table 5.4. The omnibus haplotype test resulted in a p-value of 0.348, indicating no significant overall association of this region with schizophrenia. In the specific haplotype test, the p-value for the most common haplotype was 0.04 with an odds ratio of 0.54, indicating a possible trend towards (negative) association between this particular haplotype and schizophrenia. For five of seven markers in LD region 1, the haplotype showing possible negative association consisted of alleles found to be undertransmitted in the single marker analyses (rs945713 [#5] – A; rs3924139 [#8] – C; rs4145621 [#11] – T; rs6680461 [#13] – T; and rs4657181 [#14] – T). No significant association was detected between LD region 2 (markers rs1504430 [#18] to rs348624 [#22]) and schizophrenia (data not shown).

Table 5.4: Results of the transmission disequilibrium test of the haplotype containing the ten SNPs forming LD region 1.

Haplotype #	rs945713 [#5]	rs1415263 [#7]	rs3924139 [#8]	rs4592244 [#9]	rs4657179 [#10]	rs4145621 [#11]	rs6680461 [#13]	rs4657181 [#14]	rs10800405 [#15]	rs2661818 [#16]	Frequency	Omnibus test (OT) overall p=0.348			Haplotype-specific test (HS)	
												OR_{OT}	lower limit[a]	upper limit[a]	OR_{HS}	p-value
1	A	C	T	A	G	T	T	T	C	G	0.281	*reference haplotype*			0.54	***0.04***
2	G	T	C	G	T	C	G	A	G	C	0.131	1.79	0.73	4.37	1.13	0.75
3	G	T	C	G	G	C	G	A	G	C	0.096	2.29	0.85	6.18	1.48	0.38
4	G	T	C	G	G	C	T	A	C	C	0.084	3.37	1.21	9.40	2.24	0.09
5	G	T	C	G	G	C	T	T	C	G	0.059	3.40	1.07	10.82	2.22	0.14
6	A	T	C	G	G	C	G	A	G	C	0.056	1.23	0.36	4.24	0.77	0.65
7	A	C	T	A	G	C	T	A	C	C	0.053	1.67	0.43	6.42	0.99	0.99
8	A	C	T	A	G	T	T	A	C	C	0.048	0.63	0.16	2.40	0.39	0.15
9	G	C	T	A	G	C	T	A	C	C	0.03	1.33	0.25	6.98	0.87	0.86
10	G	T	C	G	T	C	T	A	C	C	0.03	1.12	0.21	5.93	0.72	0.68
11	A	T	C	G	G	C	T	A	C	C	0.028	4.86	1.06	22.24	3.03	0.14
12	G	C	T	A	G	T	T	T	C	G	0.027	1.55	0.25	9.78	0.88	0.88
13	G	T	T	A	G	C	T	A	C	C	0.025	4.19	0.84	20.80	2.63	0.23
14	G	T	T	A	T	C	T	A	C	C	0.015	3.30	0.40	27.33	2.00	0.51
15	A	C	T	A	T	C	G	A	G	C	0.014	0.95	0.06	14.07	0.56	0.66
16	G	T	C	G	T	C	T	T	C	C	0.013	0.92	0.08	10.21	0.59	0.65
17	G	C	T	A	G	C	G	A	G	C	0.011	0.28	0.03	2.77	0.19	0.15

[a]Upper and lower limit of the 95% confidence interval for the OR of the omnibus test. WHAP does not calculate the 95% confidence intervals for the ORs of the haplotype-specific test; instead, a p-value is given for each of the tested haplotypes. Nominally significant p-values are printed in bold italics. OT, omnibus test; HS, haplotype-specific test; OR, odds ratio.

5.4.5. QTDT analysis on clinical dimensions

The results of the QTDT analysis on these clinical dimensions are summarised in Table 5.5. Since the QTDT program can only test markers for which 30 or more probands are informative, markers rs11579080 [#2], rs1572495 [#3], rs4657179 [#10], rs348624 [#22], and rs1123005 [#23] could not be included in the analysis. Nominally significant associations were obtained for the following marker-dimension combinations (the allele associated with increased trait values is given after the p-value): dimension 1 (affective flattening and alogia) with marker rs3751284 [#17] (p = 0.016; C), dimension 2 (auditory, somatic and visual hallucinations, and first rank and paranoid delusions) with marker rs1415263 [#7] (p = 0.034; C), dimension 4 (avolition and social isolation) with markers rs6680461 [#13] (p = 0.004; G) and rs10800405 [#15] (p = 0.001; G), and dimension 6 (olfactory hallucinations and other delusions) with marker rs6680461 [#13] (p = 0.048; C). Three of the markers showing nominally significant p-values in the analysis of clinical dimensions had also shown nominally significant p-values in the categorical analyses (rs3751284 [#17], rs1415263 [#7], and rs6680461 [#13]). For markers rs3751284 [#17] and rs1415263 [#7], the alleles associated with increased trait values in the quantitative analysis are different from the ones shown to be overtransmitted to schizophrenic patients in the categorical analysis (see Table 5.3 and Table 5.5), whereas for marker rs6680461 [#13], the allele shown to increase the trait value corresponds to the overtransmitted allele in the categorical analysis (allele G).

Table 5.5: Results of the QTDT analysis for 17 SNPs in the *NOS1AP* gene region.

SNP	# [a]	Informative trios	Dim. 1	Dim. 2	Dim. 3	Dim. 4	Dim. 5	Dim. 6
rs12090585	1	47	*	*	*	*	*	*
rs1538018	4	40	*	*	*	*	*	*
rs945713	5	44	*	*	*	*	*	*
rs1415263	7	43	*	0.034 (C)	*	*	*	*
rs3924139	8	38	*	*	*	*	*	*
rs4592244	9	38	*	*	*	*	*	*
rs4145621	11	39	*	*	*	*	*	*
rs6680461	13	34	*	*	*	0.004 (G)	*	*
rs4657181	14	45	*	*	*	*	*	*
rs10800405	15	31	*	*	*	0.001 (G)	*	*
rs2661818	16	32	*	*	*	*	*	*
rs3751284	17	50	0.016 (C)	*	*	*	*	*
rs1504430	18	42	*	*	*	*	*	*
rs17468951	19	44	*	*	*	*	*	*
rs12122048	20	39	*	*	*	*	*	*
rs905720	21	43	*	*	*	*	*	0.048 (C)
rs11806859	24	31	*	*	*	*	*	*

Overall Bonferroni significance level: 0.10; overall empirical significance level: 0.08

[a]SNP number as in Figure 5.1. Dim., dimension. For the explanation of the dimensions, see text and Table 5.1. P-values are shown for each marker/dimension combination; the allele that increases the trait (dimension) value is shown in parentheses. *p-value > 0.05

5.5. Discussion

We have found association of several SNPs in the *NOS1AP* gene to schizophrenia and to clinical dimensions of the disorder in the population of Antioquia, Colombia. These findings are in line with previous studies providing compelling evidence for the implication of chromosome 1q21-23, and in particular the *NOS1AP* gene, in schizophrenia susceptibility in the Canadian[331,334] and Chinese Han[379] populations. Furthermore, an association between a microsatellite marker 23.5 kb downstream of *NOS1AP*, D1S1679, and schizophrenia has been observed in the population of Antioquia[381], suggesting that the region might play a role in the aetiology of schizophrenia in this population.

NOS1AP is an interesting candidate gene for schizophrenia susceptibility because of its involvement in NMDA receptor mediated glutamatergic neurotransmission, which is thought to be implicated specifically in the aetiology of schizophrenia[232,376]. The observation of association of several SNPs in the *NOS1AP* gene to the schizophrenia phenotype and also to specific clinical dimensions of the disorder confirms and extends the previous results from Antioquia and further strengthens the link between *NOS1AP* and schizophrenia.

Of the SNPs found to be associated with schizophrenia in our study, two were also found to be significant in the study by Brzustowicz and colleagues: rs1415263 [#7] and rs4145621 [#11][334]. Moreover, the associated allele was the same in both studies: the T allele of rs1415263 [#7], and the C allele of rs4145621 [#11] (see Table 5.3). Interestingly, Xu and colleagues[339] found the expression levels of the short *NOS1AP* isoform overexpressed in the schizophrenic brain to be associated with the T allele of marker rs1415263 [#7].

Most of the SNPs found to be associated to schizophrenia in the present study are located within LD region 1. The only associated SNP outside LD region 1, rs3751284 [#17], is a synonymous change in exon 6 of the gene and has no obvious effect on NOS1AP. The markers in LD region 1 showing association are located in intron 2 of *NOS1AP*. It is possible that these markers are in LD with a further, unidentified susceptibility-conferring variant, such as a regulatory element upstream of the *NOS1AP* gene. This scenario could also explain the identification of a protective, rather than a risk-conferring, haplotype – the common protective

haplotype 1 might be in LD with the protective allele at the susceptibility locus, whereas the risk allele at the same locus might be in LD with not one, but several of the remaining, rarer haplotypes, thereby diluting the susceptibility-conferring effect in the observed haplotypes. Another possible explanation for our findings is that intronic and synonymous exonic SNPs could affect posttranscriptional mRNA processes[399]. In both scenarios, genetic variation could lead to changes in the availability of functional NOS1AP in the neuron and thereby to alterations in glutamatergic neurotransmission.

The analysis of clinical dimensions showed an association between markers rs6680461 [#13] and rs10800405 [#15] with features of avolition and social isolation (dimension 4). For both markers, the allele associated with increased symptom severity is also overtransmitted to schizophrenic patients in the single marker categorical TDT analysis (although the results do not reach statistical significance for rs10800405 [#15]; see Table 5.3 and Table 5.5). These findings are consistent with the role of NOS1AP in the NMDA receptor pathway and the NMDA receptor hypofunction theory of schizophrenia. As discussed above, NMDAR mediated neurotransmission is hypothesised to be involved in the aetiology of schizophrenia. Interestingly, NMDAR hypofunction – or generally, hypofunction of glutamatergic neurotransmission – might account for the negative symptoms of schizophrenia, as suggested by the induction of negative symptoms in healthy patients after administration of an NMDAR antagonist, as well as by the improvement of these symptoms in schizophrenics during a course of treatment with an NMDAR agonist[232,376]. It is noteworthy that the dimension found to be associated with polymorphisms within the *NOS1AP* gene, dimension 4 (avolition and social isolation), captures negative symptoms of the disorder, in accordance with the role of the *NOS1AP* gene in glutamatergic neurotransmission.

For dimension 1 (affective flattening and alogia), the C allele of marker rs3751284 [#17] was found to increase trait values. However, in the categorical single marker analysis, this allele was found to be undertransmitted to schizophrenic patients. In a similar manner, the C allele of marker rs1415263 [#7] is associated with increased values for dimension 2 (auditory, somatic and visual hallucinations, and first rank and paranoid delusions) but was found to be undertransmitted to schizophrenic patients. The results of the different analyses therefore seem somewhat contradictory.

However, the p-values from the quantitative analyses, although nominally significant, are not very small, particularly in view of the many tests carried out in this analysis. It is therefore possible that the associations found for dimensions 1 and 2 are false positives. For marker rs905720 [#21], the direction of association is the same in both categorical and quantitative analyses. However, the categorical results are not significant, and the uncorrected p-value close to 0.05 in the quantitative analysis, suggests that this association might also be a false positive. Further studies would be needed to shed light on a possible role of *NOS1AP* in dimensions 1, 2 and 6.

While there is mounting evidence for a possible link between *NOS1AP* and schizophrenia[400], not all results are consistent. A large case-control study by Puri and colleagues failed to replicate the positive findings in a British sample[380]. Instead, these authors have found an association between schizophrenia and the *UHMK1* gene, also located on chromosome 1q23, in the British sample and suggest that the original linkage signal, as well as the subsequent association results for *NOS1AP* by Brzustowicz and colleagues might be due to *UHMK1*[401]. However, the analysis of LD patterns in the European HapMap population in the region encompassing *NOS1AP* and *UHMK1* revealed no significant LD between the two genes (data not shown).

Such discordant results between studies could be accounted for by the presence of different susceptibility alleles or loci in the samples under study, especially in a heterogeneous disorder such as schizophrenia. The original study by Brzustowicz and colleagues was family-based[334]. Risk factors for a complex disease might vary between familiar and sporadic cases (the genetics of breast cancer serve as an extreme example), providing a possible explanation for the failure to replicate the original findings in the British case-control study. Although the sample used in the present study is also based on sporadic cases, it is taken from a well-described population isolate (see chapter 1.3). It is an established fact that the genetic history of population isolates can lead to the enrichment of specific risk factors[159]. It is therefore possible that *NOS1AP* represents a risk factor of specific importance in the population of Antioquia and that cases from this population are enriched for *NOS1AP* susceptibility variants.

An additional limitation to the comparison of different genetic studies of schizophrenia arises from the clinical variability of the disorder and the nosological uncertainties it might lead to. We therefore want to stress the importance of the results obtained using a dimensional approach, which should be valid independently of such possible nosological uncertainties.

Our results have to be viewed in the context of several limitations. From a conceptual point of view, it should be emphasized that the SANS and SAPS only measure positive and negative symptoms, leaving other aspects of the disease, such as cognitive and neuropsychological symptoms, unconsidered. However, these scales allowed for a hypothesis-driven exploration of a possible link between *NOS1AP* as part of the NMDA receptor pathway and negative symptoms in schizophrenia, and we therefore considered them a good starting point for our analyses.

The main limitation of our study lies in the moderate sample size. On the one hand, this puts a limit to the power of our statistical analyses; on the other hand, genetic effect sizes have been found to be overestimated in small vs. larger studies [402]. Additionally, because of high levels of LD observed between the markers under study, none of the analyses presented here have been corrected for multiple testing and the interpretation of our results is based on nominal significance only. All results should therefore be interpreted with caution and will need to be replicated in independent study samples. However, there are notable consistencies between the results of several of the analyses within this study, as well as with previously published studies. These consistencies corroborate the evidence found in this study for an association of the *NOS1AP* gene to schizophrenia and to a negatively loaded dimension (avolition and social isolation) of the disorder in the population of Antioquia.

5.6. Future Work

Future work should aim to investigate whether the association found here, particularly the association of *NOS1AP* with negative symptoms of the disease described here, can be replicated in further independent samples. To shed further light on a possible role of *NOS1AP* in the aetiology of negative symptoms, it would be of great interest to study schizophrenia deficit patients. The deficit syndrome of schizophrenia is characterised by the enduring presence of negative symptoms, even in phases of clinical remission, and it has been suggested that it might represent a separate disease entity to non-deficit schizophrenia[214,403] (see also chapter 1.5). If *NOS1AP* is truly relevant to the aetiology of negative symptoms, it should show a stronger effect size if examined in a sample selected for persistence of these symptoms. It is also of great interest that negative symptoms do not only occur in disorders of the schizophreniform spectrum (although this is where they are most common); persistent negative symptoms can also be found in mood disorders[404-406]. It would therefore be interesting to focus follow-up studies on a possible the role of *NOS1AP* in the deficit syndrome on deficit patients, both within and outside schizophrenia.

While genetic linkage and association studies can provide importance evidence for the involvement of a gene in a certain disorder, the ultimate confirmation must come from functional studies, which can help bridge the gap between genotype and phenotype and provide a model for the molecular aetiology of complex disease. It would therefore be desirable to conduct further functional studies on NOS1AP and schizophrenia. Due to the inherent restraints on functional studies in brain tissue, which is only available post mortem, one possibility could be the focus on animal studies. On the other hand, adequate animal models will always only be able to mirror specific aspects of human psychiatric disease, and the study of post-mortem brain tissue from schizophrenic (or generally, deficit) patients might complement this approach.

CHAPTER SIX

SUMMARY AND CONCLUDING REMARKS

6. Summary and Concluding Remarks

This thesis aimed at making a contribution to the elucidation of the genetic basis of two severe psychiatric conditions, bipolar disorder and schizophrenia, by studying pedigree and trio samples from the population isolate of Antioquia. Population isolates are characterised by a decreased genetic heterogeneity and therefore provide an excellent opportunity for the study of genetically complex diseases, for which sufficiently homogeneous samples are difficult to collect in an outbred population.

Although the genetic complexity of psychiatric illness has complicated the search for susceptibility loci, in recent years, a picture of genetic predisposition has begun to emerge for both schizophrenia and bipolar disorder[146,193,207]. The results obtained in this thesis add to this picture and thereby provide further reason for optimism for the future of psychiatric genetics.

While the candidate gene study of the *CLINT1* gene on chromosome 5q33 has provided negative results (chapter 3), the whole-genome linkage scan of bipolar disorder, performed to further explore the importance of other candidate regions in Antioquia, has been successful in identifying a number of candidate regions for BPI in particular, mood disorders in general, and psychosis (chapter 4). While none of the linkage signals obtained here reached the formal threshold for genome-wide significance, it is very encouraging that many of the candidate regions we identified had been previously implicated in susceptibility to BP in different populations, and that our study has therefore provided an independent replication of such prior findings. We have also identified a novel locus on chromosome 12q, which now awaits replication in additional samples from other populations.

In the light of genetically complex inheritance, most single genome-wide linkage scan might not have sufficient power to reliably detect risk-conferring variants for psychiatric illness. An important lesson from the past decade should therefore be that we might need to jointly evaluate individual linkage efforts, e.g. by the means of meta-analysis, before being able to tease out the real findings from the inevitable false positive results. Since meta-analyses will join data sets obtained in a range of different populations, this might at first seem contradictory to the emphasis I have placed on the importance of the genetic homogeneity of population isolates.

However, we do not expect most loci identified in any population to be exclusive to that population (although there might be exceptions to this). We do, however, expect that patients from population isolates will be more similar in their genetic makeup, thereby increasing the chances of detecting a signal in an initial genome scan, which will then contribute to the findings of a meta-analysis. The linkage scan performed as part of this study is a good example of this: the region on chromosome 1p, e.g., seems to be of importance in several populations, but we have increased our chances of detecting this signal in our patients by choosing them from a relatively homogeneous population.

As we have seen, population isolates provide an excellent opportunity for the study of complex diseases, including psychiatric disorders. However, as both the genome-wide linkage scan and the association study between the *NOS1AP* gene and schizophrenia (chapter 5) show, complementary strategies can be useful in further reducing the genetic heterogeneity underlying the aetiology of mental illness. In the genome scan, I have explored different diagnostic models – BPI; BPI, BPII and major depression; and psychosis –, all of which produced evidence for linkage to a different range of candidate regions. In the candidate gene analysis of the *NOS1AP* gene, we have used a dimensional approach to the phenotype of schizophrenia, thereby allowing us to dissect the heterogeneous phenotype of DSM-IV schizophrenia and to show that the *NOS1AP* gene might be of special importance in the causation of negative symptoms captured in the dimension of "avolition and social isolation". These are only two examples of phenotypic dissection; another important example is the use of endophenotypes, which allows the identification of genes responsible for physiological traits associated with disease. It is of great interest to explore such approaches to the dissection of psychiatric phenotypes further, and the identification and linkage analysis of bipolar disorder endophenotypes is indeed the next step we are taking in our BP project in Antioquia and the Central Valley of Costa Rica. Generally, it can be anticipated that the exploration of alternative approaches to phenotype definitions will represent one of the main focal points of psychiatric genetics research in the years to come.

7. Bibliography

1. Murray, C. J. L. and Lopez, A. D.(1996) *The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020.* Cambridge, MA: The Harvard School of Public Health.

2. Wells KB, Stewart A, Hays RD, Burnam MA, Rogers W, Daniels M et al (1989): The functioning and well-being of depressed patients. Results from the Medical Outcomes Study [Abstract]. *Jama* 262: 914-919.

3. Malla A, Payne J (2005): First-episode psychosis: psychopathology, quality of life, and functional outcome. *Schizophr Bull* 31: 650-671.

4. Angermeyer MC, Holzinger A, Matschinger H, Stengler-Wenzke K (2002): Depression and quality of life: results of a follow-up study. *Int J Soc Psychiatry* 48: 189-199.

5. Michalak EE, Yatham LN, Kolesar S, Lam RW (2006): Bipolar disorder and quality of life: a patient-centered perspective. *Qual Life Res* 15: 25-37.

6. Harris EC, Barraclough B (1998): Excess mortality of mental disorder. *Br J Psychiatry* 173: 11-53.

7. Das Gupta R, Guest JF (2002): Annual cost of bipolar disorder to UK society. *Br J Psychiatry* 180: 227-233.

8. Knapp M (1997): Costs of schizophrenia. *Br J Psychiatry* 171: 509-518.

9. Thomas CM, Morris S (2003): Cost of depression among adults in England in 2000. *Br J Psychiatry* 183: 514-519.

10. Avissar S, Schreiber G (2002): Toward molecular diagnostics of mood disorders in psychiatry. *Trends Mol Med* 8: 294-300.

11. American Psychiatric Association(2000) *Diagnostic and Statistical Manual of Mental Disorders.* 4th Edition, Text Revision. Washington, DC: American Psychiatric Association.

12. The World Health Organization(2004) *International Statistical Classification of Diseases and Health Related Problems (The) ICD-10.* 2nd edition. Geneva: World Health Organization.

13. Jablensky A (2006): Subtyping schizophrenia: implications for genetic research. *Mol Psychiatry* 11: 815-836.

14. Jablensky A (2007): Living in a Kraepelinian world: Kraepelin's impact on modern psychiatry. *Hist Psychiatry* 18: 381-388.

15. Craddock N, Owen MJ (2005): The beginning of the end for the Kraepelinian dichotomy. *Br J Psychiatry* 186: 364-366.

16. Craddock N, Owen MJ (2007): Rethinking psychosis: the disadvantages of a dichotomous classification now outweigh the advantages. *World Psychiatry* 6: 20-27.

17. Gould TD, Gottesman II (2006): Psychiatric endophenotypes and the development of valid animal models. *Genes Brain Behav* 5: 113-119.

18. Post RM (1992): Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. *Am J Psychiatry* 149: 999-1010.

19. Lieberman JA, Sheitman BB, Kinon BJ (1997): Neurochemical sensitization in the pathophysiology of schizophrenia: deficits and dysfunction in neuronal regulation and plasticity. *Neuropsychopharmacology* 17: 205-229.

20. Post RM, Weiss SR (1998): Sensitization and kindling phenomena in mood, anxiety, and obsessive-compulsive disorders: the role of serotonergic mechanisms in illness progression. *Biol Psychiatry* 44: 193-206.

21. Merikangas KR, Kalaydjian A (2007): Magnitude and impact of comorbidity of mental disorders from epidemiologic surveys. *Curr Opin Psychiatry* 20: 353-358.

22. McIntyre RS, Soczynska JK, Bottas A, Bordbar K, Konarski JZ, Kennedy SH (2006): Anxiety disorders and bipolar disorder: a review. *Bipolar Disord* 8: 665-676.

23. Belzer K, Schneier FR (2004): Comorbidity of anxiety and depressive disorders: issues in conceptualization, assessment, and treatment. *J Psychiatr Pract* 10: 296-306.

24. MacKinnon DF, Zamoiski R (2006): Panic comorbidity with bipolar disorder: what is the manic-panic connection? *Bipolar Disord* 8: 648-664.

25. Singh MK, DelBello MP, Kowatch RA, Strakowski SM (2006): Co-occurrence of bipolar and attention-deficit hyperactivity disorders in children. *Bipolar Disord* 8: 710-720.

26. Cassidy F, Ahearn EP, Carroll BJ (2001): Substance abuse in bipolar disorder. *Bipolar Disord* 3: 181-188.

27. Westermeyer J (2006): Comorbid schizophrenia and substance abuse: a review of epidemiology and course. *Am J Addict* 15: 345-355.

28. Jane-Llopis E, Matytsina I (2006): Mental health and alcohol, drugs and tobacco: a review of the comorbidity between mental disorders and the use of alcohol, tobacco and illicit drugs. *Drug Alcohol Rev* 25: 515-536.

29. Moore TH, Zammit S, Lingford-Hughes A, Barnes TR, Jones PB, Burke M et al (2007): Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. *Lancet* 370: 319-328.

30. Bellivier F (2005): Schizophrenia, antipsychotics and diabetes: Genetic aspects. *Eur Psychiatry* 20 Suppl 4: S335-S339.

31. Scolnick EM (2006): Mechanisms of action of medicines for schizophrenia and bipolar illness: status and limitations. *Biol Psychiatry* 59: 1039-1045.

32. Merikangas KR, Risch N (2003): Will the genomics revolution revolutionize psychiatry? *Am J Psychiatry* 160: 625-35.

33. Miyamoto S, LaMantia AS, Duncan GE, Sullivan P, Gilmore JH, Lieberman JA (2003): Recent advances in the neurobiology of schizophrenia. *Mol Interv* 3: 27-39.

34. Manji HK, Quiroz JA, Payne JL, Singh J, Lopes BP, Viegas JS et al (2003): The underlying neurobiology of bipolar disorder. *World Psychiatry* 2: 136-146.

35. Ross CA, Margolis RL, Reading SA, Pletnikov M, Coyle JT (2006): Neurobiology of schizophrenia. *Neuron* 52: 139-153.

36. Hardy J, Selkoe DJ (2002): The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297: 353-356.

37. Walsh DM, Selkoe DJ (2007): A beta oligomers - a decade of discovery. *J Neurochem* 101: 1172-1184.

38. Faraone, S. V., Tsuang, M. T., and Tsuang, D. W.(1999) *Genetics of mental disorders*. New York: The Guilford Press.

39. Shih RA, Belmonte PL, Zandi PP (2004): A review of the evidence from family, twin and adoption studies for a genetic contribution to adult psychiatric disorders. *Int Rev Psychiatry* 16: 260-283.

40. King LJ (1999): A brief history of psychiatry: millennia past and present. Part IV. *Ann Clin Psychiatry* 11: 175-185.

41. Wong ML, Licinio J (2004): From monoamines to genomic targets: a paradigm shift for drug discovery in depression. *Nat Rev Drug Discov* 3: 136-151.

42. Craddock N, Dave S, Greening J (2001): Association studies of bipolar disorder. *Bipolar Disord* 3: 284-298.

43. Farrall M, Morris AP (2005): Gearing up for genome-wide gene-association studies. *Hum Mol Genet* 14 Spec No. 2: R157-R162.

44. Hirschhorn JN, Daly MJ (2005): Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet* 6: 95-108.

45. Kruglyak L (2008): The road to genome-wide association studies. *Nat Rev Genet*

46. Risch N, Merikangas KR (1993): Linkage studies of psychiatric disorders. *Eur Arch Psychiatry Clin Neurosci* 243: 143-149.

47. Risch N, Merikangas K (1996): The future of genetic studies of complex human diseases. *Science* 273: 1516-1517.

48. Lango H, Weedon MN (2008): What will whole genome searches for susceptibility genes for common complex disease offer to clinical practice? *J Intern Med* 263: 16-27.

49. The Wellcome Trust Case Control Consortium (2007): Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447: 661-678.

50. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V et al (2007): Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. *Nat Genet* 39: 857-864.

51. Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H et al (2007): Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* 316: 1331-1336.

52. Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H et al (2007): Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science* 316: 1336-1341.

53. Stacey SN, Manolescu A, Sulem P, Rafnar T, Gudmundsson J, Gudjonsson SA et al (2007): Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. *Nat Genet* 39: 865-869.

54. Ware JH (2006): The limitations of risk factors as prognostic tools. *N Engl J Med* 355: 2615-2617.

55. Weedon MN, McCarthy MI, Hitman G, Walker M, Groves CJ, Zeggini E et al (2006): Combining information from common type 2 diabetes risk polymorphisms improves disease prediction. *PLoS Med* 3: e374-

56. Janssens AC, Aulchenko YS, Elefante S, Borsboom GJ, Steyerberg EW, van Duijn CM (2006): Predictive testing for complex diseases using multiple genes: fact or fiction? *Genet Med* 8: 395-400.

57. Biesecker BB, Marteau TM (1999): The future of genetic counselling: an international perspective. *Nat Genet* 22: 133-137.

58. Scourfield J, Owen MJ (2004) Genetic counselling. In *Psychiatric genetics and genomics,* Oxford University Press, Oxford, pp 419-427.

59. Finn CT, Smoller JW (2006): Genetic counseling in psychiatry. *Harv Rev Psychiatry* 14: 109-121.

60. Austin JC, Honer WG (2004): The potential impact of genetic counseling for mental illness. *Clin Genet* 67: 134-142.

61. Caspi A, Moffitt TE (2006): Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci* 7: 583-590.

62. Savulescu J, Foddy B (2005): Comment: genetic test available for sports performance. *Br J Sports Med* 39: 472-

63. Ban TA (2006): The role of serendipity in drug discovery. *Dialogues Clin Neurosci* 8: 335-344.

64. Broekkamp CL, Leysen D, Peeters BW, Pinder RM (1995): Prospects for improved antidepressants. *J Med Chem* 38: 4615-4633.

65. De Oliveira IR, Juruena MF (2006): Treatment of psychosis: 30 years of progress. *J Clin Pharm Ther* 31: 523-534.

66. Freeman MP, Freeman SA (2006): Lithium: clinical considerations in internal medicine. *Am J Med* 119: 478-481.

67. Tandon R, Belmaker RH, Gattaz WF, Lopez-Ibor JJ, Jr., Okasha A, Singh B et al (2008): World Psychiatric Association Pharmacopsychiatry Section statement on comparative effectiveness of antipsychotics in the treatment of schizophrenia. *Schizophr Res*

68. Kirsch I, Deacon BJ, Huedo-Medina TB, Scoboria A, Moore TJ, Johnson BT (2008): Initial Severity and Antidepressant Benefits: A Meta-Analysis of Data Submitted to the Food and Drug Administration. *PLOS Medicine* 5: e45-

69. Taylor MJ, Freemantle N, Geddes JR, Bhagwagar Z (2006): Early onset of selective serotonin reuptake inhibitor antidepressant action: systematic review and meta-analysis. *Arch Gen Psychiatry* 63: 1217-1223.

70. Agid Y, Buzsaki G, Diamond DM, Frackowiak R, Giedd J, Girault JA et al (2007): How can drug discovery for psychiatric disorders be improved? *Nat Rev Drug Discov* 6: 189-201.

71. Malhotra AK, Lencz T, Correll CU, Kane JM (2007): Genomics and the future of pharmacotherapy in psychiatry. *Int Rev Psychiatry* 19: 523-530.

72. Kerwin RW, Arranz MJ (2004) Psychopharmacogenetics. In *Psychiatric genetics and genomics,* Oxford University Press, New York, pp 397-417.

73. de Leon J, Armstrong SC, Cozza KL (2006): Clinical guidelines for psychiatrists for the use of pharmacogenetic testing for CYP450 2D6 and CYP450 2C19. *Psychosomatics* 47: 75-85.

74. Pangalos MN, Schechter LE, Hurko O (2007): Drug development for CNS disorders: strategies for balancing risk and reducing attrition. *Nat Rev Drug Discov* 6: 521-532.

75. Porsolt RD, Le Pichon M, Jalfre M (1977): Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266: 730-732.

76. Steru L, Chermat R, Thierry B, Simon P (1985): The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* 85: 367-370.

77. Seligman ME, Beagley G (1975): Learned helplessness in the rat. *J Comp Physiol Psychol* 88: 534-541.

78. Anisman H, Irwin J, Sklar LS (1979): Deficits of escape performance following catecholamine depletion: implications for behavioral deficits induced by uncontrollable stress. *Psychopharmacology (Berl)* 64: 163-170.

79. Cryan JF, Mombereau C (2004): In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. *Mol Psychiatry* 9: 326-357.

80. Cryan JF, Holmes A (2005): The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov* 4: 775-790.

81. Tordjman S, Drapier D, Bonnot O, Graignic R, Fortes S, Cohen D et al (2007): Animal models relevant to schizophrenia and autism: validity and limitations. *Behav Genet* 37: 61-78.

82. Owen MJ, McGuffin P, Gottesman II (2004) The future and post-genomic psychiatry. In *Psychiatric genetics and genomics,*Oxford University Press, New York, pp 449-467.

83. Qian Q, Wang Y, Li J, Yang L, Wang B, Zhou R et al (2007): Evaluation of potential gene-gene interactions for attention deficit hyperactivity disorder in the Han Chinese population. *Am J Med Genet B Neuropsychiatr Genet* 144: 200-206.

84. De Luca V, Tharmalingam S, Sicard T, Kennedy JL (2005): Gene-gene interaction between MAOA and COMT in suicidal behavior. *Neurosci Lett* 383: 151-154.

85. De Luca V, Tharmalingam S, Muller DJ, Wong G, de Bartolomeis A, Kennedy JL (2006): Gene-gene interaction between MAOA and COMT in suicidal behavior: analysis in schizophrenia. *Brain Res* 1097: 26-30.

86. Urwin RE, Bennetts BH, Wilcken B, Lampropoulos B, Beumont PJ, Russell JD et al (2003): Gene-gene interaction between the monoamine oxidase A gene and solute carrier family 6 (neurotransmitter transporter, noradrenalin) member 2 gene in anorexia nervosa (restrictive subtype). *Eur J Hum Genet* 11: 945-950.

87. Ma DQ, Whitehead PL, Menold MM, Martin ER, Ashley-Koch AE, Mei H et al (2005): Identification of significant association and gene-gene interaction of GABA receptor subunit genes in autism. *Am J Hum Genet* 77: 377-388.

88. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H et al (2003): Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 301: 386-389.

89. Caspi A, McClay J, Moffitt TE, Mill J, Martin J, Craig IW et al (2002): Role of genotype in the cycle of violence in maltreated children. *Science* 297: 851-854.

90. Kim-Cohen J, Caspi A, Taylor A, Williams B, Newcombe R, Craig IW et al (2006): MAOA, maltreatment, and gene-environment interaction predicting children's mental health: new evidence and a meta-analysis. *Mol Psychiatry* 11: 903-913.

91. Blennow K, de Leon MJ, Zetterberg H (2006): Alzheimer's disease. *Lancet* 368: 387-403.

92. Samanta MK, Wilson B, Santhi K, Kumar KP, Suresh B (2006): Alzheimer disease and its management: a review. *Am J Ther* 13: 516-526.

93. George-Hyslop PH, Tanzi RE, Polinsky RJ, Haines JL, Nee L, Watkins PC et al (1987): The genetic defect causing familial Alzheimer's disease maps on chromosome 21. *Science* 235: 885-890.

94. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L et al (1991): Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349: 704-706.

95. Murrell J, Farlow M, Ghetti B, Benson MD (1991): A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254: 97-99.

96. Naruse S, Igarashi S, Kobayashi H, Aoki K, Inuzuka T, Kaneko K et al (1991): Mis-sense mutation Val----Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. *Lancet* 337: 978-979.

97. Hardy JA, Higgins GA (1992): Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256: 184-185.

98. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M et al (1995): Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375: 754-760.

99. Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y et al (1995): Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature* 376: 775-778.

100. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH et al (1995): Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* 269: 973-977.

101. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS et al (1993): Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci U S A* 90: 1977-1981.

102. Hardy J (2003): Alzheimer's disease: genetic evidence points to a single pathogenesis. *Ann Neurol* 54: 143-144.

103. (1993): Apolipoprotein E genotype and Alzheimer's disease. Alzheimer's Disease Collaborative Group. *Lancet* 342: 737-738.

104. Pastor P, Roe CM, Villegas A, Bedoya G, Chakraverty S, Garcia G et al (2003): Apolipoprotein Eepsilon4 modifies Alzheimer's disease onset in an E280A PS1 kindred. *Ann Neurol* 54: 163-169.

105. Citron M (2004): Strategies for disease modification in Alzheimer's disease. *Nat Rev Neurosci* 5: 677-685.

106. Giacobini E, Becker RE (2007): One hundred years after the discovery of Alzheimer's disease. A turning point for therapy? *J Alzheimers Dis* 12: 37-52.

107. Aylsworth AS (2006) Defining disease phenotypes. In *Genetic analysis of complex disease*. 2nd edition. Wiley-Liss, Hoboken, N.J., pp 51-89.

108. Ashley-Koch A (2006) Determining genetic component of a disease. In *Genetic analysis of complex disease*. 2nd edition. Wiley-Liss, Hoboken, N.J., pp 91-115.

109. Visscher PM, Hill WG, Wray NR (2008): Heritability in the genomics era - concepts and misconceptions. *Nat Rev Genet*

110. Bearden CE, Reus VI, Freimer NB (2004): Why genetic investigation of psychiatric disorders is so difficult. *Curr Opin Genet Dev* 14: 280-6.

111. Terwilliger, J. D. and Ott, J.(1994) *Handbook of human genetic linkage.* Baltimore, London: The Johns Hopkins University Press.

112. Schmidt S (2006) Advanced parametric linkage analysis. In *Genetic analysis of complex diseases*. 2nd edition. Wiley-Liss, Hoboken, N.J., pp 255-281.

113. Morton NE (1955): Sequential tests for the detection of linkage. *Am J Hum Genet* 7: 277-318.

114. Lander E, Kruglyak L (1995): Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 11: 241-7.

115. Hauser ER, Haines J, Goldgar DE (2006) Nonparametric linkage analysis. In *Genetic analysis of complex disease*. 2nd edition. Wiley-Liss, Hoboken, N.J., pp 283-328.

116. Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES (1996): Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am J Hum Genet* 58: 1347-63.

117. Goldin LR, Weeks DE (1993): Two-locus models of disease: comparison of likelihood and nonparametric linkage methods. *Am J Hum Genet* 53: 908-915.

118. Greenberg DA, Hodge SE, Vieland VJ, Spence MA (1996): Affecteds-only linkage methods are not a panacea. *Am J Hum Genet* 58: 892-895.

119. Greenberg DA, Abreu P, Hodge SE (1998): The power to detect linkage in complex disease by means of simple LOD-score analyses. *Am J Hum Genet* 63: 870-9.

120. Abreu PC, Greenberg DA, Hodge SE (1999): Direct power comparisons between simple LOD scores and NPL scores for linkage analysis in complex diseases. *Am J Hum Genet* 65: 847-57.

121. Durner M, Vieland VJ, Greenberg DA (1999): Further evidence for the increased power of LOD scores compared with nonparametric methods. *Am J Hum Genet* 64: 281-289.

122. Ardlie KG, Kruglyak L, Seielstad M (2002): Patterns of linkage disequilibrium in the human genome. *Nat Rev Genet* 3: 299-309.

123. Martin ER (2006) Linkage disequilibrium and association analysis. In *Genetic analysis of complex disease*. 2nd edition. Wiley-Liss, Hoboken, N.J., pp 329-353.

124. Risch N, Botstein D (1996): A manic depressive history. *Nat Genet* 12: 351-353.

125. Botstein D, Risch N (2003): Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat Genet* 33 Suppl: 228-37.

126. Lewontin RC (1964): The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models. *Genetics* 49: 49-67.

127. Devlin B, Risch N (1995): A comparison of linkage disequilibrium measures for fine-scale mapping. *Genomics* 29: 311-22.

128. Kruglyak L (1999): Prospects for whole-genome linkage disequilibrium mapping of common disease genes. *Nat Genet* 22: 139-144.

129. Pritchard JK, Przeworski M (2001): Linkage disequilibrium in humans: models and data. *Am J Hum Genet* 69: 1-14.

130. Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ et al (2001): Linkage disequilibrium in the human genome. *Nature* 411: 199-204.

131. Jeffreys AJ, Kauppi L, Neumann R (2001): Intensely punctate meiotic recombination in the class II region of the major histocompatibility complex. *Nat Genet* 29: 217-222.

132. Daly MJ, Rioux JD, Schaffner SF, Hudson TJ, Lander ES (2001): High-resolution haplotype structure in the human genome. *Nat Genet* 29: 229-232.

133. Myers S, Bottolo L, Freeman C, McVean G, Donnelly P (2005): A fine-scale map of recombination rates and hotspots across the human genome. *Science* 310: 321-324.

134. The International HapMap Consortium (2005): A haplotype map of the human genome. *Nature* 437: 1299-1320.

135. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B et al (2002): The structure of haplotype blocks in the human genome. *Science* 296: 2225-9.

136. Johnson GC, Esposito L, Barratt BJ, Smith AN, Heward J, Di Genova G et al (2001): Haplotype tagging for the identification of common disease genes. *Nat Genet* 29: 233-237.

137. The International HapMap Consortium (2003): The International HapMap Project. *Nature* 426: 789-96.

138. The International HapMap Consortium (2007): A second generation human haplotype map of over 3.1 million SNPs. *Nature* 449: 851-861.

139. Lander ES (1996): The new genomics: global views of biology. *Science* 274: 536-539.

140. Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N et al (1999): Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* 22: 231-8.

141. Chakravarti A (1999): Population genetics--making sense out of sequence. *Nat Genet* 21: 56-60.

142. Reich DE, Lander ES (2001): On the allelic spectrum of human disease. *Trends Genet* 17: 502-510.

143. Pritchard JK (2001): Are rare variants responsible for susceptibility to complex diseases? *Am J Hum Genet* 69: 124-137.

144. Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH (2004): Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science* 305: 869-872.

145. Fearnhead NS, Wilding JL, Winney B, Tonks S, Bartlett S, Bicknell DC et al (2004): Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. *Proc Natl Acad Sci U S A* 101: 15992-15997.

146. Craddock N, O'Donovan MC, Owen MJ (2005): The genetics of schizophrenia and bipolar disorder: dissecting psychosis. *J Med Genet* 42: 193-204.

147. Terwilliger JD, Weiss KM (1998): Linkage disequilibrium mapping of complex disease: fantasy or reality? *Curr Opin Biotechnol* 9: 578-594.

148. Weiss KM, Terwilliger JD (2000): How many diseases does it take to map a gene with SNPs? *Nat Genet* 26: 151-7.

149. Ioannidis JP (2003): Genetic associations: false or true? *Trends Mol Med* 9: 135-8.

150. Easterbrook PJ, Berlin JA, Gopalan R, Matthews DR (1991): Publication bias in clinical research. *Lancet* 337: 867-872.

151. Cardon LR, Bell JI (2001): Association study designs for complex diseases. *Nat Rev Genet* 2: 91-9.

152. Marchini J, Cardon LR, Phillips MS, Donnelly P (2004): The effects of human population structure on large genetic association studies. *Nat Genet* 36: 512-7.

153. Cardon LR, Palmer LJ (2003): Population stratification and spurious allelic association. *Lancet* 361: 598-604.

154. Spielman RS, McGinnis RE, Ewens WJ (1993): Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52: 506-16.

155. Clayton D (1999): A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am J Hum Genet* 65: 1170-7.

156. Clayton D, Jones H (1999): Transmission/disequilibrium tests for extended marker haplotypes. *Am J Hum Genet* 65: 1161-9.

157. Escamilla MA (2001): Population isolates: their special value for locating genes for bipolar disorder. *Bipolar Disord* 3: 299-317.

158. Peltonen L, Jalanko A, Varilo T (1999): Molecular genetics of the Finnish disease heritage. *Hum Mol Genet* 8: 1913-1923.

159. Peltonen L, Palotie A, Lange K (2000): Use of population isolates for mapping complex traits. *Nat Rev Genet* 1: 182-90.

160. Wright AF, Carothers AD, Pirastu M (1999): Population choice in mapping genes for complex diseases. *Nat Genet* 23: 397-404.

161. Service S, Deyoung J, Karayiorgou M, Roos JL, Pretorious H, Bedoya G et al (2006): Magnitude and distribution of linkage disequilibrium in population isolates and implications for genome-wide association studies. *Nat Genet* 38: 556-560.

162. Service SK, Lang DW, Freimer NB, Sandkuijl LA (1999): Linkage-disequilibrium mapping of disease genes by reconstruction of ancestral haplotypes in founder populations. *Am J Hum Genet* 64: 1728-38.

163. Mathews CA, Reus VI, Bejarano J, Escamilla MA, Fournier E, Herrera LD et al (2004): Genetic studies of neuropsychiatric disorders in Costa Rica: a model for the use of isolated populations. *Psychiatr Genet* 14: 13-23.

164. Pajukanta P, Allayee H, Krass KL, Kuraishy A, Soro A, Lilja HE et al (2003): Combined analysis of genome scans of dutch and finnish families reveals a susceptibility locus for high-density lipoprotein cholesterol on chromosome 16q. *Am J Hum Genet* 72: 903-917.

165. Laitinen T, Daly MJ, Rioux JD, Kauppi P, Laprise C, Petays T et al (2001): A susceptibility locus for asthma-related traits on chromosome 7 revealed by genome-wide scan in a founder population. *Nat Genet* 28: 87-91.

166. Scott DA, Carmi R, Elbedour K, Duyk GM, Stone EM, Sheffield VC (1995): Nonsyndromic autosomal recessive deafness is linked to the DFNB1 locus in a large inbred Bedouin family from Israel. *Am J Hum Genet* 57: 965-968.

167. Reynisdottir I, Thorleifsson G, Benediktsson R, Sigurdsson G, Emilsson V, Einarsdottir AS et al (2003): Localization of a susceptibility gene for type 2 diabetes to chromosome 5q34-q35.2. *Am J Hum Genet* 73: 323-335.

168. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J et al (2006): Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nat Genet* 38: 320-323.

169. Gretarsdottir S, Sveinbjornsdottir S, Jonsson HH, Jakobsson F, Einarsdottir E, Agnarsson U et al (2002): Localization of a susceptibility gene for common forms of stroke to 5q12. *Am J Hum Genet* 70: 593-603.

170. Gretarsdottir S, Thorleifsson G, Reynisdottir ST, Manolescu A, Jonsdottir S, Jonsdottir T et al (2003): The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat Genet* 35: 131-138.

171. Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S et al (2002): Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* 71: 877-892.

172. Blouin JL, Dombroski BA, Nath SK, Lasseter VK, Wolyniec PS, Nestadt G et al (1998): Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. *Nat Genet* 20: 70-73.

173. Freimer NB, Reus VI, Escamilla M, Spesny M, Smith L, Service S et al (1996): An approach to investigating linkage for bipolar disorder using large Costa Rican pedigrees. *Am J Med Genet* 67: 254-63.

174. McInnes LA, Escamilla MA, Service SK, Reus VI, Leon P, Silva S et al (1996): A complete genome screen for genes predisposing to severe bipolar disorder in two Costa Rican pedigrees. *Proc Natl Acad Sci U S A* 93: 13060-5.

175. Escamilla MA, McInnes LA, Spesny M, Reus VI, Service SK, Shimayoshi N et al (1999): Assessing the feasibility of linkage disequilibrium methods for mapping complex traits: an initial screen for bipolar disorder loci on chromosome 18. *Am J Hum Genet* 64: 1670-8.

176. Escamilla MA, McInnes LA, Service SK, Spesny M, Reus VI, Molina J et al (2001): Genome screening for linkage disequilibrium in a Costa Rican sample of patients with bipolar-I disorder: a follow-up study on chromosome 18. *Am J Med Genet* 105: 207-213.

177. McInnes LA, Service SK, Reus VI, Barnes G, Charlat O, Jawahar S et al (2001): Fine-scale mapping of a locus for severe bipolar mood disorder on chromosome 18p11.3 in the Costa Rican population. *Proc Natl Acad Sci U S A* 98: 11485-90.

178. Garner C, McInnes LA, Service SK, Spesny M, Fournier E, Leon P et al (2001): Linkage analysis of a complex pedigree with severe bipolar disorder, using a Markov chain Monte Carlo method. *Am J Hum Genet* 68: 1061-4.

179. Ophoff RA, Escamilla MA, Service SK, Spesny M, Meshi DB, Poon W et al (2002): Genomewide linkage disequilibrium mapping of severe bipolar disorder in a population isolate. *Am J Hum Genet* 71: 565-74.

180. Carvajal-Carmona LG, Ophoff R, Service S, Hartiala J, Molina J, Leon P et al (2003): Genetic demography of Antioquia (Colombia) and the Central Valley of Costa Rica. *Hum Genet* 112: 534-41.

181. Carvajal-Carmona LG, Soto ID, Pineda N, Ortiz-Barrientos D, Duque C, Ospina-Duque J et al (2000): Strong Amerind/white sex bias and a possible Sephardic contribution among the founders of a population in northwest Colombia. *Am J Hum Genet* 67: 1287-95.

182. Bedoya G, Montoya P, Garcia J, Soto I, Bourgeois S, Carvajal L et al (2006): Admixture dynamics in Hispanics: A shift in the nuclear genetic ancestry of a South American population isolate. *Proc Natl Acad Sci U S A* 103: 7234-7239.

183. Alvarez VM (1996) Poblamiento y población en el valle de Aburrá y Medellín, 1541-1591. In *Historia de Medellín.*, Suramericana, Medellín,

184. Herzberg I, Jasinska A, Garcia J, Jawaheer D, Service S, Kremeyer B et al (2006): Convergent linkage evidence from two Latin American population

isolates supports the presence of a susceptibility locus for bipolar disorder in 5q31-34. *Hum Mol Genet* **15**: 3146-3153.

185. Muller-Oerlinghausen B, Berghofer A, Bauer M (2002): Bipolar disorder. *Lancet* 359: 241-247.

186. Benazzi F (2007): Bipolar disorder--focus on bipolar II disorder and mixed depression. *Lancet* 369: 935-945.

187. Jamison, K. R.(1994) *Touched with fire. Manic-depressive illness and the artistic temperament.* New York: Simon & Schuster.

188. Craddock N, Jones I (1999): Genetics of bipolar disorder. *J Med Genet* 36: 585-94.

189. Angst J, Cassano G (2005): The mood spectrum: improving the diagnosis of bipolar disorder. *Bipolar Disord* 7 Suppl 4: 4-12.

190. Phelps J, Angst J, Katzow J, Sadler J (2008): Validity and utility of bipolar spectrum models. *Bipolar Disord* 10: 179-193.

191. Mackin P, Young AH (2004): Rapid cycling bipolar disorder: historical overview and focus on emerging treatments. *Bipolar Disord* 6: 523-529.

192. Pini S, de Q, V, Pagnin D, Pezawas L, Angst J, Cassano GB et al (2005): Prevalence and burden of bipolar disorders in European countries. *Eur Neuropsychopharmacol* 15: 425-434.

193. Craddock N, O'Donovan MC, Owen MJ (2006): Genes for schizophrenia and bipolar disorder? Implications for psychiatric nosology. *Schizophr Bull* 32: 9-16.

194. Akiskal HS, Bourgeois ML, Angst J, Post R, Moller H, Hirschfeld R (2000): Re-evaluating the prevalence of and diagnostic composition within the broad clinical spectrum of bipolar disorders. *J Affect Disord* 59 Suppl 1: S5-S30.

195. Wittchen HU, Jacobi F (2005): Size and burden of mental disorders in Europe--a critical review and appraisal of 27 studies. *Eur Neuropsychopharmacol* 15: 357-376.

196. Leboyer M, Henry C, Paillere-Martinot ML, Bellivier F (2005): Age at onset in bipolar affective disorders: a review. *Bipolar Disord* 7: 111-118.

197. Suominen K, Mantere O, Valtonen H, Arvilommi P, Leppamaki S, Paunio T et al (2007): Early age at onset of bipolar disorder is associated with more severe clinical features but delayed treatment seeking. *Bipolar Disord* 9: 698-705.

198. McElroy SL, Kotwal R, Keck PE, Jr. (2006): Comorbidity of eating disorders with bipolar disorder and treatment implications. *Bipolar Disord* 8: 686-695.

199. Ambelas A (1979): Psychologically stressful events in the precipitation of manic episodes. *Br J Psychiatry* 135: 15-21.

200. Mortensen PB, Pedersen CB, Melbye M, Mors O, Ewald H (2003): Individual and familial risk factors for bipolar affective disorders in Denmark. *Arch Gen Psychiatry* 60: 1209-1215.

201. Smoller JW, Finn CT (2003): Family, twin, and adoption studies of bipolar disorder. *Am J Med Genet C Semin Med Genet* 123: 48-58.

202. Jones I, Kent L, Craddock N (2004) Genetics of affective disorders. In *Psychiatric genetics and genomics.*,Oxford University Press, New York, pp 211-245.

203. Craddock N, Forty L (2006): Genetics of affective (mood) disorders. *Eur J Hum Genet* 14: 660-668.

204. Badner JA, Gershon ES (2002): Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. *Mol Psychiatry* 7: 405-411.

205. Segurado R, Detera-Wadleigh SD, Levinson DF, Lewis CM, Gill M, Nurnberger JI, Jr. et al (2003): Genome scan meta-analysis of schizophrenia and bipolar disorder, part III: Bipolar disorder. *Am J Hum Genet* 73: 49-62.

206. McQueen MB, Devlin B, Faraone SV, Nimgaonkar VL, Sklar P, Smoller JW et al (2005): Combined Analysis from Eleven Linkage Studies of Bipolar Disorder Provides Strong Evidence of Susceptibility Loci on Chromosomes 6q and 8q. *Am J Hum Genet* 77:

207. Serretti A, Mandelli L (2008): The genetics of bipolar disorder: genome 'hot regions,' genes, new potential candidates and future directions. *Mol Psychiatry*

208. Kremeyer B, Herzberg I, Garcia J, Kerr E, Duque C, Parra V et al (2006): Transmission distortion of BDNF variants to bipolar disorder type I patients from a South American population isolate. *Am J Med Genet B Neuropsychiatr Genet* 141: 435-439.

209. Mueser KT, McGurk SR (2004): Schizophrenia. *Lancet* 363: 2063-72.

210. Austin J (2005): Schizophrenia: an update and review. *J Genet Couns* 14: 329-340.

211. Jablensky A (1997): The 100-year epidemiology of schizophrenia. *Schizophr Res* 28: 111-125.

212. Tamminga CA, Holcomb HH (2005): Phenotype of schizophrenia: a review and formulation. *Mol Psychiatry* 10: 27-39.

213. Carpenter WT, Jr., Heinrichs DW, Wagman AM (1988): Deficit and nondeficit forms of schizophrenia: the concept. *Am J Psychiatry* 145: 578-583.

214. Kirkpatrick B, Buchanan RW, Ross DE, Carpenter WT, Jr. (2001): A separate disease within the syndrome of schizophrenia. *Arch Gen Psychiatry* 58: 165-171.

215. Lake CR, Hurwitz N (2007): Schizoaffective disorder merges schizophrenia and bipolar disorders as one disease--there is no schizoaffective disorder. *Curr Opin Psychiatry* 20: 365-379.

216. Saha S, Chant D, Welham J, McGrath J (2005): A systematic review of the prevalence of schizophrenia. *PLOS Medicine* 2: 413-433.

217. Jablensky A (2000): Epidemiology of schizophrenia: the global burden of disease and disability. *Eur Arch Psych Clin Neurosc* 250: 274-285.

218. Mortensen PB, Pedersen CB, Westergaard T, Wohlfahrt J, Ewald H, Mors O et al (1999): Effects of family history and place and season of birth on the risk of schizophrenia. *N Engl J Med* 340: 603-608.

219. Davies G, Welham J, Chant D, Torrey EF, McGrath J (2003): A systematic review and meta-analysis of Northern Hemisphere season of birth studies in schizophrenia. *Schizophr Bull* 29: 587-593.

220. Marcelis M, Navarro-Mateu F, Murray R, Selten JP, van Os J (1998): Urbanization and psychosis: a study of 1942-1978 birth cohorts in The Netherlands. *Psychol Med* 28: 871-879.

221. Hultman CM, Sparen P, Takei N, Murray RM, Cnattingius S (1999): Prenatal and perinatal risk factors for schizophrenia, affective psychosis, and reactive psychosis of early onset: case-control study. *BMJ* 318: 421-426.

222. Jones PB, Rantakallio P, Hartikainen AL, Isohanni M, Sipila P (1998): Schizophrenia as a long-term outcome of pregnancy, delivery, and perinatal complications: a 28-year follow-up of the 1966 north Finland general population birth cohort. *Am J Psychiatry* 155: 355-364.

223. Selten JP, Cantor-Graae E, Kahn RS (2007): Migration and schizophrenia. *Curr Opin Psychiatry* 20: 111-115.

224. Weiser M, Werbeloff N, Vishna T, Yoffe R, Lubin G, Shmushkevitch M et al (2007): Elaboration on immigration and risk for schizophrenia. *Psychol Med* 1-7.

225. Owen MJ, O'Donovan M C, Gottesman II (2004) Schizophrenia. In *Psychiatric genetics and genomics.*, Oxford University Press, New York, pp 247-266.

226. Cardno AG, Gottesman II (2000): Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. *Am J Med Genet* 97: 12-17.

227. Gottesman II, Shields J (1967): A polygenic theory of schizophrenia. *Proc Natl Acad Sci U S A* 58: 199-205.

228. Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I et al (2003): Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet* 73: 34-48.

229. Owen MJ, Craddock N, O'Donovan MC (2005): Schizophrenia: genes at last? *Trends Genet* 21: 518-525.

230. Straub RE, MacLean CJ, Ma Y, Webb BT, Myakishev MV, Harris-Kerr C et al (2002): Genome-wide scans of three independent sets of 90 Irish multiplex schizophrenia families and follow-up of selected regions in all families provides evidence for multiple susceptibility genes. *Mol Psychiatry* 7: 542-559.

231. Straub RE, Jiang Y, MacLean CJ, Ma Y, Webb BT, Myakishev MV et al (2002): Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am J Hum Genet* 71: 337-348.

232. Coyle JT (2006): Glutamate and schizophrenia: beyond the dopamine hypothesis. *Cell Mol Neurobiol* 26: 365-384.

233. Gogos JA, Gerber DJ (2006): Schizophrenia susceptibility genes: emergence of positional candidates and future directions. *Trends Pharmacol Sci* 27: 226-233.

234. Lewandowski KE (2007): Relationship of catechol-O-methyltransferase to schizophrenia and its correlates: evidence for associations and complex interactions. *Harv Rev Psychiatry* 15: 233-244.

235. St Clair D, Blackwood D, Muir W, Carothers A, Walker M, Spowart G et al (1990): Association within a family of a balanced autosomal translocation with major mental illness. *Lancet* 336: 13-16.

236. Lang UE, Puls I, Muller DJ, Strutz-Seebohm N, Gallinat J (2007): Molecular mechanisms of schizophrenia. *Cell Physiol Biochem* 20: 687-702.

237. Mesa NR, Mondragon MC, Soto ID, Parra MV, Duque C, Ortiz-Barrientos D et al (2000): Autosomal, mtDNA, and Y-chromosome diversity in Amerinds: pre- and post-Columbian patterns of gene flow in South America. *Am J Hum Genet* 67: 1277-86.

238. Bedoya G, Garcia J, Montoya P, Rojas W, Amezquita ME, Soto I et al (2006): Análisis de isonimia entre poblaciones del noroeste de Colombia. *Biomedica* 26: 538-545.

239. Escamilla MA, Spesny M, Reus VI, Gallegos A, Meza L, Molina J et al (1996): Use of linkage disequilibrium approaches to map genes for bipolar disorder in the Costa Rican population. *Am J Med Genet* 67: 244-53.

240. Wang S, Ray N, Rojas W, Parra MV, Bedoya G, Gallo C et al (2008): Geographic Patterns of Genome Admixture in Latin American Mestizos. *PLoS Genetics* 4: e1000037-

241. Nurnberger JI, Jr., Blehar MC, Kaufmann CA, York-Cooler C, Simpson SG, Harkavy-Friedman J et al (1994): Diagnostic interview for genetic studies. Rationale, unique features, and training. NIMH Genetics Initiative. *Arch Gen Psychiatry* 51: 849-59.

242. Palacio CA, García J, Arbeláez MP, Sánchez R, Aguirre B, Garcés I et al (2004): Validación de la entrevista diagnóstica para estudios genéticos (DIGS) en Colombia. *Biomédica* 24: 56-62.

243. Ospina-Duque J, Duque C, Carvajal-Carmona L, Ortiz-Barrientos D, Soto I, Pineda N et al (2000): An association study of bipolar mood disorder (type I) with the 5-HTTLPR serotonin transporter polymorphism in a human population isolate from Colombia. *Neurosci Lett* 292: 199-202.

244. Hong KS, McInnes LA, Service SK, Song T, Lucas J, Silva S et al (2004): Genetic mapping using haplotype and model-free linkage analysis supports previous evidence for a locus predisposing to severe bipolar disorder at 5q31-33. *Am J Med Genet* 125B: 83-6.

245. Jasinska A, Service S, Jawahar S, Deyoung J, Levinson M, Kremeyer B et al (2008): Narrow and highly significant linkage signal for severe bipolar disorder on the chromosome 5q33 region in Latin American pedigrees. *(in preparation)*

246. Andreasen NC (1995): Symptoms, signs, and diagnosis of schizophrenia. *Lancet* 346: 477-481.

247. Alda M (2004): The phenotypic spectra of bipolar disorder. *Eur Neuropsychopharmacol* 14 Suppl 2: 94-9.

248. Mullis KB, Faloona FA (1987): Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 155: 335-350.

249. Litt M, Luty JA (1989): A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet* 44: 397-401.

250. Weber JL, May PE (1989): Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 44: 388-396.

251. The Huntington's Disease Collaborative Research Group (1993): A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72: 971-983.

252. Ellegren H (2004): Microsatellites: simple sequences with complex evolution. *Nat Rev Genet* 5: 435-445.

253. Edwards A, Civitello A, Hammond HA, Caskey CT (1991): DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am J Hum Genet* 49: 746-756.

254. Brookes AJ (1999): The essence of SNPs. *Gene* 234: 177-186.

255. Syvanen AC (2001): Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet* 2: 930-942.

256. Mukhopadhyay N, Almasy L, Schroeder M, Mulvihill WP, Weeks DE (2005): Mega2: data-handling for facilitating genetic linkage and association analyses. *Bioinformatics* 21: 2556-7.

257. Sobel E, Lange K (1996): Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker-sharing statistics. *Am J Hum Genet* 58: 1323-37.

258. Lathrop GM, Lalouel JM, White RL (1986): Construction of human linkage maps: likelihood calculations for multilocus linkage analysis. *Genet Epidemiol* 3: 39-52.

259. Lathrop GM, Lalouel JM (1984): Easy calculations of lod scores and genetic risks on small computers. *Am J Hum Genet* 36: 460-465.

260. Lathrop GM, Lalouel JM, Julier C, Ott J (1984): Strategies for multilocus linkage analysis in humans. *Proc Natl Acad Sci U S A* 81: 3443-6.

261. O'Connell JR, Weeks DE (1995): The VITESSE algorithm for rapid exact multilocus linkage analysis via genotype set-recoding and fuzzy inheritance. *Nat Genet* 11: 402-408.

262. Gudbjartsson DF, Jonasson K, Frigge ML, Kong A (2000): Allegro, a new computer program for multipoint linkage analysis. *Nat Genet* 25: 12-3.

263. Abecasis GR, Cherny SS, Cookson WO, Cardon LR (2002): Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 30: 97-101.

264. Sobel E, Papp JC, Lange K (2002): Detection and integration of genotyping errors in statistical genetics. *Am J Hum Genet* 70: 496-508.

265. Lange, K.(2002) *Mathematical and statistical methods for genetic analysis*. 2nd edition. New York, Berlin, Heidelberg: Springer.

266. Raymond M, Rousset F (1995): GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *J Hered* 86: 248-249.

267. Guo SW, Thompson EA (1992): Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48: 361-72.

268. O'Connell JR, Weeks DE (1998): PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 63: 259-66.

269. Douglas JA, Skol AD, Boehnke M (2002): Probability of detection of genotyping errors and mutations as inheritance inconsistencies in nuclear-family data. *Am J Hum Genet* 70: 487-495.

270. Boehnke M (1991): Allele frequency estimation from data on relatives. *Am J Hum Genet* 48: 22-25.

271. Lange K, Cantor R, Horvath S, Perola M, Sabatti C, Sinsheimer J et al (2001): Mendel version 4.0: A complete package for the exact genetic analysis of discrete traits in pedigree and population data sets. *Am J Hum Genet* 69S: A1886-

272. Broman KW, Murray JC, Sheffield VC, White RL, Weber JL (1998): Comprehensive human genetic maps: individual and sex-specific variation in recombination. *Am J Hum Genet* 63: 861-9.

273. Thiele H, Nurnberg P (2005): HaploPainter: a tool for drawing pedigrees with complex haplotypes. *Bioinformatics* 21: 1730-2.

274. Purcell S, Daly MJ, Sham PC (2006): WHAP: haplotype-based association analysis. *Bioinformatics*

275. Dudbridge F (2003): Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 25: 115-21.

276. Barrett JC, Fry B, Maller J, Daly MJ (2005): Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263-265.

277. Abecasis GR, Cardon LR, Cookson WO (2000): A general test of association for quantitative traits in nuclear families. *Am J Hum Genet* 66: 279-92.

278. Paunio T, Ekelund J, Varilo T, Parker A, Hovatta I, Turunen JA et al (2001): Genome-wide scan in a nationwide study sample of schizophrenia families in Finland reveals susceptibility loci on chromosomes 2q and 5q. *Hum Mol Genet* 10: 3037-3048.

279. Sklar P, Pato MT, Kirby A, Petryshen TL, Medeiros H, Carvalho C et al (2004): Genome-wide scan in Portuguese Island families identifies 5q31-5q35 as a susceptibility locus for schizophrenia and psychosis. *Mol Psychiatry* 9: 213-218.

280. DeLisi LE, Mesen A, Rodriguez C, Bertheau A, LaPrade B, Llach M et al (2002): Genome-wide scan for linkage to schizophrenia in a Spanish-origin cohort from Costa Rica. *Am J Med Genet* 114: 497-508.

281. Escamilla MA, Ontiveros A, Nicolini H, Raventos H, Mendoza R, Medina R et al (2007): A genome-wide scan for schizophrenia and psychosis susceptibility loci in families of Mexican and Central American ancestry. *Am J Med Genet B Neuropsychiatr Genet* 144: 193-199.

282. Angst J (1993): Today's perspective on Kraepelin's nosology of endogenous psychoses. *Eur Arch Psychiatry Clin Neurosci* 243: 164-170.

283. Kerner B, Brugman DL, Freimer NB (2007): Evidence of linkage to psychosis on chromosome 5q33-34 in pedigrees ascertained for bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet* 144: 74-78.

284. Pimm J, McQuillin A, Thirumalai S, Lawrence J, Quested D, Bass N et al (2005): The Epsin 4 gene on chromosome 5q, which encodes the clathrin-associated protein enthoprotin, is involved in the genetic susceptibility to schizophrenia. *Am J Hum Genet* 76: 902-907.

285. Tang RQ, Zhao XZ, Shi YY, Tang W, Gu NF, Feng GY et al (2006): Family-based association study of Epsin 4 and Schizophrenia. *Mol Psychiatry* 11: 395-399.

286. Liou YJ, Lai IC, Wang YC, Bai YM, Lin CC, Lin CY et al (2006): Genetic analysis of the human ENTH (Epsin 4) gene and schizophrenia. *Schizophr Res* 84: 236-243.

287. Gurling H, Pimm J, McQuillin A (2007): Replication of genetic association studies between markers at the Epsin 4 gene locus and schizophrenia in two Han Chinese samples. *Schizophr Res* 89: 357-359.

288. Wasiak S, Legendre-Guillemin V, Puertollano R, Blondeau F, Girard M, de Heuvel E et al (2002): Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. *J Cell Biol* 158: 855-862.

289. Kalthoff C, Groos S, Kohl R, Mahrhold S, Ungewickell EJ (2002): Clint: a novel clathrin-binding ENTH-domain protein at the Golgi. *Mol Biol Cell* 13: 4060-4073.

290. Hirst J, Motley A, Harasaki K, Peak Chew SY, Robinson MS (2003): EpsinR: an ENTH domain-containing protein that interacts with AP-1. *Mol Biol Cell* 14: 625-641.

291. Legendre-Guillemin V, Wasiak S, Hussain NK, Angers A, McPherson PS (2004): ENTH/ANTH proteins and clathrin-mediated membrane budding. *J Cell Sci* 117: 9-18.

292. Horvath CA, Vanden Broeck D, Boulet GA, Bogers J, De Wolf MJ (2007): Epsin: inducing membrane curvature. *Int J Biochem Cell Biol* 39: 1765-1770.

293. Melikian HE (2004): Neurotransmitter transporter trafficking: endocytosis, recycling, and regulation. *Pharmacol Ther* 104: 17-27.

294. Man HY, Lin JW, Ju WH, Ahmadian G, Liu L, Becker LE et al (2000): Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron* 25: 649-662.

295. Man HY, Ju W, Ahmadian G, Wang YT (2000): Intracellular trafficking of AMPA receptors in synaptic plasticity. *Cell Mol Life Sci* 57: 1526-1534.

296. Wang YT, Linden DJ (2000): Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron* 25: 635-647.

297. Chidambaram S, Mullers N, Wiederhold K, Haucke V, von Mollard GF (2004): Specific interaction between SNAREs and epsin N-terminal

homology (ENTH) domains of epsin-related proteins in trans-Golgi network to endosome transport. *J Biol Chem* 279: 4175-4179.

298. Miller SE, Collins BM, McCoy AJ, Robinson MS, Owen DJ (2007): A SNARE-adaptor interaction is a new mode of cargo recognition in clathrin-coated vesicles. *Nature* 450: 570-574.

299. Chidambaram S, Zimmermann J, von Mollard GF (2008): ENTH domain proteins are cargo adaptors for multiple SNARE proteins at the TGN endosome. *J Cell Sci* 121: 329-338.

300. Honer WG, Falkai P, Bayer TA, Xie J, Hu L, Li HY et al (2002): Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. *Cereb Cortex* 12: 349-356.

301. O'Connor JA, Hemby SE (2007): Elevated GRIA1 mRNA expression in Layer II/III and V pyramidal cells of the DLPFC in schizophrenia. *Schizophr Res* 97: 277-288.

302. Kerner B, Jasinska AJ, Deyoung J, Almonte M, Choi OW, Freimer NB (2008): Polymorphisms in the GRIA1 gene region in psychotic bipolar disorder. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*

303. Ohtsuki T, Ishiguro H, Detera-Wadleigh SD, Toyota T, Shimizu H, Yamada K et al (2002): Association between serotonin 4 receptor gene polymorphisms and bipolar disorder in Japanese case-control samples and the NIMH Genetics Initiative Bipolar Pedigrees. *Mol Psychiatry* 7: 954-961.

304. Suzuki T, Iwata N, Kitamura Y, Kitajima T, Yamanouchi Y, Ikeda M et al (2003): Association of a haplotype in the serotonin 5-HT4 receptor gene (HTR4) with Japanese schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* 121B: 7-13.

305. Petryshen TL, Middleton FA, Tahl AR, Rockwell GN, Purcell S, Aldinger KA et al (2005): Genetic investigation of chromosome 5q GABAA receptor subunit genes in schizophrenia. *Mol Psychiatry* 10: 1074-88, 1057.

306. Horiuchi Y, Nakayama J, Ishiguro H, Ohtsuki T, Detera-Wadleigh SD, Toyota T et al (2004): Possible association between a haplotype of the GABA-A receptor alpha 1 subunit gene (GABRA1) and mood disorders. *Biol Psychiatry* 55: 40-45.

307. McGuffin P, Katz R (1989): The genetics of depression and manic-depressive disorder. *Br J Psychiatry* 155: 294-304.

308. Risch N (1991): A note on multiple testing procedures in linkage analysis. *Am J Hum Genet* 48: 1058-64.

309. Ott J (1989): Computer-simulation methods in human linkage analysis. *Proc Natl Acad Sci U S A* 86: 4175-8.

310. Weeks DE, Ott J, Lathrop GM (1990): SLINK: a general simulation program for linkage analysis. *Am J Hum Genet* 47: A204 (abstr)-

311. Lindner TH, Hoffmann K (2005): easyLINKAGE: a PERL script for easy and automated two-/multi-point linkage analyses. *Bioinformatics* 21: 405-7.

312. Hoffmann K, Lindner TH (2005): easyLINKAGE-Plus--automated linkage analyses using large-scale SNP data. *Bioinformatics* 21: 3565-7.

313. Schuelke M (2000): An economic method for the fluorescent labeling of PCR fragments. *Nat Biotechnol* 18: 233-234.

314. Bull LN, Pabon-Pena CR, Freimer NB (1999): Compound microsatellite repeats: practical and theoretical features. *Genome Res* 9: 830-8.

315. Brinkmann B, Klintschar M, Neuhuber F, Huhne J, Rolf B (1998): Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. *Am J Hum Genet* 62: 1408-1415.

316. Grimaldi MC, Crouau-Roy B (1997): Microsatellite allelic homoplasy due to variable flanking sequences. *J Mol Evol* 44: 336-340.

317. Park N, Juo SH, Cheng R, Liu J, Loth JE, Lilliston B et al (2004): Linkage analysis of psychosis in bipolar pedigrees suggests novel putative loci for bipolar disorder and shared susceptibility with schizophrenia. *Mol Psychiatry* 9: 1091-1099.

318. Cheng R, Juo SH, Loth JE, Nee J, Iossifov I, Blumenthal R et al (2006): Genome-wide linkage scan in a large bipolar disorder sample from the National Institute of Mental Health genetics initiative suggests putative loci for bipolar disorder, psychosis, suicide, and panic disorder. *Mol Psychiatry* 11: 252-260.

319. Detera-Wadleigh SD, Badner JA, Berrettini WH, Yoshikawa T, Goldin LR, Turner G et al (1999): A high-density genome scan detects evidence for a bipolar-disorder susceptibility locus on 13q32 and other potential loci on 1q32 and 18p11.2. *Proc Natl Acad Sci U S A* 96: 5604-9.

320. Ekholm JM, Kieseppa T, Hiekkalinna T, Partonen T, Paunio T, Perola M et al (2003): Evidence of susceptibility loci on 4q32 and 16p12 for bipolar disorder. *Hum Mol Genet* 12: 1907-15.

321. McInnis MG, Dick DM, Willour VL, Avramopoulos D, MacKinnon DF, Simpson SG et al (2003): Genome-wide scan and conditional analysis in bipolar disorder: evidence for genomic interaction in the National Institute of Mental Health genetics initiative bipolar pedigrees. *Biol Psychiatry* 54: 1265-73.

322. Schumacher J, Kaneva R, Jamra RA, Diaz GO, Ohlraun S, Milanova V et al (2005): Genomewide scan and fine-mapping linkage studies in four European samples with bipolar affective disorder suggest a new susceptibility locus on

chromosome 1p35-p36 and provides further evidence of loci on chromosome 4q31 and 6q24. *Am J Hum Genet* 77: 1102-1111.

323. Marcheco-Teruel B, Flint TJ, Wikman FP, Torralbas M, Gonzalez L, Blanco L et al (2006): A genome-wide linkage search for bipolar disorder susceptibility loci in a large and complex pedigree from the eastern part of Cuba. *Am J Med Genet B Neuropsychiatr Genet* 141: 833-843.

324. Cassidy F, Zhao C, Badger J, Claffey E, Dobrin S, Roche S et al (2007): Genome-wide scan of bipolar disorder and investigation of population stratification effects on linkage: support for susceptibility loci at 4q21, 7q36, 9p21, 12q24, 14q24, and 16p13. *Am J Med Genet B Neuropsychiatr Genet* 144B: 791-801.

325. Ewald H, Flint T, Kruse TA, Mors O (2002): A genome-wide scan shows significant linkage between bipolar disorder and chromosome 12q24.3 and suggestive linkage to chromosomes 1p22-21, 4p16, 6q14-22, 10q26 and 16p13.3. *Mol Psychiatry* 7: 734-744.

326. Rice JP, Goate A, Williams JT, Bierut L, Dorr D, Wu W et al (1997): Initial genome scan of the NIMH genetics initiative bipolar pedigrees: chromosomes 1, 6, 8, 10, and 12. *Am J Med Genet* 74: 247-253.

327. Blackwood DH, Fordyce A, Walker MT, St Clair DM, Porteous DJ, Muir WJ (2001): Schizophrenia and affective disorders--cosegregation with a translocation at chromosome 1q42 that directly disrupts brain-expressed genes: clinical and P300 findings in a family. *Am J Hum Genet* 69: 428-433.

328. Curtis D, Kalsi G, Brynjolfsson J, McInnis M, O'Neill J, Smyth C et al (2003): Genome scan of pedigrees multiply affected with bipolar disorder provides further support for the presence of a susceptibility locus on chromosome 12q23-q24, and suggests the presence of additional loci on 1p and 1q. *Psychiatr Genet* 13: 77-84.

329. Fallin MD, Lasseter VK, Wolyniec PS, McGrath JA, Nestadt G, Valle D et al (2004): Genomewide linkage scan for bipolar-disorder susceptibility loci among Ashkenazi Jewish families. *Am J Hum Genet* 75: 204-19.

330. Savitz J, Cupido CL, Ramesar RK (2007): Preliminary evidence for linkage to chromosome 1q31-32, 10q23.3, and 16p13.3 in a South African cohort with bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet* 144: 383-387.

331. Brzustowicz LM, Hodgkinson KA, Chow EW, Honer WG, Bassett AS (2000): Location of a major susceptibility locus for familial schizophrenia on chromosome 1q21-q22. *Science* 288: 678-82.

332. Gurling HM, Kalsi G, Brynjolfson J, Sigmundsson T, Sherrington R, Mankoo BS et al (2001): Genomewide genetic linkage analysis confirms the presence of susceptibility loci for schizophrenia, on chromosomes 1q32.2, 5q33.2, and 8p21-22 and provides support for linkage to schizophrenia, on chromosomes 11q23.3-24 and 20q12.1-11.23. *Am J Hum Genet* 68: 661-673.

333. Bulayeva KB, Glatt SJ, Bulayev OA, Pavlova TA, Tsuang MT (2007): Genome-wide linkage scan of schizophrenia: a cross-isolate study. *Genomics* 89: 167-177.

334. Brzustowicz LM, Simone J, Mohseni P, Hayter JE, Hodgkinson KA, Chow EW et al (2004): Linkage disequilibrium mapping of schizophrenia susceptibility to the CAPON region of chromosome 1q22. *Am J Hum Genet* 74: 1057-63.

335. Kremeyer B, Garcia J, Kymalainen H, Wratten N, Restrepo G, Palacio C et al (2008): Evidence for a role of the *NOS1AP (CAPON)* gene in schizophrenia and its clinical dimensions: an association study in a South American population isolate. *Human Heredity* (in press):

336. Cordeiro Q, Talkowski ME, Chowdari KV, Wood J, Nimgaonkar V, Vallada H (2005): Association and linkage analysis of RGS4 polymorphisms with schizophrenia and bipolar disorder in Brazil. *Genes Brain Behav* 4: 45-50.

337. Prata DP, Breen G, Munro J, Sinclair M, Osborne S, Li T et al (2006): Bipolar 1 disorder is not associated with the RGS4, PRODH, COMT and GRK3 genes. *Psychiatr Genet* 16: 229-230.

338. Fallin MD, Lasseter VK, Avramopoulos D, Nicodemus KK, Wolyniec PS, McGrath JA et al (2005): Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. *Am J Hum Genet* 77: 918-936.

339. Xu B, Wratten N, Charych EI, Buyske S, Firestein BL, Brzustowicz LM (2005): Increased Expression in Dorsolateral Prefrontal Cortex of CAPON in Schizophrenia and Bipolar Disorder. *PLoS Med* 2: e263-

340. Cichon S, Schumacher J, Muller DJ, Hurter M, Windemuth C, Strauch K et al (2001): A genome screen for genes predisposing to bipolar affective disorder detects a new susceptibility locus on 8q. *Hum Mol Genet* 10: 2933-2944.

341. Zandi PP, Badner JA, Steele J, Willour VL, Miao K, MacKinnon DF et al (2007): Genome-wide linkage scan of 98 bipolar pedigrees and analysis of clinical covariates. *Mol Psychiatry* 12: 630-639.

342. Shink E, Harvey M, Tremblay M, Gagne B, Belleau P, Raymond C et al (2005): Analysis of microsatellite markers and single nucleotide polymorphisms in candidate genes for susceptibility to bipolar affective disorder in the chromosome 12Q24.31 region. *Am J Med Genet B Neuropsychiatr Genet* 135B: 50-58.

343. Morissette J, Villeneuve A, Bordeleau L, Rochette D, Laberge C, Gagne B et al (1999): Genome-wide search for linkage of bipolar affective disorders in a very large pedigree derived from a homogeneous population in quebec points to a locus of major effect on chromosome 12q23-q24. *Am J Med Genet* 88: 567-587.

344. Tease C, Hartshorne GM, Hulten MA (2002): Patterns of meiotic recombination in human fetal oocytes. *Am J Hum Genet* 70: 1469-1479.

345. Kong A, Gudbjartsson DF, Sainz J, Jonsdottir GM, Gudjonsson SA, Richardsson B et al (2002): A high-resolution recombination map of the human genome. *Nat Genet* 31: 241-7.

346. Sun F, Oliver-Bonet M, Liehr T, Starke H, Ko E, Rademaker A et al (2004): Human male recombination maps for individual chromosomes. *Am J Hum Genet* 74: 521-531.

347. Kelsoe JR, Spence MA, Loetscher E, Foguet M, Sadovnick AD, Remick RA et al (2001): A genome survey indicates a possible susceptibility locus for bipolar disorder on chromosome 22. *Proc Natl Acad Sci U S A* 98: 585-590.

348. Shaw SH, Mroczkowski-Parker Z, Shekhtman T, Alexander M, Remick RA, Sadovnick AD et al (2003): Linkage of a bipolar disorder susceptibility locus to human chromosome 13q32 in a new pedigree series. *Mol Psychiatry* 8: 558-564.

349. Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H et al (2002): Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc Natl Acad Sci U S A* 99: 13675-13680.

350. Hattori E, Liu C, Badner JA, Bonner TI, Christian SL, Maheshwari M et al (2003): Polymorphisms at the G72/G30 gene locus, on 13q33, are associated with bipolar disorder in two independent pedigree series. *Am J Hum Genet* 72: 1131-1140.

351. Schumacher J, Jamra RA, Freudenberg J, Becker T, Ohlraun S, Otte AC et al (2004): Examination of G72 and D-amino-acid oxidase as genetic risk factors for schizophrenia and bipolar affective disorder. *Mol Psychiatry* 9: 203-207.

352. Prata DP, Breen G, Osborne S, Munro J, St Clair D, Collier D (2007): Association of DAO and G72(DAOA)/G30 genes with bipolar affective disorder. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*

353. Kaneva RP, Chorbov VM, Milanova VK, Kostov CS, Nickolov KI, Chakarova CF et al (2004): Linkage analysis in bipolar pedigrees adds support for a susceptibility locus on 21q22. *Psychiatr Genet* 14: 101-106.

354. Lin PI, McInnis MG, Potash JB, Willour VL, MacKinnon DF, Miao K et al (2005): Assessment of the effect of age at onset on linkage to bipolar disorder: evidence on chromosomes 18p and 21q. *Am J Hum Genet* 77: 545-555.

355. Straub RE, Lehner T, Luo Y, Loth JE, Shao W, Sharpe L et al (1994): A possible vulnerability locus for bipolar affective disorder on chromosome 21q22.3. *Nat Genet* 8: 291-296.

356. Aita VM, Liu J, Knowles JA, Terwilliger JD, Baltazar R, Grunn A et al (1999): A comprehensive linkage analysis of chromosome 21q22 supports prior evidence for a putative bipolar affective disorder locus. *Am J Hum Genet* 64: 210-217.

357. Smyth C, Kalsi G, Curtis D, Brynjolfsson J, O'Neill J, Rifkin L et al (1997): Two-locus admixture linkage analysis of bipolar and unipolar affective disorder supports the presence of susceptibility loci on chromosomes 11p15 and 21q22. *Genomics* 39: 271-278.

358. Kwok JB, Adams LJ, Salmon JA, Donald JA, Mitchell PB, Schofield PR (1999): Nonparametric simulation-based statistical analyses for bipolar affective disorder locus on chromosome 21q22.3. *Am J Med Genet* 88: 99-102.

359. McQuillin A, Bass NJ, Kalsi G, Lawrence J, Puri V, Choudhury K et al (2006): Fine mapping of a susceptibility locus for bipolar and genetically related unipolar affective disorders, to a region containing the C21ORF29 and TRPM2 genes on chromosome 21q22.3. *Mol Psychiatry* 11: 134-142.

360. Ewald H, Eiberg H, Mors O, Flint T, Kruse TA (1996): Linkage study between manic-depressive illness and chromosome 21. *Am J Med Genet* 67: 218-24.

361. Vallada H, Craddock N, Vasques L, Curtis D, Kirov G, Lauriano V et al (1996): Linkage studies in bipolar affective disorder with markers on chromosome 21. *J Affect Disord* 41: 217-221.

362. Lin YM, Yang HC, Lai TJ, Fann CS, Sun HS (2003): Receptor mediated effect of serotonergic transmission in patients with bipolar affective disorder. *J Med Genet* 40: 781-786.

363. Vincent JB, Masellis M, Lawrence J, Choi V, Gurling HM, Parikh SV et al (1999): Genetic association analysis of serotonin system genes in bipolar affective disorder. *Am J Psychiatry* 156: 136-138.

364. Gerhard DS, LaBuda MC, Bland SD, Allen C, Egeland JA, Pauls DL (1994): Initial report of a genome search for the affective disorder predisposition gene in the old order Amish pedigrees: chromosomes 1 and 11. *Am J Med Genet* 54: 398-404.

365. LaBuda MC, Maldonado M, Marshall D, Otten K, Gerhard DS (1996): A follow-up report of a genome search for affective disorder predisposition loci in the Old Order Amish. *Am J Hum Genet* 59: 1343-1362.

366. Avramopoulos D, Lasseter VK, Fallin MD, Wolyniec PS, McGrath JA, Nestadt G et al (2007): Stage II follow-up on a linkage scan for bipolar disorder in the Ashkenazim provides suggestive evidence for chromosome 12p and the GRIN2B gene. *Genet Med* 9: 745-751.

367. Shink E, Morissette J, Barden N (2003): Genetic heterogeneity in a very large bipolar affective disorder pedigree from Quebec. *Am J Med Genet B Neuropsychiatr Genet* 119B: 65-68.

368. Gottesman II, Shields J (1973): Genetic theorizing and schizophrenia. *Br J Psychiatry* 122: 15-30.

369. Gottesman I, Gould TD (2003): The endophenotype concept in psychiatry: etymology and strategic intentions. *Am J Psychiatry* 160: 636-45.

370. Brzustowicz LM, Honer WG, Chow EW, Little D, Hogan J, Hodgkinson K et al (1999): Linkage of familial schizophrenia to chromosome 13q32. *Am J Hum Genet* 65: 1096-1103.

371. Cao Q, Martinez M, Zhang J, Sanders AR, Badner JA, Cravchik A et al (1997): Suggestive evidence for a schizophrenia susceptibility locus on chromosome 6q and a confirmation in an independent series of pedigrees. *Genomics* 43: 1-8.

372. Lindholm E, Ekholm B, Shaw S, Jalonen P, Johansson G, Pettersson U et al (2001): A schizophrenia-susceptibility locus at 6q25, in one of the world's largest reported pedigrees. *Am J Hum Genet* 69: 96-105.

373. Shaw SH, Kelly M, Smith AB, Shields G, Hopkins PJ, Loftus J et al (1998): A genome-wide search for schizophrenia susceptibility genes. *Am J Med Genet* 81: 364-376.

374. Hwu HG, Liu CM, Fann CS, Ou-Yang WC, Lee SF (2003): Linkage of schizophrenia with chromosome 1q loci in Taiwanese families. *Mol Psychiatry* 8: 445-452.

375. Brzustowicz LM, Hayter JE, Hodgkinson KA, Chow EW, Bassett AS (2002): Fine mapping of the schizophrenia susceptibility locus on chromosome 1q22. *Hum Hered* 54: 199-209.

376. Coyle JT, Tsai G, Goff D (2003): Converging evidence of NMDA receptor hypofunction in the pathophysiology of schizophrenia. *Ann N Y Acad Sci* 1003: 318-327.

377. Javitt DC (2004): Glutamate as a therapeutic target in psychiatric disorders. *Mol Psychiatry* 9: 984-97, 979.

378. Jaffrey SR, Snowman AM, Eliasson MJ, Cohen NA, Snyder SH (1998): CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. *Neuron* 20: 115-24.

379. Zheng Y, Li H, Qin W, Chen W, Duan Y, Xiao Y et al (2005): Association of the carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase gene with schizophrenia in the Chinese Han population. *Biochem Biophys Res Commun* 328: 809-15.

380. Puri V, McQuillin A, Thirumalai S, Lawrence J, Krasucki R, Choudhury K et al (2006): Failure to confirm allelic association between markers at the CAPON gene locus and schizophrenia in a British sample. *Biological Psychiatry* 59: 195-197.

381. Miranda A, Garcia J, Lopez C, Gordon D, Palacio C, Restrepo G et al (2006): Putative association of the carboxy-terminal PDZ ligand of neuronal nitric oxide synthase gene (CAPON) with schizophrenia in a Colombian population. *Schizophr Res* 82: 283-285.

382. Fanous AH, Kendler KS (2005): Genetic heterogeneity, modifier genes, and quantitative phenotypes in psychiatric illness: searching for a framework. *Mol Psychiatry* 10: 6-13.

383. Peralta V, Cuesta MJ (2001): How many and which are the psychopathological dimensions in schizophrenia? Issues influencing their ascertainment. *Schizophr Res* 49: 269-285.

384. Fanous AH, Neale MC, Straub RE, Webb BT, O'Neill AF, Walsh D et al (2004): Clinical features of psychotic disorders and polymorphisms in HT2A, DRD2, DRD4, SLC6A3 (DAT1), and BDNF: a family based association study. *Am J Med Genet B Neuropsychiatr Genet* 125: 69-78.

385. Cardno AG, Bowen T, Guy CA, Jones LA, McCarthy G, Williams NM (1999): CAG repeat length in the hKCa3 gene and symptom dimensions in schizophrenia. *Biol Psychiatry* 45: 1592-1596.

386. Ritsner M, Modai I, Ziv H, Amir S, Halperin T, Weizman A et al (2002): An Association of CAG Repeats at the KCNN3 Locus with Symptom Dimensions of Schizophrenia. *Biol Psychiatry* 51: 788-794.

387. Fanous AH, van den Oord EJ, Riley BP, Aggen SH, Neale MC, O'Neill FA et al (2005): Relationship between a high-risk haplotype in the DTNBP1 (dysbindin) gene and clinical features of schizophrenia. *Am J Psychiatry* 162: 1824-1832.

388. Andreasen, N. C.(1984) *The Scale for the assessment of positive symptoms (SAPS)*. Iowa City, IA: The University of Iowa.

389. Andreasen NC (1989): The Scale for the Assessment of Negative Symtoms (SANS): conceptual and theorical foundations. *Br J Psychiatry Suppl* 7: 49-58.

390. García J, Palacio CA, Garcés I, Arbeláez MP, Sánchez R, López CA et al (2003): Inferencias fenomenológicas y nosoógicas en la esquizofrenia a partir de la validación de las escalas de síntomas positivos (SAPS) y de síntomas negativos (SANS) en Colombia. *Revista de Neuro-Psiquiatría* 66: 195-214.

391. Dawson B, Trapp RG, Trapp R (2004) Statistical methods for multiple variables. In *Basic And Clinical Biostatistics*. 4th edition. Appleton & Lange, Norwalk CT, pp 245-279.

392. Kline, P.(1993) *The Handbook of Psychological Testing*. London: Routledge.

393. Kaiser HF (1974): Index of Factorial Simplicity. *Psychometrika* 39: 31-36.

394. Cattell RB (1966): The scree test for the number of factors. *Multivariate Behavioral Research* 1: 245-276.

395. Abecasis GR, Cookson WO, Cardon LR (2000): Pedigree tests of transmission disequilibrium. *Eur J Hum Genet* 8: 545-551.

396. Wratten N, Bruse SE, Azaro MA, Simone J, Hayter JE, Brzustowicz LM Identifying schizophrenia-associated non-coding variants in the CAPON gene. (2005)[Abstract]: 55th Annual Meeting of the American Society of Human Genetics, Salt Lake City, Utah, 10/25/05-10/29/05.

397. Taylor JD, Briley D, Nguyen Q, Long K, Iannone MA, Li MS et al (2001): Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. *Biotechniques* 30: 661-669.

398. Bruse SE, McGregor N, Azaro MA, Xu B, Brzustowicz LM An inexpensive bead-based oligonucleotide ligation assay for SNP genotyping. (2006)[Abstract]: 56th Meeting of the American Society of Human Genetics, New Orleans, Louisiana, 10/9/06-10/13/06.

399. Pagani F, Raponi M, Baralle FE (2005): Synonymous mutations in CFTR exon 12 affect splicing and are not neutral in evolution. *Proc Natl Acad Sci U S A* 102: 6368-6372.

400. Brzustowicz LM (2008): NOS1AP in schizophrenia. *Curr Psychiatry Rep* 10: 158-163.

401. Puri V, McQuillin A, Choudhury K, Datta S, Pimm J, Thirumalai S et al (2006): Fine Mapping by Genetic Association Implicates the Chromosome 1q23.3 Gene UHMK1, Encoding a Serine/Threonine Protein Kinase, as a Novel Schizophrenia Susceptibility Gene. *Biol Psychiatry* 61: 873-879.

402. Ioannidis JP, Trikalinos TA, Ntzani EE, Contopoulos-Ioannidis DG (2003): Genetic associations in large versus small studies: an empirical assessment. *Lancet* 361: 567-71.

403. Carpenter WT, Jr., Arango C, Buchanan RW, Kirkpatrick B (1999): Deficit psychopathology and a paradigm shift in schizophrenia research. *Biol Psychiatry* 46: 352-360.

404. Gerbaldo H, Fickinger MP, Wetzel H, Helisch A, Philipp M, Benkert O (1995): Primary enduring negative symptoms in schizophrenia and major depression. *J Psychiatr Res* 29: 297-302.

405. Herbener ES, Harrow M (2001): Longitudinal assessment of negative symptoms in schizophrenia/schizoaffective patients, other psychotic patients, and depressed patients. *Schizophr Bull* 27: 527-537.

406. Peralta V, Cuesta MJ (2004): The deficit syndrome of the psychotic illness. A clinical and nosological study. *Eur Arch Psychiatry Clin Neurosci* 254: 165-171.

URLs:

The Centre of Comparative Genomics Sequencing and Genotyping Service

http://www.ucl.ac.uk/biology/centre-for-comparative-genomics/

Ensembl	http://www.ensembl.org
FastSLINK	http://watson.hgen.pitt.edu/register/soft_doc.html
Genepop on the web	http://genepop.curtin.edu.au/
The Genome Database	http://www.gdb.org
HapMap	http://www.hapmap.org

The Mammalian Genotyping Service

http://research.marshfieldclinic.org/genetics/home/index.asp

NCBI	http://www.ncbi.nlm.nih.gov/
NCBI dbSNP	http://www.ncbi.nlm.nih.gov/projects/SNP/
Primer3	http://frodo.wi.mit.edu/

The University of California at Santa Cruz Genome Browser

http://genome.ucsc.edu

8. Appendix

8.1. Abbreviations

%	percent
°C	degrees centigrade
bp	base pairs
CEPH	Centre d'Étude du Polymorphisme Humain
dH_2O	de-ionised water
ddNPTs	di-deoxynucleosidetriphosphates
dNTPs	deoxynucleosidetriphosphates
EDTA	ethylene diamine tetraacetic acid
ELOD	expected LOD score
g	gram
Genmol	Molecular Genetics Laboratory; Prof. Andrés Ruiz-Linares' research laboratory at U de A, Medellín
GIPSI	Group of Psychiatric Investigation, our clinical collaborators at U de A, Medellín
h	hour(s)
kb	kilobases (1000 bp)
LOD	logarithm of the odds
min	minute(s)
ng	nanogram
nm	nanometer
NPL	non-parametric linkage
µg	microgram
µl	microliter
µM	micromolar
mM	millimolar
BP	bipolar disorder
PCR	polymerase chain reaction
rpm	rounds per minute
sec	second(s)

STR	short tandem repeats (microsatellite marker)
taq	*Thermus aquaticus*
TBE	tris-borate-EDTA
TS	Tourette's Syndrome
u	unit(s)
UCLA	University of California at Los Angeles
U de A	Universidad de Antioquia
UV	ultraviolet
V	Volt
W	Watt(s)

8.2. Data completeness for all markers genotyped as part of the BP linkage scan

Table 8.1: Completeness of the data obtained for chromosome 1 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D1S468	1.00	0.97	0.99
D1S214	0.93	0.92	0.93
D1S450	0.92	0.96	0.94
D1S2667	0.95	1.00	0.97
D1S2697	0.90	0.97	0.93
D1S199	0.97	0.97	0.97
D1S234	0.95	0.99	0.96
D1S255	0.96	1.00	0.98
D1S2797	0.95	0.96	0.95
D1S2890	0.97	0.96	0.96
D1S230	0.93	0.99	0.96
D1S2841	0.93	0.97	0.95
D1S207	0.88	1.00	0.93
D1S2868	0.89	0.95	0.92
D1S206	0.95	0.96	0.95
D1S2726	0.93	1.00	0.96
D1S252	0.86	1.00	0.92
D1S498	0.00	0.97	0.44
D1S484	0.92	0.97	0.95
D1S2878	0.97	0.91	0.94
D1S196	0.98	0.99	0.98
D1S218	0.90	0.95	0.92
D1S238	0.91	0.99	0.95
D1S413	0.90	0.96	0.93
D1S249	0.84	0.91	0.87
D1S425	0.99	0.99	0.99
D1S213	0.99	0.97	0.98
D1S2800	0.95	0.97	0.96
D1S2785	0.90	0.96	0.93
D1S2842	0.95	0.91	0.93
D1S2836	0.00	0.97	0.44
Average chr 1	**0.87**	**0.97**	**0.92**

[1]Weighted by the number of individuals in each data set.

Table 8.2: Completeness of the data obtained for chromosome 2 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D2S319	0.99	0.97	0.98
D2S2211	1.00	0.92	0.96
D2S162	0.98	0.96	0.97
D2S168	1.00	0.99	0.99
D2S305	0.98	0.99	0.98
D2S165	0.91	0.97	0.94
D2S367	0.97	0.92	0.95
D2S2259	0.98	1.00	0.99
D2S391	0.96	1.00	0.98
D2S337	0.99	0.95	0.97
D2S2368	0.98	0.91	0.95
D2S286	1.00	1.00	1.00
D2S2333	0.97	0.97	0.97
D2S2216	0.90	0.97	0.93
D2S160	0.98	0.93	0.96
D2S347	0.98	0.96	0.97
D2S112	0.96	0.95	0.95
D2S151	0.96	0.97	0.96
D2S142	0.97	0.99	0.98
D2S2330	0.99	1.00	0.99
D2S335	0.99	0.99	0.99
D2S364	0.95	0.92	0.93
D2S117	1.00	0.95	0.98
D2S325	0.98	0.97	0.98
D2S2382	0.98	0.96	0.97
D2S126	0.99	0.96	0.98
D2S396	0.81	0.99	0.89
D2S206	1.00	0.97	0.99
D2S338	0.99	0.99	0.99
D2S125	1.00	1.00	1.00
Average chr 2	**0.97**	**0.97**	**0.97**

[1]**Weighted by the number of individuals in each data set.**

Table 8.3: Completeness of the data obtained for chromosome 3 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D3S1297	0.96	0.96	0.96
D3S1304	0.96	0.99	0.97
D3S1263	0.81	0.89	0.85
D3S2338	0.93	0.97	0.95
D3S1266	0.98	0.96	0.97
D3S1277	0.92	0.93	0.93
D3S1289	0.98	0.97	0.98
D3S1300	0.97	0.89	0.93
D3S1285	0.97	0.96	0.96
D3S1566	0.84	0.96	0.89
D3S3681	0.84	0.97	0.90
D3S1271	0.99	1.00	0.99
D3S1278	0.99	0.96	0.98
D3S1267	0.99	0.97	0.98
D3S1292	1.00	0.99	0.99
D3S1569	0.96	1.00	0.98
D3S1279	0.96	0.95	0.95
D3S1614	0.87	1.00	0.93
D3S1565	0.97	1.00	0.98
D3S1262	0.97	0.95	0.96
D3S1580	0.93	0.92	0.93
D3S1601	0.98	0.99	0.98
D3S1311	0.98	1.00	0.99
Average chr 3	**0.94**	**0.96**	**0.95**

[1]**Weighted by the number of individuals in each data set.**

Table 8.4: Completeness of the data obtained for chromosome 4 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D4S412	0.88	1.00	0.93
D4S2935	0.97	1.00	0.98
D4S403	0.90	0.96	0.93
D4S419	0.89	0.97	0.93
D4S391	0.99	1.00	0.99
D4S405	0.96	0.99	0.97
D4S1592	0.97	0.95	0.96
D4S392	0.98	0.97	0.98
D4S2964	0.90	1.00	0.95
D4S1534	0.93	1.00	0.96
D4S414	0.87	0.99	0.92
D4S1572	0.97	0.97	0.97
D4S406	1.00	0.99	0.99
D4S402	0.88	0.96	0.92
D4S1575	1.00	1.00	1.00
D4S424	0.93	0.93	0.93
D4S413	0.80	0.84	0.82
D4S1597	0.87	0.96	0.91
D4S1539	0.86	0.97	0.91
D4S415	0.93	0.96	0.95
D4S1535	0.98	0.99	0.98
D4S426	1.00	0.99	0.99
Average chr 4	**0.93**	**0.97**	**0.95**

[1]**Weighted by the number of individuals in each data set.**

Table 8.5: Completeness of the data obtained for chromosome 5 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D5S1981	0.99	0.97	0.98
D5S406	0.98	0.99	0.98
D5S630	0.96	1.00	0.98
D5S416	0.97	0.97	0.97
D5S419	0.96	1.00	0.98
D5S426	0.99	0.99	0.99
D5S418	0.00	1.00	0.45
D5S407	0.93	0.91	0.92
D5S647	0.99	1.00	0.99
D5S424	0.98	0.99	0.98
D5S641	0.95	0.97	0.96
D5S428	0.95	0.91	0.93
D5S644	0.96	1.00	0.98
D5S433	1.00	1.00	1.00
D5S2027	0.99	1.00	0.99
D5S471	0.99	0.97	0.98
D5S2115	0.91	0.95	0.93
D5S436	0.95	1.00	0.97
D5S410	0.98	0.96	0.97
D5S422	1.00	1.00	1.00
D5S400	1.00	1.00	1.00
D5S408	0.98	0.96	0.97
Average chr 5	**0.93**	**0.98**	**0.95**

[1]**Weighted by the number of individuals in each data set.**

Table 8.6: Completeness of the data obtained for chromosome 6 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D6S1574	0.97	1.00	0.98
D6S309	0.96	0.97	0.96
D6S470	0.99	0.93	0.96
D6S289	0.99	1.00	0.99
D6S422	0.97	0.99	0.98
D6S276	0.96	0.96	0.96
D6S1610	0.95	0.96	0.95
D6S257	0.99	0.99	0.99
D6S460	0.98	0.99	0.98
D6S462	0.96	0.99	0.97
D6S434	0.87	0.95	0.90
D6S287	1.00	0.97	0.99
D6S262	0.00	1.00	0.45
D6S292	0.96	0.99	0.97
D6S308	0.87	0.97	0.92
D6S441	1.00	0.99	0.99
D6S1581	0.85	0.99	0.91
D6S264	0.90	0.97	0.93
D6S446	0.91	0.96	0.93
D6S281	0.90	0.99	0.94
Average chr 6	**0.90**	**0.98**	**0.93**

[1]**Weighted by the number of individuals in each data set.**

Table 8.7: Completeness of the data obtained for chromosome 7 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D7S531	0.91	0.92	0.92
D7S517	0.99	1.00	0.99
D7S513	0.92	0.97	0.95
D7S507	0.98	0.97	0.98
D7S493	0.95	0.97	0.96
D7S516	0.98	1.00	0.99
D7S484	0.98	0.97	0.98
D7S510	0.95	1.00	0.97
D7S519	0.92	0.97	0.95
D7S502	0.98	0.95	0.96
D7S669	0.95	0.99	0.96
D7S630	0.97	1.00	0.98
D7S657	0.98	1.00	0.99
D7S515	0.98	0.93	0.96
D7S486	0.95	0.88	0.92
D7S530	0.89	0.99	0.93
D7S640	0.91	0.99	0.95
D7S684	1.00	1.00	1.00
D7S661	1.00	1.00	1.00
D7S636	0.92	0.95	0.93
D7S798	1.00	1.00	1.00
D7S2465	0.98	0.97	0.98
Average chr 7	**0.96**	**0.97**	**0.96**

[1]**Weighted by the number of individuals in each data set.**

Table 8.8: Completeness of the data obtained for chromosome 8 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D8S264	0.97	1.00	0.98
D8S277	0.93	0.96	0.95
D8S550	0.97	1.00	0.98
D8S549	0.99	0.93	0.96
D8S258	0.99	0.95	0.97
D8S1771	0.98	0.97	0.98
D8S505	0.96	0.95	0.95
D8S285	0.99	0.97	0.98
D8S260	0.92	1.00	0.96
D8S270	1.00	0.97	0.99
D8S1784	0.77	0.95	0.85
D8S514	0.99	1.00	0.99
D8S284	0.98	0.99	0.98
D8S272	0.99	0.88	0.94
Average chr 8	**0.96**	**0.97**	**0.96**

[1]**Weighted by the number of individuals in each data set.**

Table 8.9: Completeness of the data obtained for chromosome 9 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D9S288	1.00	0.99	0.99
D9S286	0.90	0.96	0.93
D9S285	1.00	0.97	0.99
D9S157	0.98	1.00	0.99
D9S171	0.90	0.99	0.94
D9S161	1.00	0.95	0.98
D9S1817	0.99	0.96	0.98
D9S273	0.90	1.00	0.95
D9S175	0.99	0.99	0.99
D9S167	0.96	1.00	0.98
D9S283	0.96	0.99	0.97
D9S287	1.00	0.99	0.99
D9S1690	0.99	0.99	0.99
D9S1677	0.97	1.00	0.98
D9S1776	0.96	0.99	0.97
D9S1682	0.95	1.00	0.97
D9S290	0.93	0.99	0.96
D9S164	0.95	0.99	0.96
D9S1826	0.91	0.92	0.92
D9S158	0.90	0.93	0.92
Average chr 9	**0.96**	**0.98**	**0.97**

[1]**Weighted by the number of individuals in each data set.**

Table 8.10: Completeness of the data obtained for chromosome 10 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D10S249	0.99	0.97	0.98
D10S591	0.97	0.99	0.98
D10S189	0.97	1.00	0.98
D10S547	0.88	0.91	0.89
D10S1653	0.97	1.00	0.98
D10S548	0.99	0.96	0.98
D10S197	0.93	1.00	0.96
D10S208	0.97	1.00	0.98
D10S196	0.97	0.95	0.96
D10S1652	0.97	0.93	0.95
D10S537	0.97	0.95	0.96
D10S1686	0.98	0.97	0.98
D10S185	0.98	1.00	0.99
D10S192	0.97	1.00	0.98
D10S597	0.99	0.95	0.97
D10S1693	0.97	0.97	0.97
D10S587	0.98	0.95	0.96
D10S217	1.00	0.96	0.98
D10S1651	1.00	0.99	0.99
D10S212	1.00	1.00	1.00
Average chr 10	**0.97**	**0.97**	**0.97**

[1]**Weighted by the number of individuals in each data set.**

Table 8.11: Completeness of the data obtained for chromosome 11 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D11S4046	0.92	0.99	0.95
D11S1338	0.85	0.97	0.90
D11S902	1.00	0.99	0.99
D11S904	0.93	1.00	0.96
D11S935	0.93	0.95	0.94
D11S905	0.00	0.97	0.44
D11S4191	0.93	0.99	0.96
D11S987	1.00	0.99	0.99
D11S1314	1.00	1.00	1.00
D11S937	0.92	0.99	0.95
D11S901	1.00	1.00	1.00
D11S4175	0.89	0.96	0.92
D11S898	0.96	0.99	0.97
D11S908	0.90	0.99	0.94
D11S925	0.87	0.96	0.91
D11S4151	0.99	0.97	0.98
D11S1320	0.97	0.93	0.95
D11S968	0.93	0.97	0.95
Average chr 11	**0.89**	**0.98**	**0.93**

[1]**Weighted by the number of individuals in each data set.**

Table 8.12: Completeness of the data obtained for chromosome 12 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D12S352	0.95	0.99	0.96
D12S99	0.98	0.91	0.95
D12S336	0.95	0.95	0.95
D12S364	0.95	0.97	0.96
D12S310	0.91	0.92	0.92
D12S1617	0.98	0.97	0.98
D12S345	0.91	0.91	0.91
D12S85	0.90	0.96	0.93
D12S368	0.90	0.96	0.93
D12S83	0.90	0.93	0.92
D12S326	0.93	0.96	0.95
D12S351	0.99	0.92	0.96
D12S346	0.99	0.99	0.99
D12S78	1.00	0.92	0.96
D12S79	0.84	0.99	0.90
D12S86	0.93	0.99	0.96
D12S324	0.92	0.92	0.92
D12S1659	1.00	0.99	0.99
D12S1723	1.00	0.91	0.96
Average chr 12	**0.94**	**0.95**	**0.95**

[1]**Weighted by the number of individuals in each data set.**

Table 8.13: Completeness of the data obtained for chromosome 13 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D13S175	1.00	0.99	0.99
D13S217	0.96	0.88	0.92
D13S171	0.97	0.95	0.96
D13S218	0.98	0.99	0.98
D13S263	0.96	1.00	0.98
D13S153	0.85	0.97	0.90
D13S156	0.97	0.96	0.96
D13S170	0.93	0.99	0.96
D13S265	0.95	0.97	0.96
D13S159	0.00	0.93	0.42
D13S158	0.96	0.95	0.95
D13S173	0.95	0.96	0.95
D13S1265	0.91	0.89	0.90
D13S285	0.97	1.00	0.98
Average chr 13	**0.88**	**0.96**	**0.92**

[1]**Weighted by the number of individuals in each data set.**

Table 8.14: Completeness of the data obtained for chromosome 14 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D14S261	0.96	0.96	0.96
D14S283	1.00	0.95	0.98
D14S275	0.98	0.97	0.98
D14S70	0.95	0.92	0.93
D14S288	0.91	1.00	0.95
D14S276	0.92	1.00	0.96
D14S63	0.97	0.97	0.97
D14S258	1.00	0.97	0.99
D14S74	0.91	1.00	0.95
D14S68	0.93	0.89	0.92
D14S280	0.98	0.92	0.95
D14S65	0.90	0.97	0.93
D14S985	0.93	0.91	0.92
D14S292	1.00	0.93	0.97
Average chr 14	**0.95**	**0.96**	**0.95**

[1]Weighted by the number of individuals in each data set.

Table 8.15: Completeness of the data obtained for chromosome 15 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D15S128	1.00	1.00	1.00
D15S1002	0.97	0.89	0.93
D15S165	0.97	0.93	0.95
D15S1007	0.93	0.95	0.94
D15S1012	0.99	1.00	0.99
D15S994	0.00	0.97	0.44
D15S978	1.00	1.00	1.00
D15S117	0.98	0.95	0.96
D15S153	0.95	0.99	0.96
D15S131	0.95	0.99	0.96
D15S205	0.91	0.97	0.94
D15S127	0.95	0.97	0.96
D15S130	0.99	0.95	0.97
D15S120	1.00	0.99	0.99
Average chr 15	**0.90**	**0.97**	**0.93**

[1]Weighted by the number of individuals in each data set.

Table 8.16: Completeness of the data obtained for chromosome 16 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D16S423	0.89	0.91	0.90
D16S404	0.92	0.93	0.93
D16S3075	0.96	0.92	0.94
D16S3103	0.96	0.97	0.96
D16S3046	1.00	1.00	1.00
D16S3068	0.95	1.00	0.97
D16S3136	0.97	0.96	0.96
D16S415	0.95	0.97	0.96
D16S503	0.97	0.92	0.95
D16S515	0.89	0.92	0.90
D16S516	0.96	0.83	0.90
D16S3091	0.92	0.97	0.95
D16S520	0.95	0.97	0.96
Average chr 16	**0.94**	**0.94**	**0.94**

[1]**Weighted by the number of individuals in each data set.**

Table 8.17: Completeness of the data obtained for chromosome 17 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D17S849	0.82	0.92	0.87
D17S831	0.96	0.97	0.96
D17S938	0.98	0.99	0.98
D17S1852	0.99	0.97	0.98
D17S799	0.99	0.93	0.96
D17S921	0.98	0.97	0.98
D17S1857	1.00	0.95	0.98
D17S798	0.00	0.92	0.42
D17S1868	0.90	0.93	0.92
D17S787	1.00	0.96	0.98
D17S944	0.98	0.97	0.98
D17S949	0.91	0.95	0.93
D17S785	1.00	0.99	0.99
D17S784	0.91	0.96	0.93
D17S928	0.99	0.97	0.98
Average chr 17	**0.89**	**0.96**	**0.92**

[1]**Weighted by the number of individuals in each data set.**

Table 8.18: Completeness of the data obtained for chromosome 18 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D18S59	0.95	0.96	0.95
D18S63	1.00	0.99	0.99
D18S452	0.92	0.95	0.93
D18S464	0.96	0.99	0.97
D18S53	0.99	0.99	0.99
D18S478	0.90	0.97	0.93
D18S1102	0.98	0.88	0.93
D18S474	1.00	0.99	0.99
D18S64	0.96	0.97	0.96
D18S68	0.97	0.88	0.93
D18S61	0.98	0.95	0.96
D18S1161	0.95	0.96	0.95
D18S462	0.98	0.95	0.96
D18S70	0.78	0.96	0.86
Average chr 18	**0.95**	**0.96**	**0.95**

[1]**Weighted by the number of individuals in each data set.**

Table 8.19: Completeness of the data obtained for chromosome 19 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D19S209	0.96	0.96	0.96
D19S216	1.00	0.99	0.99
D19S884	0.97	0.99	0.98
D19S221	0.00	0.87	0.39
D19S226	0.99	0.99	0.99
D19S414	0.95	0.99	0.96
D19S220	0.96	1.00	0.98
D19S420	1.00	0.99	0.99
D19S902	0.98	0.93	0.96
D19S571	0.80	0.92	0.86
D19S418	0.87	0.97	0.92
D19S210	0.99	0.88	0.94
Average chr 19	**0.87**	**0.96**	**0.91**

[1]**Weighted by the number of individuals in each data set.**

Table 8.20: Completeness of the data obtained for chromosome 20 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D20S117	0.93	1.00	0.96
D20S889	0.96	0.99	0.97
D20S115	0.98	0.99	0.98
D20S186	0.96	0.97	0.96
D20S112	0.90	0.91	0.90
D20S195	0.99	0.97	0.98
D20S107	0.97	0.92	0.95
D20S119	1.00	0.96	0.98
D20S178	0.86	0.95	0.90
D20S196	0.97	0.97	0.97
D20S100	0.98	0.92	0.95
D20S171	0.85	0.95	0.89
D20S173	0.99	0.95	0.97
Average chr 20	**0.95**	**0.96**	**0.95**

[1]Weighted by the number of individuals in each data set.

Table 8.21: Completeness of the data obtained for chromosome 21 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D21S1256	0.84	0.89	0.86
D21S1914	0.98	0.93	0.96
D21S263	0.00	0.95	0.43
D21S1252	0.90	0.99	0.94
D21S266	0.90	0.97	0.93
Average chr 21	**0.72**	**0.95**	**0.82**

[1]Weighted by the number of individuals in each data set.

Table 8.22: Completeness of the data obtained for chromosome 22 in the genome-wide linkage scan of fifteen extended pedigrees segregating severe bipolar disorder from Antioquia.

marker name	data set 1	data set 2	weighted average[1]
D22S420	1.00	0.97	0.99
D22S539	0.91	0.97	0.94
D22S315	0.87	0.96	0.91
D22S280	0.95	0.97	0.96
D22S283	0.96	0.97	0.96
D22S423	0.99	0.96	0.98
D22S274	0.89	0.96	0.92
Average chr 22	**0.94**	**0.97**	**0.95**

[1]Weighted by the number of individuals in each data set.

8.3. Published Papers and Manuscripts

In this section of the appendix, I have attached all papers on which I have collaborated during the time of my Ph.D. studies in Professor Ruiz-Linares' laboratory.