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Genetic Factors in Schizophrenia

Studies on Treatment Response to Typical Neuroleptics and Age at Onset

■

ACADEMIC DISSERTATION

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To my wife

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Introduction

Pharmacogenetics is about 50 years old as a field of medicine. In the 1950s, clinicians observed inherited differences in drug effects (Evans and Johnson 2001). The discovery of the genetic variation in CYP2D6 in the late 1980s was considered to be of major importance in psychiatry, as most antipsychotics and antidepressants at that time were metabolized via this drug metabolizing enzyme (Kawanishi et al. 2000). However, drug target pharmacogenetics proper began only about ten years ago (Evans and Johnson 2001).

Several lines of evidence suggest that genetic factors, in part, underlie observed differences in treatment response in schizophrenia (Catalano 1999, Basu et al. 2004, Malhotra et al. 2004). The effects of antipsychotics may be apparent only after several weeks, and this delay may have serious consequences. Thus tools for predicting the response, as well as adverse effects, may be of great importance in the treatment of schizophrenia (Malhotra et al. 2004).

Until now, several polymorphisms in the genes in drug metabolizing enzymes and drug targets have been associated with treatment response to antipsychotic drugs (Kirchheiner et al. 2004, Malhotra et al. 2004). However, relatively few of these results have been replicated in independent samples (Arranz and Kerwin 2003).

In this thesis, the polymorphisms of genes affecting brain development (BDNF, EGF, and NOTCH4) or genes modulating brain functioning (APOE and COMT) were chosen for pharmacogenetic study. The present study focuses on treatment response to typical neuroleptics, but the associations between patients and healthy controls as well as age at onset were also studied.

Abstract

Background: In schizophrenia, pharmacogenetics may provide the clinician with a useful tool in deciding which antipsychotic drug may suit the patient best. Besides this, ample data suggests that differences in treatment response to typical antipsychotics may help to create clinically meaningful, genetic-based subgroups in schizophrenia.

Aims: To study the association of the polymorphisms of five genes brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), NOTCH4, catechol-O-methyltransferase (COMT), and apolipoprotein E (APOE) between poor and good treatment response to typical neuroleptics, between patients with schizophrenia and controls, and in age at onset in schizophrenia.

Subjects and methods: The sample comprised 94 Finnish patients with a DSM-IV diagnosis of schizophrenia. Of these patients 43 were good responders and 51 poor responders to typical antipsychotics. There were 98 controls of similar age and sex, who were healthy blood donors. DNA was isolated from blood. Genotypes were determined using polymerase chain reaction (PCR) and applying either the 5' nuclease assay or specific restriction enzyme treatment and electrophoresis for allelic discrimination.

Results: The main result was the predictive effect of the combination of two polymorphisms (NOTCH4: SNP2 and COMT: V108/158M) on treatment response to typical neuroleptics. In addition, EGF polymorphism was associated with schizophrenia. Polymorphisms of three genes (NOTCH4, EGF, and APOE) were associated with age at onset of schizophrenia.

Conclusions: These results provide preliminary data of the association of the genes studied with either risk of schizophrenia, treatment response or age at onset in schizophrenia. Interestingly, studying genes associated with the development of the brain may provide more precise prediction of treatment response to typical antipsychotics. However, these results need to be replicated in other independent studies.

Contents

Abbreviations

ACE	angiotensin-converting enzyme
ADRA1A	alpha1A-adrenergic receptor
ADRA2A	alpha1A-adrenergic receptor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
apoE	apolipoprotein E
BDNF	brain-derived neurotrophic factor
Bp	base pair
BPRS	Brief Psychiatric Rating Scale
CA	clozapine induced agranulocytosis
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CGI	Clinical Global Impression Scale
CI	confidence interval
CNS	central nervous system
COMT	catechol-O-methyltransferase
CP	chlorpromazine
CYP	cytochrome P450
D2	dopamine2
DAT	dopamine transporter
Del	deletion
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DRD2	dopamine2 receptor
DRD3	dopamine3 receptor
DRD4	dopamine4 receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, fourth edition
DTNBP1	dystrobrevin-binding protein 1
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	extensive metabolizer
ER	estrogen receptor
GABA	gamma-aminobutyric acid
GH3	a rat pituitary tumor line cell expressing prolactin and growth hormone
GH4C1	a rat anterior pituitary cell line
Gi3α	adenylate cyclase inhibitory G protein i3 alpha
GRIN2B	N-methyl D-aspartate receptor subunit 2B
GSK-3β	glycogen synthase kinase-3 beta
H1	histamine1 receptor
H2	histamine2 receptor
HLA	Human Leukocyte Antigen
5-HT	5-hydroxitryptamine, serotonin
5-HTT	serotonin transporter

5-HTTLPR	polymorphism within the promoter region of the serotonin transporter gene
ICD-10	International Classification of Disease, tenth edition
IM	intermediate metabolizer
Ins	insertion
MAO-A	monoamine oxidase A
MGB	minor groove binder
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase
n	number
NGF	nerve growth factor
NICD	Notch intracellular domain
NRG1	neuregulin1
NS	not significant
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
OR	odds ratio
PANSS	positive and negative syndrome scale
PCR	polymerase chain reaction
PET	positron emission tomography
PI3-K	phosphatidylinositol 3-kinase
PM	poor metabolizer
RFLP	restriction fragment length polymorphism
RGS4	regulator of G-protein signalling 4
RNA	ribonucleic acid
SD	standard deviation
SNP	single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SSRI	selective serotonin reuptake inhibitor
TD	tardive dyskinesia
TNF alpha	tumor necrosis factor alpha
trkB	tyrosine kinase receptor B
UM	ultrarapid metabolizer
UV	ultraviolet
VCFS	velocardiofacial syndrome
VNTR	variable number of tandem repeats
WCST	Wisconsin Card Sort Test

List of original publications

This thesis is based on the following publications, referred to in the text by their Roman numerals I-V. Some additional data is also presented.

I. Anttila S, Illi A, Kampman O, Mattila KM, Lehtimäki T, Leinonen E. Lack of association between two polymorphisms of brain-derived neurotrophic factor and response to typical neuroleptics. (J Neural Transmission, in press)

II. Anttila S, Illi A, Kampman O, Mattila KM, Lehtimäki T, Leinonen E. Association of EGF polymorphism with schizophrenia in Finnish men. Neuroreport 2004;15(7):1215-1218. (Copyright 2004, with permission from Lippincott Williams & Wilkins)

III. Anttila S, Kampman O, Illi A, Roivas M, Mattila KM, Lassila V, Lehtimäki T, Leinonen E. NOTCH4 gene promoter polymorphism is associated with the age of onset in schizophrenia. Psychiatr Genet 2003;13(2):61-64. (Copyright 2004, with permission from Lippincott Williams & Wilkins)

IV. Anttila S, Illi A, Kampman O, Mattila KM, Lehtimäki T, Leinonen E. Interaction between NOTCH4 and catechol-O-methyltransferase genotypes in schizophrenia patients with poor response to typical neuroleptics. Pharmacogenetics 2004;14(5):303-307. (Copyright 2004, with permission from Lippincott Williams & Wilkins)

V. Kampman O, Anttila S, Illi A, Mattila KM, Rontu R, Leinonen E, Lehtimäki T. Apolipoprotein E polymorphism is associated with age of onset in schizophrenia. J Hum Genet 2004;49(7):355-359. (Copyright 2004, with permission from Springer-Verlag)

Review of the literature

1. Schizophrenia

Schizophrenia is a devastating psychiatric syndrome, which affects about one percent of people world-wide (Schultz and Andreasen 1999). The symptoms of schizophrenia usually appear at young age, by the second and third decades of life (Meltzer et al. 1997). Schizophrenia is a strongly familial disease and recent studies suggest that the risk of schizophrenia is increased about ten-fold in first-degree relatives of schizophrenic probands (Riley and Kendler 2004). However, several environmental factors such as viral exposure, nutritional deficiencies, and obstetric complications, may interact with numerous genetic variations, and modify the disease (Schultz and Andreasen 1999).

1.1 Diagnosis

The diagnostic criteria of schizophrenia are laid down in the International Classification of Disease, tenth edition (ICD-10) and the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV). There are some differences between these criteria. In ICD-10, severe symptoms should have been present for 1 month, but DSM-IV requires 6 months' duration (Schultz and Andreasen 1999). ICD-10 is the official system for clinical diagnoses in the European countries while DSM-IV is used in the United States (Breier 2004). Schizophrenia is characterised by three broad types of symptoms: positive symptoms, negative symptoms and cognitive impairment (Mueser and McGurk 2004). Positive or psychotic symptoms include hallucinations and delusions such as suspiciousness, unusual thoughts and incoherence or looseness of associations in thought and speech. Negative symptoms refer to flat or blunted affect and emotions, amotivation, avolition, anhedonia, or alogia.

1.2 Epidemiology

The lifetime prevalence of schizophrenia is about one percent throughout the world (Jablensky et al. 1992). The same prevalence has been reported in Finland but there may

be a decline in the incidence (Suvisaari et al. 1999). However, in an isolated region (some isolated region of Finland) the incidence on schizophrenia may be three-fold (Hovatta et al. 1999).

Epidemiological studies have suggested some risk factors for schizophrenia in Finland. Urban birth is a risk factor for schizophrenia (Haukka et al. 2001) and males have about 30 % higher incidence than females (Suvisaari et al. 1999). Patients with schizophrenia seem to have had a higher incidence of obstetric complications than their nonpsychotic siblings (Rosso et al. 2000).

1.3 Age at onset

Kraepelin was the first to report that male patients of schizophrenia were younger than females at the time they were admitted to the hospital for the first time (Salokangas et al. 2003). In male patients, the onset of schizophrenia is usually at the age of 15-24 years and about 3-5 years earlier than in female patients (Angermeyer and Kuhn 1988, Häfner et al. 1993). Women present with a second increase of the incidence between 45 and 54 years, which is suggested to be caused by reduced estrogen levels (Häfner 2003, Rao and Kolsch 2003). There are also several reports of differences in the age of onset in different subpopulations in schizophrenia (Salokangas et al. 2003, Schürhoff et al. 2004).

The definition of the age at onset of schizophrenia is usually the time at which positive psychotic symptoms or disorganization first appear (Meltzer et al. 1997). The onset of clinical symptoms of schizophrenia is usually preceded by prodromal symptoms, including signs of behavioural dysfunction and subclinical psychotic symptoms (Lieberman et al. 2001).

Early age at onset is associated with poorer response to treatment with antipsychotic drugs, poorer outcome and a higher familial risk of schizophrenia (Meltzer et al. 1997, Schürhoff et al. 2004). Early age at onset may also be associated with impairment in verbal learning and memory and with more severe negative symptoms (Bellino et al. 2004, Tuulio-Henriksson et al. 2004).

1.4 Neuropathology

Kraepelin and Bleuler were the first to suggest that schizophrenia is a brain disease, which has significant cognitive deficits (Antonova et al. 2004). Research has now focused especially on three regions in the brain: prefrontal cortex, thalamus and medial temporal lobe. The most interesting features in these regions are grey matter volume, neuron density, somal size and the neuropil (the structures between neuronal cell bodies consisting of neuronal processes and synapses) (Cho et al. 2004).

Several brain abnormalities in schizophrenia have been reported and replicated in magnetic resonance imaging (MRI) studies (Shenton et al. 2001). These findings include ventricular enlargement and abnormalities in some medial temporal lobe structures (amygdala, hippocampus, and parahippocampal gyrus, and neocortical temporal lobe) (Shenton et al. 2001). Grey matter deficits are reported in dorsal prefrontal cortex in the majority of the 50 studies reviewed by Shelton et al. (2001). A recent study by Callicott et al. (2003) shows that cognitively intact siblings of patients with schizophrenia may have a primary physiological abnormality in dorsolateral prefrontal cortex function.

The dopamine hypothesis of hyperdopaminergia is largely based to the efficacy of dopamine receptor blocking antipsychotics (Carlsson and Lindqvist 1963, van Rossum 1966, Seeman et al. 1975, Carlsson 1978). This suggestion has led to a large number of studies focused on dopamine and its metabolites (Siever and Davis 2004). Dopamine receptor binding potency, as well as receptor occupancy was shown to predict the effectiveness of antipsychotics (Creese et al. 1976, Farde et al. 1988). The dopamine hypothesis was also supported by reports that amphetamine-induced release of dopamine resulted in schizophrenia-like symptoms (Randrup and Munkvad 1972, Snyder 1973). Decreased levels of dopamine metabolites have been reported in patients with poor outcome, and increased levels in patients with more severe psychotic symptoms (Siever and Davis 2004).

Numerous studies have led to pathophysiological models of schizophrenia. Temporal volume reductions and functional abnormalities are among the most consistently observed findings in schizophrenia (Davidson and Heinrichs 2003). These

abnormalities are hypothesized to form a primary abnormality which may lead to prefrontal functional deficits, and consequently to striatal hyperdopaminergia (Siever and Davis 2004). This pathology may emerge from genetic susceptibilities interacting with adverse environmental events, such as hypoxia from birth complications (Boksa and El-Khodor 2003, Siever and Davis 2004).

Several neurotransmitters (dopamine, glutamate, serotonin and GABA) and interactions between some brain regions (thalamus, hippocampus, and prefrontal cortex) seem to be significantly involved in the neuropathology of schizophrenia (Harrison 1999, Schultz and Andreasen 1999). Recently, it has been proposed that schizophrenia is associated with strongly interconnected abnormalities of dopamine and glutamate transmission (Kegeles et al. 2000, Laruelle et al. 2003).

Using a genetic approach, Egan et al. (2001) showed that schizophrenia patients who had a low-activity met allele of the catechol-O-methyltransferase (COMT) gene (and thus elevated levels of dopamine in prefrontal cortex), had enhanced cognitive performance in the Wisconsin Card Sort Test (WCST) and a more efficient physiological response in prefrontal cortex measured with functional MRI. The authors suggested that this common functional polymorphism (Val108/158 Met) in COMT gene may have an effect on prefrontal cognition and physiology. The high-activity val allele leads to lower dopamine levels in prefrontal cortex, which may lead to a slightly increased risk of schizophrenia (Egan et al. 2001). However, we have earlier suggested that met allele carriers have increased risk of schizophrenia with poor response to typical neuroleptics (Illi et al. 2003b).

1.5 Antipsychotic drugs

All antipsychotic drugs so far share the capacity to block dopamine-2 (D2) receptors (Tamminga 2004). Chlorpromazine, the first antipsychotic drug, was discovered in 1952 when tested as a sedative drug in schizophrenia in France (Tamminga 2004). Several other D2 receptor blocking agents were introduced during the following years, and as a group they are called typical (or conventional or traditional) neuroleptics. In the course of time psychiatrists observed that typical neuroleptics were at their best in reducing positive

symptoms (hallucinations, delusions, and thought disorder). However, their effect on negative symptoms (i.e., affective flattening, alogia, or avolition) was poor, and they had several, significant and potentially serious side effects. Several studies suggested that typical neuroleptics may also result in cognitive impairment, but this consequence may still be controversial (Mishara and Goldberg 2004).

Typical antipsychotics are compared to each other using chlorpromazine-equivalent ratios. For haloperidol chlorpromazine-equivalent ratio is 50 i.e., "2 mg of haloperidol equals 100 mg of chlorpromazine". Chlorpromazine-equivalent ratios for typical antipsychotics and clozapine are shown in Table 1 (Kane 1996).

Table 1. Chlorpromazine-equivalent doses for typical antipsychotics and clozapine (Kane 1996).

Antipsychotic	Chlorpromazine-equivalent doses
Chlorpromazine	100 mg
Chlorprothixene	100 mg
Fluphenazine	2 mg
Haloperidol	2 mg
Levopromazine	100 mg
Perphenazine	10 mg
Thioridazine	100 mg
Clozapine	50 mg

Clozapine was the first atypical antipsychotic first introduced for clinical use in Finland in 1977 (Tamminga 2004). It has a significantly higher affinity to 5-hydroxytryptamine 2A (5-HT2A) than D2 receptors and consequently, the newer atypical antipsychotics have tried to mimic these properties. However, clozapine still remains superior to typical and other atypical antipsychotics in schizophrenia (Tamminga 2004). Clozapine has shown an antipsychotic effect with only 30-60% D2 occupancy level while typical neuroleptics, risperidone and olanzapine need a 60-70% D2 occupancy level (Kapur et al. 1999). This raises the possibility that clozapine has a fundamentally different mechanism of action than other antipsychotics (Kapur et al. 1999).

In the search for new antipsychotics, the greatest interest has focused on the neurotransmitters and their receptors in the frontal cortex (Roth et al. 2004). Clozapine is thought to normalize glutaminergic and dopaminergic neurotransmission via complex interactions with large numbers of molecular targets (Roth et al. 2004). The second step was high 5-HT2A/D2 affinity ratio antipsychotics: risperidone, olanzapine, and quetiapine (Roth et al. 2004).

There are some differences between the brain region of action of typical neuroleptics and clozapine. Typical neuroleptics result in a depolarization block of the neostriatum as well as the medial prefrontal cortex, while clozapine acts only on midbrain dopaminergic cells that project to the medial prefrontal cortex (Lambe and Aghajanian 2004, Tamminga 2004) (Figure 1).

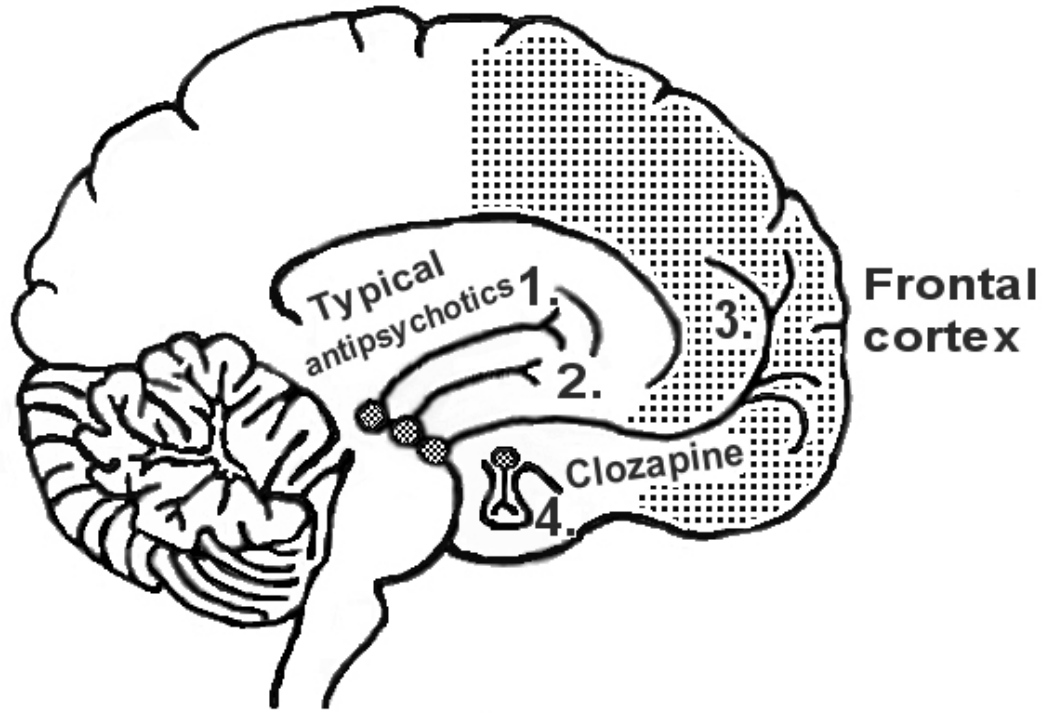


Figure 1. Dopaminergic pathways in the brain and the major regions of action of typical antipsychotics (striatum) and clozapine (frontal cortex)

1. nigrostriatial tract from the substantia nigra to the striatum
2. mesolimbic tract from the ventral tegmental area to many parts of the limbic system
3. mesocortical tract from the ventral tegmental area to the neocortex, particularly the prefrontal area.
4. tuberoinfundibular tract from the arcuate nucleus of the hypothalamus to the pituitary stalk

1.6 Treatment-resistant schizophrenia

The prevalence of treatment resistance is difficult to determine given the lack of agreement on defining the term. It has been estimated that 20-45 % of people with schizophrenia of over two years' duration are only partially responsive to antipsychotic medication and 5-10 % of patients derive no benefit at all (Pantelis and Lambert 2003). Schizophrenia patients with treatment resistant disease have been found to have increased cortical atrophy and lower levels of catecholamines in cerebrospinal fluid (McMahon et

al. 2002). A recent study by Arango et al. (2003) suggests that a larger right prefrontal cortex grey matter volume may be associated with poor response to haloperidol. Thus, it has been suggested that schizophrenia patients with treatment response to typical neuroleptics may constitute a distinct subtype of the disease (Joober et al. 2002, McMahon et al. 2002).

Kane et al. (1988) introduced their definition to treatment-resistant schizophrenia:

1. Persistent positive psychotic symptoms: Items score ≥ 4 (moderate) on at least two of four positive symptom items (rated on a 1-7 scale) on the Brief Psychiatric Rating Scale (BPRS) - hallucinatory behaviour, suspiciousness, unusual thought content, and conceptual disorganization.
2. Current presence of at least moderately severe illness: Total BPRS score ≥ 4 (moderate) on the Clinical Global Impression Scale (CGI).
3. Persistence of illness: No period of good social or occupational functioning within the last 5 years.
4. Drug-refractory condition: At least three periods in the preceding 5 years of treatment with conventional antipsychotics from at least two chemical classes at doses ≥ 1000 mg per day of chlorpromazine equivalents for 6 weeks, each without significant symptom relief, and failure to improve by at least 20 percent as measured by total BPRS score or intolerance of haloperidol at 10 to 60 mg per day during a 6-week prospective trial.

Treatment-resistance is usually defined as failure to respond to the usual drug treatment (Wahlbeck et al. 1998). Pantelis and Lambert (2003) suggest that patients should be treated for a minimum of two trials in which they receive 300-600 mg equivalents/day of chlorpromazine for 4-6 weeks instead before they can be considered non-responders.

2. Genetics of schizophrenia

Ample research suggests that schizophrenia is a strongly familial disorder (Riley and Kendler 2004). However, the exact genes have still not been identified. It has been suggested that single genetic factors in one subject will act in combination with other

genes and environmental factors, and a single gene may not account for a more than 1.5-fold increase in the risk of schizophrenia (Weinberger 2002).

2.1 Twin, adoption and family studies

Twin studies provide a tool to evaluate the contribution of genetic and environmental risk factors (Riley and Kendler 2004). They show consistently higher concordance rates in monozygotic (about 50 %) than dizygotic (about 17 %) twins. In most studies the heritability is 60-80 %. Moreover, a recent twin study suggests that there is overlap between schizophrenia, schizo-affective disorder and manic syndromes (Cardno et al. 2002).

So far, all adoption studies have shown that biological relatives of schizophrenia patients have a higher risk of schizophrenia (Riley and Kendler 2004), and of schizophrenia-spectrum disorders (Tienari et al. 2004).

In several European studies, the risk of schizophrenia has been about ten times higher in the siblings or offspring of schizophrenia patients when compared to general population (Riley and Kendler 2004).

2.2 Genome-wide studies

In genome-wide scanning hundreds of polymorphic markers are spaced throughout the chromosomal DNA and linkage analysis determines whether the marker and the disease are linked (Gelernter and Lappalainen 2004).

Linkage studies in schizophrenia have proved difficult for several reasons. When compared to monogenic (or Mendelian) disorder, the penetrance is usually incomplete in schizophrenia and schizophrenia-like symptoms may be caused by other diseases or illegal drugs. In addition, the diagnostic boundaries in schizophrenia are uncertain, and one locus may be associated with the disease in one family but not in another (Riley and Kendler 2004).

The first strong evidence for a linkage in schizophrenia was presented by Sherrington et al. (1988), but it could not be replicated in independent samples (Baron 2001). Subsequently, linkage studies have suggested several chromosomal regions as a candidate locus for schizophrenia: 1q21-22, 1q32-41, 4q31, 5p13-14, 5q22-31, 6p22-24, 6q21-22, 8p21-22, 9q21-22, 10p11-15, 13q14-32, 15q15, 22q11-13, and Xp11 (Baron 2001). In a Finnish isolate, Hovatta et al. (1999) reported linkage to 1q32.2-q41, 4q31, 9q21, and Xp11.4-p11.3.

In a recent meta-analysis, genome scans of 20 studies were analysed (Lewis et al. 2003). The study produced significant genomewide evidence for linkage on chromosome 2q, but also regions of chromosomes 5q, 3p, 11q, 6p, 1q, 22q, 8p, 20q, and 14p.

2.3 Candidate gene studies: genes studied

Although the aetiology of schizophrenia is still unknown, some genes are considered as suitable candidate genes on the basis of biochemical, pharmacological, immunological, animal models, and functional imaging studies (Harrison and Owen 2003). Researchers have been especially interested in such genes located in a candidate locus, having relevant functions and functional polymorphism. The most promising and already replicated findings are NRG1 (neuregulin1, locus 8p12-21), COMT (catechol-O-methyltransferase, 22q11), RGS4 (regulator of G-protein signalling 4, 1q21-22), DTNBP1 (dystrobrevin-binding protein 1, 6p22), and G72 (13q34) (Harrison and Owen 2003).

2.3.1 Brain-derived neurotrophic factor (BDNF) gene

The BDNF gene is located on chromosome 11p13. The BDNF G196A polymorphism in the 5' pro-region leads to an amino acid substitution (valine to methionine) at codon 66 (val66met) (Egan et al. 2003). This SNP is located in the pro-BDNF sequence and has been suggested to effect on BDNF secretion (Egan et al. 2003). Two studies suggest that met allele is associated with reduced BDNF secretion (Egan et al. 2003, Chen et al. 2004).

BDNF C270T polymorphism is located in a non-coding region of the gene (Riemenschneider et al. 2002). This polymorphism has been suggested to be associated with impaired BDNF production (Riemenschneider et al. 2002, Kanemoto et al. 2003).

In the study by Egan et al. (2003) the BDNF G196A (val66met) polymorphism was not associated with schizophrenia. However, two recent studies showed an association between the C270T polymorphism of BDNF and schizophrenia (Nanko et al. 2003, Szekeres et al. 2003). In both studies, the C/T genotype and the T allele were more frequent in schizophrenia patients (Szekeres et al. 2003).

Nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) serve as major neuronal survival factors. BDNF has an important role in the regulation of synaptic transmission and synaptogenesis (Lessmann et al. 2003). BDNF is involved in the development of dopaminergic systems and interacts with the meso-limbic dopaminergic systems (Hyman et al. 1991, Altar et al. 1992, Thoenen 1995, Blöchl and Sirrenberg 1996, Altar et al. 1997). BDNF also increases the survival of glutamate neurons and stimulates the growth of dendrites and increases the spine density of glutamate pyramidal neurons in neocortex (McAllister et al. 1995, McAllister et al. 1996). In addition, BDNF induces normal expression of the dopamine D3 receptor in nucleus accumbens both during development and in adulthood (Guillin et al. 2001). Several studies have suggested that BDNF is associated with glutaminergic pathways. BDNF expression is increased by an NMDA antagonist (MK-801) in cingulate and entorhinal cortices (Castrén et al. 1993, Hughes et al. 1993) and prevented in entorhinal cortex by haloperidol and clozapine (Lindén et al. 2000).

In several studies, haloperidol has downregulated BDNF expression in hippocampus (Angelucci et al. 2000, Lipska et al. 2001, Chlan-Fourney et al. 2002, Bai et al. 2003, Fumagalli et al. 2003). High-dose risperidone significantly downregulates BDNF, but clozapine and lower doses of risperidone have no effect when compared to controls (Angelucci et al. 2000, Lipska et al. 2001, Chlan-Fourney et al. 2002, Xu et al. 2002). Treatments with quetiapine, olanzapine, and clozapine have upregulated and attenuated decreased BDNF levels (Lindén et al. 2000, Xu et al. 2002, Bai et al. 2003, Fumagalli et al. 2003). The results may imply that the pharmacological effects of antipsychotic treatment are modulated by BDNF expression (Dawson et al. 2001). Taken

together, typical and atypical antipsychotics affect neuronal survival and death differently, which may result in differences in adverse effects or treatment response (Ukai et al. 2004).

2.3.2 Epidermal growth factor (EGF) gene

The EGF gene is located on chromosome 4q25-q27 and, so far, only one study has evaluated the polymorphism of the gene (Shahbazi et al. 2002). In this original work, promoter and 5' untranslated regions of the EGF gene were screened for polymorphism. In position 61, a G to A polymorphism was significantly associated with EGF production in peripheral-blood mononuclear cell cultures. Cells from individuals with AA genotype produced less EGF protein than individuals with other genotypes.

EGF has a major role in the development of the brain (Futamura et al. 2002). EGF may also have a neuromodulatory or neurotransmitter role, and has significant affects in dopaminergic, serotonergic, and glutaminergic functions in the brain (Ferrari et al. 1991, Plata-Salaman 1991, Yamada et al. 1997, Futamura et al. 2003, Gil et al. 2003). Mice lacking epidermal growth factor receptor (EGFR) demonstrate defects in cortical neurogenesis which may suggest that EGFR has a role in neuronal migration (Wong 2003).

EGF protein levels in the prefrontal cortex and putamen were lower in schizophrenic patients than in controls (Futamura et al. 2002). Serum EGF levels were also lower in the patients with schizophrenia than in controls (Futamura et al. 2002).

EGF is synthesized as a precursor which may have an important role in the cell-cell interactions (Yamada et al. 1997). EGF is a specific ligand for a receptor tyrosine kinase EGFR (ErbB1) but stronger signalling is allowed by heterodimers with other ErbB receptors (ErbB2, ErbB3 and ErbB4) (King et al. 1988, Yamada et al. 1997).

In CNS, EGF protein and/or mRNA is located in cerebrospinal fluid and several brain regions (e.g. brainstem, cerebellum, cerebral cortex, hippocampus) (Schaudies et al. 1989, Yamada et al. 1997). In dopaminergic neurons, EGF stimulates neurite outgrowth, increases dopamine uptake and enhances long-term survival (Yamada et al. 1997). EGF also increases dopamine synthesis and induces the expression of dopamine 2 (D2)

receptors in GH-3 cells (a rat pituitary tumor line cell expressing prolactin and growth hormone), which normally lack functional D2 receptors (Missale et al. 1994, Futamura et al. 2003).

2.3.3 NOTCH4 gene

NOTCH4 gene is located in the region of 6p21.3. Four Notch genes differ in the number of EGF repeats and the length of intracellular domain (Artavanis-Tsakonas et al. 1999). Notch receptor is a transmembrane receptor which is activated by ligands of neighbouring cells (Justice and Jan 2002). Ligand binding leads to the proteolytic cleavage of Notch, and the Notch intracellular domain (NICD) is cleaved (Fortini 2001). NICD then enters nucleus and modulates the expression of various target genes (Fortini 2001). The best known cell-fate effect of Notch is lateral inhibition, during which Notch signalling inhibits all but one of a group of equivalent precursor cells (Harper et al. 2003).

Notch signalling has a significant role in the development of CNS and regulates the generation of neurons and glia from neural stem cells (Grandbarbe et al. 2003). Upregulation of Notch activity also increases the number of interneuronal contacts in cortex (Sestan et al. 1999). In addition, Notch signalling regulates the differentiation of GABAergic neurons and has a role in the maintenance of synapses and the neuroglial stem cell lineages in hippocampus (Justice and Jan 2002, Kabos et al. 2002). Two recent studies suggest that Notch signalling has a significant effect on long-term memory in adult brain (Ge et al. 2004, Presente et al. 2004). However, the postnatal neurological functions of Notch signalling remain largely unknown (Nickoloff et al. 2003).

Notch and ErbB signalling are associated with some neurobiologically interesting pathways. Notch, as well as EGF, can activate PI3-K signalling causing phosphorylation of the Akt kinase (Rangarajan et al. 2001, Yarden and Sliwkowski 2001). In addition, Notch signalling is in close interaction with Presenilin1, and NICD may be protected by GSK-3 β (Foltz et al. 2002, Hitoshi et al. 2002). Interestingly, BDNF-dependent spatial learning is associated with TrkB/PI3-K signalling pathway (Mizuno et al. 2003, Yamada and Nabeshima 2003).

Wei and Hemmings (2000) reported a strong association between NOTCH4 polymorphism and schizophrenia. However, their highly significant result could not be replicated in the majority of subsequent studies (Skol et al. 2003). Interestingly, NOTCH4 (CTG)n polymorphism was correlated with differences in measures of frontal lobe cognitive performance and frontal lobe brain tissue volumes (Wassink et al. 2003).

The knowledge of the biological effects of NOTCH4 SNP2 polymorphism is scanty. This polymorphism is located near (CTG)n polymorphism and may thus be associated with morphological and functional changes in the brains of patients with schizophrenia (Wassink et al. 2003). In addition, the original finding by Wei and Hemmings (2000) showed that SNP2-(CTG)n haplotype had the strongest association of all haplotypes with schizophrenia.

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is a rare disease caused by mutations in NOTCH3 gene (Joutel et al. 1996). All mutations associated with CADASIL result in changes in EGF-like repeats but it is not known if these changes effect Notch3 signalling (Gridley 2003).

2.3.4 Catechol-O-methyltransferase (COMT) gene

COMT is the major enzyme in the brain in metabolizing dopamine, and norepinephrine (Männistö and Kaakkola 1999). The COMT gene has a functional polymorphism, Val108/158Met (Lachman et al. 1996). Met/met genotype is associated with 3- to 4-fold lower enzyme activity than val/val genotype. Thus, lower activity COMT of met allele carrying subjects may lead to higher dopamine levels in CNS. However, studies on COMT knockout mice have likewise demonstrated that dopamine levels are increased only in prefrontal cortex, where dopamine transporters have lower expression level and where they are not located in synapses (Egan et al. 2001).

Several lines of evidence have made COMT a strong candidate gene in psychiatry, and in particular, schizophrenia. The COMT gene is located in 22q11, a susceptibility locus for schizophrenia (Shifman et al. 2002). Velocardiofacial syndrome (VCFS) is associated with microdeletion in this same region, and patients with VCFS are at significantly increased risk for schizophrenia and other psychoses (Murphy et al. 1999).

The results by Egan et al. (2001) suggested that increased prefrontal dopamine catabolism may slightly increase the risk for schizophrenia. Shifman et al. (2002) also reported that Val allele carriers (i.e. those who have higher COMT activity) had a slightly increased risk for schizophrenia. Moreover, Glatt et al. (2003) suggested in their meta-analysis that Val allele may be a risk factor for schizophrenia in Europeans. However, lower dopamine catabolism may be associated with treatment-resistant schizophrenia (Illi et al. 2003b, Inada et al. 2003), and hostility in schizophrenia (Volavka et al. 2004).

There are only two reports of an association between COMT polymorphism and age at onset in schizophrenia. Both of them suggest that COMT Val/Met genotype is associated with later age at onset of schizophrenia (Liou et al. 2002, Tsai et al. 2004).

2.3.5 Apolipoprotein E (APOE) gene

APOE gene is located on chromosome 19q13.2 and many mutations and polymorphisms in both exons, introns and the promoter region have been described (Nickerson et al. 2000). The most widely studied polymorphisms are located at positions 3937 and 4075 in exon 4, and they result in three common alleles - APOE ε2, APOE ε3, and APOE ε4 (Gerdes 2003). They are coding for apoE isoforms whose amino acid sequences differ at positions 112 and 158; apoE2 has cysteine in both positions, whereas apoE3 has cysteine and arginine respectively, and apoE4 has arginine in both positions (Gerdes 2003).

APOE is expressed in humans as three isoforms coded by three different alleles, APOE ε2, ε3, and ε4 resulting in six genotypes (ε2/2, ε2/3, ε2/4, ε3/3, ε 3/4, and ε4/4) (Lehtimäki et al. 1990). Individuals carrying ε4 allele have lower serum apoE concentrations than those not carrying this allele, but APOE polymorphism does not affect CSF apoE concentrations (Lehtimäki et al. 1995, Nickerson et al. 2000, Siest et al. 2000). However, ε4 carriers have lower rates of glucose metabolism in the posterior cingulate, parietal, temporal, and prefrontal cortex (Reiman et al. 2004).

The strong association between APOE ε4 and the risk of Alzheimer's disease has led to ample research of the neurobiological effects of apoE and APOE polymorphisms (Siest et al. 2000). ApoE is involved in the mobilization and redistribution of cholesterol during neuronal growth and after injury (Mahley 1988). ApoE4 is associated with

inhibition of neurite outgrowth in embryonic neurons, in neuronal cell lines, and in cultured adult mouse cortical neurons (Nathan et al. 2002). ApoE may also have a regulatory role in hippocampal synapses (Veinbergs et al. 1999).

Association studies between APOE polymorphism and schizophrenia have mostly yielded conflicting results (Sutcliffe and Thomas 2002, Dean et al. 2003, Schürhoff et al. 2003). The meta-analysis by Schürhoff et al. (2003) suggested that ε3 allele frequency may be increased in schizophrenia patients in Asian population. The ε4 allele has been associated with the risk of schizophrenia in two studies (Harrington et al. 1995, Liu et al. 2003). Interestingly, Liu et al. (2003) reported that ε4 was a significant risk for those born during two periods in recent Chinese history of extreme food deprivation, suggesting thus a gene-environmental interaction. In addition, schizophrenia patients with ε4 allele may have fewer psychotic symptoms than patients without ε4 allele (Pickar et al. 1997). Earlier age at onset of schizophrenia has been associated with higher frequency of ε4 allele in two Caucasian samples but not in an Asian sample (Arnold et al. 1997, Igata-Yi et al. 1997, Martorell et al. 2001). However, there was no association between APOE polymorphism and age at onset in two Spanish samples (Durany et al. 2000, Saiz et al. 2002). Two recent functional polymorphisms in the ApoE transcriptional regulatory area were not associated with the risk of schizophrenia (Shinkai et al. 1998).

In two recent studies, APOE polymorphism was not associated with treatment response to typical neuroleptics (Durany et al. 2000) or to clozapine (Hong et al. 2000). However, in a postmortem analysis patients with schizophrenia had higher levels of apoE in the Brodmann's area 9 than control subjects (Dean et al. 2003). In rats, apoeE levels were lower in an analogous cortical region to Brodmann's area 9 in haloperidol treated rats than in vehicle treated rats, thus suggesting that antipsychotic drugs may decrease apoE levels as part of their therapeutic action (Dean et al. 2003). In addition, APOE ε4 may be associated with a reduced hippocampal volume in patients with schizophrenia (Plassman et al. 1997, Hata et al. 2002).

3 Environmental risk factors and gene-environment interaction

Schizophrenia seems to have a polygenic model of inheritance, which may interact with environmental factors (Mednick et al. 1998, Schultz and Andreasen 1999, Sullivan et al. 2003). The most studied environmental factors include season of birth, viral infections, and obstetric complications (Geddes and Lawrie 1995, Torrey et al. 1997, Verdoux et al. 1997, Suvisaari et al. 2000, Cannon et al. 2002a, Suvisaari et al. 2003, Koponen et al. 2004). In addition, psychological traumas during childhood and adolescence may increase the risk of schizophrenia (Parnas et al. 1985, Corcoran et al. 2002, Mueser et al. 2002, Read and Ross 2003).

More than 200 studies have investigated seasonality of birth in schizophrenia (Tochigi et al. 2004). Most of the studies have reported an excess of winter-early spring births and/or a decrease of late spring-summer births in the disease (Torrey et al. 1997, Tochigi et al. 2004). Tsuang (2000) has suggested that higher rates of infections during winter is the most likely cause of this birth excess but several other reasons have also been evinced (Tochigi et al. 2004). Some preliminary studies have implicated interactions between season of birth and candidate gene polymorphisms (Chotai et al. 2003). Vulnerability to viral infections may be associated with some genetic factors such as Human Leukocyte Antigen (HLA) A9 or HLA-DR1 histocompatability alleles (Narita et al. 2000, Tsuang 2000).

Obstetric complications have repeatedly been shown to be associated with increased risk of schizophrenia (Cannon et al. 2002a). MRI studies have shown several significant correlations between obstetric complications and brain abnormalities (Falkai et al. 2003, Gilbert et al. 2003, Schulze et al. 2003). Some results suggest that obstetric complications may be associated with genetic or autoimmune factors (Cannon et al. 2002a, Cannon et al. 2002b). Animal models might provide insights into the mechanisms by which specific obstetric complications have long-term influence on brain development leading to increased risk of schizophrenia (Boksa and El-Khodor 2003).

4. Pharmacogenetics

Different people respond in different ways to drug treatment, and the first reports of inherited differences were presented in the 1950s. It is estimated that genetics may account for 20 to 95 percent of variability in drug disposition and effects (Evans and McLeod 2003). These observations have led to ever growing fields of pharmacogenetics and pharmacogenomics. In practice, these terms are synonymous. However, pharmacogenomics uses genome-wide approaches and pharmacogenetics studies individual genes (Evans and McLeod 2003). Goldstein et al. (2003) used the term pharmacogenetics in its broadest meaning: heritable variation to inter-individual variation in drug response. Recently, Malhotra et al. (2004) defined pharmacogenetics as "the study of genetically determined inter-individual differences in response to pharmacological agents" and pharmacogenomics as "the application of genome-wide approaches to the study of inter-individual differences in response to pharmacological agents".

Pharmacogenetics may provide an important tool for the pharmaceutical industry (Roses 2002). More targeted drug development may also provide safer and more efficient drug treatment for patients (Schmith et al. 2003).

4.1 Pharmacogenetics of drug disposition

Pharmacogenetics focused first on drug metabolism. The classical study in debrisoquine metabolism in 1977 finally led to the characterization of polymorphisms that eliminate cytochrome P450 (CYP) 2D6 activity (Goldstein et al. 2003). Later on pharmacogenetics expanded to the broader field of drug disposition including transporters that influence drug absorption, distribution, and excretion (Evans and McLeod 2003, Meisel et al. 2003, Oscarson 2003).

So far, CYP2D6 is the most widely studied enzyme involved in drug metabolism. More than 75 different alleles have been identified in the CYP2D6 gene, and ultra rapid metabolizers are known to have multiple copies of this gene (Weinshilboum 2003). CYP2D6 poor metabolizers (PM) would thus have significantly elevated levels of some drugs while other drugs (e.g. codeine) may have reduced efficacy (Roden and George

2002). The CYP2D6 genotype has been shown to predict plasma concentrations of some selective serotonin reuptake inhibitors (SSRI) and tricyclic antidepressants in healthy volunteers (Malhotra et al. 2004). Although there are recommendations for drug therapy based on CYP2D6 genotype, these guidelines may be based on limited research (Malhotra et al. 2004).

There are recommendations of dose adjustment in poor, intermediate (IM), extensive (EM), and ultrarapid (UM) metabolizers of CYP2D6 and CYP2C19 in subjects using common antidepressants and antipsychotics (Kirchheiner et al. 2004).

There are considerable ethnic variations in the frequencies of CYP2D6 mutations leading to PM phenotype which are more common in Caucasians (7 %) and Africans (7-8 %) than in the Asian population (1 %). By contrast, the incidence of PMs of CYP2C19 substrates is much higher in Asians (15-30 %) than in Caucasians (Bondy and Zill 2004). When compared to CYP2D6 polymorphism, considerably fewer studies have evaluated the impact of CYP2C19 on drug metabolism (Kirchheiner et al. 2004).

4.2 The candidate gene approach in pharmacogenetics

There are several important and replicated results in pharmacogenetics concerning drug metabolism. However, finding candidate genes which may predict pharmacodynamic drug actions has proved much more difficult (Meisel et al. 2003). Some of the most interesting results in pharmacogenetics and pharmacogenomics are briefly reviewed in following text.

4.2.1 Alzheimer's disease

Apolipoprotein E (APOE) gene ε4 is a well-known genetic risk factor of Alzheimer's disease. In addition, ε4 allele carrying patients with Alzheimer's disease have poorer treatment response to cholinergic enhancer tacrine than those not carrying that allele (Cacabelos 2002). However, patients with ε3/ε4 genotype responded better than other patients to a combination of three different neuroimmunotrophic drugs (Cacabelos 2002).

4.2.2 Major depression

In major depression, the long variant in the promoter region of the serotonin transporter gene (5-HTTLPR) has predicted response to several SSRIs (Malhotra et al. 2004). This result is reasonable as serotonin transporter protein is the target of SSRIs, and the inhibition of the more active form of the gene may, at least theoretically, lead to increased level of serotonin in synapse (Bondy and Zill 2004). However, the results are consistent only in Caucasian population. In Asian population, the long variant has been associated with poor response to SSRIs (Kim et al. 2000, Yoshida et al. 2002). These conflicting results are puzzling, but may suggest different interactions between gene variants in different populations (Bondy and Zill 2004). Also, recent data suggest that the phenotype of drug response is very complex, and may be dependent on several gene-gene or gene-environment interactions (Bondy and Zill 2004).

Zill et al. (2000) reported that G-protein β3 subunit gene C825T polymorphism was associated with treatment response to various pharmacological treatments and to electroconvulsive therapy. In this relatively small (n=88) and heterogeneous patient sample T/T genotype was associated with treatment response. In a large patient sample in a study by Serretti et al. (2003) T/T genotype was associated with better response to fluvoxamine 300 mg/day (n=362) or paroxetine 40 mg/day (n=128). T allele carriers we associated with better treatment response to various antidepressants in an Asian sample (n=106) (Lee et al. 2004).

4.2.3 Cancer

The ErbB receptors and their ligands that belong to the epidermal growth factor (EGF) family of peptides are involved in the pathogenesis of different types of carcinomas. Overexpression of ErbB2/HER2 is associated with enhanced tumour aggressiveness and a high risk of relapse and death (Roses 2004). Overexpression of ErbB2 predicts poor response to hormonal therapy (Dowsett 2001) but better response to trastuzumab (Vogel et al. 2002). This diagnostic test allowed trastuzumab to progress through the pipeline to approval (Roses 2004).

5. Pharmacogenetics of schizophrenia

5.1 General aspects

Pharmacogenetics has received a great deal of interest in psychiatric research for several reasons. First, psychotropic drug efficacy may not be apparent until weeks after the initiation of drug treatment. This delay in treatment response may cause significant consequences, such as persisting psychiatric symptoms, loss of employment, social dysfunction, medical morbidity, and even suicide (Malhotra et al. 2004). Pharmacogenetic data may also reveal meaningful subtypes of the psychiatric disorders (Joober et al. 2002, Kerwin and Arranz 2002).

Applying pharmacogenetic tools in psychiatry has raised some important ethical questions. Most notably, genetic tests may indicate susceptibility to a psychiatric disorder or a patient may be stigmatised as a non-responder (Morley and Hall 2004).

5.2 Cytochrome P450 enzymes

CYP2D6 is the major metabolizer of risperidone and most of the typical antipsychotics (Kirchheiner et al. 2004). Thus, several studies have tried to evaluate the impact of the genetic variation of CYP2D6 gene on antipsychotic drug response. However, most antipsychotic drugs are metabolized by more than one enzyme. Because of this, a significant relationship between CYP2D6 genotype and steady-state concentrations was only shown for a few drugs (e.g. perphenazine, zuclopenthixol, risperidone and haloperidol) in some individuals, and only when used as monotherapy. The clinical impact of these polymorphisms with respect to therapeutic response and dosing remains scanty and is largely based on case reports (Bondy and Zill 2004).

Clozapine and olanzapine are metabolized primarily by CYP1A2. The CYP1A2 gene polymorphism is not suggested to have a significant effect on the metabolism of these atypical antipsychotics (Prior and Baker 2003, van der Weide et al. 2003).

5.3 Candidate gene approach; typical antipsychotics

The candidate gene approach is the most widely used way of studying the effect of genetic variability on treatment response. Candidate genes include the target receptors of antipsychotic drugs, neurotransmitter transporters and metabolizing enzymes (Kirchheiner et al. 2004). This strategy has had some success in detecting genes with even minor influence in clinical response. Its major drawback is the difficulty in replicating positive findings (Kerwin and Arranz 2002). This may, at least partly, be due to ethnic origins and assessment criteria. One possible way is to make the comparison between the very poor and very good responders (Kerwin and Arranz 2002). Also, standardized rules for pharmacogenetic studies may increase the chance of replication (Cichon et al. 2000).

As all antipsychotic drugs today block dopamine receptors, the genes of dopamine receptors have been the subject of extensive research (Kerwin and Arranz 2002). The dopamine 2 type receptors (D2, D3, and D4) are the most widely studied, but most studies have focused on clozapine. The association studies between treatment response to typical antipsychotics and polymorphisms of dopamine receptors in schizophrenia patients are listed in Table 2.

Table 2. Association studies between treatment response to typical neuroleptics and dopamine receptor polymorphisms in schizophrenia patients.

Gene Reference	Mutation	N	Antipsychotic(s)	Ethnicity	Result
DRD2 [1]	–141C Ins/Del	146	several typical	Chinese	NS
DRD2 [2]	–141C Ins/Del	170	several typical?	Japanese	NS
DRD2 [2]	–141C Ins/Del	170	several typical?	Japanese	NS
DRD2 [3]	–141C Ins/Del	94	several typical	Finnish	NS
DRD2 [4]	–141C Ins/Del Taq1A	49	bromperidol, nemonapride	Japanese	NS
DRD2 [5]	Taq1A	26	haloperidol	?	NS
DRD3 [6]	allele 2	80, 87	several typical?	Israeli, Italian	2-2: poor response
DRD3 [7]	BalI	76	several typical	Swedish	homozygotic: good response
DRD4 [8]	48-bp VNTR	28	several typical	USA	7 repeat: poor response?
DRD4 [9]	48-bp VNTR	638	several typical	German	NS

[1] Arranz et al. 1998a, [2] Ohara et al. 1998, [3] Kampman et al. 2003, [4] Kondo et al. 2003, [5] Schäfer et al. 2001, [6] Ebstein et al. 1997, [7] Jönsson et al. 1993, [8] Cohen et al. 1999, [9] Kaiser et al. 2000

ABBREVIATIONS: NS, not significant; DRD2, dopamine2 receptor gene; DRD3, dopamine3 receptor gene; DRD4, dopamine4 receptor gene; Ins, insertion; Del, deletion; VNTR, variable number of tandem repeats

The functional polymorphism of COMT gene was associated with poor response to typical neuroleptics in two studies (Illi et al. 2003b, Inada et al. 2003). Illi et al. (2003b) also reported an additional synergistic effect of promoter polymorphism in MAOA gene,

which resulted in a six-fold higher risk of being a non-responder to typical neuroleptics. Illi et al. (2003a) also reported that interaction between COMT and angiotensin-converting enzyme (ACE) polymorphisms shows an increased risk of being a non-responder.

5-HT2A gene T102C polymorphism and response to typical neuroleptics have been evaluated in one study (Joober et al. 1999). In male schizophrenia patients, C allele carriers had better response than those not carrying that allele. Because typical neuroleptics down-regulate brain-deriver neurotrophic factor (BDNF) in hippocampus, polymorphisms of this gene have been studied in one study. Krebs et al. (2000) reported an excess of the 172-176 bp alleles of BDNF in neuroleptic-responding patients with schizophrenia. Methylenetetrahydrofolate reductase (MTHFR) gene polymorphism (C677T) has been linked to treatment response to typical neuroleptics (Joober et al. 2000).

5.4 Candidate gene approach; atypical antipsychotics

Pharmacogenetic studies in clozapine have focused on dopamine4 receptors and serotonin 2A and 2C receptors (Tables 3, 4 and 5). The majority of the positive association results could not be replicated. However, a meta-analysis suggested that the C allele of 5-HT2A gene is associated with poor response to clozapine (Arranz et al. 1998b). Recently, this effect was estimated to be minor, the weighted mean of odds ratios being 1.7 (Kirchheiner et al. 2004). A combination of six polymorphisms in serotonin and histamine related genes resulted in 76.7 % success in the prediction of clozapine response (Arranz et al. 2000). However, not even this result could be replicated (Schumacher et al. 2000).

Table 3. Clozapine response and serotonin receptor polymorphism

Gene	Polymorphism	Result	Reference
5-HT2A	A1438G	significant association	Arranz et al. 1998c
	A1438G	NS	Masellis et al. 1998
	His452Tyr	significant association	Masellis et al. 1998
	His452Tyr	NS	Malhotra et al. 1996a
	His452Tyr	NS	Arranz et al. 1996
	His452Tyr	NS	Nöthen et al. 1995
	His452Tyr	NS	Schumacher et al. 2000
	His452Tyr	NS	Arranz et al. 1998c
	T102C	significant association	Arranz et al. 1995
	T102C	NS	Masellis et al. 1998
	T102C	NS	Lin et al. 1999
	T102C	NS	Malhotra et al. 1996a
	T102C	NS	Nöthen et al. 1995
	T102C	NS	Schumacher et al. 2000
	T102C	NS	Masellis et al. 1995
	Thr25Asn	NS	Nöthen et al. 1995
5-HT2C	Cys23Ser	significant association	Sodhi et al. 1995
	Cys23Ser	NS	Masellis et al. 1998
	Cys23Ser	NS	Schumacher et al. 2000
	Cys23Ser	NS	Malhotra et al. 1996b
	Cys23Ser	NS	Rietschel et al. 1997
	G330T, C244T	NS	Schumacher et al. 2000
5-HT3A	C178T, A1596G	NS	Gutierrez et al. 2002
5-HT3B	CA repeat	NS	Gutierrez et al. 2002
5-HT5A	-G19C	NS	Birkett et al. 2000
	A12T	NS	Birkett et al. 2000
5-HT6	T267C	significant association	Yu et al. 1999
	T267C	NS	Masellis et al. 2001

NS = non-significant association

Modified from the original summary of Kirchheiner et al. (2004).

Table 4. Clozapine response and dopamine receptor polymorphisms

Gene	Polymorphism	Result	Reference
D1	promoter	significant association	Potkin et al. 2003
D2	141C Ins/Del	NS	Arranz et al. 1998b
D3	Ser9Gly	significant association	Scharfetter et al. 1999
	Ser9Gly	NS	Malhotra et al. 1998
	Ser9Gly	NS	Shaikh et al. 1996
D4	12-bp VNTR	NS	Kohn et al. 1997
	12-bp VNTR	NS	Rietschel et al. 1996
	48-bp VNTR	NS	Rao et al. 1994
	48-bp VNTR	NS	Kohn et al. 1997
	48-bp VNTR	NS	Rietschel et al. 1996
	48-bp VNTR	NS	Shaikh et al. 1993
	48-bp VNTR	NS	Shaikh et al. 1995
	13-bp del	NS	Rietschel et al. 1996
	Gly11Arg	NS	Rietschel et al. 1996

NS = non-significant association

Modified from the original summary of Kirchheiner et al. (2004).

Table 5. Clozapine response and serotonin transporter, histamine receptor, adrenoceptor, glutamate receptor, BDNF and APOE polymorphisms

Gene	Polymorphism	Result	Reference
5-HTT	44-bp ins/del	NS	Tsai et al. 2000
H1	several	NS	Mancama et al. 2002
H2	several	NS	Mancama et al. 2002
ADRA1A	Arg492Cys	NS	Bolonna et al. 2000
ADRA2A	C1291G	NS	Bolonna et al. 2000
	C1291G	NS	Tsai et al. 2001
	G261A	NS	Bolonna et al. 2000
GRIN2B	C2664T	NS	Hong et al. 2001
BDNF	Val66Met	NS	Hong et al. 2003
APOE	ε4	NS	Hong et al. 2000
TNFalpha	G-308A	NS	Tsai et al. 2003

NS = non-significant association

Modified from the original summary of Kirchheiner et al. (2004) and updated.

There are three pharmacogenetic studies with conflicting results concerning response to risperidone in schizophrenia and 5-HT2A gene T102C polymorphism. In the study by Lane et al. (2002), C allele was associated with better response to risperidone. However, another study obtained the opposite result, suggesting that those patients not carrying C allele had better response to treatment with risperidone (Herken et al. 2003a). The third study did not find any association between 5-HT2A gene polymorphism and response to risperidone (Yamanouchi et al. 2003).

Szekeres et al. (2004) studied the association between dopamine3 receptor polymorphisms (Ser9Gly and VNTR) and dopamine transporter (DAT) polymorphisms

and response to clozapine, olanzapine, quetiapine and risperidone. The authors reported that Ser/Ser genotype was associated with poor response to antipsychotic treatment.

In conclusion, the results of pharmacogenetic studies of treatment response to antipsychotics have so far been inconclusive. This may result in methodological reasons or because the researchers may also have chosen to study the wrong genes or polymorphisms (Kirchheiner et al. 2004).

5.5 Pharmacogenetics of adverse effects in schizophrenia

Genetic variation behind adverse effects in schizophrenia has been quite widely studied (Kirchheiner et al. 2004). Here some of the most important results are briefly reviewed.

Several groups have reported that the Ser9Gly DRD3 gene polymorphism is associated with risk for tardive dyskinesia (TD) (Badri et al. 1996, Steen et al. 1997, Basile et al. 1999, Segman et al. 1999, Lovlie et al. 2000, Liao et al. 2001, Lerer et al. 2002, Woo et al. 2002, Zhang et al. 2003). In each study, either the glycine/glycine genotype or the glycine allele was associated with the risk of TD. These results could not be replicated in some other studies (Inada et al. 1997, Rietschel et al. 2000, Garcia-Barcelo et al. 2001, Chong et al. 2003).

5-HT2A receptor gene T102C polymorphism has also been associated with TD in two studies (Segman et al. 2001, Tan et al. 2001). Two other studies could not replicate these results (Basile et al. 2001, Herken et al. 2003b).

Two studies in Asia have evaluated the effect of 5-HT2C -759C/T polymorphism in antipsychotic drug-induced weight gain (Reynolds et al. 2002, Reynolds et al. 2003). In their first study (n=123), C allele carriers had more weight gain than those not carrying C allele (Reynolds et al. 2002). In the second study with patients using clozapine (n=32), drug-induced weight gain was observed only in male C allele carriers (Reynolds et al. 2003). However, these results could not be replicated in a Caucasian population (Theisen et al. 2004).

Lahdelma et al. (2001) reported that HLA-A1 is associated not only with a good response to clozapine but also with a low risk of clozapine induced agranulocytosis (CA) using schizophrenia patients. Risk of CA is also associated with HLA-B16, B38, DR4, DR2, and DQ1 as well as NQO2 gene (Meged et al. 1999, Lahdelma et al. 2001, Ostrousky et al. 2003). In the study by Turbay et al. (1997), CA was associated with several haplotypes in MCH region on chromosome 6p21.

Aims of the study

There were two lines in the selection of genes concerning neuroleptic drug response in this study. The first focused on genes which may modulate dopaminergic activity and the second on genes which have an effect on brain development.

The aims of this thesis were:

To test the association of five candidate gene polymorphisms (BDNF, EGF, NOTCH4, COMT and APOE) and:

1. the treatment response to typical neuroleptics
2. the age at onset of schizophrenia
3. the risk of schizophrenia.

Subjects and methods

1. Patients and ethics

The patients were recruited between 1st January 1999 and 31st December 2000. The study was carried out in compliance with the code of ethics of the World Medical Association (Declaration of Helsinki) and the standards established by the local medical ethics committee. The participants gave written informed consent.

1.1 Diagnosis

An experienced psychiatrist interviewed all the patients and checked the diagnoses according to the DSM-IV criteria by evaluating hospital records.

1.2 Inclusion and exclusion criteria

The inclusion criteria were defined as diagnosis of schizophrenia. Criteria for response or non-response to typical neuroleptics are defined in 1.3 and 1.4. Only patients who gave written informed consent were included.

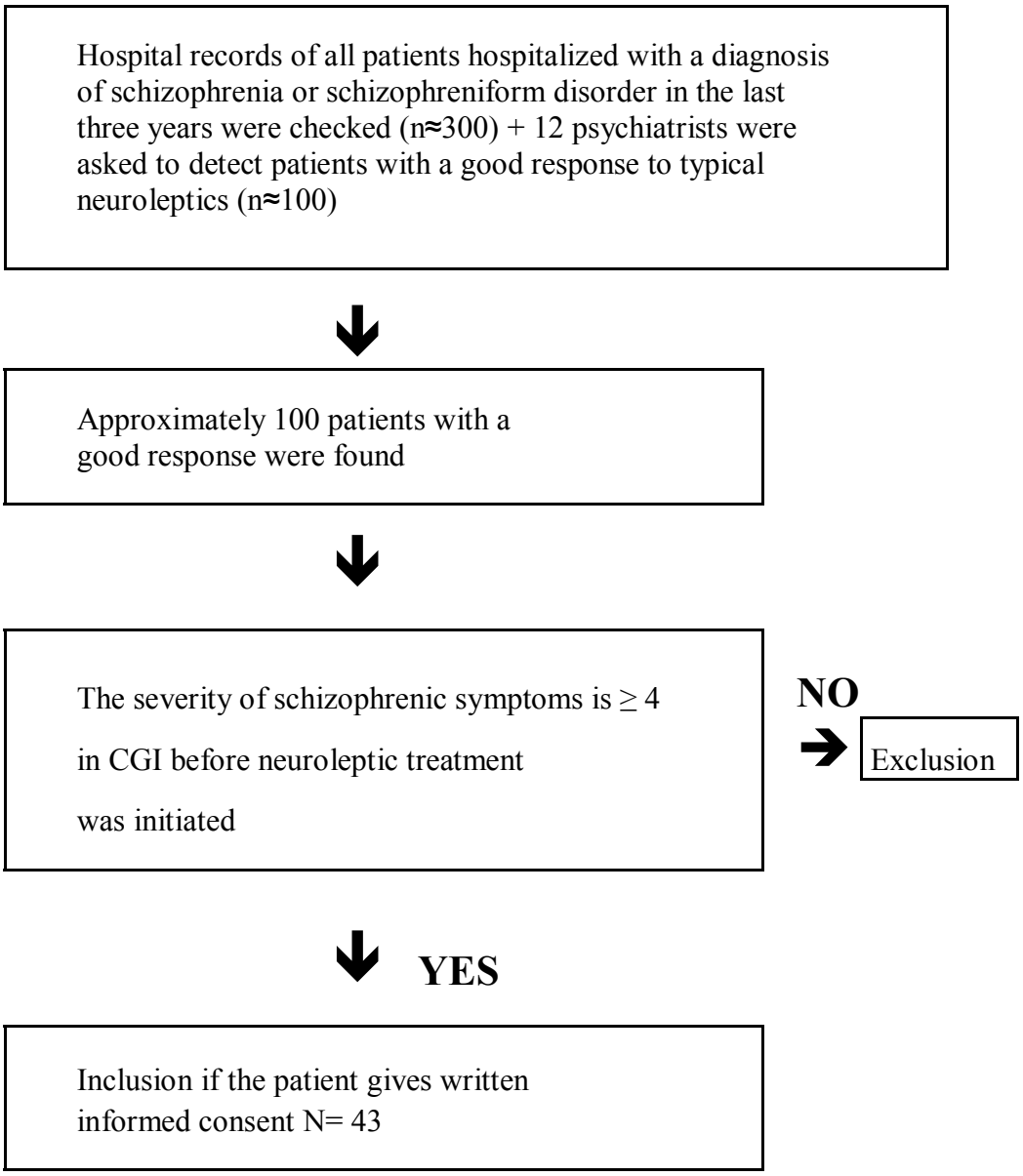
The first exclusion criterion was major affective disorders and schizo-affective disorder. In addition, patients with drug or alcohol abuse were excluded. If a patient had a neurological or somatic disease likely to cause psychotic symptoms, the patient was excluded from the study.

1.3 Criteria for the responders

The patients in the responder group (group I) had experienced a sufficient and long-lasting response to treatment with conventional neuroleptics. Assessment of response was based on information in hospital and mental health care records and a personal interview with each patient. Before the initiation of neuroleptic treatment, the severity of schizophrenic

symptoms had to be ≥4 according to the Clinical Global Impression scale (CGI). A flowchart of inclusion and exclusion criteria for responders is presented in Figure 2.

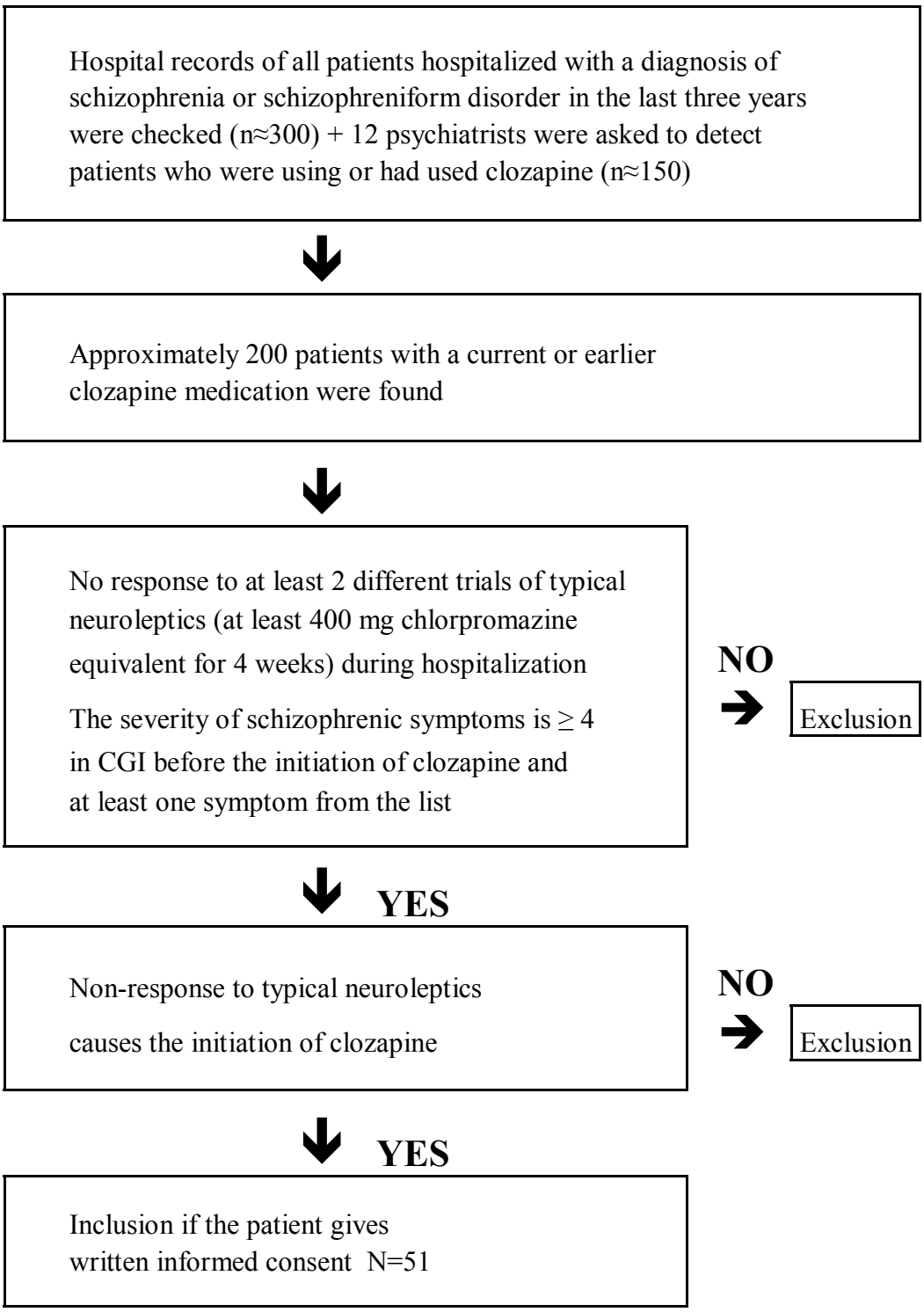
Figure 2. Flowchart of inclusion and exclusion criteria for responders



1.4 Criteria for the non-responders

The patients in the non-responder group (group II) were those with clozapine medication who had failed to respond on at least two different occasions to treatment with two different conventional antipsychotics during hospitalization. In each treatment period the lowest accepted daily dose was 400 mg chlorpromazine equivalent for a minimum of four weeks. Prior to the initiation of clozapine treatment the severity of schizophrenic symptoms had to be ≥ 4 on the CGI scale and at least one of the following symptoms had to be present: conceptual disorganization, suspiciousness, hallucinatory behaviour, or unusual thought content. A flowchart of inclusion and exclusion criteria for non-responders is shown in Figure 3.

Figure 3. Flowchart of inclusion and exclusion criteria for non-responders



1.5 Definition of the age at onset

Age at onset was determined as the patient's age during the first hospitalization at which the diagnosis of schizophrenia or schizophreniform psychosis was used. This data was obtained from the Hospital Discharge Register.

1.6 Details of the study model in practice

We used two different forms, one for responders and another for non-responders. In these forms both inclusion and exclusion criteria were taken together. These forms were completed by the author and four other psychiatrists before the patients were asked to participate in the study.

Unfortunately, we did not save data of those who fulfilled the criteria but could not be located or those who declined to participate in the study. However, quite many patients refused to participate because of suspiciousness.

1.6.1 How this study started

In order to find the best responders to typical neuroleptics, we went through the charts of all patients hospitalized because of schizophrenia in the Department of Psychiatry in Tampere University Hospital during the last three years. If a patient experienced a good response to typical neuroleptics and no exclusion criteria were found, he/she was interviewed by one of the investigators. The CGI before antipsychotic treatment was estimated on the basis of this interview and hospital records. If CGI was ≥ 4 and the patient gave an informed written consent, he/she was asked to give a blood sample.

Because the recruitment of the patients in the University Hospital Clinic was too slow, we expanded the recruitment to some mental health centres and one other psychiatric hospital. We also contacted 12 psychiatrists and asked them to nominate patients likely to meet our criteria. The selection was continued until 43 patients fulfilled inclusion and exclusion criteria and gave informed consent.

The doses of typical neuroleptics were registered weekly during the patients' hospitalization for up to 4 weeks. The mean chlorpromazine (CP) equivalent doses (±SD) of typical neuroleptics during the first weeks of hospitalization for the patients considered good responders were following: 312±146 mg in the first week, 339±140 mg in the second week, 335±149 mg in the third week and 339±194 mg in the fourth week. Of these patients, 43 gave informed consent, and finally participated in the study. Due to strict criteria, the patients finally participating to the study were selected from a large sample of patients with schizophrenia.

To find non-responders to typical neuroleptics, we also first collected the hospital records in the Department of Psychiatry in Tampere University Hospital of all patients for the last three years and selected those receiving clozapine treatment for schizophrenia. In addition, we contacted 12 psychiatrists and expanded our recruiting as described previously. The patients who, according to the chart, seemed to fulfil the criteria were then interviewed. E.g. we registered the starting and ending dates for both, at least 4-week treatment periods and used certain codes for typical neuroleptics used and checked that their chlorpromazine equivalent doses were at least 400 mg for all days of the period. We also checked that during these two treatment periods, at least two different typical neuroleptics were used. The depot neuroleptics were changed to comparable doses/day according to manufactures' guidelines. In about 50% of the clozapine patients, our criteria for non-response were not met and the patients were therefore excluded. In some clozapine patients, clozapine was initiated because of intolerance of typical neuroleptics, and these patients were excluded. On the basis of hospital records and personal interview, the CGI was estimated before the initiation of clozapine. Only patients with CGI ≥4, were included.

At the end of the study we had collected blood samples from 98 patients. However, we did not have adequate data on four patients, and they were excluded.

1.6.2 Details of the study groups

The patient sample included 43 patients who were considered good responders and 51 patients who were non-responders to typical neuroleptics. Table 6 shows mean age and mean age at onset of the patients. The patients were followed after their first

hospitalization because of schizophrenia or schizophreniform psychosis for 16.4±7.3 years before they entered the study.

Table 6. Mean ages and ages at onset of schizophrenia in responders and non-responders (years ±SD)

	Responders	Non-responders
Mean age (years) ±SD		
Men	46.9±10.7 (N= 17)	45.9±10.9 (N= 29)
Women	48.8±12.0 (N=26)	42.1±9.6 (N=22)*
All	48.1±11.4 (N= 43)	44.3±10.4 (N= 51)
Age at onset (years) ±SD		
Men	30.0±7.8	27.9±10.9
Women	33.6±10.3	26.8±9.1*
All	32.2±9.5	27.4±10.1*

* $p<0.05$ (between responders and non-responders; ANOVA)

For the responder group, we included only patients who had CGI ≥4 before the neuroleptic treatment was initiated. In this group, the mean CGI was 5.26±0.82, and only 8 patients had CGI=4.

In the non-responder group, CGI was 5.67±0.77 before clozapine treatment was initiated. For the non-responder group, we registered the chlorpromazine (CP) equivalent doses for two different at least 4 week long periods. In the first week the mean CP equivalent dose was 704±301 mg and during the second week 777±310 mg. The most common typical neuroleptic was chlorpromazine, which was initiated in 45% of the patients. The second and third most common antipsychotics were zuclopenthixol and chlorprothixene. During the second four-week period, the most common antipsychotics were levomepromazine and perphenazine. The gender differences are shown in Table 7.

Table 7. The most common neuroleptics during the first and second treatment periods in the whole sample of non-responders, and in male and female patients separately.

	First period			Second period		
	1st most common	2nd most common	3rd most common	1st most common	2nd most common	3rd most common
Male	CP	Z	P	P	L	several
Female	CP	CPX	Z	Z	L	CP
All	CP	Z	CPX	P	L	Z

CP=chlorpromazine; Z=zuclopenthixol; CPX=chlorprothixene; L=levomepromazine; P=perphenazine.

2. Controls

The controls were 98 age and gender-matched healthy blood donors from the Finnish Red Cross. These 98 controls were chosen from 400 randomly selected blood donors. The only selection criteria were gender and age. In Finland, the subjects complete a written health statement, including information on neurological and mental health at every blood donation session. They are also asked about medication, allergies, heart diseases, infectious diseases and other chronic diseases. All subjects are also interviewed by a qualified nurse before each donation. In Finland, blood donors are not paid.

3. Methods

3.1 Genetic methods - DNA isolation and genotyping

From all patients 10 ml of venous blood was drawn into EDTA vacuum tubes and immediately frozen at -20 degrees Celsius for later DNA isolation. Genomic DNA was extracted from controls from peripheral blood leukocytes (buffy coats) and from patients from venous whole blood using commercially available kit (Qiagen Inc., Hilden, Germany).

Genotypings were planned by two biochemists (Kari M Mattila and Riikka Rontu) and performed by laboratory technicians in the Department of Clinical Chemistry

(Laboratory of Atherosclerosis Genetics), Tampere University Hospital and the Medical School, University of Tampere.

3.1.1 Brain-derived neurotrophic factor genotyping

The genotyping of the G196A (val66met) polymorphism of the BDNF was carried out as described by Ventriglia et al. (2002). A 171 bp sequence of the gene containing the polymorphism was amplified using the primers 5'-ACT CTG GAG AGC GTG AAT GG-3' (forward) and 5'-ACT ACT GAG CAT CAC CCT GGA-3' (reverse). This was followed by digestion of the PCR products with PmaCI restriction enzyme, separation of the fragments generated by agarose gel electrophoresis and after ethidium bromide staining the genotypes were established under UV light as follows: G/G (99 bp, 72 bp); G/A (171 bp, 99 bp, 72 bp) and A/A (171 bp).

The C270T polymorphism of the BDNF was genotyped as reported by Kunugi et al. (2001). The following pair of primers was used to amplify a 223 bp fragment of the BDNF: 5'-CAG AGG AGC CAG CCC GGT GCG-3' (forward) and 5'-CTC CTG CAC CAA GCC CCA TTC-3' (reverse). The PCR products were subsequently digested with the HinfI restriction enzyme, the fragments generated separated by agarose gel electrophoresis and after ethidium bromide staining the genotypes were established under UV light as follows: C/C (127 bp, 78 bp); C/T (127 bp, 78 bp, 63 bp); and T/T (127 bp, 63 bp).

3.1.2 Epidermal growth factor genotyping

The genotyping of the EGF polymorphism was carried out as described by Shahbazi et al. (2002). Briefly, a 242 bp sequence of the EGF gene containing the polymorphism at position 61 (A-G) was amplified using the following pair of primers: 50-TGTCACTAAAGGAAAGGAGGT-30 (forward) and 50-TTCACAGAGTTTAACAGCCC-3' (reverse). The PCR product was digested with AluI restriction enzyme and the fragments generated separated using agarose gel electrophoresis (A allele 15, 34, 91 and 102 bp fragments; G allele: 15, 34 and 193 bp fragments).

3.1.3 NOTCH4 genotyping

Oligonucleotide primers designed for the identification of the T-C substitution in the promoter region (at position - 25) of NOTCH4 [Notch (Drosophila) homolog 4] were 50-ACT CAG GAA ACA GCT CAG ACG T-30 (forward) and 50-CAC TGA ACATCC TCC TAA GGG A-30 (reverse). The amplification product [283 base pairs (bp)] was digested with the MspI restriction enzyme, which cuts the C allele (generating 153 bp and 130 bp fragments) but not the T allele.

3.1.4 Catechol-O-methyltransferase genotyping

A 217-bp fragment of the COMT gene containing the G to A polymorphism at position 1947 was amplified using the primers 50-AGG TCT GAC AAC GGG TCA GGC-30 and 50-TCG TGG ACG CCG TGA TTC AGG-30 as described by Kunugi et al. (1997). After digestion of the PCR product with the restriction enzyme NlaIII, the COMT H/H, COMT H/L, and COMT L/L genotypes were established by identifying the restriction fragments using agarose gel (4 %) electrophoresis. As a control, water samples and known sequenced control samples were run in parallel with unknown DNA samples.

3.1.5 Apolipoprotein E genotyping

For the APOE 112 genotyping, we used fluorogenic allele-specific TaqMan probes and primers as previously described (Koch et al. 2002). APOE 158 genotypes were determined using allele-specific fluorogenic probes with conjugated minor groove binder (MGB) group (Livak 1999). The nucleotide sequences of the APOE 158 primers and probes used in the PCR were deduced from published sequences deposited in the GenBank database and were chosen and synthesized in conjunction with Applied Biosystems (Foster City, CA, USA) using the Assay-by-Design tool. DNA samples were genotyped by employing the 5' nuclease assay for allelic discrimination using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR reaction containing genomic DNA, 1 Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe was performed in 96-well plates using the standard protocol in a total volume of 25 µl. Water controls and known control samples previously typed by RFLP-PCR analysis were run in parallel with unknown DNA samples. After

cycling, end-point fluorescence was measured and genotype calling was carried out by the allelic discrimination analysis module.

3.2 Statistical methods

The frequence distributions of the genotypes and alleles of the genes studied between responders and non-responders (in original Publications II-V), the distribution of NOTCH4 polymorphism and the month of birth in patients (I) as well as the differences in gene and genotype frequencies between patients and controls (I-V) were compared using Pearson Chi-Square test.

The association of the distribution of the BDNF polymorphisms between responders and non-responders and between patients and controls were studied using a Fisher exact test (I).

The association between the age at onset and the gene polymorphism was studied using the Kaplan-Meier method and the log rank test for the analysis of survival (I-III, V). In addition, one-way ANOVA was used to compare the age at onset of schizophrenia between different genotypes (II, V). The age at onset was also compared between APOE genotype groups (carries vs. non-carriers of certain alleles) using non-parametric tests (Mann-Whitney U-test and median test) (V).

The calculations of the haplotype analysis were made with the Arlequin version 2.000 software (I).

Multiple logistic regression was used in the interaction analysis and calculation of odds ratios and their CIs (IV).

The statistical analysis was carried out using SPSS/Win [Versions 10.0 (I), 11.0 (II-IV), and 11.5 (V), SPSS Inc., Chicago, IL] on a microcomputer.

Results

1. Brain-derived neurotrophic factor: Lack of association with response to typical neuroleptics (I)

The BDNF G196A (val66met) and C270T polymorphisms were not associated with response to typical neuroleptics, or the age at onset of schizophrenia. Moreover, these polymorphisms of the BDNF gene were not associated with the risk of schizophrenia.

In the haplotype analysis, these polymorphisms did not show any significant association with treatment response to typical neuroleptics or risk of schizophrenia.

2. Epidermal growth factor polymorphism: association with the age at onset and the risk of schizophrenia in men (II)

The EGF gene G61A polymorphism was significantly associated with the risk of schizophrenia in male patients only. G allele was more than 3.5 times more frequent in male patients with schizophrenia than in male controls. Additional data not presented in the original publication indicate that G allele was more frequent in male responders than male controls (OR=10.322 (95% CI 1.268-84.046), p=0.010). In addition, G allele carrying male patients with schizophrenia had significantly later age at onset than those male patients not carrying G allele.

3. NOTCH4 polymorphisms: association with the age at onset and month of birth in schizophrenia (III)

NOTCH4 SNP2 (T-25C) polymorphism was significantly associated with the age at onset in schizophrenia. Additional data not presented in the original publication indicate that both T allele carrying male responders (p=0.0003) and male non-responders (p=0.001)

had an earlier age at onset of schizophrenia than those not carrying T allele (Kaplan-Meier log-rank test).

In this sample, NOTCH4 SNP2 polymorphism was not associated with the risk of schizophrenia.

4. NOTCH4 and catechol-O-methyltransferase polymorphisms: association with response to typical neuroleptics and determination of a subpopulation of poorly responding patients (IV)

For treatment response to typical neuroleptics, there was a significant interaction between NOTCH4 SNP2 and COMT V108/158M pooled polymorphisms (P=0.003). Patients with both NOTCH4 C/C genotype and COMT low/low genotype were significantly more often non-responders to conventional antipsychotics [OR=10.25 (95% CI 2.21-47.53), p<0.001]. In addition, this combination was significantly more frequent in non-responding patients than controls [OR 3.00 (95% CI 1.33-6.76), p=0.007].

5. Apolipoprotein E polymorphism: association with age at onset in schizophrenia (V)

APOE ε4/ε4 genotype was associated with earlier age at onset in schizophrenia (p=0.0015). However, no association was found between APOE polymorphism and treatment response to typical neuroleptics. Moreover, APOE polymorphism was not associated with the risk of schizophrenia.

A summary of the results is shown in Table 8.

Table 8. Summary of the present results

	Gene polymorphism					combination	
	BDNF G196A	BDNF C270T	EGF G61A	NOTCH4 SNP2	COMT V108/158M	NOTCH4 and COMT SNP2 and V108/158M	APOE §
association with treatment response in schizophrenia	-	-	-	-	+	+	-
association with risk of schizophrenia	-	-	+*	-	+	+	-
association with age at onset	-	-	+*	+*	not studied	not studied	+

§ APOE polymorphisms in positions 3937 and 4075 in exon 4, resulting in three common alleles – APOE ε2, APOE ε3, and APOE ε4

* association was found among male subjects only

Discussion

1. Selection of subjects and genetic polymorphisms

1.1 Selection of subjects

Our method was a retrospective study model where the aim was to ascertain the extremes of treatment responses to typical antipsychotics. The patients were carefully selected from about 400 patients with schizophrenia. We chose only patients who were admitted to a psychiatric hospital for schizophrenia or schizophreniform psychosis and this inclusion criterion, partly, ensures that these patients were severely ill. We also estimated the CGI before antipsychotic drug treatment and registered the doses of antipsychotics used.

We wanted to have as homogenous a schizophrenia patient sample as possible. For this reason, we excluded patients with major affective disorders or schizoaffective disorder at any time. In addition, all patients whose psychotic symptoms may have resulted from neurological diseases or drug abuse were excluded.

The patients have been followed up on an average for more than 16 years before they entered the study. For non-responders this may sound familial. But for a responder this certainly means that despite good and long-lasting response, they were still occasionally sent to the psychiatric hospital, and were still meeting a psychiatrist. This may mean that our responders represent those patients with schizophrenia who need antipsychotic drug treatment for a long time and not those who have recovered and do not need any treatment.

1.2 Selection of genetic polymorphisms

We applied several principles in selecting the genes and polymorphisms for this study. The genes of the target receptors of antipsychotics were of major interest. However, very few studies focusing on them have been replicated. For example, there are fourteen

pharmacogenetic studies on dopamine receptor (D1, D2, D3, and most notably D4) gene variation. Only two of them reported a significant association (Kirchheiner et al. 2004). So far, there are only nine studies on dopamine receptor gene variation and response to typical antipsychotics (Table 2). In these studies, only four, small to moderate, have reported positive results.

We also considered genes associated with neuroimmunology because there is some evidence of their involvement in the response to antipsychotics (Leykin et al. 1997, Joffe et al. 1998, Pollmächer et al. 2000, Goldsmith 2002). In addition, some studies of antipsychotic induced gene expression in the prefrontal cortex were very interesting but the data on the functional polymorphism of these genes is limited (Kontkanen et al. 2002).

In a recent study, larger right prefrontal grey matter volume was associated with better treatment response to clozapine and with poorer treatment response to haloperidol (Arango et al. 2003). In another study, patients with high dorsolateral prefrontal cortex volume and metabolic activity were more likely to respond to clozapine (Molina et al. 2003a). However, these parameters did not predict response to risperidone (Molina et al. 2003b).

There was mounting evidence suggesting that genes associated with brain development may be worth studying and that is why the following genes were selected: BDNF, EGF, APOE, and NOTCH4. Most of these gene products have been present in different concentrations in the brains of patients with schizophrenia than in controls. BDNF and EGF levels were decreased in the prefrontal cortex of schizophrenia patients (Futamura et al. 2002, Weickert et al. 2003). In schizophrenia, there are increased levels of apoE in frontal cortex (Dean et al. 2003). In addition, NOTCH4 polymorphism is associated with prefrontal functioning and volumes (Wassink et al. 2003).

Neuregulin1 (NRG1) is a strong candidate gene for schizophrenia, and thus worth studying even in antipsychotic response (Corfas et al. 2004). NRG1 expression has been shown to be increased in schizophrenia patients' dorsolateral prefrontal cortex and it was positively correlated with antipsychotic medication dosage (Hashimoto et al. 2004). A preliminary result of our study group suggests that SNP8NRG221533 polymorphism is associated with treatment response to typical neuroleptics (Kampman et al. 2004).

Another good candidate gene for a pharmacogenetic study is RGS4, which is also associated with the risk of schizophrenia (Williams et al. 2004). In addition, RGS4 regulates the D2/Galphao/AC5 pathway (Taymans et al. 2003). In future, AKT1 gene polymorphisms may be worth studying (Emamian et al. 2004).

2. Genetic polymorphisms and treatment response to typical neuroleptics

The main goal of this study was to find genetic polymorphisms which would predict treatment response to typical neuroleptics. The aim was challenging because the majority of positive results in the pharmacogenetics of schizophrenia have not been possoble to replicate in independent samples (Kirchheiner et al. 2004). There are only few pharmacogenetic studies concerning treatment response to typical neuroleptics.

The traditional goals of pharmacogenetic studies in schizophrenia have been dopamine receptor genes and genes associated with dopamine transport or metabolism. However, some data suggested that neurodevelopmentally active proteins and the related genes may also be significant in the treatment response to different classes of antipsychotics (Altar et al. 1997, Dean et al. 2003, Futamura et al. 2003, Wassink et al. 2003, Weickert et al. 2003).

Of all the genes studied here, BDNF has been most frequently associated with antipsychotic induced alterations in CNS. In the present study, however, neither polymorphisms of BDNF gene (G196A and C270T) nor their haplotypes were associated with treatment response to typical neuroleptics. However, there may be pharmacogenetic relevant polymorphisms in other areas of BDNF gene e.g. in its several promoter regions. Fluoxetine, desipramine and electroconvulsive therapy, for example seem to regulate these promoters in different ways (Dias et al. 2003). Thus, possible functional polymorphisms in these promoter regions of BDNF may be worth studying in relation to antipsychotic treatment response.

Our results suggested that EGF G61A polymorphism may be associated with treatment response to typical neuroleptics in male patients with schizophrenia. Moreover, in the present population G allele was more frequent in male responders than male controls. Both EGF and neuregulin1 signal through ErbB receptors, and one neuregulin1 polymorphism is also associated with treatment response to typical neuroleptics (Kampman et al. 2004). These preliminary results together suggest that signalling via ErbB receptors may be important in antipsychotic treatment. However, these results should be replicated and the functional importance of the polymorphisms should be studied as well. Studying several other polymorphisms in these genes may also provide a more precise estimation of their value in the pharmacogenetics of schizophrenia.

NOTCH4 SNP2 polymorphism was not significantly associated with treatment response to antipsychotics but showed a significant interaction with COMT polymorphism. Our result suggested that the combined effect of certain NOTCH4 and COMT polymorphisms significantly predicted poor response to typical neuroleptics. However, caution should be exercised when interpretating these results. NOTCH4 SNP2 polymorphism itself was not studied by Wassink et al. (2003). Albeit SNP2 polymorphism is located only 1732 bp to (CTG)n polymorphism (Skol et al. 2003), the association between NOTCH4 SNP2 polymorphism and the performance in the Wisconsin Card Sort Test (WCST) and with frontal gray matter volume remains open. Thus, our results on the combined risk of COMT and NOTCH4 polymorphisms should be taken to be tentative and replications are needed to evaluate its true value in clinical decision making.

Only one study has evaluated the association between NOTCH4 SNP2 polymorphism and treatment response to antipsychotics (Carmine et al. 2003). They found no association but, unfortunately, did not report which type of antipsychotics the patients used.

Only one previous study has investigated the association between APOE polymorphism and response to typical neuroleptics. Durany et al. (2000) reported a tendency for poor response to neuroleptics to be associated with a lower ε2 allele frequency. Again, there is plenty of data suggesting an important role for APOE in treatment response to antipsychotics and other polymorphisms of the APOE gene should also be studied. In addition, APOE allele frequency is influenced by environmental factors

such as vitamin D supply, and nutrition may interact with genetic variation of APOE gene (Gerdes 2003, Liu et al. 2003).

3. Genetic polymorphisms and subgroups in schizophrenia

Schizophrenia is a heterogeneous disease involving both genetic and environmental factors (Schultz and Andreasen 1999). It has also been estimated that one single gene may have only a very limited effect on the total risk of schizophrenia (Weinberger 2002). In addition, more relevant subgroups of schizophrenia are needed for genetic studies (Garver 1997, Kirkpatrick et al. 2001, Tienari et al. 2003). Pharmacogenetic studies may even lead to meaningful subgroups in schizophrenia (Joober et al. 2002, Kerwin and Arranz 2002).

Our preliminary results suggest that EGF polymorphism as well as the combination of COMT and NOTCH4 polymorphisms may be associated with a meaningful subgroup of schizophrenia. A combination of NOTCH4 C/C genotype and COMT low/low genotype was significantly more common in non-responding than in responding patients and in controls. In male subjects, G allele of EGF was significantly more frequent in patients responding well than in controls. However, because of the small sample size and several other limitations of the study, these results should be considered speculative.

4. Age at onset

Early onset is associated with poor response to neuroleptic treatment in schizophrenia (Meltzer et al. 1997). Not surprisingly, responders had a significantly earlier age at the first hospitalization than non-responders in the present patient sample (27.4±10.1 vs. 32.2±9.5 years). We reported associations between the age at first hospitalization and NOTH4, EGF and APOE gene polymorphisms.

According to our results, patients carrying G allele of EGF had a later age at the time of the first hospitalization than those patients who not carrying this allele. Shahbazi et al. (2002) reported that the G allele of EGF was associated with increased production of EGF. EGF has also been shown to have estrogen-like effects, and thus G allele may be associated with an increased estrogen-like effect which may delay age at onset of the

disease. Although there is plenty of research of the EGF signalling as well as the disease modifying effects of estrogen (Halbreich et al. 2003), this explanation remains speculative.

A Swedish group reported no association between NOTCH4 SNP2 polymorphism and age at first hospitalization (Carmine et al. 2003). However, their sample size was relatively small (n=74) and they did not study differences between male and female patients. In an Asian sample, Takahashi et al. (2003) reported that early (< 19 years) age at onset was significantly associated with SNP_A, a SNP near SNP1. Unfortunately, Takahashi et al. (2003) did not report the bp distance between SNP_A and SNP2, which makes the comparison of the results difficult.

We also found that APOE polymorphism was associated with age at first hospitalization because of schizophrenia or schizophreniform psychosis. Patients who had ε4/ε4 genotype had significantly earlier age at onset. This result is, at least partly, in line with three other reports (Arnold et al. 1997, Durany et al. 2000, Martorell et al. 2001). However, some studies have reported no association between APOE polymorphism and age at onset (Ohara et al. 1997, Pickar et al. 1997, Sorbi et al. 1998, Thibaut et al. 1998, Saiz et al. 2002, Schürhoff et al. 2003). In addition, Kimura et al. (1997) reported that ε2 may protect against early onset schizophrenia. Igata-Yi et al. (1997) reported that ε4 allele frequency was significantly lower in early onset schizophrenia. As the meta-analysis of Schürhoff et al. (2003) suggested that APOE ε4 allele may be a risk factor of schizophrenia only in an Asian population, the modifying effect of APOE polymorphism on schizophrenia may also vary in different populations and/or because of different environmental factors. Thus, our result may be true only in Northern European population and replication of this result should be attempted here.

5. Gender differences

Schizophrenia seems to be more common in men (Aleman et al. 2003) and some major features of the disease differ between male and female patients (Meltzer et al. 1997, Salokangas et al. 2003, Schürhoff et al. 2004). The differences may, in part, depend on hormonal reasons (Halbreich et al. 2003) but genetic factors may also play a significant

role in the course of the disease between male and female patients (Chen et al. 1996, Gourion et al. 2004, Schürhoff et al. 2004). Thus, it may be relevant to analyze the results in genetic studies separately in male and female subjects. However, dividing a sample into subgroups weakens the statistical power and confounds the interpretation of the results.

For the reason mentioned above, our results concerning differences between males and females should be seen as preliminary. EGF polymorphism was associated with age at onset and response to typical neuroleptics in males only. However, there is some research supporting the meaningfulness of studying the effect of EGF polymorphism separately in both genders. EFG signalling is clearly related to estrogen-like effects (Apostolakis et al. 2000) and EGF secretion is regulated differently in males and females (Stern et al. 2000).

In addition, NOTCH4 polymorphism was associated with age at onset only in male patients. However, there is no clear explanation why NOTCH4 polymorphism has a different effect in male and female patients on the age at onset of schizophrenia.

6. Limitations of the study

The relatively small sample size constitutes the major limitation of the present study. However, we selected the good responders carefully, with the help of 12 psychiatrists in the Tampere Region. Moreover, the definition of non-response was strict and clozapine had been initiated with all the non-responders because of poor response to typical neuroleptics.

The retrospective study model did not allow the use of standardized psychiatric rating scales such as BPRS (Brief Psychiatric Rating Scale) or PANSS (Positive and Negative Syndrome Scale) in defining the treatment response. The results might be easier to replicate if standardized psychiatric rating scales were used.

There was some selection because the most suspicious patients did not want to participate in this kind of study. This is probably an important factor which may even result in different patient populations in different study designs. On the whole, Finnish

people are considered to be very positively disposed towards research. Such factors may have an impact on which patients are selected for studies.

There may be some selection bias because patients with drug or alcohol abuse were excluded. Substance abuse is particularly common in male patients, which may lead to a significant selection in male study population. However, drugs or alcohol abusing patients are less compliant than schizophrenia patients without substance abuse problems (Margolese et al. 2004). If these substance abusing patients were included in the study population, it would have made the estimation of treatment response to antipsychotic drugs more difficult and unreliable.

In the present study, the age at onset is defined as the beginning of the first hospitalization due to schizophrenia or schizophreniform disorder. This definition is not commonly used in studies on schizophrenia. In fact, there may be several years' delay from the beginning of the schizophrenic symptoms to the first hospital admission. However, this definition was chosen because of our retrospective study model and the definition was equal for all patients. The data of the first hospitalization was collected from the Finnish Hospital Discharge Register and the accuracy of the psychiatric diagnoses is considered to be excellent (Suvisaari et al. 2000). Alternative ways to estimate the age at onset (onset of psychotic symptoms or first visit to the physician because of mental problems) would also entail severe sources of error in this study model.

Because early age at onset is associated with poorer treatment response to antipsychotics in schizophrenia (Meltzer et al. 1997), there might also be connection in genetic level. The present research frame could also clarify this question. NOTCH4 SNP2 polymorphism was significantly associated with age at onset in both responding and non-responding male patients. EGF and APOE gene polymorphisms were not equally distributed between responders and non-responders. However, the very small subgroups did not allow us to perform meaningful statistical analysis in order to find out if there is a difference between these polymorphisms and age at onset separately in responders and non-responders.

Blood donors served as a control group in this study. Controls were chosen from a randomly selected group of blood donors (n=400) and they underwent no specific

examination for psychiatric status. However, the eligibility of blood donors in Finland entails a written statement regarding health status at every donation session. Thus, the blood donors represent a part of general population without chronic diseases or regular medications.

The results in all five publications of this thesis are exploratory in nature and therefore no correction for multiple testing was carried out. This is a predefined decision in this study model. If multiple statistical comparisons were made in this kind of study, some important and significant results might be lost. Such studies need to be replicated in any case and only several replication studies can provide adequate proof of the usefulness of the genetic tests.

Summary and conclusions

The major findings of the study were:

1. BDNF gene polymorphisms (G196A and C270T) were not associated with the treatment response to typical neuroleptics, with the age at onset of schizophrenia and these two studied polymorphisms are not risk factors for schizophrenia in Finnish population (I).

2. EGF gene polymorphism (A61G) was associated with the age at onset of schizophrenia, but there was no association with treatment response to typical neuroleptics, or with the risk of schizophrenia in the whole study population. However, the G allele of the EGF gene was more frequent in male patients with schizophrenia than in male controls (OR=3.594 (95% CI 1.347-9.591), p=0.008) (II).

3. NOTCH4 gene promoter polymorphism (SNP2) was associated with the age at onset of schizophrenia, but there was no association with treatment response to typical neuroleptics, or with the risk of schizophrenia in our study population (III).

4. NOTCH4 (SNP2) and COMT (V108/158M) polymorphisms in combination were associated with the treatment response to typical antipsychotics (IV).

5. APOE gene polymorphism was associated with the age of onset in schizophrenia, but there were no association with treatment response to typical neuroleptics, or with the risk of schizophrenia (V).

The main result of the present dissertation was the predictive effect of the combination of two polymorphisms (NOTCH4: SNP2 and COMT: V108/158M) on treatment response to typical antipsychotics. This result is especially interesting as it combines several important results of the functional genetic variation of a dopamine metabolizing enzyme and the association between brain morphology and treatment response.

The present studies also report genetic variations associated with modifying effect in schizophrenia. The polymorphisms of NOTCH4, EGF, and APOE were associated with age at onset of schizophrenia. As notch signalling and the effects of EGF are highly significant in the development of the brain, these results may be promising. Along with other studies, these reports may provide a better understanding of the disease.

This thesis may, at its best, recommend that one focus of pharmacogenetic studies in schizophrenia be on the genetic variation of CNS growth factors. Most probably, these genetic changes occur in interaction between several other genes and/or environmental factors. However, these results should be seen as preliminary, and only after several replication studies will thus possibly show their real value.

Some researchers have suggested that treatment response to typical neuroleptics may provide a tool in confirming the existence of a subtype of schizophrenia. The results of my thesis also suggest that certain genetic polymorphisms may be associated with typical antipsychotic responding or non-responding patient subtypes.

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