Whole genome analysis of genetic susceptibility factors in psychotic disorders

Lavinia Athanasiu

Department of Medical Genetics

Division of Mental Health and Addiction

Oslo University Hospital

Faculty of Medicine

University of Oslo

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1. Preface

1.1 Acknowledgements

This present work has been performed at the TOP study group Department of Mental Health and Addiction and Department of Medical Genetics, Oslo University Hospital-Ullevål and Institute of Clinical Medicine, University of Oslo during the period 2007 and 2011. The PhD fellowship was financed by the South East Health Authority of Norway.

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Last but not least, I want to thank *Kim* for all support and encouragement to keep researching, and my *mum* and *dad* for teaching me to have high standards and big aims. I love you.

1.2 Abbreviations

ACSM1	*acyl- CoA synthetase medium-chain family member 1*
ADHD	Attention deficit hyperactivity disorder
ANK3	*ankyrin 3, node of Ranvier*
ASW	African ancestry in Southwest USA
BDNF	*brain-derived neurotrophic factor*
BMI	Body mass index
CDCV	Common disorder - common variant
CDRV	Common disorder - rare variant
CEU	Central European ancestry Utah
CGH	Comparative genomic hybridization
CHB	Han Chinese in Beijing, China
CHD	Chinese in Metropolitan Denver, Colorado
CMYA5	*cardiomyopathy associated 5*
CNV	Copy number variation
CNP	Copy number polymorphism
CVD	Cardiovascular disease
DAOA	*D-amino oxidase activator*
DISC1	*disrupted in schizophrenia*
DNA	Deoxyribonucleic acid
DNTBP1	*dystrobrevin binding protein*
DSM-IV-TR	Diagnostic and Statistical Manual for Mental Disorders (Revised 4th ed.)
FISH	Fluorescence *in situ* hybridization
GAF	Global Assessment of Functioning Scale –Split version
GIH	Hujarati Indians in Houston, Texas
GWAS	Genome Wide Association Study

HDL-C	High density lipoprotein cholesterol
ICS	International classification of diseases
IQ	Intelligence Coefficient
ISC	The International Schizophrenia Consortium
JPT	Japanese in Tokyo, Japan
LD	Linkage disequilibrium
LDL-C	Low density lipoprotein cholesterol
LWK	Luhya in Webuye, Kenya
MAF	Minor allele frequency
MKK	Maasai in Kinyawa, Kenya
MMP16	*matrix metallopeptidase 16*
MRI	Magnetic resonance imaging
MXL	Mexican ancestry in Los Angeles, California
NMDA	N-methyl-D-aspartate
NRG1	*neuregulin*
NRGN	*neurogranin*
NRXN1	*neurexin 1*
NTRK3	*neurotrophic tyrosine receptor 3*
OPCML	*opioid binding protein/ cell adhesion molecule-like*
OR	Odds ratio
PCLO	*piccolo*
PDE4B	*phosphodiesterase 4B*
PGC	Psychiatric GWAS Consortium
PLAA	*phospholipase A-2 activating protein*
PLAP	phospholipase A2- activating protein
PRSS16	*protein, serine 16*

RELN	*reelin*
SNPs	Single nucleotide polymorphisms
TCF4	*transcription factor 4*
TG	Triglycerides
TOP	Tematisk Område Psykoser (Thematically Organized Psychosis Study)
TSI	Toscani in Italy
YRI	Yoruba people trios in Ibadan, Nigeria
ZNF804A	*zinc finger protein 804A*

1.3 Genetic terms

Allele	One of two or more forms of a genetic variant or a gene
Codon	A sequence of three consecutive nucleotides that codes for one amino acid or chain termination (stop signal)
Complex disease	Illness where no single locus contains alleles that are necessary or sufficient for disease. The disease is caused by the combined action of (several) genetic and environmental factors
Dominant inheritance	A trait or disorder that is phenotypically expressed in heterozygotes
Endophenotype	A relatively well-specified physiological or behavioral measure, that may be linked to the disease phenotype
Exon	A transcribed sequence of a gene, forming a part of the mature mRNA that encodes and specifies the protein
Genome	The total genetic material of an organism
Genotype	The genetic constitution of an cell, also used for the specific set of alleles at a certain locus
Genotyping	The process of determining a genotype
Haplotype	The specific combination of alleles that occurs together at closely linked loci on one of the two chromosomes, and therefore tend to be inherited together as one unit
Hardy Weinberg Equilibrium	Describes the principle that under certain assumptions, the allele and genotype frequencies in a population remain stable from one generation to the next
Heritability	The proportion of variation in a trait or disease in a population that is attributable to genetic variation among the individuals
Heterozygote	Individual having two different alleles at a locus
Homozygote	Individuals having identical alleles at a locus
Intron	The non-coding regions of a gene
Locus	The physical localization of a gene, or other DNA sequence on a chromosome

Missense mutation	Point mutation altering the codon, leading to an amino acid substitution in the protein
Monogenic disease	Illness caused by mutation in a single gene
Nonsense mutation	A point mutation that alters an amino acid codon to a stop codon, usually resulting in premature termination of translation and a truncated protein
Penetrance	The proportion on individuals with a certain genotype who express the associated phenotype
Phenotype	Observable physical and/or biochemical characteristics
Recessive inheritance	A trait or disorder that is phenotypically expressed in homozygotes
Susceptibility gene	A gene in which common variation is associated with a disease, *i.e.* carrier of this variant have an increased risk for the disease
Synonymous mutation	Also called silent mutation. Describes a point mutation that alter a codon to another codon that encodes the same amino acid (due to redundancy of the genetic code), with no change in the protein

1.4 List of papers

This thesis is based on the following publications, henceforth referred to by their roman numerals:

Paper I:
Athanasiu L, Mattingsdal M, Kähler AK, Brown A, Gustafsson O, Agartz I, Giegling I, Muglia P, Cichon S, Rietschel M, Pietiläinen OP, Peltonen L, Bramon E, Collier D, Clair DS, Sigurdsson E, Petursson H, Rujescu D, Melle I, Steen VM, Djurovic S, Andreassen OA. Gene variants associated with schizophrenia in a Norwegian genome-wide study are replicated in a large European cohort. J Psychiatr Res. 2010 Sep;44(12):748-53. Epub 2010 Feb 24. PubMed PMID: 20185149.

Paper II
Athanasiu L, Mattingsdal M, Melle I, Inderhaug E, Lien T, Agartz I, Lorentzen S, Morken G, Andreassen OA, Djurovic S. Intron 12 in NTRK3 is associated with bipolar disorder. Psychiatry Res. 2011 Feb 28;185(3):358-62. Epub 2010 Jun 15. PubMed PMID: 20554328.

Paper III
Athanasiu L, Brown AA, Birkenaes AB, Mattingsdal M, Agartz I, Melle I, Steen VM, Andreassen OA, Djurovic S. Genome-wide association study identifies genetic loci associated with body mass index and HDL-cholesterol levels during psychopharmacological treatment. A cross-sectional naturalistic study. Submitted to Psychiatry Research.

Associated papers:

Tesli M, **Athanasiu L**, Mattingsdal M, Kähler AK, Gustafsson O, Andreassen BK, Werge T, Hansen T, Mors O, Mellerup E, Koefoed P, Jönsson EG, Agartz I, Melle I, Morken G, Djurovic S, Andreassen OA. Association analysis of PALB2 and BRCA2 in bipolar disorder and schizophrenia in a scandinavian case-control sample. Am J Med Genet B Neuropsychiatr Genet. 2010 Oct 5;153B(7):1276-82. PubMed PMID: 20872766.

Kähler AK, Djurovic S, Rimol LM, Brown AA, **Athanasiu L**, Jönsson EG, Hansen T, Gústafsson O, Hall H, Giegling I, Muglia P, Cichon S, Rietschel M, Pietiläinen OP, Peltonen L, Bramon E, Collier D, St Clair D, Sigurdsson E, Petursson H, Rujescu D, Melle I, Werge T, Steen VM, Dale AM, Matthews RT, Agartz I, Andreassen OA. Candidate gene analysis of the human natural killer-1 carbohydrate pathway and perineuronal nets in schizophrenia: B3GAT2 is associated with disease risk and cortical surface area. Biol Psychiatry. 2011 Jan 1;69(1):90-6. Epub 2010 Oct 15. PubMed PMID: 20950796.

Djurovic S, Gustafsson O, Mattingsdal M, **Athanasiu L**, Bjella T, Tesli M, Agartz I, Lorentzen S, Melle I, Morken G, Andreassen OA. A genome-wide association study of bipolar disorder in Norwegian individuals, followed by replication in Icelandic sample. J Affect Disord. 2010 Oct;126(1-2):312-6. Epub

2010 May 7. PubMed PMID: 20451256.

Wirgenes KV, Djurovic S, Sundet K, Agartz I, Mattingsdal M, **Athanasiu L**, Melle I, Andreassen OA. Catechol O-methyltransferase variants and cognitive performance in schizophrenia and bipolar disorder versus controls. Schizophr Res. 2010 Sep;122(1-3):31-7. Epub 2010 Jun 1. PubMed PMID: 20605701.

Rimol LM, Agartz I, Djurovic S, Brown AA, Roddey JC, Kähler AK, Mattingsdal M, **Athanasiu L**, Joyner AH, Schork NJ, Halgren E, Sundet K, Melle I, Dale AM, Andreassen OA; Alzheimer's Disease Neuroimaging Initiative. Sex-dependent association of common variants of microcephaly genes with brain structure. Proc Natl Acad Sci U S A. 2010 Jan 5;107(1):384-8. Epub 2009 Dec 22. PubMed PMID: 20080800; PubMed Central PMCID: PMC2806758.

Steinberg S, Mors O, Børglum AD, Gustafsson O, Werge T, Mortensen PB, Andreassen OA, Sigurdsson E, Thorgeirsson TE, Böttcher Y, Olason P, Ophoff RA, Cichon S, Gudjonsdottir IH, Pietiläinen OP, Nyegaard M, Tuulio-Henriksson A, Ingason A, Hansen T, **Athanasiu L**, Suvisaari J, Lonnqvist J, Paunio T, Hartmann A, Jürgens G, Nordentoft M, Hougaard D, Norgaard-Pedersen B, Breuer R, Möller HJ, Giegling I, Glenthøj B, Rasmussen HB, Mattheisen M, Bitter I, Réthelyi JM, Sigmundsson T, Fossdal R, Thorsteinsdottir U, Ruggeri M, Tosato S, Strengman E; Genetic Risk and Outcome in Psychosis, Kiemeney LA, Melle I, Djurovic S, Abramova L, Kaleda V, Walshe M, Bramon E, Vassos E, Li T, Fraser G, Walker N, Toulopoulou T, Yoon J, Freimer NB, Cantor RM, Murray R, Kong A, Golimbet V, Jönsson EG, Terenius L, Agartz I, Petursson H, Nöthen MM, Rietschel M, Peltonen L, Rujescu D, Collier DA, Stefansson H, St Clair D, Stefansson K. Expanding the range of ZNF804A variants conferring risk of psychosis. Mol Psychiatry. 2011 Jan;16(1):59-66. Epub 2010 Jan 5. PubMed PMID: 20048749.

2. Abstract

Schizophrenia is a complex neurodevelopmental illness affecting about one percent of the world's population, throughout countries, cultural groups, and sexes. It is characterized by abnormal mental function and disturbed behavior, mostly appearing after puberty as a diverse mixture of positive and negative symptoms, and cognitive impairment. The severity of the symptoms and the chronic pattern of schizophrenia often cause a high degree of disability. Although extensive effort has been made in the quest of finding the origin of the disease, the new progress is hampered by the complexity and diversity of the symptoms combined with the fact that most symptoms of schizophrenia are usually noticed quite late in the disease process. Most of the genetic components of schizophrenia remain unknown ("the missing heritability"), as with the underlying biological mechanisms.

The present study includes three papers and is based upon naturalistic data from the cross-sectional part of the Thematically Organized Psychosis Research (TOP) Study, carried out in joint collaboration between the University of Oslo and University Hospitals of Oslo (now Oslo University Hospital).

Through genome-wide association analysis in a homogenous Norwegian case-control sample, several potential susceptibility genes for schizophrenia were identified and replicated in a larger North-European case-control sample. The combined analysis identified *phospholipase A-2 activating protein* (*PLAA*), *acyl-CoA synthetase medium-chain family member 1* (*ACSM1*) and *ankyrin 3, node of Ranvier* (*ANK3*) as putative candidate genes for schizophrenia.

It has been hypothesized that genetic risk might overlap for different psychotic disorders. *Neurotrophic tyrosine receptor 3* (*NTRK3*) has an important role in brain development and plasticity, and has been associated with hippocampal function in schizophrenia patients. By comparing allele frequencies of markers in *NTRK3* between bipolar patients and controls, we found markers in intron 12 significantly associated with bipolar disorder. The markers were in close proximity to reported linkage regions reported in schizophrenia, early-onset major depressive disorder and eating disorder, further supporting the hypothesis of genes influencing risk beyond traditional diagnostic boundaries.

Further, antipsychotics, antidepressants and mood-stabilizers are still cornerstones in the treatment of psychotic disorders, but the treatment is associated with serious clinical problems, like metabolic and cardiovascular side effects. Genetic variation could explain the difference in observed adverse effects in patients. Twelve indicator variables for metabolic side effects and cardiovascular risk factors were analyzed to identify genetic variants that mediate the effect of psychopharmacological agents on these variables. For body mass index and high density lipoprotein cholesterol, three loci were identified, two upstream of *matrix metalloproteinase 16* and one on 12q21 respectively, that were found to significantly mediate drug-induced side effects.

3. General introduction

3.1 Schizophrenia

3.1.1 Phenotype

Schizophrenia is one of the major psychiatric disorders, affecting approximately one percent of the general population, and the incidence is slightly higher in men than in women (1). Characterization of the schizophrenia phenotype includes a collection of clinical features, usually subdivided into positive symptoms (delusions, hallucinations and other reality distortions) and negative symptoms (loss of motivation, inability to experience pleasure, poverty of speech, lack of initiative, apathy and reduced social drive), as well as cognitive impairment, and mood symptoms (2;3). The symptoms vary widely among patients, and diagnosing the disorder is solely based on the symptoms presented, implemented in the diagnosis systems *Diagnostic and Statistical Manual of Mental disorders, 4th edition*, text revision (DSM-IV-TR) (4) and *International Classification of Diseases, 10th revision* (ICD-10) (http://www.who.int/classifications/icd/en).

The devastating neuropsychiatric disorder is ranked as one of the most costly disorders to afflict humans. Onset is generally during late adolescence or early adulthood when the prefrontal cortex is still developing, but subtle cognitive and behavioral signs are often present from childhood. Several longitudinal population-based studies demonstrate that schizophrenia patients have a history of delayed maturation including delayed developmental milestones in the first year (5), and a reduced Intelligence Coefficient (IQ) score early in childhood (6;7). The cognitive deficits are among major features of schizophrenia, and domains effected are e.g. speed of processing, attention, working memory, verbal learning and memory, visual learning and memory, reasoning and problem solving, verbal comprehension and social cognition (8). Another important feature of schizophrenia is the mood symptoms, which often precede the onset of psychosis.

3.1.2 Aetiology and epidemiology

After a century of studying schizophrenia, the etiology has not yet been established, but a strong genetic component is confirmed from both twin studies and adoption studies. One of the main hypotheses implicates multiple neurotransmitter systems. Abnormal dopamine transmission in the subcortical meso-limbic and meso-cortical systems is closely linked to the “positive” and “negative" symptoms, and the dopamine hypothesis is further strengthened by the ability of the dopamine antagonist amphetamine to induce psychosis (9). The dopamine hypothesis appeared when the mechanisms of action of antipsychotic medication were discovered. These drugs that were discovered in the early 1950’s are strong antagonists of dopaminergic neurotransmission (Dopamine D_2 blockers), and subsequently led to the

neurochemical view of schizophrenia. Important roles have also been found for serotonin and acetylcholine.

Some of the cognitive symptoms have together with positive and negative symptoms been observed in healthy individuals given low doses of N-methyl-D-aspartate (NMDA) receptor antagonist (10-12), while conversely, compounds modulating the glycine modulatory site on the NMDA receptor have been reported to reduce some of the cognitive symptoms of schizophrenia. These observations led to the glutamate hypothesis of schizophrenia, where hypofunction of the NMDA receptor may contribute to the pathophysiology of schizophrenia.

Structural magnetic resonance imaging (MRI) and functional MRI have revealed changes in global anatomical measures such as smaller cortical grey matter, thinner cortices, especially in the frontal and temporal lobe, and increased ventricle-to-brain volume (13). Other MRI studies have been performed to investigate the changes in normal and abnormal cortical development. Longitudinal neuroimaging studies have depicted changes in grey matter density, with the prefrontal cortex being last to mature in mid-twenties (14), and there is now strong MRI evidence for abnormalities in bipolar disorder also (13;15;16). However, new technology can reveal more subtle but functionally important abnormalities as very little is known about disease characteristics and clinical outcome in relationship to brain phenotypes, or how susceptibility genes relate to the neuronal substrates. Recent studies have provided indications of genetic effects on brain function (17-20), which together with the high heritability estimates of brain phenotypes (21), suggest that combining brain imaging and genetics could be a fruitful approach to understand underlying mechanisms as well as develop objective biomarkers of outcome.

Epidemiology of schizophrenia is complex, and studies have identified migrant status, urbanicity, maternal malnutrition during famine and infections in the second trimester, winter/spring birth, perinatal injury or cytokine exposure, birth complications and older paternal age as environmental risk factors for schizophrenia (22). These early adverse experiences might leave epigenetic “scares”, that could potentially explain the inconsistent findings in risk genes, as patients could have sequential or epigenetic alteration increasing risk for schizophrenia (23). Recent genetic studies have revealed some new genetic markers in schizophrenia (24), but most of the genetic factors are not yet identified (see section 3.4.5).

3.1.3 Treatment

Antipsychotics have been in use for nearly a half century and are still cornerstones in treatment of psychotic illnesses, while antidepressants and mood-stabilizers can also be used for some aspects of the phenotype. The antipsychotic medications are classified into the so-called typical or first generation (e.g. chlorpromazine, haloperidol), and the atypical or second generation (e.g. clozapine, olanzapine) antipsychotics. While the clinical effect may be similar between the two groups of drugs (25), many typical antipsychotics are linked to extrapyramidal side effects (EPS), such as tardive dyskinesia (TD), and elevated serum

prolactin levels, while the atypical antipsychotics have less motor side effects, but are instead associated with metabolic disturbances including dyslipidemia, elevated glucose levels and weight gain (26), all important cardiovascular risk factors. A large proportion of patients experience these adverse effects, possibly resulting in discontinuation of treatment. A recent meta-analysis on weight gain and first time psychosis was recently published, and the results indicated that lower pre-treatment body mass index (BMI), younger age, triglyceride levels, more negative symptoms, more co-medication and antidepressants predicted an increase in weight after antipsychotic treatment (27).

Although both typical and atypical antipsychotics adequately reduce delusions and hallucinations, they have minimal or no effect on cognitive deficits (problems with attention and working memory) associated with schizophrenia. Therefore, schizophrenia remains a very disabling illness, with a need for new therapeutics.

3.1.4 Mortality

Increased mortality rates have been documented from the pre-antipsychotic era (28), and are still high in severe mentally ill today. In the pre-antipsychotic period, there were no efficient treatment for schizophrenia or severe affective disorder available, and patients with family unable to care for them, were confined to so- called lunatic asylums, with poor conditions. Two large Scandinavian studies investigated death rates and causes of death in the asylums, and reported significantly elevated mortality rates, where the main cause of death was found to be tuberculosis and pneumonia (29;30). A study later attributed most of the excess deaths in schizophrenia to indirect effects of the illness, for example refusal of food, suicide, injuries and the general way of life (31). The introduction of more efficient symptom control with lithium in 1948 and chlorpromazine in 1952 revolutionized the treatment of schizophrenia, and the asylums were substituted by community based care. However, no decrease in the mortality rates was observed. The major health problems were no longer poverty or infectious diseases, but had shifted to cardiovascular disease (CVD) together with increased risk of suicide (32). On average, people with serious mental illness have a 25-years shorter life expectancy compared to the general population (33). The cardiovascular mortality in patients with schizophrenia increased from 1976 to 1995, with the greatest increase seen in the last few years of that period (34), and the introduction of second-generation antipsychotic drugs during the 1990s was suggested to have had a negative effect on the mortality in these patients. However, a recent study comparing the cause-specific mortality in 66,881 patients versus the total Finish population (5.2 million) from 1996 to 2006 concluded that long-term treatment of antipsychotic drugs is associated with lover mortality compared with no antipsychotic use (35).

3.1.5 Other psychotic disorders

Bipolar disorder is a severe mental illness characterized by severe mood swings; periods with distinctly depressed and elevated mood in between periods with normal mood (euthymia). The lifetime prevalence of bipolar disorder is generally stated to be one to two percent (36) and is believed to be relatively consistent across cultures and regions. The understanding of the pathophysiology of bipolar disorder remains limited. However, several neurobiological abnormalities have been identified that are likely to be underlying features of the disorder, such as immunological, neuroendocrinological, and molecular biological deviations (37). Furthermore, neuroimaging and post mortem studies have identified both structural and functional disturbances in the prefrontal and orbitofrontal cortex, the amygdala and the ventral striatum (38). These brain regions are involved in the regulation of emotions and motivated behavior as well as cognition.

The etiology of bipolar disorder remain to a large extent unknown, but family and twin studies have provided strong evidence of high heritability (39) and multiple genes appear to be involved. Recent genetic association studies have identified some gene variations conferring risk for bipolar disorder, but the effect sizes are small (40;41).

3.2 Human genetics and inheritance

The inheritance of traits from one generation to the next was first described by the Austrian monk Gregor Johann Mendel (1822-1884). His pea breeding allowed him to notice that certain traits, such as flower colour and seed shape, were inherited in certain patterns and derived laws of heredity based on these observations. However, the importance of his work was not recognized until three European biologists independently rediscovered his work 15 years after his death and formed the basis of modern genetics.

The molecular basis of how genetic instruction is passed from one generation to the next was not solved until the DNA structure was elucidated in the 1950's (42). With the completion of the sequencing effort of the entire human genome in 2001, hope for new progress in elucidating the genetic bases of complex disease was high. Since then, researchers have gained an increased understanding of the role of the environment, especially of infectious agents, in shaping our genetic architecture and present-day disease. Knowledge on the plasticity of the human genome and large chromosomal rearrangements, that play a more substantial role in disease aetiology than previously appreciated, is emerging.

Despite huge breakthroughs in genetics the last years, the major challenges are still to identify the genetic variants that contribute to complex disease, as most of these factors still remain unknown.

3.2.1 Genetic variation

Even though the human genome is about 99.5 percent identical between any given people, there are also millions of base pairs that differ between individuals (43). These natural genome variants can be classified in several ways. The simplest type is single nucleotide variants that involve substitution of only one base pair, and they are usually denoted a polymorphism if the minor allele frequency (MAF) is above one percent in the population. Single Nucleotide Polymorphisms (SNPs) with a MAF above five percent are referred to as common (44).

In the last ten years information on location and frequency of SNPs have been deposited in the Single Nucleotide Polymorphism database (dbSNP), including over 24.6 million validated SNPs (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi, accessed June 2011). The international HapMap project enables large-scale investigation of the contribution of common variation to phenotypic diversity, with on-going identification of new SNPs, including the pattern of inheritance among the SNPs (www.hapmap.org) (ref). Phase I, finalized in 2005, included 270 DNA samples from four geographically diverse populations; 30 trios (two parents and an adult child) from Utah, USA, with northern and western European ancestry (CEU), 30 Yoruba people trios in Ibadan, Nigeria (YRI), 45 unrelated Japanese in Tokyo, Japan (JPT), and 45 unrelated Han Chinese in Beijing, China (CHB), resulting in 1.2 million SNPs. Since then, the HapMap data set has increased, with a total of over 4.6 million SNPS in Phase II that was completed in 2006 (45), and 1.6 million SNPs genotyped in 1,301 individuals from 7 additional populations including Chinese in Metropolitan Denver, Colorado (CHD), Hujarati Indians in Houston, Texas (GIH), African ancestry in Southwest USA (ASW), Luhya in Webuye, Kenya (LWK), Mexican ancestry in Los Angeles, California (MXL), Maasai in Kinyawa, Kenya (MKK), Toscani in Italy (TSI) in Phase III completed in 2008.

SNPs represent a large amount of the genetic variation in a human genome, with about three million SNPs in any individual human genome as compared to the reference sequence (45;46), but structural variants, including inversions, insertions, deletions and Copy Number Variants (CNVs), account for at least 20 percent of all genetic variation (45). These structural variants can change the number of copies of a gene or piece of regulatory DNA or jam two genes together, possibly resulting in gene disruption, altered gene expression and altered regulation of nearby genes.

3.2.2 Linkage disequilibrium

All SNPs are the result of mutation events that occurred in the past. The mutations are surrounded by distinct set of genetic variants on the ancestral chromosome regions. These sets of nearby variants along a certain region on one chromosome are called haplotypes, which if compatible with life, will increase in frequencies in the population. New haplotypes will form when new mutations or recombination events occur. Linkage disequilibrium (LD) describes

the non-random association of alleles at two or more loci, in other words, the genotypes at two or more loci are not independent of one another, they are inherited together more often than expected by chance. In a population the result of LD between two nearby polymorphisms will give certain combinations of alleles that deviate from the values they would have if the alleles at each locus were randomly combined based on their frequencies. On the contrary, a population is said to be in linkage equilibrium when there is no such deviation. LD between alleles at two loci can be defined by several measures that all depend on the quantity of D:

$D_{AB} = P_{AB} - (P_A \cdot P_B)$

in which D_{AB} is the difference between the frequency of gametes carrying the pair of alleles A and B at two loci (P_{AB}) and the product of the frequencies of those alleles (P_A and P_B). When $D = 0$ there is perfect linkage equilibrium in the population. The quantity D does not only vary with the extent of LD, but is also constrained by the allele frequencies. The measure D' was introduced by Lewontin (47) as the ratio of D to its maximum possible absolute value, given the allele frequencies. Another commonly used way to quantify LD is with the squared correlation coefficient r^2:

$$r^2 = \frac{D_{AB}^2}{P_A(1 - P_A)P_B(1 - P_B)}$$

which is the measure of the correlation of alleles at two loci. An r^2 value of one is known as perfect LD and occurs if exactly two of the four possible haplotypes exist (48-50).

The feasibility of association studies is critically dependent upon the extent of LD across the genome, as these studies are dependent on associations of polymorphisms in LD with causative polymorphisms. In Paper I, the quantity r^2 was used to find surrogate markers for polymorphisms genotyped on the Affymetrix array that were not on the Illumina array used to genotype the replication sample.

In general, LD is affected by several genetic factors such as natural selection, mutation rate, marker recombination rate, and population migration. Of these genetic factors, recombination rate plays a pivotal role in shaping the pattern of linkage disequilibrium.

3.3 Association study design

Genetic association studies investigate the association between one or more genetic polymorphisms and disease, predisposition to disease or some other quantitative characteristic (51). Compared with linkage studies, the power and genetic resolution is greater in association studies (52), which together with the rapidly decreasing genotyping cost has made association studies the mainstream choice when searching for genetic susceptibility of complex diseases today. Genetic association studies can be divided into two groups: population-based case-

control studies and family-based studies, each with separate strengths and limitations. Family-based studies, usually using trios (cases and their parents), are well suited for finding rare variants underlying rare conditions or rare sub-phenotypes of a common disorder. In contrast, population-based studies use unrelated cases and controls, and have become the method of choice for finding common polymorphisms thought to affect predisposition for a complex phenotype. Subsequently, population-based studies can be hypothesis driven candidate gene studies or genome wide association analysis undertaken without prior hypotheses.

In a population, an association between a genetic polymorphism and a trait might exist if the polymorphism: (i) has a causal role or (ii) is in LD with a nearby causal variant, or (iii) the association is due to population stratification or admixture. The first of these types of associations are called direct associations and are the most powerful and straightforward to analyze, as the polymorphisms that are investigated are considered to be putative causal variants. The second type of association is an indirect association, *i.e.* the polymorphism is a surrogate for the causal locus. Indirect association studies are less powerful and are more difficult to analyze than direct studies, and it is generally necessary to type several surrounding markers in order to have a high chance of detecting them. The third type of association is a result of confounding by population stratification. Population stratification refers to the presence of a systematic differences in allele frequencies between subpopulations in a study due to ancestry difference among the subjects, resulting in both positive- and negative confounding, with the former generating positive findings while the latter obscuring true associations.

An appropriate protocol for both candidate- and genome wide studies has been published in the *Nature protocols* journal (53), with the important steps listed below:

A. Define the phenotype in sufficient detail

B. Evaluate the heritability of the illness in question

C. Determine if the conditions for a population-based study are fulfilled

D. Select the controls properly

E. Calculate the required sample size

F. Consider whether your study is a *de novo* or replication

In psychiatric genetics, the phenotypes (clinical diagnosis) are solely based on the symptoms presented (A), and objective biological measures are not available. The different diagnoses and alternative phenotypes used in this thesis are further described in the “Material and Method” section. The heritability of the disorders (B) has already been established, with a heritability estimate of 80 percent for schizophrenia (54). Schizophrenia is a fairly common disorder, and the genetic variants conferring risk are postulated to be common in the population, known as the Common Disease - Common Variant hypothesis (CDCV) (55). The main study design (C) used in the thesis was to search for common variants throughout the

genome, using a case-control design (**Paper I**, **Paper II**). In **Paper II**, common variants in a specific gene, *neurotrophic tyrosine kinase, receptor, type* 3 (*NTRK3*), were investigated for association with bipolar disorder. The controls (D) used in the thesis are selected from the same catchment area as the cases, ensuring same ethnicity between cases and controls. Also, the homogeneity of the sample is illuminated in "Material and Method" section. Power calculations (E) for the studies are also described in the "Materials and Method" section. Both genome wide studies of this thesis (**Paper I**, **Paper III**) are to some extent pioneers. No other genome-wide case –control association study on schizophrenia using a homogenous Norwegian population was published at the time (F). Regarding **Paper III**, few genome wide analyses on the association between drug treatment and genetic factors are available.

3.3.1 Candidate gene studies

In candidate gene studies, a specific gene, based on knowledge from animal models or known biological pathways or a chromosomal region identified through linkage study is examined. Genetic variants in the gene of interest are examined in a group diagnosed with schizophrenia, (the cases) and in a group of healthy individuals (the controls), and the allele frequencies are compared between the groups. The allele is said to be associated with the disease if it is observed significantly more or less frequently in the cases. In addition, a study usually investigates several SNPs in the gene of interest, making it necessary to correct for multiple testing to not make type I error.

3.3.2 Genome wide studies

Genome wide association study (GWAS) became feasible when new technology enabled rapid and cost efficient genotyping of several hundreds of thousands of DNA polymorphisms spread across the genome simultaneously, in combination with knowledge, gathered in the International Hap Map Project, on common SNPs in different population. The technical advance that has made this possible is the availability of chip-based genotyping products. Ideally, complete information at every variable point in the genome should be provided, but usually current chips captures 80 % of variant sites where the minor allele frequency (MAF) is above one percent in at least on population.

A well defined phenotype, which can be sensitively and specifically diagnosed or measured, is critically important. Also, robustly large sample sizes are needed, mandating collaborations among groups.

The GWAS approach has now been successfully applied to many complex diseases and a plethora of studies are published.

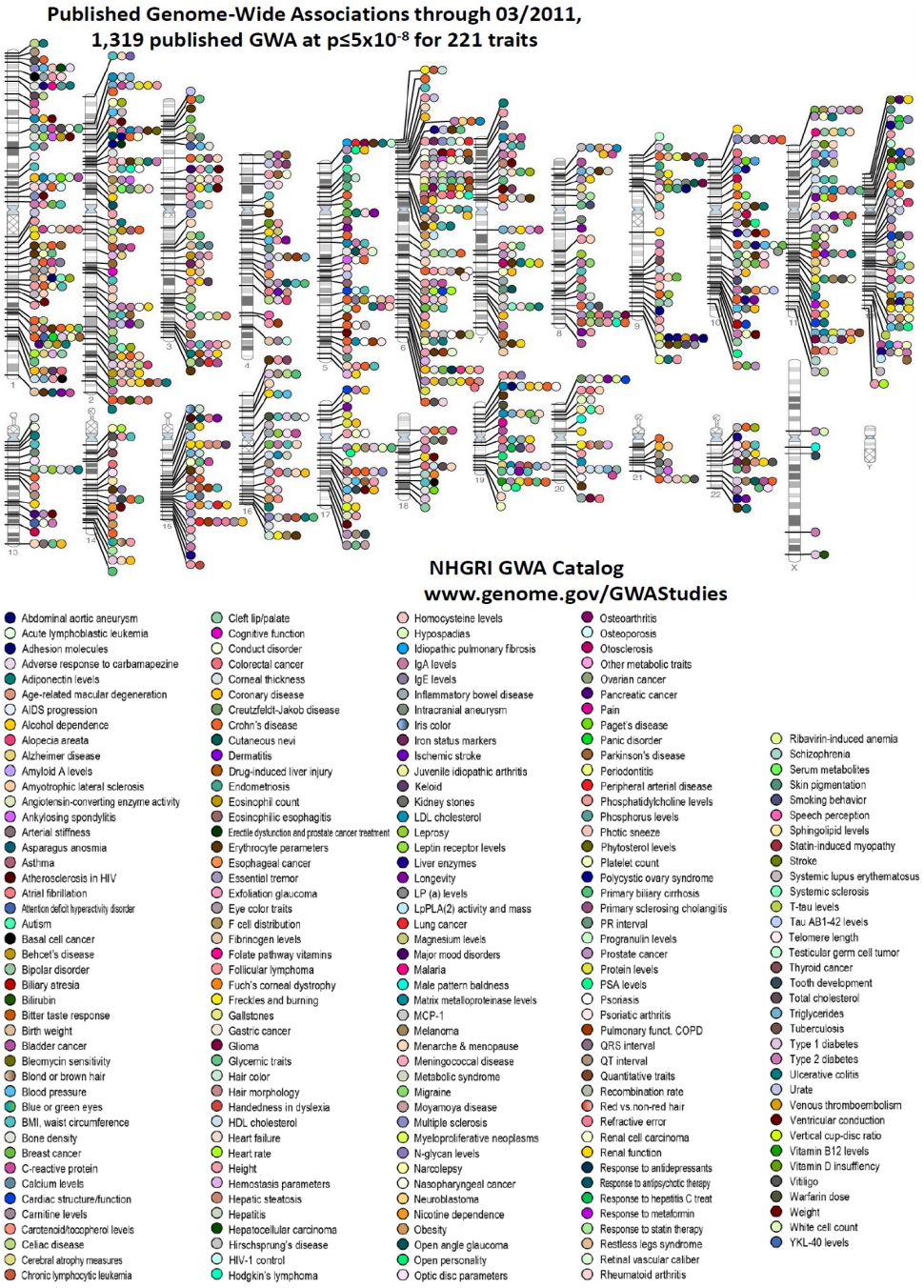

Figure 1. All published genome-wide associations studies prior to 2nd quarter of 2011.

GWAS is advantageous over candidate association design, as the study is not limited to predefined biological hypotheses, but are rather hypothesis generating. As the underlying biological mechanisms of schizophrenia remains unclear, the ultimate goal of GWAS is to identify genetic variants' contributing effects to complex phenotypes in order to improve our understanding of the biological architecture underlying the trait.

3.3.3 Copy number variation studies

The human genome contains genetic variation not only at the base-pair level, but also at larger stretches of duplicated, deleted or inverted pieces of DNA. Large genomic imbalances are visible by light microscopy, while shorter submicroscopic alterations are detected by molecular techniques, like fluorescence *in situ* hybridization (FISH) or array- based comparative genomic hybridization (array-CGH). Recent years' development in microarray technology has enabled genome-wide screens for submicroscopic deletions and duplications, revealing these genetic aberrations as abundant in the human genome, accounting for more genetic differences between individuals than those observed for SNPs (56;57). These chromosomal aberrations are called copy number variant (CNV). CNV is defined as gain or loss of genomic segments, ranging from 1 kb up to several megabases, compared to a reference genome. They account for at least five to ten percent of the structural variation in the genome (58) and can be classified as rare or common, the later if the MAF is above one percent in the population, also called copy number polymorphisms (CNPs). CNV are inherited from one generation to the next or formed *de novo*, with an estimated frequency of 0.14 for new insertion or deletions events per generation (59). Some CNVs are observed to have the same breakpoints in unrelated individuals (recurrent), while others are non-recurrent. Observations that CNVs could impact transcriptional or translational levels of overlapping or nearby genes sparked an interest in these chromosomal aberrations (60). Further, reports that certain CNVs were associated with differential susceptibility to complex disease further fuelled the interest (61-63).

3.4 The search for schizophrenia genes

3.4.1 Heritability of schizophrenia

Understanding the genetic basis of schizophrenia continues to be a major challenge. It is well established that schizophrenia aggregates in families. Family studies conducted over many years have documented an increased risk of schizophrenia in relatives of probands with the disorder (64). The lifetime risk for schizophrenia in the general population is 1 percent, while the risk of siblings and offspring is 10 times this. The heritability (the observed variance in illness in the population due to additive genetic causes) estimates may be as high as 80 percent (65). Family, twin and adoption studies have been vital for establishing an important genetic contribution to the etiology of schizophrenia (66). In addition, twin and adoption

studies have also shown a shared familial, and probably genetic, liability for schizophrenia and a range of other psychotic illnesses (67) as well as personality disorders (68). It is now recognized that schizophrenia is a complex genetic disorder (69), where several genes, each with modest effect, contribute in interaction with environmental factors to susceptibility (70). On one hand, reduced fecundity is often seen in individuals suffering from schizophrenia. Therefore, it has been speculated if schizophrenia represents a complex disease caused by multiple, rare, *de novo* mutations rather than common variations, as natural selection would eliminate such common variants (71). On the other hand, common risk alleles in the general population may have relatively weak individual effects, be pleiotropic, and interact with other variants. Epistatic interactions between risk genes and between their products, as well as interaction with environmental risk factors are considered to explain the polygenic illness (72).

In the early faces, the schizophrenia genetic research was based on linkage studies, then progressing to association analysis of biological or positional candidates. More recently, GWAS and CNV analysis have become the method of choice. There has been an explosion of research examining genetic variants underlying schizophrenia in the last year. A number of risk genes have been identified in schizophrenia, and substantial research is now focused on the molecular mechanisms through which these genes may confer risk. Given that the majority of identified genetic variants are noncoding single nucleotide polymorphisms (SNPs), it is hypothesized that these SNPs are changing gene expression, perhaps by altering transcriptional activity or alternative splicing (73). Due to the serious disabling condition and enormous socioeconomic consequences of schizophrenia, identification of susceptibility genes and the underlying pathophysiology remains of great importance (74).

3.4.2 Observations from linkage studies

Linkage mapping involves modeling the correlation between disease status and the pattern of allele segregation within families, and has been successful in identifying the genetic basis of many human diseases in which the disease penetrance follows a simple Mendelian model (monogenic diseases), *i.e.* Huntington's disease, cystic fibrosis and some forms of cancer (52). Quite a few linkage studies have been performed to interrogate the genetic bases of schizophrenia, and although the replication of these have proven difficult, some potential regions of linkage have surfaced (75). Among the promising candidates are *dysbindin* (*DNTBP1*), *neuregulin 1* (*NRG1*), and *D-amino oxidase activator* (*DAOA*), identified through LD mapping of linked regions 6p24-22, 8p21-22 and 13q34 respectively (76-78), and further supported by follow up studies(79).

In addition, other promising regions and candidates have emerged through studying chromosomal abnormalities segregating with psychiatric disease in families, such as *disrupted in schizophrenia* (*DISC1*) and *phosphodiesterase 4B* (*PDE4B*) (80).

3.4.3 Candidate gene studies

A plethora of genetic association studies have been performed to investigate the contribution of genetic variants in schizophrenia susceptibility, and several reviews are at hand (81;82). Unfortunately the field has experienced difficulty in replicating initial findings, leaving researchers with results difficult to validate and interpret. The leading candidate gene targets have been genes encoding proteins involved in neurotransmission, predominantly dopaminergic and serotonergic, although probably most neurotransmitter system have been investigated (81). Genes related to brain development have also been examined extensively for association with schizophrenia (81). Due to the rapidly evolving field, with vast and inconsistent literature, the freely online accessible SzGene database was established (83) (www.schizophreniaforum.org) to collect association data and perform systematic meta-analyses. The SzGene database included (by 3rd of July, 2011) 1,727 eligible studies investigating 8,788 SNPs in 1,008 genes, and the gene ranked highest on the result list was *protein, serine, 16* (*PRSS16*). *PRSS16* is a gene exclusively expressed in the thymus and is located on the large histone gene cluster on chromosome 6, near the major histocompatibility complex class 1 region.

To increase power in the genetic association studies, collaborative efforts between several research centers have evolved in order to obtain large cohorts of cases and controls, e.g. the SGENE consortium (www.sgene.ue). In 2007 The Psychiatric GWAS Consortium (PGC) was formed, conducting meta-analysis of individual genotype data from European subjects with various neuropsychiatric phenotypes, such as attention deficit hyperactivity disorder (ADHD), autism, bipolar disorder, major depressive disorder and schizophrenia (84).

3.4.4 Genome wide association studies

Genome-wide association studies (GWAS) have become widely popular for exploring the genetics of complex disorders, as the method incorporates the power to detect small effects and requires no prior knowledge of the pathogenesis. The design has proven successful for some phenotypes (85). The early GWAS were based on DNA pooling (an approach where two single quantitative assays are made from pools of patients and controls respectively) and small sample sizes (200-1,000 individuals), but as the genotyping cost decreased dramatically, the studies are now often based on individual genotyping and larger sample sizes (>2,000). The first study based on individual genotyping (86), performed on 178 cases and 144 controls, indentified the genes *colony simulating factor 2 receptor alpha*, *short stature homeobox isoform b*, but replication studies are lacking and they need to be further examined in regard to schizophrenia. A second GWAS was performed on a larger sample (738 cases and 733 controls) (87), but none of the findings achieved genome wide significance (88). The study sample was ethnically heterogeneous, and that might have adversely impacted upon power. Another study, based on an initial GWAS of 479 UK cases and 2937 UK controls and sequential follow-up of loci surpassing a threshold ($P < 10^{-5}$) in 6,829 cases and 9,897

controls, resulted in the identification of a significant association (P value meta-analyses = 1.61×10^{-7}, OR = 1.12) of the gene *zinc finger protein 804 A* (*ZNF804*). This association has since been replicated in several studies (89-91), and is emerging as a true susceptibility gene for schizophrenia.

3.4.5 Copy number variants

A short review of the literature on CNVs and mental disorders, reveal a consistency in findings beyond doubt that CNVs confer risk of developing neuropsychiatric disorders, like autism, mental retardation and schizophrenia. The total burden of CNVs in schizophrenia patients is markedly increased compared to the general population (92-94), especially the combination of large CNV with high number of genes per CNV. Several cytogenetic studies have implicated structural abnormalities in schizophrenia (80;95-97), highlighting a translocation of the gene *disrupted in schizophrenia* (*DISC1*) in a large Scottish pedigree (98). Today, several studies have implicated that rare deletions on 1q21.1, 15q13.3, 15q11.2, 17p12 and 22q11.2, as well as duplications at 16q11.2, 16p13.1 and 22q13.3 all increase the risk of developing schizophrenia and autism (99).

The deletion on 22q11.2 is one of the first CNVs to be associated with schizophrenia. Originally identified on the basis of a macroscopic deletion visible on a karyotype (100), the deletion is known as the DiGeorge syndrome or velocardiofacial syndrome with a observed frequency of up to 25 percent of all schizophrenia patients (101;102).

The 1q21.1 was first observed in schizophrenia by Wash *et al.* (92) and later independently identified by two larger studies (24;93). Interestingly, deletions at 1q21.1 have also been implicated in autism, microcephaly, heart defects and cataracts (103). Deletions within the *neurexin 1* gene (*NRXN1*) on 2p16.3 were first reported in schizophrenia cases by Kirov *et al.* (104). They found a 0.25 Mb large deletion in two affected siblings, spanning the promoter and the first exon of *NRXN1*, but no CNV was found for the gene in the control sample. Another study by Walsh *et al.* observed a 115kb deletion in identical twins with schizophrenia (92). Interestingly, partially overlapping deletions have been identified in two siblings with autism (105) and in a mentally retarded patient (106). Several other studies have investigated deletions in the 2p16.3 region, and overall, deletions were found in 0.19 percent of the cases and in 0.04 percent of the controls, giving an OR of 4.78 (95% CI:2.44-9.37) (107). Further, deletions at 15q11.2 have been reported in several studies (24;108), with a combined frequency of 0.6 percent in cases and 0.22 percent in controls (95 % CI: 2.0-3.9). Another deletion on 15q13.3 was reported in the two large independent schizophrenia studies (24;93). The pathogenicity of this locus is heterogeneous, with a variety of phenotypes associated, like mental retardation and seizures, idiopathic generalized epilepsy and autism (107). Moreover, CNVs on 16p13.1 increase susceptibility to autistic phenotypes, mental retardation, and other clinical abnormalities in addition to schizophrenia (107).

3.5 Genetic overlap of mental disorder

The dichotomous classification of psychotic disorders from the early 19th century by Emil Kraepelin is still reflected in current psychiatric diagnostic systems (DSM-IV and ICD-10), where schizophrenia and bipolar disorder are still handled as distinct disease entities. Kraepelin used the terms dementia praecox and manic depression insanity for schizophrenia and bipolar disorder respectively, but doubted this separation later (109). In addition, patterns of co-aggregation, or co-morbidity of psychiatric disorders in family studies, suggest a causal relationship between schizophrenia, other psychotic disorders and certain personality disorders (67). The debate on psychiatric continuum, with shared underlying biological mechanisms, is still ongoing (110), with substantial molecular evidence indicating that disease mechanism and genetic susceptibility do not respect current diagnostic boundaries (40;111-115). The diseases are inherited in a complex fashion and is further compounded by the conundrum of the phenotypic complexity seen for psychiatric illnesses, where both schizophrenia and bipolar disorder have broadly similar outcome and response to treatment (116). Therefore, the diagnostic categories are prone to be heterogeneous and the boundaries between them to some extent capricious.

The strong indications of overlapped genetic causes and pathways in schizophrenia and other mental disorders was also proved in a large Swedish population based study (112) and several GWASs (113;117;118). In general, common SNP alleles have been shown to have a role in all disorders (99), while recent CNV studies have shown an overlap between the genetic loci that predispose to particularly autism and schizophrenia, where some loci seem to be acting in a reciprocal manner, such that deletions are associated with autism, and duplications are associated with schizophrenia and vise versa (99).

Reports have emerged on both similarities and differences between various psychiatric phenotypes in key biological substrates, such as immune activation (119) and brain structures (13). Findings from studies using endophenotype measures have also reported overlap of the disorders, including reduced white matter densities (120;121), reduced anterior thalamic grey matter (122), aberrant gene expression in post mortem brain tissue of both schizophrenia and bipolar disorder (123), common epidemiological risk factors, like winter/ spring birth (124) and similarities in cognitive impairment (125). Also, the clinical features that define psychotic disorders (*e.g.*, delusions, hallucinations and affect dysregulation) are present to different degree in bipolar disorder and schizophrenia. These disorders may therefore be perceived as dimensionally different rather than categorically separate entities (125). This suggests that there are behavioral and neurobiological phenotypes across schizophrenia and bipolar disorder. If this is correct, studies of quantitative phenotypes across diagnosis may give more direct link to underlying neurobiological substrates, and increase the statistical power compared to the usual descriptive categories. The same picture is emerging from studies of pharmacological treatment, which show that antipsychotic drugs are effective in both schizophrenia and bipolar disorder, supported by recent findings of similar mechanisms related to metabolic side-effects across diagnosis (126;127).

3.6 Genetic variation and adverse effects of treatment

There are several adverse effects linked to second generation psychotropic agents, like increased body weight (128;129), lipid and glucose abnormalities (130) and possibly hypertension, with medical consequences ranging from cosmetic concerns to increased rates of cardiovascular disease *e.g.* hypertension, coronary artery disease and diabetes (131). Unfortunately, these adverse effects are often the limiting factor for compliance in patients treated with psychotropic agents. It is therefore important to understand individual differences in the susceptibility to metabolic side effects to optimize treatment.

The underlying molecular mechanisms responsible for antipsychotic drug-induced weight gain remain unknown. One theory is that second generation antipsychotic drugs (*e.g.*, clozapine, olanzapine, risperidone, quetiapine and ziprasidone) affect the hypothalamic control of appetite regulation and energy expenditure. Both first generation- and second generation antipsychotic drugs have a complex pharmacology, interacting with several serotonergic (*e.g.*, 5-HT_{1A}, 5-HT_{2C}, 5-HT_6 and 5-HT_7 (132-134)), dopaminergic (*e.g.*, D_2, D_3 and D_4 (135-137)), histaminergic (like H_1 (138) and H_4 (139)) and muscarinic acetylcholine receptors (140;141)). A study conducted by Kroeze and colleagues examined a large number of first generation- and second generation antipsychotic drugs and indentified H_1-histamine receptor affinity to be significantly correlated with short-term weight gain (142).

Pharmacogenomics is the study of the role of inherited and acquired genetic variation in drug response. Several candidate genes have been examined for association to drug-induced weight gain, with most promising findings for the 5-HT_{2C} receptor (143), leptin (144) and insulin-induced gene 2 (145). A recent pharmacogenomic GWAS found several SNPs to mediate the effects of psychotropic agents on hip circumference, waist circumference and triglycerides (146), but replication is warranted to firmly establish these genetic variants as true mediators of psychotropic agents. There is a need for more knowledge on pharmacogenomics of adverse effects associated with psychopharmacological agents, and the study by Adkins *et al.*(146) indicate that GWAS can discover genes and pathways that potentially affect individual adverse effects of antipsychotic medication.

4. Aims of the study

The main goal of this thesis was to expand current knowledge on inherited genetic variants as susceptibility factors of psychotic disorders, by performing case-control genome wide association studies.

Specifically the aims were to

1) identify putative susceptibility genes for schizophrenia in a homogenous Norwegian sample by genome-wide association study, and potentially validate true associations in a larger European sample (**Paper I**)

2) assess the potential involvement of *neurotrophic tyrosine kinase, receptor, type 3* (*NTRK3*) in bipolar disorder etiology, testing for both single marker associations and haplotype associations, to test if the gene influence risk beyond traditional diagnostic boundaries (**Paper II)**

3) explore the role of variation in adverse effects of psychotropic drugs, using a genome wide approach on a large number of subjects, diagnosed with psychiatric disorders and with well characterized drug treatment and response(**Paper III**).

5. Materials and Methods

5.1 The thematic research area psychosis

The Thematically Organized Psychosis Study (TOP) is a large, translational, ongoing multi-site research study, carried out by the University of Oslo in collaboration with several psychiatric hospitals in the Oslo area. The main diagnostic groups are schizophrenia and bipolar disorder, and the patients are mainly included from the outpatients units of each health care sector, but also from intermediate and long-term treatment units. The health care system is catchment area based and free of charge. The patients were invited to participate in the study by the clinician responsible for their treatment. The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate approved the study, and the biobank was approved by the Ministry of Health.

In this thesis, data are based on patients and controls included in the study from the start-up in October 2002 through June 2010.

5.1.1 Subjects

In order to participate in the studies performed in this thesis, subjects had to be between 18 and 65 years old, speak a Scandinavian language, obtain an IQ score above 70, have no history of severe head trauma or neurological disease, fulfill the DSM-IV criteria for any major psychotic or bipolar disorder and be willing and able to give informed consent. Diagnosis was established using the Structured Clinical Interview for DSM-IV-TR-axis I disorders (SCID-I) (147). All interviewers finished a training course in SCID assessment based on the UCLA training program (148), and participated in diagnostic evaluation meetings on regular basis led by an experienced clinical researcher in the field of diagnostics in severe mental disorder. To assess reliability for the actual study interviews, a stratified random sample was drawn, consisting of cases from each of the raters. Anonymous vignettes describing symptoms and development of the illness were then rated by two experts blind to the study ratings. For the 28 vignettes the overall agreement for the DSM-IV diagnostic categories was 82% and the overall $\kappa = 0.77$ (95% CI: 0.60–0.94). Global Assessment of Functioning Scale (GAF) (149) was utilized to measure psychosocial functioning and split into scales of symptoms (GAF-S) and function (GAF-F) to improve psychometric properties (149;150). The inter-rater reliability of the investigators was good for the GAF with an intra class correlation, ICC 1.1, of 0.86 (149;151). The majority (90%) of the patients was ethnically Norwegian, i.e. the patient and both parents were born in Norway, while in a minor fraction of the cases (10%), one parent was born outside Norway in another North-Western European country.

This led to inclusion of the following diagnosis: schizophrenia, schizophreniform, schizoaffective (in this thesis referred to as schizophrenia spectrum disorders) and bipolar spectrum disorder (including bipolar I, II and NOS).

The healthy subjects were randomly selected using records of people from the same catchment areas as the patient groups. Only subjects born in Norway were contacted by letter and invited to participate. All controls were of Caucasian origin; around 85% had two Norwegian parents, the rest had one parent of other European origin. Moreover, all participants had to have Norwegian as their first language or have received their compulsory schooling in Norway.
The control subjects were screened by interview for severe mental illness and substance abuse, and with the Primary Care Evaluation of Mental Disorders (PRIME-MD). None of the control subjects had a history of moderate/severe head injury, neurological disorder, mental retardation or an age outside the age range of 18–65 years. Healthy subjects were excluded if they or any of their close relatives had a lifetime history of a severe psychiatric disorder (schizophrenia, bipolar disorder and major depression), a history of medical problems thought to interfere with brain function (hypothyroidism, uncontrolled hypertension and diabetes), or significant illicit drug use.

5.1.2 Measurements

Information on age, gender and country of birth was recorded. After the inclusion interview, a physical examination was performed. Height and weight for BMI (kg/m^2), waist circumference and heart rate (beats/minute) were obtained. Blood pressure was recorded manually in a sitting position after resting, and waist circumference was measured midway between the lower rib and the iliac crest in the upright position using non elastic tape. Prior to the physical examination, blood samples were drawn after an over-night fasting and analyzed for fasting plasma glucose, triglycerides, high density lipoprotein cholesterol, low density lipoprotein cholesterol, total cholesterol and C-reactive Peptide. All serum analyses were performed at the Department of Clinical Chemistry, Oslo University Hospital, Oslo, Norway, on an Integra 800 (Roche Diagnostics, IN, USA), using standard methods. In addition, information on smoking habits was recorded. In order to obtain normally distributed variables, all outcome measures besides LDL-C and TC were log transformed.

To assure proper compliance, the serum concentrations of all psychopharmacological agents were determined by the Laboratory of Clinical Psychopharmacology, St. Olav Hospital, Trondheim, Norway. Further, patients' drug intake was also measured with a self report. For more details, see Jonsdottir et al (152).

5.1.3 Genetic features of the TOP sample

The TOP sample was genotyped on the Affymetrix Genome-Wide Human SNP Array 6.0 at Expression Analysis Inc, Durham, USA and Oslo University Hospital, Oslo, Norway. When visualizing the overall genetic landscape, we observe that the TOP study sample primarily clusters with the CEU sample of the HapMap sample (Figure 2). Evidently there is similar genetic structure between the schizophrenia spectrum and bipolar disorder cases, as well as the healthy controls (Figure 3). The figures are based on the TOP 5 dataset (October 2010), containing 1023 samples.

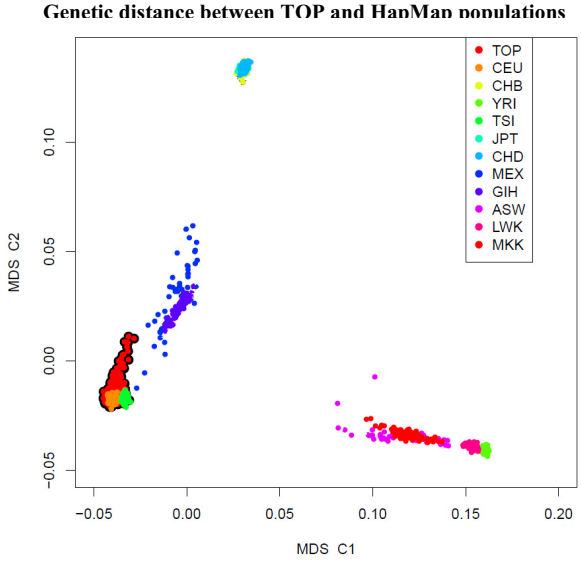

Figure 2. Multidimensional scaling (MDS) based on 550,000 SNPs genotyped in the Norwegian TOP sample (n=1023) and eleven separate HapMap samples. The first component (x-axis) is plotted against the second component (y-axis). TOP: the TOP sample after main quality control; CEU: Utah residents with Northern and Western European ancestry from the CEPH collection; CHB: Han Chinese in Beijing, China; YRI: Yoruban in Ibadan, Nigeria; TSI: Toscans in Italy; JPT: Japanese in Tokyo; CHD: Chinese in Metropolitan Denver, Colorado; MEX: Mexican ancestry in Los Angeles, California; GIH: Gujarati Indians in Houston, Texas; ASW: African ancestry in Southwest USA; LWK: Luhya in Webuye, Kenya; MKK: Maasai in Kinyawa, Kenya.

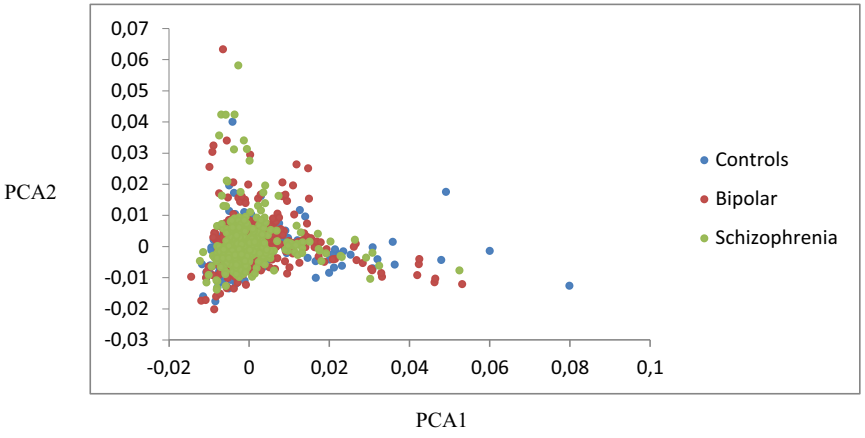

Figure 3. Principal components analysis (PCA) on 550,000 SNPs genotyped in the Norwegian TOP sample (n=1023), including schizophrenia spectrum patient, bipolar patients and controls. Principal component 1 (PCA1, x-axis) is plotted against principal component 2 (PCA2, y-axis), showing a complete overlap between the three groups.

5.1.4 Sub-studies sampling procedures

Paper I:

For the purpose of investigating genetic variants associated with schizophrenia, data from healthy controls and individuals diagnosed with schizophrenia spectrum disorders were included in the study.

Paper II:

For the purpose of exploring an association between bipolar spectrum disorder and genetic variants in the gene *NTRK3*, genetic data from healthy controls and subjects with bipolar spectrum disorder were included in the study.

Paper III:

For the cause of investigating genetic variants that affect adverse side effects of psychopharmacological agents, subjects with a severe mental disorder were included in the study. In addition, we excluded patients who had received psychopharmacological treatment for less than four weeks, or if the serum concentration of the drugs were not in concordance with the prescribed daily dose.

5.2 Replication sample description (SGENE-plus)

A total of 1,321 patients affected with schizophrenia and 12,277 controls from Germany (Munich), England, Finland, Iceland, Italy and Scotland (The SGENE sample; http://SGENE.eu) with an additional 859 cases and 854 controls from Germany (Munich) and Scotland, 483 cases and 367 controls from Germany (Bonn) as well as 282 controls from Finland, giving a total of 2,663 schizophrenia cases and 13,780 controls (SGENE-plus) were used in the replication analysis in ***Paper I***. Cases were diagnosed with schizophrenia according to DSM-IV or ICD-10 criteria. The controls were part of several genetic studies conducted by DeCode (Reykjavik, Iceland) and were not screened for psychiatric diseases.

5.3 Methods in the association studies

All papers in this thesis are genetic association studies. ***Paper I*** and ***Paper III*** are genome-wide investigations, while ***Paper II*** had a candidate gene approach.

5.3.1 Candidate gene study

In ***Paper II*** the gene *NTRK3*, that had been associated with schizophrenia previously, was investigated for genetic association with bipolar disorder. 149 markers, extracted from the genome wide genotyping of the TOP sample, were tested for frequency differences between cases and controls using both allelic and genotype-based Cochran-Armitage trend tests implemented in PLINK (153). Logistic regression was used to analyze associations of haplotype with disease status, using the sliding window approach with window sizes between 2 and 10, also implemented in PLINK (153). In addition, a graphical assessment of P-values from the sliding windows haplotype tests (GrASP) (154) was used to visualize haplotypes associated with bipolar disorder.

5.3.2 Genome wide study

A genome wide approach was selected for ***Paper I*** and ***Paper III***. The study design is well suited as an exploratory study in **Paper I** where 201 schizophrenia cases and 305 controls were used in the discovery sample. The approach allows search for genetic risk variant without prior hypothesis. The replication sample was also genotyped using genome-wide based chip, consisting of 1,321 cases and 12,277 controls with European origin (described in section 5.2). In **Paper III**, 594 subjects diagnosed with a psychiatric disease were used in the GWAS on metabolic outcomes. Each SNP that survived quality control and cleaning was tested for association with the disease trait (schizophrenia) in **Paper I**, and the metabolic outcome measure in **Paper III**. A Manhattan plot was generated for each GWAS performed, where the negative log of the P value was plotted against the chromosomal position, using the plot function in R (155). In addition, LocusZoom was used to make so called forest plots, where the negative log of the P value and the recombination rates are plotted against chromosomal position. The measure of LD between the highest scoring SNP and its neighboring markers are also depicted.

5.3.3 Linkage disequilibrium as a tool

In order to investigate SNPs across the two genotyping platforms (Affymetrix and Illumina) used to genotype the discovery and replication sample in **Paper I**, respectively, LD was used to identify surrogate SNPs with an r^2 value of above 0.8, using HaploView. LD was also used to evaluate obtained association signals in a region, to differentiate SNPs capturing the same association signal, and SNPs capturing independent association signals.

5.3.4 Genotyping technology and quality control

A new era was entered when chip-based technology enabled genotyping of more than 500,000 SNPs simultaneously in one sample, and it has revolutionized the search for the genes underlying human complex diseases. DNA was extracted from whole blood drawn at inclusion, and genotyped at Expression Analysis Inc. (Durham, USA) or at Oslo University Hospital, (Oslo, Norway) using the Affymetrix Genome-Wide Human SNP array 6.0 (Affymetrix Inc., Santa Clara; CA, USA). As with any genotyping method, a proper, strict quality control is necessary. Individuals with discrepancies between reported and genotyped sex (based on X and Y genotypes) were removed. We removed all mitochondrial DNA SNPs, as well as those on the sex chromosome or whose location was unknown. To control for population stratification, identical by state (IBS) scores were calculated. This step allows the removal of samples from ethnically distant subjects and tuning for any systematic differences between genotyping runs. Initially, individuals with a call rate less than 90% were removed, followed by all SNPs with a call rate less than 95%. Secondly, we excluded individuals with a call rate less than 97 %, and then SNPs with less than 97% and any remaining SNPs with a minor allele frequency of less than 0.05. Finally, individuals with outlying (greater than three standard deviations) levels of heterozygosity were removed. Following quality control, 608,239 SNPs from 837 individuals remained for statistical analysis.

5.4 Statistics

5.4.1 Statistical analyses

The essential intention of an association study is to compare allele or genotype frequencies of affected subjects to the frequencies of a control group. The common test for genetic association is the standard χ^2 test, based on either a 2 x 2 (Table 1) or a 2 x 3 (Table 2) table, where the test for independence of the phenotype is from alleles or genotypes, respectively. To reject the null hypothesis of no association, the χ^2test statistic has to be large enough.

The allelic association analysis is calculated as

$$\chi_{\text{allelic}}^2 = \frac{(A2 \cdot C1 - A1 \cdot C2)^2}{A_{tot} \cdot C_{tot} \cdot A \cdot a}$$

see table 1 for parameters. The assumptions for an allelic test are that the alleles are in Hardy Weinberg Equilibrium and that the risk allele has an additive effect. The same table can be used when the mode of inheritance is dominant or recessive, as the genotypes are dichotomized. In the dominant model, the homozygote with two risk alleles and the heterozygote with one risk allele are grouped together and compared to the homozygote with no risk alleles. In the recessive model, the homozygote with two risk alleles is compared to the heterozygote with one risk allele and the homozygote with no risk alleles.

Table 1. 2 x 2 contingency table for allelic association test

Allele		A	a	Total
Frequency/ Allele count	Cases	A1	A2	A_{tot}
	Controls	C1	C2	C_{tot}
	Total	A	a	N

We used the allele based 2 x 2 test (**Paper I**) and the dominant model (**Paper III**).

For the genotypic association test, the observed genotype frequencies between cases and controls are compared with those expected under the null hypothesis of no association, in a χ^2 test:

$$\chi_{geno}^2 = \frac{\left(A1-AA\frac{A_{tot}}{N}\right)^2}{AA\frac{A_{tot}}{N}} + \frac{\left(A2-Aa\frac{A_{tot}}{N}\right)^2}{Aa\frac{A_{tot}}{N}} + \frac{\left(A3-aa\frac{A_{tot}}{N}\right)^2}{aa\frac{A_{tot}}{N}} + \frac{\left(C1-AA\frac{C_{tot}}{N}\right)^2}{AA\frac{C_{tot}}{N}} + \frac{\left(C2-Aa\frac{C_{tot}}{N}\right)^2}{Aa\frac{C_{tot}}{N}} + \frac{\left(C3-aa\frac{C_{tot}}{N}\right)^2}{aa\frac{C_{tot}}{N}}$$

see Table 2 for each included factor.

Table 2. 2 x 3 contingency table for genotypic association test

Genotype		AA	Aa	aa	Total
Frequency/ Allele count	Cases	A1	A2	A3	A_{tot}
	Controls	C1	C2	C3	C_{tot}
	Total	AA	Aa	aa	N

In the follow-up study of the initial discovery GWAS (**Paper I**), the multi-national case-control SGENE-plus sample was used, consisting of individuals from eight North-European countries. The statistical method used to perform the stratified association analysis was the Cochran-Maentel Haenszel (CMH) χ^2 test for independence of i x j x k contingency tables, where k is the number of strata (the number of subsamples in this case). The SGENE sample was analyzed for association using the allele-based 2 x 2 x k test. To evaluate the effect size of risk conferred by the allele with potential susceptibility, the odds ratio (OR) was calculated. For a given SNP with alleles A and a, the OR is the difference between the odds of having the disease when carrying the A allele, compared to the odds of having the disease when carrying the a allele. Using the allele frequencies or allele counts given in Table 1,

$OR = (A1/C1)/ (A2/C2) = (A1 \cdot C2)/(C1 \cdot A2)$

In other words, the OR is calculated by comparing the allele frequencies for each of the two alleles between cases and controls. The calculated risk measure has an uncertainty, and a confidence interval is used to indicate the reliability of the estimate, so usually the 95 percent confidence interval is accompanying the OR.

Also, a combined analysis using Fischer's combined probability test was used to calculate combined χ^2 values to obtain combined P values for each marker from the discovery case-control GWAS and the follow-up study (**Paper I**).

5.4.1 Power considerations

Genetic susceptibility loci in psychiatric diseases generally have a low effect size, hence large studies are needed to detect differences in SNP frequencies between cases and controls. The statistical power in an association study is the probability to detect true underlying genetic associations. A well powered study usually uses a parameter of 80 percent, meaning that if there is a true underlying SNP versus disease association, there is an 80 percent change of detecting it. Power calculations were done using the online available tool Genetic Power Calculator (156). Parameters used in the calculation are prevalence of the illness in the population, significance and power levels, the assumed effect size of the underlying causal variant, the allele frequencies of both the SNP and underlying causal variant and the LD measure between these two variants and last the assumed genetic model of inheritance for the risk SNP.

5.4.2 Correction for multiple testing

Because of the high number of statistical tests that are performed in a GWAS, there is a high false positive discovery rate, *i.e.*, the type I error is inflated. There are several ways to correct for multiple testing, and some common methods are Bonferroni, permutation and false discovery rate (FDR) (157).The significance α value is usually set at five percent, *i.e.* we are willing to accept one false positive association for every 20 SNPs tested. When testing approximately 600,000 SNPs, that would result in 30,000 SNPs obtaining a P value below α (0.05 x 600,000) just by chance. Therefore, one should consider setting a more stringent α level. Depending on the study design, genome wide statistical significance is set at P values of approximately 5×10^{-8} or less in most studies, representing the significance level α divided by number of SNPs tested for association with the trait, called the Bonferroni correction. The FDR approach controls the number of false discoveries in those tests that result in a discovery (*i.e.* a significant result). Therefore, FDR is less conservative than the Bonferroni correction.

In **Paper I**, a more liberal threshold was used to identify top scoring markers to be tested in the replication study. Here, the 1,000 SNPs with lowest P values were selected for further

analysis in the replication sample. In **Paper III** a FDR of 0.1 was used to declare genome wide significance. Both approaches are more liberal than the conservative Bonferroni correction, but all the SNPs tested are not independent of one another (some SNPs are in LD), advocating for a more liberal correction. To declare significance in **Paper II**, the Bonferroni correction was used, correcting for 142 SNPs tested.

5.4.3 The statistical software

The software used in this thesis is open-source tools that are free to download from their respective web pages, with the exception of SPSS.

PLINK
PLINK is a toolset designed for whole genome genetic association analysis (pngu.mgh.harvard.edu/purcell/plink/) (153) that can be used for single marker associations, as well as haplotype or epistatic tests. The Windows/MSDOS command line application was used for genome wide association analysis (**Paper I, III**) and single marker and haplotype association analysis (**Paper II**). In addition quality control and cleaning of the data was undertaken using commands implemented in the program.

R
R is an integrated suite of software for statistical computing and graphics (www.r-project.org/), which was used for data management (**Paper I**, **II**, **III**), for drawing Manhattan and quantile-quantile plots (**Paper I**, **III**), and calculate combined P values for the case-control GWAS (**Paper I**).

Affymetric Power Tools
The Affymetrix Power Tools are a set of cross-platform command line programs that implement algorithms for analyzing Affymetrix GeneChip arrays (Affymetrix Inc, CA, USA), and was used to determine the genotypes for the GWAS sample.

SPSS
The SPSS inc. (www.spss.com/) *SPSS statistics* (renamed *Predictive Analytical Software (PASW) Statistics*) was used to determine if metabolic outcome variables were normally distributed, and if not, log transformed.

HaploView
The program was used for calculation and visualization of LD and to pick surrogate markers on the Illumina chip for SNPs on the Affymetrix chip. The GUI for Windows version 4.1 (**Paper I, II, III**) was used (www.broad.mit.edu/haploview/haploview) (158).

LocusZoom
LocusZoom is a tool to plot regional association results from genome wide association scans or candidate gene studies (http://csg.sph.umich.edu/locuszoom/) (159).

6. Results in brief

All three papers included in this thesis are genetic association studies, either candidate-based (**Paper II**) or genome wide (**Paper I, III**) of psychotic disorders, with focus on disease susceptibility or presence of adverse effects related to psychopharmacological agents.

6.1 Paper I

Gene variants associated with schizophrenia in a Norwegian genome-wide study are replicated in a large European cohort

The aetiology of schizophrenia is still unclear, but a strong genetic component is unequivocal and several genes with modest effects are thought to act jointly to increase risk. We wanted to search for and identify susceptibility genes for this disabling illness, by exploring a well characterized Norwegian case-control sample.

We genotyped over 570,000 markers throughout the genome on the Affymetrix Genome-Wide Human SNP Array 6.0 in 201 cases and 305 controls. SNP versus disease associations were investigated using the allele based test in the Norwegian discovery sample, and a focused follow-up of the 1,000 highest scoring markers (or HapMap-based surrogates) were tested for disease association in a large, multi-centre European cohort consisting of 1,321 schizophrenia cases and 12,277 controls to validate the potential risk variants. Further, a combined analysis of the findings from the discovery and replication analysis was performed.

In the TOP discovery GWAS, no association reached the predefined genome wide significance level of 8.7×10^{-8}, but the GWAS revealed seven loci that were moderately associated with schizophrenia ($P < 1 \times 10^{-5}$). The strongest signal was found on 7q21, 12 kb downstream of the *piccolo* (*PCLO*) gene, encoding a presynapitc cytomatrix protein. In the follow-up study, 1,000 markers were selected and attempted to replicate in the SGENE-plus sample, but for 300 markers no surrogate match was available. Replication of 32 associations was observed with uncorrected P value < 0.05 and concurring OR between the two samples, and one marker reached the experimental significance value $P < 2.4 \times 10^{-4}$. The combined analysis highlighted three loci with P values in the 10^{-6} range, on 9p21, 16p12 and 10q21 near the genes *PLAA*, *ACSM1* and *ANK3* respectively. In addition, the 30 genes or gene regions that ranked highest in the SzGene database (accessed June 2009) were examined for SNP-disease association in the discovery GWAS to investigate if the same susceptibility genes confers risk in the Norwegian population. Marginally significant associations were found for *DISC1*, *RELN*, *NRG1* and *OPCML*.

In conclusion, potential susceptibility genes for schizophrenia were identified in the discovery GWAS and these findings were further supported by replication in the large European cohort. As anticipated, potential true associations that were successfully replicated in the SGENE-plus sample were not necessarily among the apparent top hits in the discovery sample.

Moreover, a potential genetic overlap of bipolar disorder and schizophrenia is advocated with the association of *ANK3* as susceptibility gene for schizophrenia, as the same gene has repeatedly been associated with bipolar disorder in several populations. Further replications in other populations are warranted to validate these genetic variants as true susceptibility loci for schizophrenia.

6.2 Paper II

Intron 12 in NTRK3 is associated with bipolar disorder

Neurotrophic factors have an important role in brain development and plasticity. Aberrant expression of the *neurotrophic tyrosine kinase receptor 3* (*NTRK3*) has been implicated in psychiatric disorders, and a previous study by our group suggested the *NTRK3* gene influences hippocampal function and may modify the risk for schizophrenia. In this study, *NTRK3* polymorphisms were analyzed for association with bipolar disorder, as several studies now advocate a genetic overlap in the aetiology of the two disorders. Patients diagnosed with bipolar spectrum disorders and healthy controls were investigated for 149 polymorphisms, spanning the *NTRK3* gene, with the basic allele-based test and the genotype-based Cochran-Armitage trend test. In addition, haplotypes were investigated for inflation in the cases.

Several genetic markers, with non-random location, reached study-wide significance with corrected P value 0.004 (correcting for 149 tests). The same region was highlighted with the haplotype association analysis. Interestingly, our markers appeared to be located close to or within the linkage regions reported in studies including schizophrenia, early-onset depression and eating disorder.

In conclusion, these findings support the hypothesis that some genes influence risk for severe mental illnesses beyond traditional diagnostic boundaries.

6.3 Paper III

Genome-wide association study identifies genetic loci associated with body mass index and HDL-cholesterol levels during psychopharmacological treatment. A cross-sectional naturalistic study

Metabolic and cardiovascular side effects are serious clinical problems related to psychopharmacological treatment. The underlying mechanisms are mostly unknown, and these adverse effects are often a limiting factor for compliance. Genetic variation is thought to influence the risk of adverse effects. We investigated genetic variants in a cross-sectional GWAS, focusing on body weight and other related metabolic factors in a relative large, chronic population of psychiatric patients, with robust phenotypic indices, treated with multiple psychotropic agents. The patients were assigned to three different medication groups

based on the reported side effects liability of psychopharmacological agents used in their treatment. Drug naïve patients as well as those receiving medication with low liability for adverse effects were selected for medication group I. The patients selected for medication Group II were receiving drugs with some liability for adverse effects, while those in medication group III were treated with psychopharmacological drugs with strong liability for adverse side effects. We analyzed if the effect of drugs in group II relative to group I and group III relative to group I, on the twelve metabolic and cardiovascular outcome variables were dependent on genetic variants. The genome wide significance level was defined as FDR below 0.01.

Only minor differences in metabolic and cardiovascular outcomes among the different psychopharmacological groups were detected. However, there was a graded effect of various drugs on blood measurements, such as lipid profiles. Interactions between medication and genetic variants were found to affect BMI and high density lipoprotein cholesterol (HDL-C) levels. For genetic interactions affecting BMI the gene *matrix metallopeptidase 16* (*MMP16*) was identified and an intergenic region on 12q21.

Interesting results indicate that genetic variation influence the level of adverse effects experienced. The genes identified in this setting of more tolerable long term treatment could have impact on the longevity of patients with severe mental disorders, as they often receive pharmacological treatment for long periods of their life span, and have increased mortality. These results await replication once the suitable sample with naturalistic design will be available.

7. General discussion

7.1 Findings, interpretations and clinical implications

The three papers that form the basis of this thesis are all genetic association studies aimed at investigating the role of common variants in the susceptibility for psychotic disorders, with special focus on schizophrenia. In addition genetic variants influencing the levels of adverse effects of psychotropic agents were investigated.

7.1.1 Susceptibility genes for schizophrenia

In **Paper I** the GWAS identified 32 SNPs associated with schizophrenia in the discovery sample that were also associated in the follow up study in the larger SGENE sample, with OR in the expected range for complex disorders of 1.1 - 1.2. The markers draw attention to 16 loci harboring potential susceptibility genes for schizophrenia.

The strongest finding for genetic variants conferring risk for schizophrenia was rs7045881 located in the first intron of *PLAA* on 9p21, with experiment-wide significance level of association in the combined analysis ($P = 2.12 \times 10^{-6}$, OR = 1.16). This gene encodes a phospholipase A2-activating protein (PLAP). The putative role of this protein is in regulation of the metabolism of phospholipids in the cell membrane, by activating the protein phospholipase A2 that is found in excess in cytosol in cellular samples obtained from patients suffering from schizophrenia. A polymorphism in the first intron of *cytosolic phospholipase a2* was previously also associated with schizophrenia (160).

Another strong association was observed for markers located on 16p12, within the *ACSM1* locus (rs433598, $P = 3.27 \times 10^{-6}$, OR = 1.13) and within the *ACSM2* locus (rs1234972, $P = 9.09 \times 10^{-5}$, OR = 1.12). Interestingly these genes have been among the top findings in another schizophrenia GWAS (87). However, the risk conferring SNPs in the CATIE study do not overlap with the ones in the TOP study. This could be related to differences in the sample ethnicity of the two studies. The CATIE study is based on an ethnically more heterogeneous sample that could have different ancestries, while the TOP study is based on a more homogenous population.

Markers within the first intron in *ANK3* were associated with schizophrenia in the combined analysis (rs10761482, $P = 7.68 \times 10^{-6}$, OR = 1.16). *ANK3* is one of the top scored susceptibility genes for bipolar disorder, encoding a protein involved in several cell functions, like proliferation and cell mobility. The protein was first found in axonal initial segments and nodes of Ranvier of neurons in the central and peripheral nervous system (161). The aetiology of schizophrenia and bipolar disorder are converging as new findings support the hypothesis of a psychiatric spectrum rather than the dichotomized view first proposed by Kraepelin.

7.1.2 Common genetics for mental disorders

In **Paper II**, genetic variants in *NTRK3* previously associated with schizophrenia were investigated for their possible association to bipolar disorder, to explore if this gene might be part of the suggested genetic overlap between the two disorders (112). Of the 143 investigated markers, 20 were SNPs significantly associated with bipolar disorder (Bonferroni corrected P-values). These markers were located in intron five through twelve. In addition, we investigated *in silico* the possible effect of the intronic markers on gene expression. The best scoring transcription factor modules were a combination of Nkx2.2, Pax6, Zic3 and PUO2F1.

Neurotrophins are a class of proteins that include brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), and the proteins are involved in regulation of several neuronal functions (162;163) and synaptic plasticity (164). The *NTRK3* gene is highly expressed in both the developing and the adult hippocampus in mammals (165). Our group previously identified genetic variants associated with schizophrenia and two variants also had an effect on hippocampal activation during a memory encoding task (18). The present results seem to implicate *NTRK3* in aetiology of bipolar disorder, and to confer risk for both schizophrenia and bipolar disorder.

Other genes have also been implicated in several mental disorders, for example *ANK3*. The gene has repeatedly been associated with bipolar disorder (166;167), and was associated with schizophrenia in the discovery GWAS and replicated in the follow-up.

7.1.3 Genetic variation and risk for adverse effects

Metabolic and cardiovascular side effects are serious clinical problems related to psychopharmacological treatment, but the underlying mechanisms remain mostly unsolved. In **Paper III**, a genome-wide association study of metabolic and cardiovascular risk factors during pharmacological therapy in patients with severe mental disorders, in a naturalistic setting, was undertaken to elucidate this conundrum. Twelve indicators of metabolic side effects (BMI, waist circumference, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), triglycerides TG, fasting glucose, HDL-C/TC ratio and C-reactive protein) as well as cardiovascular risk factors (blood pressure, heart rate) were analyzed in a naturalistic sample of 594 patients of Norwegian ancestry. We analyzed interactions between gene variants and three categories of psychopharmacological agents based on their reported potential for side effects and defined genome-wide significance based on FDR of 0.1. Dividing the pharmacological agents in three medication groups was no arbitrary task. It was based on accumulated evidence on risk for adverse metabolic side effects, and this may be subject to publication bias. Further, the pharmacological agents in the three different groups are likely to exert their effect through different molecular mechanisms, resulting in reduced power to find true associations.

The results highlight three genomic regions potentially harboring susceptibility genes for drug induced metabolic side effects, identifying *matrix metalloproteinase 16* as a candidate gene. For BMI, two significantly associated loci were identified on 8q21.3, highlighted by 13 markers. There were seven markers in a 30kb region and the strongest signal was rs7838490 ($P = 6.07 \times 10^{-8}$, FDR= 0.051) where the G allele in combination with medications in group II confers risk of increased BMI. In another locus 140 kb away, six markers were significant, and rs6989402 obtained the strongest signal ($P = 1.54 \times 10^{-7}$, FDR= 0.018). Both of these loci are located upstream of the gene *matrix metalloproteinase 16*. For HDL-C, marker rs11615274 on 12q21 was significant ($P = 9.01 \times 10^{-8}$, FDR =0.051) and the T allele is associated with lower HDL-C levels in combination with medications in group II. Individuals with the C allele will thus tolerate treatment with medications in group II better than those with the T allele. This locus on 12q21 is located in an intergenic region, but regulatory elements in the region might effect expression of nearby genes.

Few GWAS studies regarding the metabolic and cardiovascular side effects of psychopharmacological drugs and genetic factors are available. Follow-up studies of our results are necessary to strengthen and validate our findings. In the future, hopes for individualized treatment based on the individual's genetic make up, regarding both treatment response and adverse side effects of antipsychotics are high.

7.1.4 Current large scale GWAS findings

Large scale endeavors were undertaken during the last three years, with consistent and partially concordant results. Stefansson *et al.* (24) performed a GWAS on 2,663 cases and 13,498 controls, while the second was conducted on 3,322 cases and 3,587 controls by the International Schizophrenia Consortium (ISC) (113). Both studies identified association of schizophrenia with SNPs located in the major histocompatibility complex that is located on chromosome 6p21.3-22.1. The strongest signal was located around the gene *NOTCH4*. The association was further confirmed in an independent GWAS of schizophrenia (168). Family linkage studies have previously also identified this region to be a potential locus for the disease (169) and an budding hypothesis linking schizophrenia to immunity also supports the importance of the MHC in the pathogenesis of the disease (170;171). Several other associations were detected in these two studies. Stephansson *et al.* identified associated markers in *neurogranin* (*NRGN*) and *transcription factor 4* (*TCF4*) (24) genes that are important in brain development, while *ZNF804A* was further strengthened as an schizophrenia associated gene by the ISC's study (113).

Finally, a new wave of potentially associated markers has emerged from recent meta-analyses. The meta-analysis reported by Chen *et al.* used data from a combined dataset of 17,198 familial and sporadic cases and a control dataset of 11,380 individuals (172). The highest scoring associations were two markers in the *cardiomyopathy associated 5* (*CMYA5*) gene. Interestingly, familial haplotype analysis has linked *dystrobrevin binding protein 1*

(*DTNBP1*) to schizophrenia, and the protein products of these two genes interact directly (173). Another meta-analysis, undertaken by Wang *et al.*, used a combined dataset of individuals diagnosed with either schizophrenia or bipolar disorder (1,171 cases of schizophrenia) (118), identified five new loci that potentially could be associated with the two diseases.

Our GWAS is one of the first studies in genome-wide molecular genetics of psychiatric disorders conducted in Norway. The project intended to further enhance collaboration between psychiatry and basic research in the Oslo region, and develop transdisciplinary research collaboration between the different departments.

As for clinical value, blood typing for the A, B, O system may serve as an analogy of how specific therapeutic interventions are guided by a specific laboratory test. Similarly, we believe that it will be possible to develop clinical guidelines in psychopharmacology in order to clarify requirements and consequences of individual pharmacogenetic tests. The future project, which will continue on the basis of this thesis, will provide a platform for the use of genetics in the care of psychiatric patients. This could contribute to replace conventional "trial and error" treatment strategies with individualized drug therapy, and could have significant impact on the treatment and care of patients with severe mental disorders.

7.2 General methodological issues; strengths and limitations

7.2.1 Materials

The studies included in this thesis had several strengths. A multi-site approach and broad inclusion criteria permitted us to gather information in a representative sample of individuals receiving "treatment as usual". The sample was well characterized, and reliability testing was performed for all essential items. The confounding effects of ethnicity, long term hospitalization and chronic disability were reduced as the subjects were ethnically homogenous and overall young, with a short duration of treatment and a high level of general functioning. Most subjects were on stable medication, and adherence was ascertained by measuring serum concentration of all psychotropic drugs. In addition, complete medication history is obtained for all patients, thereby minimizing the confounding effects of previous medication.

The ethical aspects of the studies in this PhD project is highly relevant as it implied research involving sensitive personal information and use of biological materials from patients with severe psychiatric disorders. Consideration of objectives, the methodology and the possible implications of results were taken. The participants knew how their information and blood samples would be used for genetic analysis and that they were protected by measures to ensure confidentiality. Adult individuals were recruited to the TOP study, capable of giving informed consent. The clinical investigators, in collaboration with the clinician in charge of treatment, were responsible of evaluating if individual patients were competent of giving

consent. The genetic studies, conducted on the TOP study sample, were approved by the local Committees for Medical Research Ethics. The Data Inspectorate had approved the database and the handling of data, and were based on very strict rules about handling information of such sensitive character, and controlled by the local "Personvernombudet". Further, the TOP biobank was approved by the Health Directorate.

Somatic assessments of the TOP patients were performed by physicians according to a standard protocol, as described in the Methods section. Blood samples in the study were drawn by one specially trained project nurse, collected between 8.00 and 12.00 am, after at least eight hours of fasting. The clinical chemical and hormonal analyses from the study in **Paper III** were performed at the Department of Clinical Chemistry and the Hormone Laboratory at Oslo University Hospital. Both these laboratories are subjected to regular internal precision and accuracy controls, and both national and international quality controls are in concordance with the recommendations of the Nordic committee on Quality Control of the Scandinavian Society for Clinical Chemistry.

BMI is a widely used measure of obesity as it is easy and cheap to measure. One disadvantage of BMI is that it does not discriminate between muscle and fat mass. A person with large muscle mass will have a high BMI, even in the absence of excess fat. To minimize the bias, we controlled for age and gender, variables that could affect the BMI measure.

Psychiatric assessments were performed by clinically experienced psychologists or psychiatrists. The SCID-I interview was used and is known to yield highly reliable diagnosis of Axis I disorders (174). Similarly, the PANSS scale has excellent psychometric properties for assessing psychotic symptoms (175). The GAF scale is viewed as the primary instrument for assessing changes in psychotic symptoms and functioning, although its reliability in clinical settings has been questioned, it has proved highly agreeable in research situations (176).

One limitation of the case-control studies are the diagnosis of severe mental disorders. The diagnosis of schizophrenia and bipolar disorder are both merely based on descriptive criteria on the basis of clinical symptoms. The lack of objective biological measures makes validation of diagnosis unfeasible. Accurate phenotyping of cases is indispensable to the quality of a genetic association study. The lack of biological measures in psychiatric research results in inconsistent findings that do not replicate in subsequent analyses. The inherent phenotypic complexity of psychiatric disorders further obstructs the studies.

A limitation in **Paper III** is the naturalistic design that could have impacted the results. The lack of randomized design could result in unknown factors confounding the results. A range of psychopharmacological agents were grouped together, which could have made it difficult to detect an association signal. The study design was cross-sectional and naturalistic, wherein the possibility that clinicians would change the pharmacological treatment in patients who experience severe side effects, thus reducing the effect of susceptibility genes. Furthermore, the groups were defined based on literature of side effect liability, which could also induce noise. The results (interaction effects) must thus be replicated in independent samples.

7.2.2 Association; study design and analyses

Genetic association studies have experienced inappropriate methodology and poor reporting, which could lead to misleading results and incorrect conclusions. Guidelines for a successful design and reporting are at hand and should be adhered too. The quality of a genetic study is determined by several factors and sample size has long been recognized as one of the major determinants. The sample size needed depends on the preferred power of the study, level of significance, effect size and frequency of the predisposing allele (177;178). Required sample size decreases with increasing frequency of the susceptibility allele. Potential limitation in the GWAS (**Paper I**) was the small sample size used in the discovery GWAS. However, a homogenous sample has been advocated as a crucial factor to minimize false positive results. In addition, we were able to replicate our initial findings through the access to the large multi-centre SGENE-plus sample.

Genotyping error might arise due to many reasons, such as unrecognized tri-allelic SNPs (179) and co-existing mutations in close proximity to the polymorphic site (180), but with stringent quality control measures the error rate is small and will not impact the quality of the study.

Preliminary studies reporting positive findings of significant associations need to be replicated in subsequent studies to be validated as true positive findings. The necessity of these follow-up studies has become an unconditional criterion for publishing genetic association studies in most journals. Replication studies are needed to identify bias and to strengthen the confidence in individual studies, and will reduce type I errors where an effect is found when in fact non true association is present (181).

Population stratification can lead to false positives due to differences in genotyping frequencies and disease prevalence among sub-populations in the total sample, and is caused when samples with different genetic ancestries and/or different genetic mechanisms underlying the disease are combined in the same study (182). The TOP sample is examined for population stratification, and PCA plots show a complete overlap between the schizophrenia, bipolar disorder and control groups (see Figure 2 in the Material & Method section).

Functional studies will be important for the validation of susceptibility genes. The functional effects of candidate genes can be examined *in vivo*, using neuroimaging endophenotypes (17) or animal models, but *in vitro* studies of specific molecular pathways could shed light on the underlying biology of psychiatric disorders.

7.2.3 Correction for multiple testing

Genetic association studies performed with thousands of markers will just by chance give markers where the null hypothesis is rejected, *i.e.* false positive associations. To control for these false positives the family-wise error rate requires very strict α adjustment. The balance of controlling for false positive associations and false negative associations is not arbitrary. Using a too stringent adjustment prevents many true associations with small effect sizes from being discovered and followed up. To find true associations, we looked well beyond the top hit list, and selected 1,000 markers with the strongest association signal to be followed up in a replication study (**Paper I**). In the candidate gene study we used the more stringent Bonferroni correction to declare significance (**Paper II**), while in the GWAS on adverse effects, a FDR of 0.1 was employed to declare significance (**Paper III)**. The latter method is less stringent than Bonferroni correction where the percentage reflects the number of false positives among all tests, while the FDR adjusted P-value implies that a certain percentage of significant tests will result in false positives and is thus a smaller quantity. FDR adjustment is suitable as all markers that were examined were not independent of one another.

7.3 Implications for further research

The possibility to undertake GWAS in large, well-characterized samples is now generating a stream of putative disease susceptibility genes, highlighting new etiological pathways and revealing novel ways of understanding the molecular basis of human health and disease. The TOP study group has together with other partners, been involved in several major recent genetics discoveries for the complex disorder schizophrenia. The results from GWAS are regarded as a major breakthrough in psychiatric genetics, and these findings and others support the involvement of both rare variants with modest or large effect sizes, as well as common variants with low effect sizes, in schizophrenia and bipolar disorder susceptibility.

However, the current susceptibility genes only explain a fraction of the heritability of these severe mental disorders which motivated a search for the "hidden heritability". Searching for rare variants by new sequencing technologies has been proposed as a fruitful new approach to discover the "missing heritability". The emergence of such next generation sequencing platforms within the last five years, has revolutionized DNA sequencing, and now enables efficient sequencing of large stretches of DNA at high throughput and relatively low cost. The development of sequence capture of both custom targeted and predefined sequence areas has enabled researchers to look into their particular area of interest. For instance, exome sequencing, *i.e.* sequencing of most exons in the human genome, has emerged as a technique to identify the underlying disease causing locus behind monogenic diseases as well as a method to diagnose patients for diseases loci already known. The discovery list now extends to the previously unidentified genes being more than ten Mendelian diseases. The identification of true disease-causing alleles is crucial for the development of sequencing as a diagnostic tool in psychiatric illnesses.

8. Future perspectives

Despite the excellent advances in knowledge that a genomic approach to medicine has achieved, there are limitations due to the fact that many phenotypes are highly polygenic and susceptible to genetic interactions. Typical examples are common human diseases. Additionally, in contrast to traditional Mendelian analyses of disease state with high relative risk of disease (λ), and an OR (well over ten), the relative risk of common, chronic, and late-onset diseases is typically much lower. Common alleles, that are usual findings from association studies, almost never have large effects (ORs typically < 1.5, and often < 1.2) (183), and therefore could only explain a small proportion of the variance in disease. On the other hand, rare variants may have ORs between two and ten, but also only explain a small proportion of the variance in disease susceptibility in the population because of their rarity. Moreover, genetic variants with low penetrance can be quite common. In response to this problem, explicit addressing of low penetrance mechanisms, such as genotype–environment interaction and epistasis are required (184).

9. Concluding remarks

The field of genetics has evolved rapidly from the start of this thesis in 2007. The research area started out with candidate studies and evolved rapidly, with high-throughput genotyping tools emerged and improved, to enable genome wide studies and CNV studies delivering huge amounts of exciting data. Presently, new technology has brought the field to deep sequencing.

We have identified putative novel susceptibility genes for schizophrenia. The identification of genetic risk factors for schizophrenia remains a very challenging task. The complex nature of its aetiology and pathophysiology is complicating the picture with heterogeneous phenotype, environmental factors, allelic and locus heterogeneity, interaction of multiple genes, small effect of risk alleles, mixture of common and rare variants, sequence and structural variants.

In conclusion, direct study of genotypes with minimal reference to phenotypes is insufficient to elucidate genetic phenomena of complex diseases. Genetic association studies alone have important limitations that reduce the understanding of phenotypic causation. Therefore, phenomics, the comprehensive study of phenotypes, is crucial for understanding the biology of complex disorders.

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Paper I

Gene variants associated with schizophrenia in a Norwegian genome-wide study are replicated in a large European cohort

Journal of Psychiatric Research. 2010. Sep; 44(12):748-53

Paper II

Intron 12 in *NTRK3* variants is associated with bipolar disorder

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Paper III

Genome-wide association study identifies genetic loci associated with body mass index and HDL-cholesterol levels during psychopharmacological treatment. A cross-sectional naturalistic study

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Genome-wide association study identifies genetic loci associated with body mass index and HDL-cholesterol levels during psychopharmacological treatment. A cross-sectional naturalistic study.

Lavinia Athanasiu[1,2,3,] Dr. Andrew A. Brown[1,4], Dr. Astrid B. Birkenaes[1], M.Sc Morten Mattingsdal[3,5] Prof. Ingrid Agartz[1,6], Prof. Ingrid Melle[1,3], Prof. Vidar M Steen[7,8], Prof. Ole A Andreassen[1,3], Dr. Srdjan Djurovic[1,2,3]

[1]Institute of Clinical Medicine, University of Oslo; Address: Postboks 1130 Blindern N-0318 Oslo, Norway, [2]Department of Medical Genetics, and [3]Division of Mental Health and Addiction, Oslo University Hospital, Address: Kirkeveien 166, N-0407 Oslo, Norway, [4]Department of Biostatistics, University of Oslo. Address: Blindern, N-0318 Oslo, Norway, [5]Research Unit, Sorlandet Hospital, Kristiansand, Norway, [6]Department of Psychiatric Research, Diakonhjemmet Hospital, Address: Postboks 85 Vinderen, N-0319 Oslo, Norway, [7]Dr. Einar Martens Research Group for Biological Psychiatry, Department of Clinical Medicine, University of Bergen, Bergen, Norway, [8]Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, Bergen, Norway,

Corresponding author

Lavinia Athanasiu M.Sc.

Psychosis Research Section - TOP, Building 49, Division of Mental Health and Addiction,Oslo University Hospital- Ulleval, Kirkeveien 166, N-0407 Oslo, Norway

Ph: +47 23 02 73 32

Fax: +47 23 02 73 33

lavinia.athanasiu@medisin.uio.no

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Abstract

Metabolic and cardiovascular side effects are serious clinical problems related to psychopharmacological treatment, but the underlying mechanisms are mostly unknown. We performed a genome-wide association study of metabolic and cardiovascular risk factors during pharmacological therapy.

Twelve indicators of metabolic side effects (body mass index (BMI), waist circumference, low density lipoprotein cholesterol, high density lipoprotein cholesterol (HDL-C) , total cholesterol (TG), triglycerides, fasting glucose, HDL-C/TC ratio and C-reactive protein as well as cardiovascular risk factors (blood pressure, heart rate) were analyzed in a naturalistic sample of 594 patients of Norwegian ancestry. We analyzed interactions between gene variants and three categories of psychopharmacological agents based on their reported potential for side effects and defined genome-wide significance based on false discovery rate of 0.1.

For BMI, two significantly associated loci were identified on 8q21.3. There were seven markers in one 30kb region and the strongest signal was rs7838490 ($P = 6.07 \times 10^{-8}$, FDR= 0.051). In another locus 140 kb away, six markers were significant, and rs6989402 obtained the strongest signal ($P = 1.54 \times 10^{-7}$, FDR= 0.018). Both of these loci are located upstream of the gene *matrix metalloproteinase 16*. For HDL-C, marker rs11615274 on 12q21 was significant ($P = 9.01 \times 10^{-8}$, FDR =0.051).

The results highlight three genomic regions potentially harboring susceptibility genes for drug induced metabolic side effects, identifying *matrix metalloproteinase 16* as a candidate gene. This deserves to be replicated in additional populations to provide more evidence for molecular genetic mechanisms of side effects during psychopharmacological treatment.

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Authors LA, SD, VMS, and OAA participated in design of the study and wrote the protocol. Authors SD, LA, and AAB managed the literature searches and analyses, Authors IA, IM, and ABB contributed materials and analysis tools. Authors LA, AAB, and MM undertook the statistical analysis, and authors LA, OAA, VMS, and SD wrote the first draft of the manuscript. All authors have read, contributed and approved the final manuscript.

All authors declare that they have no conflicts of interest.

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Introduction

Effective psychopharmacological treatment for schizophrenia and affective disorders was introduced with the discovery of antipsychotics in the 1950s. Antipsychotics, antidepressants and mood-stabilizers are still cornerstones in their treatment. However, a large proportion of patients experience adverse effects, which are often a limiting factor in obtaining successful treatment effects.

First generation antipsychotics are typically associated with extrapyramidal syndromes (Correll and Schenk 2008), while several of the second generation antipsychotics are associated with metabolic disturbances including dyslipidemia, elevated glucose levels and weight gain (Leucht et al., 2009; Meyer et al., 2008; Meyer and Koro 2004), as well as cardiovascular disturbances (Ray et al., 2009). Similar adverse effects are also observed for several mood-stabilizing drugs (Andersohn et al., 2009).

Metabolic and cardiovascular adverse effects are of major clinical importance because of their relevance for the increased mortality seen in severe mental disorders, which is mainly due to increased somatic morbidity (Colton and Manderscheid 2006; Tiihonen et al., 2009). A better understanding of the mechanisms underlying metabolic and cardiovascular adverse effects associated with current psychopharmacological drugs is therefore imperative, and can lead to development of more tolerable drugs. Most studies of the mechanisms related to adverse effects of psychopharmacological agents are based on randomized controlled trials (RCT). These studies are necessary to establish effect. However, RCT studies have limitations in terms of representativity due to strict inclusion and exclusion criteria. As an example, in the CATIE study approximately ¾ of the patients discontinued the treatment (Lieberman et al., 2005). Thus, it is important to also investigate the mechanisms of adverse drug effects in a

naturalistic setting, since in clinical practice treatment is adjusted in order to limit side effects. It is of interest to identify the mechanisms related to adverse effects in the context of satisfaction with the treatment from both the patient and clinician, and with time spans that goes far beyond the duration of RCTs. It is this long-term aspect of treatment that contributes to the most important outcome, mortality (Tiihonen et al., 2009). Recent genome-wide association studies have successfully identified genetic variants related to BMI (Thorleifsson et al., 2009; Willer et al., 2009), waist circumference (Fox et al., 2007), triglycerides (TG) (Kathiresan et al., 2008; Waterworth et al., 2010), HDL-C (Ma et al., 2010; Willer et al., 2009), LDL-C (Wallace et al., 2008; Willer et al., 2009) and total cholesterol (TC) (Aulchenko et al., 2009; Ma et al., 2010), as well as heart rate (Eijgelsheim et al., 2010; Newton-Cheh et al., 2007) and blood pressure (Levy et al., 2009; Newton-Cheh et al., 2009) in the general population. It would be expected that some of the same genetic factors influence the risk of metabolic disturbances in patients with severe mental disorders. In addition, genetic susceptibility factors that are specific for adverse effects of psychopharmacological drug treatment could also be involved.

Twin studies have also indicated that part of the risk of metabolic adverse effects of antipsychotics may be heritable (Theisen et al., 2005). Several candidate genes have been examined for association to drug-induced weight gain, with most promising findings for the 5-HT_{2C} receptor (Reynolds et al., 2002), leptin (Zimmermann et al., 2003) and insulin-induced gene 2 (LeHellard S. et al., 2009). Recently, a genome-wide approach in the CATIE study identified 21 markers related to antipsychotic-induced side effects involving *MEIS2*, *PRKAR2B*, *GPR98*, *FHOD3*, *RNF144A*, *ASTN2*, *SOX5*, and *ATF71P2*, as well as various intergenic regions (Adkins et al., 2010). This study was conducted in an ethnically heterogeneous sample from USA. Scandinavians constitute more ethnically homogenous

populations, since these countries have only recently experienced non- Caucasian immigration. In the current study, heterogeneity was further reduced by including only patients with Norwegian heritage.

The aim of the present study was thus to identify genetic variants associated with psychopharmacological-induced metabolic and cardiac side effects in a naturalistic setting, using a genome-wide cross-sectional approach in a genetically homogenous sample of Norwegian patients. Our sample consisted of 594 patients with a severe mental disorder (schizophrenia or bipolar disorder) from the Thematically Organized Psychosis (TOP) Study. The TOP study is a naturalistic study, were patients due to good clinical practice tend to be preselected according to individual drug preferences. Thus, differences in metabolic or cardiovascular adverse effects expected to be found in study samples randomized to different treatments are minimized (Birkenaes et al., 2009). We have previously shown in a subsample of this population, that drug-induced increase in body mass was minimal, probably because of awareness of this treatment hazard among clinicians. However, independently of body mass, more "hidden" adverse effects were significantly associated with psychopharmacological treatment (Birkenaes et al., 2008). In the current study, the patients were examined for twelve indicators of metabolic side effects (BMI, waist circumference, TC, HDL-C, LDL-C, HDL-C/TC ratio, TG, glucose, and C-reactive protein), and cardiovascular variables (blood pressure and heart rate) were measured.

Material and method

Study sample

A total of 837 Caucasian individuals with severe mental disorders were recruited and successfully genotyped on Affymetrix Genome-Wide Human SNP array 6.0 (Affymetrix Inc., Santa Clara, CA, USA) and passed quality control measures. Of these, we had 594 patients with information on their psychopharmacological treatment that passed our inclusion criteria. The subjects participate in a large ongoing study on schizophrenia and bipolar disorder, the Thematically Organized Psychosis (TOP) Study, from May 2003 through May 2009.

The patients were mainly included from the outpatients units of Oslo University Hospital, but also from intermediate- and long-term treatment units. The health care system is catchment area based and free of charge. The patients were invited to participate in the study by the clinician responsible for their treatment. The sample is previously described in detail (Athanasiu et al., 2010; Djurovic et al., 2010). In brief, the subjects in the TOP study had to be registered in one of the psychiatric services at Oslo University Hospital, aged 18 to 65 years, meet DSM-IV criteria for any major psychotic or bipolar disorder, understand and speak a Scandinavian language, have no history of severe head trauma or neurological disease; and have an Intelligence Coefficient (IQ) score over 70. In addition, we excluded patients who had received psychopharmacological treatment for less than four weeks. All patients were born in Norway and the vast majority had two Norwegian-born parents. To assure proper compliance, the serum concentrations of all psychopharmacological agents were determined by the Laboratory of Clinical Psychopharmacology, St. Olav Hospital, Trondheim, Norway. Further, patients' drug intake was also measured with a self report. For more details, see Jonsdottir et al (Jonsdottir et al., 2010). Patients were excluded if no

appropriate serum concentration was detected in blood samples, or they reported non-compliance.

In total, 594 patients were successfully screened for the metabolic and cardiovascular outcome measures and genotyped. These subjects were included in the analysis, consisting of schizophrenia spectrum disorders (n= 283), bipolar disorder (n= 213) and psychosis NOS (n=98).

Participants received a detailed description of the study, and they signed a written informed consent. The study was approved by the Ethics Board.

Metabolic and cardiovascular outcome measures

All patients were subjected to a physical examination by a physician at inclusion into the TOP study. Height and weight for BMI (kg/m^2), waist circumference and heart rate (beats/minute) were obtained. Blood pressure (BP) was recorded manually in a sitting position after resting, and waist circumference was measured midway between the lower rib and the iliac crest in the upright position using non elastic tape.

Before the physical examination, blood samples were drawn after an over-night fasting and analyzed for fasting plasma glucose, TG, HDL-C, LDL-C, TC and C-reactive Peptide. All serum analyses were performed at the Department of Clinical Chemistry, Oslo University Hospital, Oslo, Norway, on an Integra 800 (Roche Diagnostics, IN, USA), using standard methods. In addition, patients were asked about their smoking habits. To obtain normally distributed variables, all outcome measures besides LDL-C and TC were log transformed.

For more information, see Birkenaes et al., 2008, 2009 (Birkenaes et al., 2008; Birkenaes et al., 2009).

Psychopharmacological agents

The patients recruited used antipsychotics, mood stabilizers and/or antidepressants. The individual medications were divided in three groups with respect to their potential to induce metabolic- and cardiovascular side effects, based on recent reviews (Andersohn et al., 2009). Group I included drugs with the lowest potential, group II medium, and group III the highest. Group I also included patients using no medication (n=107). The medications investigated in the current study should have indications for all patient groups (schizophrenia, bipolar disorders and psychosis NOS), thus patients receiving lithium were excluded from the study. Table 1 shows the number of patient on each drug in the three different medication groups. Some patients received more than one pharmacological agent. They were grouped based on the drug with the most severe side effect potential. The average metabolic and cardiovascular risk factors in each medication group are presented in Table 2.

Genotyping

The genome-wide genotyping and genotyping quality control for the sample have been described earlier (Athanasiu et al., 2010). To briefly restate, DNA was extracted from whole blood drawn at inclusion, and genotyped at Expression Analysis Inc. (Durham, USA) and at Oslo University Hospital, (Oslo, Norway) using the Affymetrix Genome-Wide Human SNP array 6.0 (Affymetrix Inc., Santa Clara; CA, USA). Individuals with discrepancies between reported and genotyped sex were removed. We removed all mitochondrial DNA SNPs, as well as those on the sex chromosome or whose location was unknown. To control for population stratification, identical by state (IBS) scores were calculated. Initially, individuals with a call rate less than 90% were removed, followed by all SNPs with a call rate less than 95%. Secondly, we excluded individuals with a call rate less than 97 %, and then SNPs with less than 97% and any remaining SNPs with a minor allele frequency of less than 0.05.

Finally, individuals with outlying (greater than three standard deviations) levels of heterozygosity were removed. Following quality control, 608,239 SNPs from 837 individuals remained for statistical analysis, of whom 594 subjects also passed the inclusion criteria with respect to available information about the psychopharmacological treatment (see above).

Statistics

Model: We investigated interactions between SNP and the medication groups I, II, and III defined in the Psychopharmacological agents section with respect to differences in the level of metabolic -and cardiovascular side effects, using linear regression models implemented in PLINK (Purcell et al., 2007) applied to the twelve metabolic and cardiovascular phenotypes. The dominant regression model included two variables of interest: interaction terms which captures whether the effect of being on medication in group II in contrast to group I, and in group III in contrast to group I depends on genotype. In addition we included age, gender, smoking habits, SNP as well as groups II and III variables as possible confounding factors to control for.

Correction of multiple testing: To address the problem of multiple testing, we calculated a FDR value for each P value, estimating the proportions of false discoveries among all significant markers. We used a predefined threshold of 0.1 to declare genome-wide significance.

Software tools for data analysis

Affymetrix Power Tools (APT), version 1.10.0 and the embedded birdseed-v2 algorithm were used to genotype the raw data. Subsequent output files were modified and combined with marker annotation and so-called transposed .ped and .fam files were created as ready input

files for PLINK (Purcell et al., 2007). PLINK was then used for quality control and association analysis. R (R Development Core Team 2008) was used to draw Manhattan plots and calculating FDR values. SPSS Release 18.0.0 was used for descriptive statistics, one-way between-groups analysis of variance, test for normality and log-transformation of the variables. LocusZoom Version 1.1 was used to plot and visualize regional association results (Pruim et al., 2010).

Cross-outcome comparisons

We investigated common mechanisms across outcomes, by taking advantage of information on multiple related phenotypes in the sample. We examined if the identified genome-wide significant SNPs were associated to any other metabolic measures.

Results

Similar to the results from a sub-sample of the current study population (Birkenaes et al., 2008; Birkenaes et al., 2009), there were only minor differences in metabolic and cardiovascular adverse effects among the different psychopharmacological groups (table 2). There were also little differences in those adverse effects that are easy to observe, such as BMI. However, there was a graded effect of various drugs on non-visual adverse effects, such as lipid profiles. This could be due to the naturalistic setting, where clinicians continuously adjusted the treatment in order to limit the amount of adverse effects (Birkenaes et al., 2008; Birkenaes et al., 2009).

Genome-wide association analysis

We analyzed if the effect of drugs in group II relative to group I and group III relative to group I, on the twelve metabolic and cardiovascular outcome variables were dependent on genotype (see table 1 for overview of the groups). The analyses revealed 14 significant interactions (FDR<0.1), one where the interaction acted on HDL-C levels and 13 interactions acting on BMI. All interactions were related to differential SNP effects between medication groups I against II. There were several interaction effects between group I and III, but none reached statistical significance. Graphic representations of the genome-wide analysis are found in Figure 1 for the interaction between SNP and medication group II compared to group I on BMI, and in Figure 2 for the interactions between SNP and medication group II compared to group I on HDL-C levels. For the other ten phenotypes, no markers reached genome-wide significance. All markers with a declared genome-wide significant FDR value (FDR < 0.1) are listed in Table 3. The table presents significant results grouped by phenotype.

Our most significant result was for marker rs7838490 located on 8q21.3 mediating the effect on BMI (P= 6.1 x 10^{-8}, FDR = 0.018, coefficient = -0.10). Figure 5 shows the fitted values from the regression model for adjusted BMI values for each medication group, broken down by genotype. In addition, seven other genome-wide markers are found in the same 30kb region, also mediating the effect of group II on BMI. This region is 200 kb upstream of the gene *MMP16*. In addition, another region closer but likewise upstream of *matrix metalloproteinase 16* (*MMP16*) also mediates the effect of group II medications on BMI. SNP rs6989402 obtained the smallest P-value in this region (P= 1.5 x 10^{-7}, FDR= 0.018, coefficient = -0,097), whose importance is highlighted by five additional genome-wide significant markers close by. These two loci (within the 200 kb region) upstream of *MMP16* are in linkage disequilibrium (see Figure 3).

Our second strongest result involved marker rs11615274 on 12q21 (P= 9.01 x 10^{-8}, FDR=0.051, coefficient = -0.14) mediating the effect of group II on HDL-C levels. The marker is intergenic and several other neighboring markers display independent nominally significant association (P < 9 x 10^{-5}) (see Figure 4). Figure 6 shows the adjusted HDL-C values for each medication group, broken down by the three different genotypes for rs11615274. The line represents the fitted values from the regression model, where the SNP is assumed to have a dominant effect.

Secondary analysis

We investigated if the 14 genome-wide significant interactions were associated to other outcomes. We identified 19 secondary associations, listed in Table 4.

For marker rs11615274 that interacts with medication group II compared to group I on HDL-C levels, a secondary nominal significant association was found for fasting glucose levels.

Likewise, all of the genome-wide significant SNP- medication group II interactions for BMI were nominally associated for SNP-medication group III interactions. The same was observed for the interactions affecting HDL-C levels.

In addition, four of the SNPs associated to BMI (rs7838490, rs10504857, rs7819913, rs1580508) obtained nominally significant P values for diastolic blood pressure.

Discussion

We report genome-wide significant interaction effects for 14 SNPs on metabolic- and cardiovascular parameters, across groups of psychopharmacological agents with different liabilities for adverse effects. The markers highlight three genomic regions potentially harboring susceptibility genes for drug- mediated increase in HDL-C levels and BMI. In the current naturalistic sample, there were some significant differences in metabolic- and cardiovascular parameters between the groups of psychopharmacological drugs, but many factors showed no differences, suggesting that the treatment had been adjusted in order to limit the amount of adverse effects to a tolerable level. Thus, the genes identified in this setting of more tolerable long term treatment could have impact on the longevity of patients with severe mental disorders, as they often receive pharmacological treatment for long periods of their life span, and have increased mortality (Tiihonen et al., 2009).

The metabolic measures BMI and HDL-C were most strongly influenced by genetic variation. Our most significant finding was the association of rs7838490 on 8q21.3 with increasing BMI levels for patients in group II, compared to group I. This seems to be an important locus, as six other neighboring markers also obtained genome-wide significant FDR values. In addition, in a locus 140 kb away, six additional markers reached genome-wide significance. The two loci are in LD and are located upstream of metalloproteinase *MMP16*. The markers are intergenic, but might affect regulatory elements that potentially effect the expression of *MMP16*. This suggests a role of *MMP16* in the development of side effects of psychopharmacological agents. Thus could be related to the effect of metalloproteinases on plasma soluble tumor necrosis factor receptor superfamily, member 1A (sTNFRSF1A), which is hypothesized to be involved in lipid regulation. A genome-wide scan for adiposity in 18 Dutch dyslipidemic families, revealed genetic linkage (LOD 2.8) of sTNFRSF1A

concentration and marker D8S1110 on chromosome 8 (Van Der Kallen et al., 2000), a region previously reported to show evidence of linkage with leptin levels in Mexican Americans (Comuzzie et al., 1997). Notably, the transmembrane TNFRSF1A receptor regulates leptin secretion by adipocytes (Yamaguchi et al., 1998). In addition, metalloproteinases are responsible for activation of TNFRSF1A, that is essential for induction of leptin production by tumor necrosis factor-α (Finck and Johnson 2000). One such metalloproteinase is *MMP16* (Nagase and Woessner, Jr. 1999). Therefore, rs7838490 on 8q21.3upstream of *MMP16* could potentially mark a region that affects the expression of this protein, resulting in aberrant TNFRSF1A activation and altered leptin production that could lead to obesity. Further support for a role of this region on 8q21.3 comes from recent studies that identified markers on 8q21.1 and 8q21.11 associated with waist circumference (Fox et al., 2007) and BMI ($P = 6 \times 10^{-8}$) (Speliotes et al., 2010) respectively.

Another main finding was the SNP rs11615274 on 12q21 associated with decreased HDL-C level. This SNP is located in an intergenic region, with no identified neighboring genes. However, there are several lines of evidence implicating this region in the development of metabolic abnormalities. Several publications have identified risk variants on 12q21 for diabetes type 2 (Voight et al., 2010; Zeggini et al., 2008). It is well known that the diabetes course is associated with complex alterations in plasma lipoproteins (Kreisberg 1998). At the level of the adipocyte, impaired insulin action leads to increased rates of intracellular hydrolysis of triglycerides with the release of non-esterified fatty acids, resulting in reduced plasma HDL-C levels. Diabetic hyperglycemia reduces cholesterol efflux from the tissues and reverse cholesterol transport, thus lowering the circulating levels of antioxidant and antiatherogenic HDL-C (Krentz 2003).

The current findings of significant interaction effects can have clinical implications. Our statistical model was designed to identify genes with differing effects on cardiovascular or metabolic phenotypes in the different pharmacological groups. Thus, as illustrated in figure 5 and figure 6, the identified gene variants affect BMI and HDL-C differently in the two medication groups. In particular, the naturalistic design allowed us to identify genes that are associated with adverse effects in patients on long term treatment. In a real life clinical setting, this suggests that during treatment that is acceptable for both patients and their doctors, the currently identified genes increase the liability of adverse effects. This suggests that patients with these gene variants may be treated for long periods of time with drugs that interact to increase the risk of cardiovascular mortality. Since many patients receive psychopharmacological treatment for a significant period of their lives (Colton and Manderscheid 2006; Ray et al., 2009; Tiihonen et al., 2009), identifying subsets with specific risks would be of great interest.

Some limitations of our study must be considered. The study was cross sectional, which can make it difficult to assess the relationship to drug treatment. The definitions of the three different adverse-effect groups were based on accumulated evidence on risk for adverse metabolic side effects, and this may be subject to publication bias. Further, the medications in these groups might exert their pharmacological effects by different mechanisms. If so, this would reduce the power to detect associations. Therefore, the present results could indicate common mechanisms. Confounding factors as physical exercise and dietary regime were not taken into consideration, but it is highly unlikely that there is a systematic bias between the different groups. The same could be argued for the different diagnosis included in the present study. However, we did not find any interaction with diagnosis and therefore analyzed the whole sample together. Also, it is suboptimal that side effects were based on one

measurement, and not repeated. However, this would also reduce the power to detect associations. The same argument can be used for the naturalistic design. Proper clinical follow up will most likely reduce the rate of adverse effects, and only patients who are comfortable with the treatment are continued.

Despite these limitations, we report here multiple alleles representing risk for metabolic side effects after treatment with psychopharmacological agents with known metabolic- and cardiovascular side effects. We did a GWAS on an ethnic homogenous and clinically well characterized sample, which increased the likelihood of identifying genome-wide significant signals. We suggest that the present findings could be useful in studies of predicting individual metabolic side effects. However, to further validate these loci as susceptibility genes for adverse metabolic and cardiovascular effects, replication in additional populations are needed. Further work is also required to study the genes' expression and regulation to further elucidate their role.

Legends

Figure 1: Genome-wide association analysis of SNP-medication interaction for body mass index

Manhattan plot showing significance of association of all quality-control-positive SNPs in the TOP sample as -log10 of the dominant linear regression P value. SNPs are plotted on the x axis according to their position on each chromosome. Chromosomes are shown in alternating colors for clarity. Associations with body mass index are indicated on the y axis as -log10 P value.

Figure 2: Genome-wide association analysis of SNP-medication interaction for high density lipoprotein cholesterol

Manhattan plot showing significance of association of all quality-control-positive SNPs in the TOP sample as -log10 of the dominant linear regression P value. SNPs are plotted on the x axis according to their position on each chromosome. Chromosomes are shown in alternating colors for clarity. Associations with high density lipoprotein cholesterol are indicated on the y axis as -log10 P value.

Figure 3: Regional association results of the –log10 (P values) for SNPS near *MMP16*. The LD between the highest-scoring marker and the surrounding markers (based on Hap Map) is color indicated. Recombination rates are indicated by the blue line, peaks are hot spots.

Figure 4: Locus zoom plot showing the high density lipoprotein cholesterol- associated region on chromosome 12.

Figure5: Adjusted logBMI plotted by rs7838490 and medication group

logBMI values, adjusted for age, gender and smoking, plotted separately for the three different medication groups and broken down by genotype. The line represents the fitted values from the regression model, where the SNP is assumed to have a dominant effect.

Figure 6: Adjusted HDL-C plotted by rs11615274 and medication group

logHDL-C values, adjusted for age, gender and smoking, plotted separately for the three different medication groups for each genotype. The line represents the fitted values from the dominant regression model.

Table 1: Psychopharmacological agents

Overview of the medications used by the patients in the TOP study and their assigned medication groups.

Table2: Descriptive statistics (means and *s.d.*) and total number of assessments per drug

Descriptive statistics for all 12 phenotypes. Analyses of clinical data were performed in SPSS (http:(//www.spss.com) All test were two-tailed. The ones marked with an asterix were significant after correcting for multiple testing.

Abbreviations: ANOVA, univariate analysis of variance; BMI, body mass index; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive peptide

Table 3: Genome-wide significant results (FDR< 0.1)

All genome-wide significant interactions of the dominant regression model.

Table4: Number of alternate outcomes associated (P< 0.05) to genome-wide significant SNPs, by metabolic phenotype

Number of alternate outcomes associated (P = 0.05) to genome-wide significant SNPs. If an interaction including the SNP is observed for the phenotype the score 1 is given, if two interactions are observed then the score 2 is given. To interactions indicate that both medication groups interact with the SNP and affect the phenotype. The ones marked with an asterisk indicate that the SNP was genome-wide significant associated to the phenotype.

Tables:

Table 1 Psychopharmacological agents

Medication group	**Count**	**Medication group**	**Count**	**Medication group**	**Count**
Group I		***Group II***		***Group III***	
Escitalopram	14	Zuclopenthixol	7	Clozapine	15
Citalopram	7	Venlafaxine	9	Levomepromazine	8
Fluoxetine	3	Mirtazapine	10	Valproic acid	29
Lamotrigine	21	Risperidone	35	Carbamazepine	3
Methylphenidate	3	Amitriptyline	1	Chlorprothixene	6
Paroxetine	1	Quetiapine	69	Olanzapine	182
Amisulpride	3	Mianserin	3	**Total**	**243**
Topiramate	1	Perfenazin	12		
Ziprasidone	18	Sertindole	1		
Sertraline	3	**Total**	**147**		
Aripiprazole	21				
Bupropion	2				
No medication	107				
Total	**204**				

Table 2 Descriptive statistics (means and *s.d.*) and total number of assessments per group

	Descriptive statistics										
	Group I			*Group II*			*GroupIII*			ANOVA	
Phenotype:	Mean	*s.d.*	N	Mean	*s.d*	N	Mean	*s.d.*	N	F	P
BMI (kg/m^2)	25.64	*.39*	190	26.49	*.42*	137	25.98	*.29*	218	1.21	.298
Waist circumference (cm)	88.74	*1.27*	139	92.63	*1.45*	103	92.54	*1.10*	137	3.21	.014
Systolic BP (mmHg)	121.24	*1.16*	197	117.89	*1.15*	140	123.87	*1.05*	224	6.55	.002
Diastolic BP (mmHg)	77.82	*.79*	197	77.62	*.82*	140	79.39	*.72*	223	1.66	.192
Heart rate (bpm)	71.6	*.82*	191	77.02	*1.41*	135	74.29	*.88*	208	6.65	.001*
Glucose	5.02	*.07*	195	5.07	*.07*	134	5.13	*.05*	230	.90	.409
Triglycerides	1.25	*.06*	194	1.53	*.11*	136	1.66	*.08*	234	7.12	.001*
HDL-C	1.44	*.03*	195	1.34	*.04*	136	1.30	*.02*	235	5.45	.005
LDL-C	3.17	*.07*	192	3.11	*.09*	134	3.36	*.06*	224	3.28	.038
Total cholesterol	5.13	*.08*	195	5.12	*.09*	136	5.36	*.072*	234	3.13	.044
Ratio (cholesterol/HDL-C)	3.8	*.12*	120	4.11	*.18*	92	4.52	*.13*	147	7.38	.001*
CRP	3.33	*.52*	191	3.39	*.44*	132	3.32	*.53*	226	.00	.996

Table 3 Genome-wide significant results (FDR< 0.1)

Outcome	CHR	SNP	Interacting drug group	Minor allele	Association coefficient	P value	FDR	Cytoband	Gene
log BMI	8	rs7838490	Group II	A	-0,10	6,07E-08	1,81E-02	q21.3	intergenic
HDL-C	12	rs11615274	Group II	T	-0,14	9,01E-08	5,18E-02	q21	intergenic
log BMI	8	rs6989402	Group II	G	-0,10	1,54E-07	1,81E-02	q21.3	MMP16
log BMI	8	rs1012116	Group II	A	-0,10	1,54E-07	1,81E-02	q21.3	MMP16
log BMI	8	rs10504857	Group II	G	-0,10	1,63E-07	1,81E-02	q21.3	intergenic
log BMI	8	rs7819913	Group II	G	-0,10	1,93E-07	1,81E-02	q21.3	intergenic
log BMI	8	rs4369037	Group II	T	-0,10	2,88E-07	1,81E-02	q21.3	MMP16
log BMI	8	rs35175011	Group II	A	-0,10	2,95E-07	1,81E-02	q21.3	MMP16
log BMI	8	rs10504845	Group II	T	-0,09	3,30E-07	1,81E-02	q21.3	MMP16
log BMI	8	rs1580508	Group II	A	-0,10	3,56E-07	1,81E-02	q21.3	intergenic
log BMI	8	rs16884251	Group II	T	-0,10	3,57E-07	1,81E-02	q21.3	intergenic
log BMI	8	rs16884273	Group II	C	-0,10	3,61E-07	1,81E-02	q21.3	intergenic
log BMI	8	rs35385383	Group II	G	-0,10	3,78E-07	1,81E-02	q21.3	intergenic
log BMI	8	rs11994538	Group II	A	-0,09	1,01E-06	4,44E-02	q21.3	MMP16

Table 4 Number of alternate outcomes associated (P< 0.05) to genomewide significant SNPs, by metabolic phenotype

			Weight		*Lipids*					*Bloodpressure*			
SNP	*GENE*	*P value*	*BMI*	*Waist*	*Total Chol*	*TG*	*LDL-C*	*HDL-C*	*Glucose*	*Dia*	*Sys*	*HR*	*CRP*
rs7838490	intergenic	6,07E-08	2*	0	0	0	0	0	0	1	0	0	0
rs11615274	intergenic	9,01E-08	0	0	0	0	0	2*	1	0	0	0	0
rs6989402	MMP16	1,54E-07	2*	0	0	0	0	0	0	0	0	0	0
rs1012116	MMP16	1,54E-07	2*	0	0	0	0	0	0	0	0	0	0
rs10504857	intergenic	1,63E-07	2*	0	0	0	0	0	0	1	0	0	0
rs7819913	intergenic	1,93E-07	2*	0	0	0	0	0	0	1	0	0	0
rs4369037	MMP16	2,88E-07	2*	0	0	0	0	0	0	0	0	0	0
rs35175011	MMP16	2,95E-07	2*	0	0	0	0	0	0	0	0	0	0
rs10504845	MMP16	3,30E-07	2*	0	0	0	0	0	0	0	0	0	0
rs1580508	intergenic	3,56E-07	2*	0	0	0	0	0	0	1	0	0	0
rs16884251	intergenic	3,57E-07	2*	0	0	0	0	0	0	0	0	0	0
rs16884273	intergenic	3,61E-07	2*	0	0	0	0	0	0	0	0	0	0
rs35385383	intergenic	3,78E-07	2*	0	0	0	0	0	0	0	0	0	0
rs11994538	MMP16	1,01E-06	2*	0	0	0	0	0	0	0	0	0	0

Figures:

Figure 1

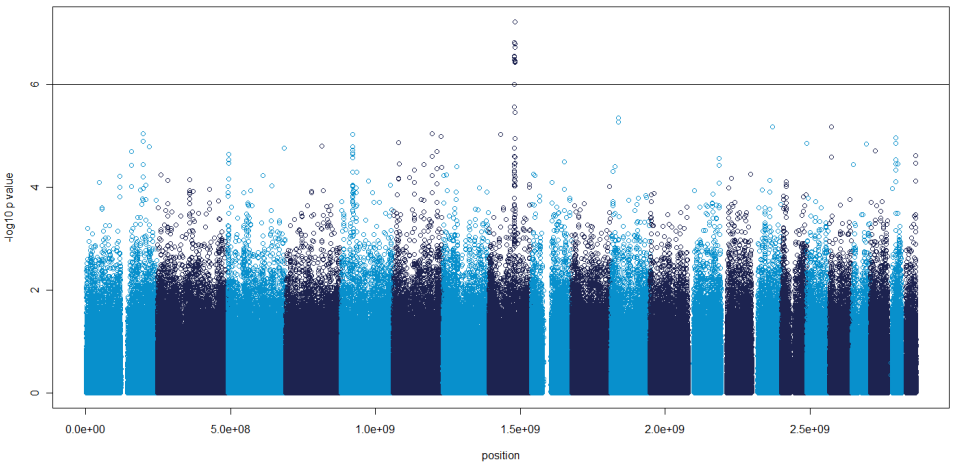


Figure 2

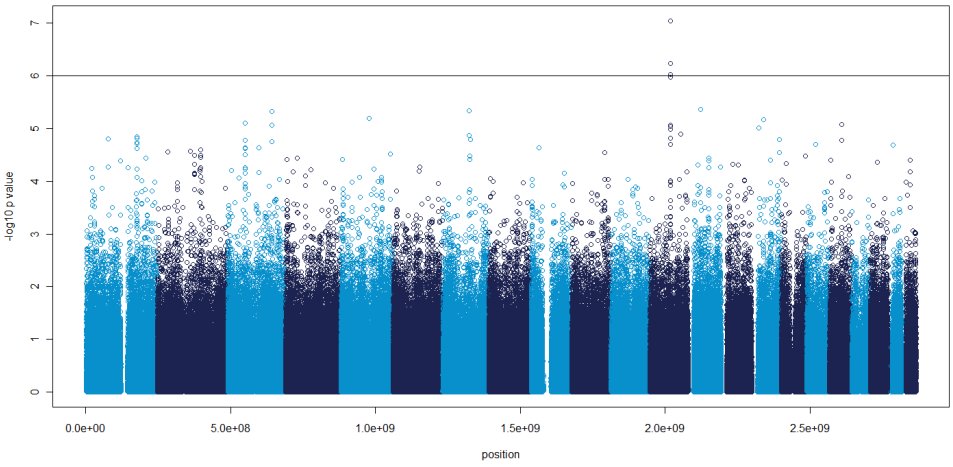


Figure 3

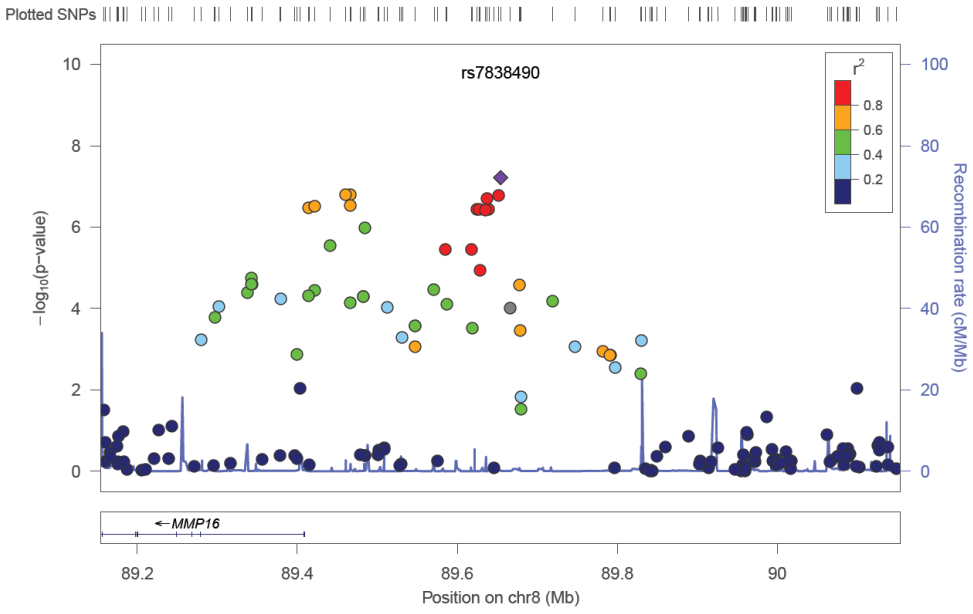


Figure 4

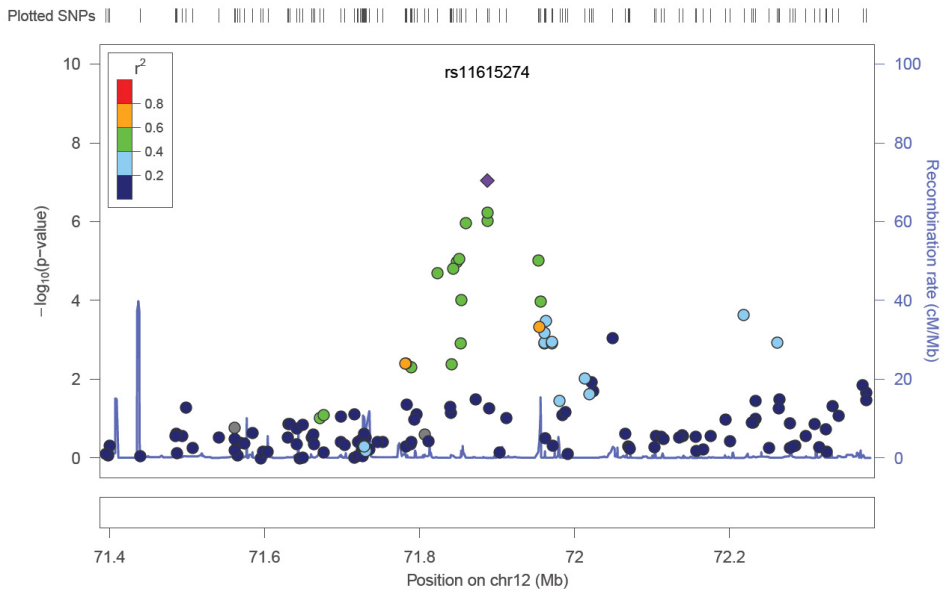


Figure 5

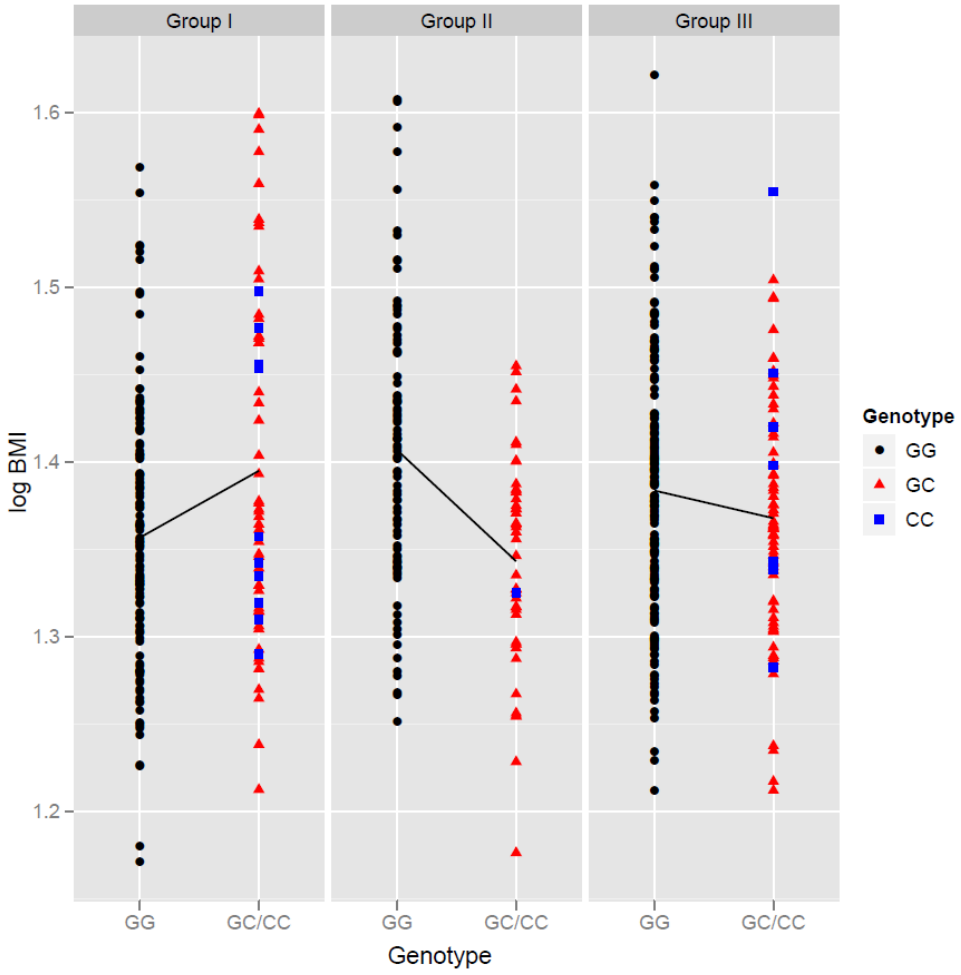
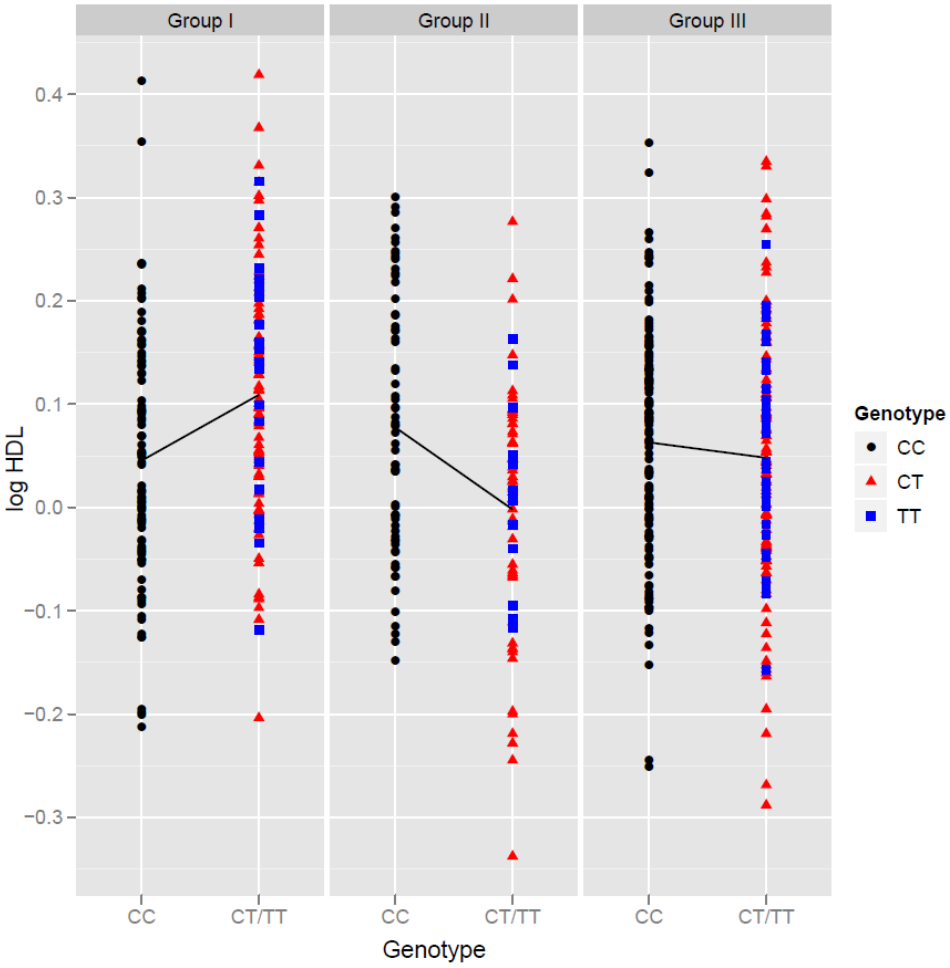

Group I
Group II
Group III
log BMI
1.6
1.5
1.4
1.3
1.2
GG
GC/CC
Genotype
Genotype
GG
GC
CC

Figure 6


Group I
Group II
Group III
log HDL
0.4
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
CC
CT/TT
Genotype
Genotype
CC
CT
TT

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