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Pharmacogenetics of Adverse Effects in Clozapine Treatment

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To my grandmother Ritva and my late grandfather Erkki

ABSTRACT

Background and aims. Clozapine is currently regarded as the most efficient antipsychotic drug for patients with schizophrenia, but its adverse effects limit its use. Sedation is the most common adverse effect and leading reason for discontinuing clozapine. Clozapine-induced sialorrhea (CIS) is the second most common adverse effect, which may be bothersome, especially at night-time. Constipation and gastrointestinal hypomotility are also common, and may lead to severe, even life-threatening complications such as ileus. The dose-standardized concentrations of clozapine and norclozapine vary between patients due to environmental and genetic reasons. Higher concentrations are associated with a higher burden of adverse effects and especially the risk of seizures. With the aid of pharmacogenetic information adverse effects could be predicted in the future. Pharmacogenetic findings could possibly also lead to better understanding of the mechanisms behind the adverse effects. The aims of this study were to study if 1) sialorrhea, 2) sedation, 3) constipation and other anticholinergic symptoms and 4) clozapine and norclozapine concentrations, concentration/dose-ratio or clozapine dose are associated with single nucleotide polymorphisms (SNPs) in genes related to the pharmacological mechanisms of the adverse effect.

Material and methods. A total of 237 Finnish clozapine-treated patients with schizophrenia participated in the study. Patients completed the Liverpool University Neuroleptic Side Effect Rating Scale (LUNSERS). Blood samples were taken, from which clozapine and norclozapine concentrations were analysed and patients genotyped. SNPs were analysed in three heritability models (additive, recessive and dominant) separately and in groups forming weighted genetic risk scores (GRSs). Permutation tests were performed for estimating the null distribution of the GRSs and for validating the GRS results.

Results. Adrenoceptor α_{2a} gene (*ADRA2A*) rs1800544 was associated with CIS. Eight SNPs in high linkage disequilibrium (LD, $r^2 = 1$) in the histamine *N*-methyltransferase (*HNMT*) gene were associated with sedation. Anticholinergic symptoms were associated with cholinergic muscarinic receptor 3 gene (*CHRM3*)

rs685548 and two GRSs with SNPs from 5-hydroxytryptamine (serotonin) receptor 5-HT$_4$ and 5-HT$_7$ genes (*HTR4*, *HTR7*), tryptophan hydroxylase 1 gene (*TPH1*), cholinergic muscarinic receptor M$_2$ and M$_3$ genes (*CHRM2* and *CHRM3*, ATP binding cassette subfamily B member 1 gene (*ABCB1*) and opioid receptor µ$_1$ gene (*OPRM1*). No associations were found between studied SNPs from cytochrome P450 enzyme genes, *ABCB1* gene and ATP binding cassette subfamily G member 2 gene (*ABCG2*) and variations in clozapine pharmacokinetics or clozapine dose. A trend-level association between a GRS of 4 SNPs and the sedation factor score (p=0.066) was observed. A GRS of 5 SNPs also predicted on a trend-level whether the patient received a lower (100-300mg) or higher (350-800mg) dose of clozapine (p=0.074). This might be related to variation in treatment response.

Conclusions. Associations were found between CIS, sedation and anticholinergic symptoms and the genetic polymorphisms studied. The findings in this study are not directly applicable into tools for clinical use, but in combination with other findings in the future could aid in the development of such tools. Pharmacogenetics and pharmacogenomics have an important role in promoting drug safety and better adherence to drug therapy, possibly preventing severe complications.

TIIVISTELMÄ

Tausta ja tavoitteet. Klotsapiinia pidetään tällä hetkellä tehokkaimpana antipsykoottisena lääkkeenä. Haittavaikutukset rajoittavat kuitenkin sen käyttöä. Sedaatio on yleisin haittavaikutus ja syy klotsapiinihoidon keskeyttämiselle. Klotsapiinin aiheuttama syljenerityksen lisääntyminen on toiseksi yleisin kiusallinen haittavaikutus, jota voi ilmetä etenkin öisin. Ummetus ja suolen toiminnan hitaus ovat myös yleisiä, ja nämä voivat johtaa pahimmillaan vaikeisiin komplikaatioihin kuten suolitukokseen. Annosvakioiduissa klotsapiinin ja norklotsapiinin seerumin pitoisuuksissa on vaihtelua potilaiden välillä johtuen geneettisistä ja ympäristövaikutteisista syistä. Korkeammat klotsapiinipitoisuudet ovat kaiken kaikkiaan yhteydessä haittaoireiden lisääntymiseen ja kouristusriskiin. Farmakogeneettisen tiedon avulla voidaan mahdollisesti ennustaa tulevaisuudessa lääkehoidon haittavaikutuksia ja tämä tieto voi myös auttaa haittavaikutusten mekanismien tunnistamisessa. Tämän tutkimuksen tavoitteena oli tutkia, ovatko 1) syljenerityksen lisääntyminen, 2) sedaatio, 3) ummetus ja muut antikolinergiset oireet ja 4) klotsapiini- ja norklotsapiinipitoisuudet, pitoisuusannossuhde tai klotsapiiniannos yhteydessä yhden nukleotidin polymorfismeihin (single nucleotide polymorphism, SNP) geeneissä, jotka liittyvät haittavaikutusten farmakologisiin mekanismeihin.

Aineisto ja menetelmät. Yhteensä 237 suomalaista skitsofreniaa sairastavaa potilasta, joilla oli käytössä klotsapiinilääkitys, osallistui tutkimukseen. Potilaat täyttivät arviointiasteikon haittaoireiden voimakkuuksien arvioimiseksi (Liverpool University Neuroleptic Side Effect Rating Scale, LUNSERS). Verinäytteistä määritettiin klotsapiini- ja norklotsapiinipitoisuudet sekä potilaiden genotyypit. SNP:t analysoitiin kolmella periytyvyysmallilla (additiivinen, resessiivinen ja dominantti) erikseen ja ryhmissä muodostaen painotettuja geneettisiä riskipisteytyksiä (weighted genetic risk score, GRS). GRS:ien nollajakauman arvioimiseksi ja GRS-tulosten validoimiseksi käytettiin permutaatiotestausta.

Tulokset. Adrenoseptori α_{2a} -geenin (*ADR2A*) rs1800544 oli yhteydessä lisääntyneeseen syljeneritykseen. Kahdeksan SNP:ia histamiini-*N*-

metyylitransferaasigeenissä (*HNMT*), jotka olivat voimakkaassa kytkentäepätasapainossa keskenään (LD, $r^2=1$), olivat yhteydessä sedaatioon. Antikolinergisten oireiden ja kolinergisen muskariinisen M_3 -geenin (*CHRM3*) rs685548:n välillä ja kahden GRS:n välillä löytyi assosiaatiot. Kahdessa GRS:ssä oli SNP:ejä seuraavista geeneistä: serotoniinireseptori 5-HT_4 ja 5-HT_7 (*HTR4*, *HTR7*), tryptofaanihydroksylaasi 1 (*TPH1*), *CHRM2* ja *CHRM3*, ATP binding cassette subfamily B member 1 (*ABCB1*) ja opioidireseptori μ_1 (*OPRM1*). Assosiaatioita klotsapiinin farmakokineetisen vaihtelun tai klotsapiiniannoksen ja tutkittujen SNP:ien sytokromi P450 geeneistä, *ABCB1*-geenistä ja ATP binding cassette subfamily G member 2 (*ABCG2*) -geenistä välillä ei löytynyt. 4 SNP:n GRS oli trenditasolla yhteydessä sedaation määrän vaihteluun ja havaittiin myös trendi 5 SNP:n GRS:n yhteydestä siihen, oliko potilaalla käytössä matalampi (100-300mg) vai korkeampi (350-800mg) annos klotsapiinia, mikä voi liittyä vaihteluun hoitovasteen saavuttamisessa.

Johtopäätökset. Tutkimuksessa löydettiin assosiaatioita klotsapiinin aiheuttaman syljenerityksen lisääntymisen, sedaation ja antikolinergisten oireiden ja tutkittujen geneettisten polymorfismien välillä. Löydettyjä markkereita ei voida suoraan soveltaa työkaluina kliinisessä työssä, mutta yhdistettynä mahdollisiin tuleviin löydöksiin näitä työkaluja on mahdollista kehittää. Farmakogenetiikalla ja farmakogenomiikalla tulee olemaan tulevaisuudessa tärkeä rooli lääketurvallisuuden ja hoitomyöntyvyyden edistämisessä estäen mahdollisia vakavia komplikaatioita.

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LIST OF ORIGINAL STUDIES

The thesis is based on the following original studies, referred throughout the text by Roman numerals I-IV:

I   Solismaa A, Kampman O, Seppälä N, Viikki M, Mäkelä KM, Mononen N, Lehtimäki T, Leinonen E. Polymorphism in alpha 2A adrenergic receptor gene is associated with sialorrhea in schizophrenia patients on clozapine treatment. Hum Psychopharmacol. 2014 Jul;29(4):336-41.

II   Solismaa A, Kampman O, Lyytikäinen LP, Seppälä N, Viikki M, Mononen N, Lehtimäki T, Leinonen E. Histaminergic gene polymorphisms associated with sedation in clozapine-treated patients. Eur Neuropsychopharmacol. 2017 May;27(5):442-449.

III   Solismaa A, Kampman O, Lyytikäinen LP, Seppälä N, Viikki M, Mononen N, Lehtimäki T, Leinonen E. Genetic polymorphisms associated with constipation and anticholinergic symptoms in patients receiving clozapine. Clin Psychopharmacol. 2018 Jun;38(3):193-199.

IV   Solismaa A, Kampman O, Lyytikäinen LP, Seppälä N, Viikki M, Mononen N, Lehtimäki T, Leinonen E. Genetic variations affecting clozapine pharmacokinetics in patients with schizophrenia. [Submitted to The Pharmacogenomics Journal on 28th Mar 2018]

ABBREVIATIONS

ABCB1	ATP binding cassette subfamily B member 1 gene
ABCG2	ATP binding cassette subfamily G member 2 gene
ADRA2A	adrenoceptor α_2 gene
AIC	Akaike information criteria
AOC1	aminase oxidase, copper containing 1 gene
APA	American Psychiatric Association
BMI	body mass index
C/D-ratio	concentration/dose-ratio
CBT	cognitive behavioural therapy
CHRM1-3	cholinergic receptor muscarinic 1-3 gene
CLOCK	Circadian Locomotor Output Cycles Kaput gene
CIS	clozapine-induced sialorrhea
CNS	central nervous system
COMT	catechol-o-methyl transferase gene
CYP	cytochrome P450 enzymes
DAO	diamine oxidase
DNA	deoxyribonucleic acid
DSM-5	diagnostic and statistical manual of mental disorders, fifth edition
ECT	electroconvulsive therapy
FDR	false discovery rate
FMO3	flavin-containing monooxygenase 3 gene
GLM	generalized linear univariate model
GRS	genetic risk score
GWAS	genome-wide association study
HDC	histidine decarboxylase
HNMT	histamine-*N*-methyltransferase
HRH1-4	histamine receptor H_1-H_4 genes
HTR1-7	5-hydroxytryptamine (serotonin) receptor 1-7 genes
ICD-10	International Statistical Classification of Diseases and Related Health Problems, Tenth Revision
LD	linkage disequilibrium
LUNSERS	Liverpool University Neuroleptic Side Effect Rating Scale
MAF	minor allele frequency
MDR1	multi-drug resistance gene, also known as *ABCB1*
mRNA	messenger ribonucleic acid

NIH	National Institutes of Health (US)
OPRM1	opioid receptor μ_1 gene
rs	reference SNP
sd	standard deviation
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
SNRI	serotonin-norepinephrine reuptake inhibitor
TPH1	tryptophan hydroxylase 1 gene
UGT	uridine diphosphateglycuronosyl-transferase gene
UKU	Udvalg for Kliniske Undersogelser
VIF	variance inflation factor
VTE	venous tromboembolism
WHO	World Health Organization

1 INTRODUCTION

Schizophrenia is a psychiatric disorder with complex cognitive and behavioural symptoms. It occurs worldwide and is currently estimated to have a lifetime morbid risk of 0.7% (McGrath, Saha, Chant, & Welham, 2008) and a considerable heritability (Sullivan et al. 2003). The disorder profoundly affects the lives of the individuals diagnosed with schizophrenia and their family members. It impairs patients' cognitive, occupational and social functioning (Lewandowski, Cohen, & Ongur, 2011), reduces their life expectancy of patients by 10-20 years (Chesney, Goodwin, & Fazel, 2014) and is associated with a high suicide risk (Hor & Taylor, 2010).

Antipsychotic medication has a major role in the treatment of schizophrenia (Owen, Sawa, & Mortensen, 2016). Between 20 and 30% of the patients have a poor response to the treatments (Kennedy, Altar, Taylor, Degtiar, & Hornberger, 2014). Clozapine is the drug of choice for treatment resistant schizophrenia (Kane, Honigfeld, Singer, & Meltzer, 1988), where approximately 60% of patients respond to clozapine treatment (Laursen, Mortensen, MacCabe, Cohen, & Gasse, 2014). Clozapine has several adverse effects that may inhibit the use of the medication and sometimes lead to discontinuation of the clozapine treatment (Legge et al., 2016; Miller, 2000). The leading causes for clozapine discontinuation are sedation, neutropenia or agranulocytosis, tachycardia, dizziness, nausea and vomiting, weight gain, fever and hypersalivation (sialorrhea) (Legge et al., 2016). Some of the adverse effects, such as agranulocytosis and gastrointestinal hypomotility, are potentially life threatening (Idanpaan-Heikkila, Alhava, Olkinuora, & Palva, 1975; Palmer, McLean, Ellis, & Harrison-Woolrych, 2008). There is individual variation in the adverse effects (Seppälä, N. et al., 2015). Monitoring the adverse effects in clinical practice is important and proper cautions such as hematological monitoring are needed in preventing agranulocytosis. Some of the adverse effects can be managed with symptomatic mediations.

Pharmacogenetics and pharmacogenomics have been a topic of wide interest recently. Genotyping methods are becoming more cost-effective and in the future

extensive genetic information about patients may be accessible as an aid for clinical decisions (Dickmann & Ware, 2016; Relling & Evans, 2015). The purpose of this dissertation is to study a subset of well recognized and clinically relevant adverse effects during clozapine treatment and their association with genetic markers. A better understanding of this would yield new pharmacogenetic information on the genetic mechanisms behind variations in the adverse effects in clozapine treatment. Ideally, the new information could also be used in clinical tools in predicting these adverse effects by genetic testing, thereby making it easier to prevent and manage these adverse effects and promote drug safety and adherence to the treatment.

2 REVIEW OF THE LITERATURE

2.1 Schizophrenia

Schizophrenia is a complex psychiatric disorder having a profound impact on affected individuals and their families. The origins of schizophrenia are still partly unknown, but genetic and environmental factors seem to be involved in disrupted brain development (Owen et al., 2016). The psychopathology of schizophrenia has been traditionally divided into positive and negative symptoms. Positive symptoms are so-called psychotic symptoms, delusions, hallucinations and incoherence, while negative symptoms include social withdrawal, diminished motivation and reduction in emotional responses and spontaneous speech (Joyce & Roiser, 2007; World Health Organization, 1992). These symptoms overlap with the cognitive symptom domain. Currently the neurocognitive deficits are regarded as core symptoms of schizophrenia. The severity of the deficits, especially in the verbal domain, are associated with functional outcome in schizophrenia (Heinrichs & Zakzanis, 1998; Lepage, Bodnar, & Bowie, 2014). Positive symptoms are generally intermittent psychotic episodes, although chronic psychotic symptoms may be present, whereas the neurocognitive symptoms are more persistent and have long-term effects on the individual.

The first psychotic episode is usually preceded by a prodromal phase and occurs in late adolescence or early adulthood (Addington & Heinssen, 2012; Lieberman et al., 2001). Cognition and social functioning may be mildly impaired many years before the first episode of psychosis (Lewandowski et al., 2011), but a more marked decline in the neurocognitive functions develops at or near the first episode psychosis (Mesholam-Gately, Giuliano, Goff, Faraone, & Seidman, 2009). Life expectancy of patients diagnosed with schizophrenia is reduced by 10-20 years (Chesney et al., 2014) and lifetime suicide risk is approximately 5% (Hor & Taylor, 2010). Medical comorbidities and high levels of cardiovascular risk factors are common in individuals with schizophrenia (Mitchell et al., 2013). Overall mortality at older age, in patients over 65 years old, is about threefold compared to general

population. Causes of death are otherwise similar, but accidents and suicides are more common (Talaslahti et al., 2012). The overall social and occupational functioning is poor. There is some regional variation in the unemployment rate of patients with schizophrenia, but it is generally estimated to be very high, 80-90% (Kooyman, Dean, Harvey, & Walsh, 2007). In a Finnish cohort study, more than half (56%) of patients diagnosed with schizophrenia were on disability pension by the age of 34 (Miettunen et al., 2007) and 21 % were in remission and only 3.4 % had recovered (Lauronen et al., 2005; Lauronen et al., 2007).

2.1.1 Aetiology and epidemiology

According to information gathered from twin studies, the heritability of schizophrenia is considerable, approximately 80% (Sullivan, Kendler, & Neale, 2003). However, the genetic aetiology of schizophrenia is polygenic. In 2014 an extensive genome-wide association study (GWAS) was published: 108 distinct common genetics variants were found to be associated with schizophrenia, which explained less than 5% of the genetic variance of the risk of schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Sullivan, Daly, & O'Donovan, 2012). Rare variants associated with higher risk of schizophrenia have also been identified, and newly occurring (de novo) copy number variant mutations also seem to play a role in schizophrenia (Fromer et al., 2014; Singh et al., 2017).

The neurodevelopmental hypothesis has been the leading paradigm for understanding how environmental factors contribute to the aetiology of schizophrenia. The hypothesis is that the neurodevelopment of the foetus is disturbed in the late first trimester of pregnancy, which leads to development of pathological neural circuits. These circuits then are activated during adolescence or young adulthood, sometimes owing to stressful events, causing the emerging symptoms of schizophrenia to manifest. (Fatemi & Folsom, 2009) Several risk factors have been identified for schizophrenia: maternal infections, severe stress to a mother during the first trimester, nutritional deficiencies, intrauterine growth restriction, pregnancy and birth complications (Owen et al., 2016). Higher rates of schizophrenia have also been linked to socio-economic factors. Individuals born in late winter or early spring, born or brought up in cities and individuals who have a relatively old father, or both of whose parents are relatively young, have a higher

risk for schizophrenia (Owen et al., 2016). In the Northern Finland 1966 Birth Cohort Study, the most significant risk factors for schizophrenia were parental psychosis, unwanted pregnancy, low birth weight, male gender, perinatal brain damage, late age at learning to stand and walk, not being potty-trained by the age of one, infections in the central nervous system by the age of 14, and not attending normal school grade (Isohanni et al., 2006). Younger age at onset of schizophrenia is associated with more negative symptoms, more relapses, more hospitalizations, poorer social and occupational functioning and worse global outcome (Immonen, Jaaskelainen, Korpela, & Miettunen, 2017). Furthermore, it has been reported that earlier onset of schizophrenia is associated with more severe cognitive impairment (Rajji, Ismail, & Mulsant, 2009).

2.1.2   Symptoms and diagnosis

Schizophrenia is a heterogeneous entity based on a syndromic concept. It is currently diagnosed with the operational criteria described in the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) published by the American Psychiatric Association (APA) or the International Statistical Classification of Diseases and Related Health Problems (ICD-10) published by the World Health Organization (WHO) (American Psychiatric Association & American Psychiatric Association DSM-5 Task Force, 2013; World Health Organization, 1992). The general diagnostic criteria and syndromes, symptoms and signs of schizophrenia as described in ICD-10 are presented in Table 1.

There are some differences between the DSM-5 and ICD-10 criteria: minimum duration of the illness is six months in DSM-5 and one month in ICD-10. Functional impairment is included in the diagnostic criteria in DSM-5 whereas in ICD-10 it is not. Furthermore, in DSM-5 and in the ICD-11 published in June 2018, the subtypes of schizophrenia have been omitted: it was considered that the subtypes explained the heterogeneity of schizophrenia poorly and were not useful for therapeutic or prognostic purposes (Sampogna, 2017; Tandon et al., 2013). Because the manifestations of symptoms are diverse and attempts to group the symptoms into valid subtypes have repeatedly failed, the current view is that the traditional descriptive diagnoses of schizophrenia and other psychiatric disorders are poorly related to the underlying processes in the brain, the aetiology and pathogenesis of the disorder (Owen et al., 2016). A transition to a dimensional

approach to psychiatric diagnoses has been suggested (Craddock & Owen, 2010; Owen, 2014). In some aspects, schizophrenia and other psychiatric diagnoses overlap; the boundaries between diagnoses and further between the healthy and those diagnosed are indistinct (Owen et al., 2016). It has been realized that psychotic symptoms, such as paranoid thinking or hallucinations, are at times present in 5-8 % of the healthy population as well (van Os, Linscott, Myin-Germeys, Delespaul, & Krabbendam, 2009).

Table 1. General diagnostic criteria for schizophrenia in ICD-10 (World Health Organization, 1992)

Criteria	Description of the syndromes, symptoms and signs
Duration ≥ 1 month	(1) a. Thought echo, thought insertion or withdrawal, or thought broadcasting.
G1. At least one of the syndromes, symptoms and signs listed below under (1), or at least two of the symptoms and signs listed under (2)	(1) b. Delusions of control, influence or passivity, clearly referred to body or limb movements or specific thoughts, actions, or sensations; delusional perception.
G2. If the patient also meets criteria for manic episode (F30) or depressive episode (F32), the criteria listed under G1.1 and G1.2 above must have been met before the disturbance of mood developed.	(1) c. Hallucinatory voices giving a running commentary on the patient's behavior, or discussing him between themselves, or other types of hallucinatory voices coming from some part of the body.
G3. The disorder is not attributable to organic brain disease, or to alcohol- or drug-related intoxication, dependence or withdrawal	(1) d. Persistent delusions of other kinds that are culturally inappropriate and completely impossible (e.g. being able to control the weather, or being in communication with aliens from another world).
	(2) e. Persistent hallucinations in any modality, when occurring every day for at least one month, when accompanied by delusions (which may be fleeting or half-formed) without clear affective content, or when accompanied by persistent over-valued ideas.
	(2) f. Neologisms, breaks or interpolations in the train of thought, resulting in incoherence or irrelevant speech.
	(2) g. Catatonic behaviour, such as excitement, posturing or waxy flexibility, negativism, mutism and stupor.
	(2) h. "Negative" symptoms such as marked apathy, paucity of speech, and blunting or incongruity of emotional responses (it must be clear that these are not due to depression or to neuroleptic medication).

2.1.3 Treatment

Antipsychotic medication is the cornerstone of schizophrenia treatment (Owen et al., 2016). The main antipsychotic effect from almost all antipsychotic drugs is attributed to blockade of dopamine D_2 receptors (Creese, Burt, & Snyder, 1976; Seeman, Lee, Chau-Wong, & Wong, 1976). Drug therapy promotes recovery, prevents relapses, mortality and impairment of function and lowers the risk of developing treatment resistant schizophrenia. Antipsychotic medications are generally comparable in their effectiveness with the exceptions of clozapine and long-acting injectable antipsychotic medications being superior in efficacy in preventing relapses of schizophrenia. Using long-acting injectable antipsychotic medications has been suggested to be recommendable for all patients, including those suffering their first psychosis, based on the finding that the risk of rehospitalization is 20 to 30 % lower compared to per os antipsychotic treatment (Tiihonen et al., 2017).

In addition to pharmacotherapy, a multi-disciplinary approach and ample psychological and social support are needed. Educational and vocational support, psychoeducation, psychotherapy and rehabilitation may all play a significant role in the treatment of schizophrenia. (Owen et al., 2016) In the treatment guideline from National Institute for Health and Care Excellence (NICE, UK), cognitive behavioural therapy is recommended to be offered to all those with psychosis or schizophrenia. Psychoeducational family interventions are also recommended as they reduce relapses and encourage medication compliance (Xia, Merinder, & Belgamwar, 2011). Arts therapies provided by registered arts therapists with experience of treating schizophrenia patients can be considered (National Institute for Health and Care Excellence, 2014).

2.1.4 Treatment resistant schizophrenia

Twenty to thirty percent of patients tend to have poor response to schizophrenia treatments (Kennedy et al., 2014). The poor response to treatment often manifests early: approximately 80% of the patients with treatment resistant schizophrenia do not respond to treatment after the first psychosis (Demjaha et al., 2017). The costs of treatment resistant schizophrenia are large, approximately 60-80% of all the

schizophrenia related costs (Kennedy et al., 2014). The definition of treatment resistant schizophrenia varies widely in the literature. A consensus guideline for determining treatment resistance was recently presented (Howes et al., 2017). This guideline suggests that the symptoms should be at least moderate with a duration of at least 12 weeks and include moderate to severe functional impairment. Essential for the definition is, that at least two different antipsychotic medications have been tried, with an effective dosing (600mg chlorpromazine equivalents or more) and duration long enough for each (six weeks or more). Adherence to the treatment should also have been monitored systematically. After well-executed trials with two antipsychotic medications, only a small portion of patients will respond to medications other than clozapine (Dold & Leucht, 2014). Clozapine treatment is effective and recommended for treatment resistant schizophrenia (Chakos, Lieberman, Hoffman, Bradford, & Sheitman, 2001; Kane et al., 1988). Thirty to sixty percent of patients will respond to clozapine treatment, but the remaining non-responders can be described as clozapine resistant or ultraresistant schizophrenias (Kane et al., 1988; Miyamoto, Jarskog, & Fleischhacker, 2014). In these cases, combination strategies can be attempted, for example, combining clozapine treatment with another antipsychotic medication, mood stabilizer or electro-convulsive therapy (ECT). There is so far no comprehensive evidence as to which combination strategies are the most effective or recommended (Barber, Olotu, Corsi, & Cipriani, 2017). Very high doses of antipsychotic medication are not generally recommended, firstly, because of the higher health risks and adverse effects, and secondly due to the lack of evidence (Dold & Leucht, 2014; Lindenmayer, Citrome, Khan, Kaushik, & Kaushik, 2011; Meltzer et al., 2014). Although high doses and combined antipsychotic medications are sometimes administered, it has been suggested that excessive medication with D_2 blockers may in fact worsen the treatment response (Yamanaka et al., 2016; Yin, Barr, Ramos-Miguel, & Procyshyn, 2017).

2.2 Clozapine

2.2.1 Pharmacology

Clozapine was first synthesized by the Swiss pharmaceutical company Wander AG in 1958. It was named clozapine in 1959 and first commercially used in 1972. Clozapine was initially classified as a tricyclic antidepressant with neuroleptic medication properties, but later it was grouped among second-generation antipsychotic agents. It differed from other previously known neuroleptic agents in not having extrapyramidal side effects. Psychiatrists even questioned its antipsychotic efficacy because it lacked these effects. (Crilly, 2007; Hippius, 1989) Clozapine led to the subsequent development of other second-generation, atypical antipsychotic agents which shared the property of low extrapyramidal adverse effects. Clozapine has a broad spectrum of action. It has high affinities for muscarinic M_1-M_5, serotonin $5\text{-}HT_{2a}$, $5\text{-}HT_{2c}$, $5\text{-}HT_6$, $5\text{-}HT_7$, alpha$_1$-adrenergic, histamine H_1, and dopamine D_4 receptors (Meltzer & McGurk, 1999). The receptor affinities of clozapine in comparison to several other antipsychotics are presented in Table 2.

Table 2. Receptor affinities for antipsychotics at therapeutic doses (adapted from Koulu & Mervaala, 2013)

Receptor	Clozapine	Olanzapine	Quetiapine	Risperidone	Aripiprazole	Haloperidol
D_1	+	++	-	+	+	+
D_2	+	++	+	+++	++++	+++
D_3	+	++	+	+++	++++	+++
D_4	++	++	-	+++	++	+++
$5\text{-}HT_{1A}$	+	-	+	-	+++	-
$5\text{-}HT_{2A}$	+++	+++	+	++++	+++	+
$5\text{-}HT_{2C}$	++	+++	-	+++	++	-
$5\text{-}HT_7$	++	+	-	+++	++	-
α_1	+++	++	++	+++	++	+++
α_2	++	++	+	+++	+	-
H_1	+++	+++	+++	++	++	+
M_1	+++	+++	+	-	-	-

K_i affinity values: - = > 1 000nM, + = 100-1 000nM, ++ = 10-100nM, +++ = 1-10nM, ++++ = 0,1-1nM

There is variation between patients in the plasma clozapine levels at a constant dose and also notably within patients (Diaz, de Leon, Josiassen, Cooper, & Simpson, 2005; Schaber et al., 2001). Age, sex, body mass index (BMI), caffeine use and especially smoking contribute towards the variation of concentration/dose-ratio (C/D-ratio) of clozapine (Bowskill, Couchman, MacCabe, & Flanagan, 2012; Carrillo, Herraiz, Ramos, & Benitez, 1998; Seppälä, N. H., Leinonen, Lehtonen, & Kivisto, 1999). Cytochrome P450 enzymes CYP1A2, CYP2C19 and CYP3A4 are the most functional in the *N*-demethylation of clozapine, whereas the role of CYP2D6 and CYP2C9 is smaller but nevertheless present (Olesen & Linnet, 2001). Uridine diphosphateglycuronosyl-transferase (UGT) contributes to glucuronidation of clozapine metabolites and flavin-containing monooxygenase 3 (FMO3) participates in the *N*-oxidation of clozapine (Erickson-Ridout, Sun, & Lazarus, 2012; Sachse et al., 1999). ATP-binding cassette (ABC) transporters function as transmembrane proteins transporting clozapine and other drugs across intra- and extracellular membranes. Genetic variation, such as ATP binding cassette subfamily G member 2 (*ABCG2*) gene polymorphisms, may also affect the C/D-ratio of clozapine (Akamine, Sugawara-Kikuchi, Uno, Shimizu, & Miura, 2017). *CYP* enzymes, *UGT*, *FMO3* and ABC-transporter genes have been studied in regard to clozapine concentrations, but the results so far are inconsistent (Krivoy, Gaughran, Weizman, Breen, & MacCabe, 2016).

2.2.2   Indications and effectiveness

Clozapine is the drug-of-choice for treatment resistant schizophrenia. Its superior efficacy has been consistently shown in studies and it has retained its position as newer antipsychotic medications have entered the market (Kane et al., 1988; Leucht et al., 2009; Leucht et al., 2013; McEvoy et al., 2006; Siskind, McCartney, Goldschlager, & Kisely, 2016). There is regional variance in clozapine use prevalence, but in general clozapine remains underutilized globally especially in the treatment of suicidal patients (Warnez & Alessi-Severini, 2014). In a study on international trends in clozapine use in 17 countries in 2005-2014, the prevalence of clozapine use was greatest in Finland (189.2/100,000 persons) and lowest in Japan (0.6/100,000) (Bachmann et al., 2017). In addition to being underutilized, clozapine treatment should also in many cases be initiated earlier than in the current practice as it could help in preventing suicide, impairment of the

functioning and exacerbation of negative cognitive symptoms (Hor & Taylor, 2010). The delayed initiation of clozapine treatment is probably due to its adverse effects and the haematological monitoring required. These are addressed in the next chapter. There are several hypotheses about the pharmacological mechanisms accounting for the superior efficacy of clozapine. When comparing clozapine to other antipsychotics, possible explanations for its superior efficacy include that clozapine has a relatively low occupancy at D_2 dopamine receptors (<70%), clozapine has a high dissociation constant for D_2, high ratio of serotonin 5-hydroxytryptamine 5-HT_{2A} receptor to D_2 receptor affinity and a high affinity for the D_4 receptor. Other antipsychotic agents also have some of the abovementioned properties, but it has been suggested that it is these properties in combination which make clozapine superior in efficacy (Nucifora, Mihaljevic, Lee, & Sawa, 2017).

Pharmacogenetic studies on clozapine effectiveness and treatment response of clozapine have also been carried out. Most of the genes investigated are within the dopaminergic and serotonergic system, but the relations between treatment response and tumour necrosis factor alpha (*TNFA*) and G protein subunit beta 3 (*GNB3*) genes have also been studied. In a meta-analysis, two SNPs in *HTR2A* (rs6313 and rs6314) and one SNP in *HTR3A* (rs1062613) were associated with treatment response (Gressier, Porcelli, Calati, & Serretti, 2016). Studies so far have had moderate sample sizes, heterogenous definition of treatment response and clozapine dosages and compliance were not considered (Gressier et al., 2016). Further studies on the subject are warranted.

2.2.3   Adverse effects

Adverse effects are the most common reason for discontinuation of clozapine treatment, accounting over half of cases when medication is discontinued either by a clinician-led or patient-led decision. Adverse effects leading to discontinuation and their frequencies are presented in Figure 1. Clozapine cessation due to adverse effects is particularly common during the first months of treatment and 45% of clozapine treatments are discontinued during the first two years (Legge et al., 2016; Pai & Vella, 2012). Higher doses of clozapine are associated with higher burden of adverse effects in general (Yusufi et al., 2007). The adverse effects on which this

study focuses are presented in detail in the following subchapters and other clinically important adverse effects are described in this chapter.

Agranulocytosis is a life-threatening adverse effect, defined as the granulocyte count falling below $500/mm^3$. Cases of agranulocytosis were first reported in 1975 in Finland (Idanpaan-Heikkila et al., 1975). This led to clozapine being withdrawn from the market worldwide until it was later gradually reintroduced for those who did not respond to other treatments. Since the reintroduction of clozapine, regular haematological monitoring of white blood cell count has been deemed obligatory for safe use of clozapine. In a large register study, the incidence of agranulocytosis was 0.38 % and 0.012 % of patients died from complications of agranulocytosis (Honigfeld, Arellano, Sethi, Bianchini, & Schein, 1998). The highest risk for agranulocytosis is during the first three months of treatment and 95 % of the agranulocytosis develops within six months of clozapine initiation (Lieberman & Safferman, 1992). Agranulocytosis is slightly more common in the elderly, women and patients under 21 years old (Alvir & Lieberman, 1994).

Myocarditis may occur in 0.015% to 0.188% patients, with a mortality rate up to 50% (Merrill, Ahmari, Bradford, & Lieberman, 2006). Ninety percent of the myocarditis incidents occur within the two first months of clozapine treatment (Nucifora et al., 2017). Cases of venous thromboembolisms (VTEs) associated with clozapine treatment have also been reported with a mortality rate of 44%, although the incidence is very rare and it is not known if the risk of VTE is related to prothrombotic effect (Paciullo, 2008). Elevated risk of seizures is also a known adverse effect of clozapine. Although there is limited information on which adverse effects of clozapine are associated with higher doses, the seizure risk has been observed to be clearly dose dependent: the risk of seizure rises with higher clozapine serum concentrations (Tiihonen et al., 1991; Varma, Bishara, Besag, & Taylor, 2011). Seizures related to clozapine treatment are not a contraindication, and clozapine may be continued with co-administration of antiepileptic drugs, the recommended drugs being valproate and lamotrigine (Varma et al., 2011). Weight gain is a common adverse effect of clozapine and patients taking clozapine have the highest rates of metabolic syndrome among those taking other antipsychotic medications (Mitchell et al., 2013). Clozapine use is associated with a high risk of developing diabetes, with 43% patients developing diabetes in a ten-year follow-up study (Henderson et al., 2005).

Figure 1. Adverse effects cited as reason for clozapine discontinuation, patient n = 80, adverse effect n = 130 (Legge et al., 2016, with permission under Creative Commons Attribution License)
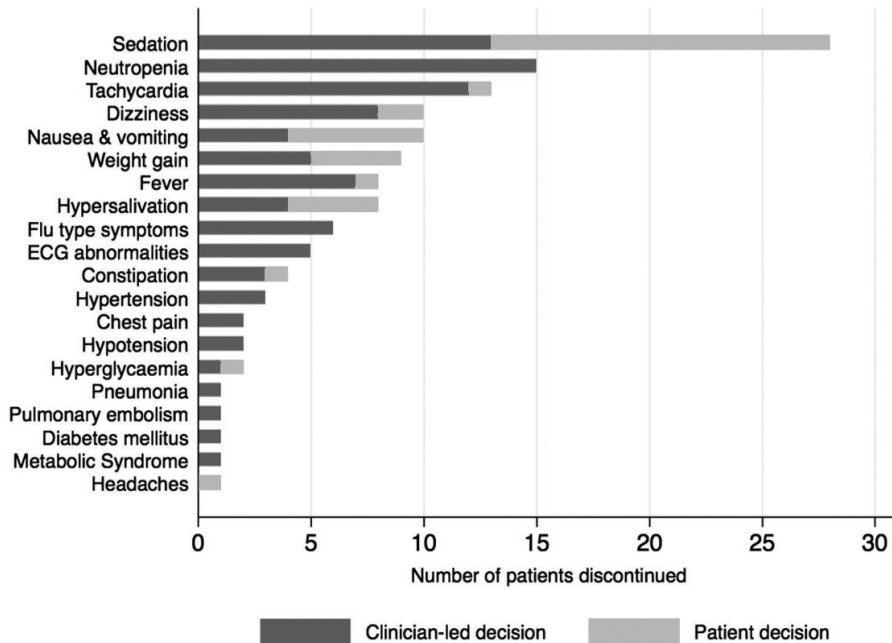
2.2.3.1 Clozapine-induced sialorrhea (CIS)

Approximately 30 % of patients taking clozapine experience excess salivation, sialorrhea, as an adverse effect (Praharaj, Arora, & Gandotra, 2006). CIS is an inconvenient adverse effect that is also socially stigmatizing (Praharaj et al., 2006). CIS may occur in daytime but it is most severe at night: the adverse effect commonly causes a wet pillow during the night (Ben-Aryeh, Jungerman, Szargel, Klein, & Laufer, 1996). Patients suffering from CIS may wake up to a sensation of choking and saliva may irritate the vocal chords causing chronic cough (Boyce & Bakheet, 2005). In the most severe cases it can lead to complications like aspiration pneumonia (Hinkes, Quesada, Currier, & Gonzalez-Blanco, 1996).

The function of the salivary glands is regulated by the autonomic nervous system (Proctor & Carpenter, 2007). The mode of action by which clozapine causes sialorrhea is still unknown. Several ways by which clozapine increases salivation have been proposed instead of a single mechanism. Clozapine seems to cause

salivation by muscarinic M_4-receptor agonism and M_1-receptor antagonism (Ekstrom, Godoy, & Riva, 2010; Zorn, Jones, Ward, & Liston, 1994). Clozapine may also affect the swallowing-respiration co-ordination via alpha-2-adrenoceptor antagonism (Ashby & Wang, 1996; Yamanishi et al., 2010). Decreased laryngeal peristalsis has also been proposed to contribute to CIS (Praharaj et al., 2006). Furthermore, the salivary flow follows circadian rhythm patterns and *CLOCK* gene expression has been detected in salivary gland cells (Thie, Kato, Bader, Montplaisir, & Lavigne, 2002; Zheng, Seon, McHugh, Papagerakis, & Papagerakis, 2012). Clozapine may affect salivation indirectly by altering the circadian patterns through its sedative effects.

There is lack of evidence for efficient treatment methods for managing CIS. Several treatments have been used: tricyclic antidepressants (such as amitriptyline), amisulpride, clonidine (alpha-2 agonist), local anticholinergic agents, scopolamine patches and botulinum toxin have been used to manage CIS (Copp, Lament, & Tennent, 1991; Gaftanyuk & Trestman, 2004; Sockalingam, Shammi, & Remington, 2007). Hence high quality, randomized placebo-controlled studies assessing the effectiveness of treatment methods for CIS are needed (Syed et al., 2008).

2.2.3.2 Sedation

Another common adverse effect during clozapine treatment is sedation (Safferman, Lieberman, Kane, Szymanski, & Kinon, 1991). Among the reasons for discontinuation of clozapine treatment, sedation seems to be the most common single cause (Legge et al., 2016). In maintenance treatment no association between serum clozapine concentrations and sedation has been found (Yusufi et al., 2007). Serum clozapine concentrations have been reported to be associated with sedation at 6 weeks after clozapine initiation, but no longer at 12 weeks (VanderZwaag et al., 1996), which suggests that some tolerance develops.

In the CNS, the histaminergic network regulates sleep and wakefulness (Panula & Nuutinen, 2013). The sedative properties of clozapine have mainly been regarded as a result of inverse agonist binding of clozapine on histamine H_1-receptors (Ashby & Wang, 1996; Humbert-Claude, Davenas, Gbahou, Vincent, & Arrang, 2012). The H_3-receptor agonist effect of clozapine may also cause sedation (Lin,

2000). The histaminergic system in relation to clozapine and the metabolism of histamine are presented in Figure 2. Histamine decarboxylase (HDC), diamine oxidase (DAO) and histamine-N-methyltransferase (HNMT) are the enzymes involved in histamine metabolism and rate limiting of histamine levels (Haas, Sergeeva, & Selbach, 2008).

Most patients tolerate the sedation after initiation (Marinkovic, Timotijevic, Babinski, Totic, & Paunovic, 1994), but 17-67% of patients still experience sedative adverse effects in long-term use (Safferman et al., 1991). To manage this sedation a single evening dose is recommended and reducing the dose may be considered (Taylor, Paton, Kapur, & South London and Maudsley NHS Trust, 2015). Adding aripiprazole as an adjuvant antipsychotic medication, or fluvoxamine to alter the clozapine/norclozapine ratio, may be effective, but more evidence on the efficacy and tolerability of these treatments is still needed (Barbui et al., 2011; Lu, Chen, Kuo, Hsu, & Chen, 2018; Perdigues et al., 2016). Prescribing fluvoxamine as an adjuvant must be approached with caution as there is a substantial increase in clozapine plasma levels and a risk for toxic levels (Fabrazzo et al., 2000). Use of stimulant medication has been proposed, but it may impair the treatment balance (Young, Bowers, & Mazure, 1998).

Figure 2.   The functions of histamine receptors on regulating sleep and wakefulness and the metabolic pathway of histamine (II, with permission)
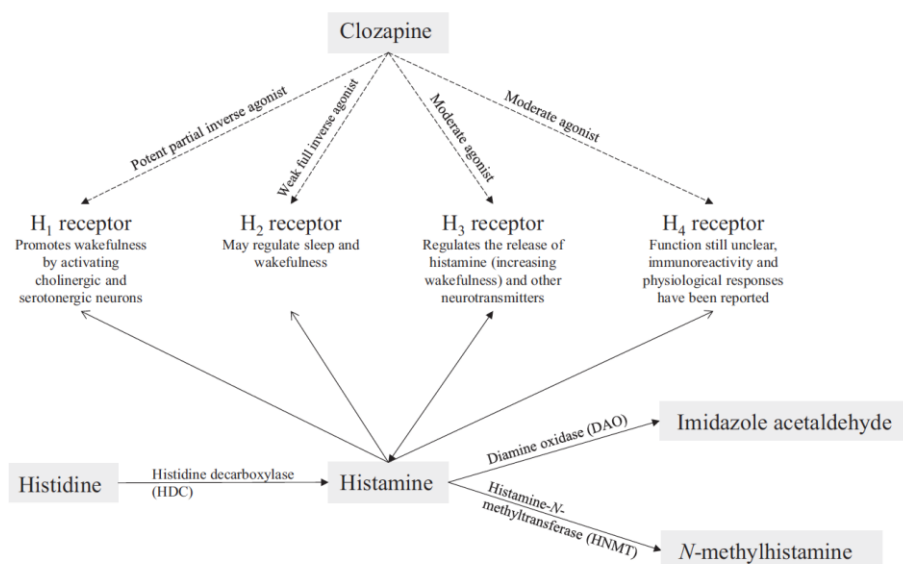
2.2.3.3   Constipation and anticholinergic symptoms

The prevalence of constipation is 14-60% in patients taking clozapine (Hayes & Gibler, 1995; Safferman et al., 1991). Laxative medication is often needed to manage the adverse effect (John, Chengappa, Baker, Gupta, & Mortimer, 1995). Clozapine is associated with gastrointestinal hypomotility: the bowel movements of clozapine patients are significantly slower than those of controls (Baptista et al., 2015; Every-Palmer et al., 2016). This may lead to constipation and further to other complications such as ileus, intestinal obstruction, bowel ischaemia, toxic megacolon and dysphagia. In comparison with other antipsychotics, clozapine is associated with the highest risk for ileus (Bender et al., 2004). Clozapine-induced gastrointestinal hypomotility may currently be under-recognized among clinicians. It is noteworthy that the risk of mortality in complications caused by clozapine-induced gastrointestinal hypomotility is the highest among adverse effects, including agranulocytosis (De Hert et al., 2011; Palmer et al., 2008). For patients taking clozapine, careful monitoring of symptoms of gastrointestinal hypomotility is recommended (Cohen, Bogers, van Dijk, Bakker, & Schulte, 2012).

Gut motility is regulated via muscarinic M_2 and M_3 receptors in the interstitial cells of Cajal and smooth muscle cells (Epperson et al., 2000). Furthermore, serotonergic signalling has a crucial but complex and still controversial role in gut function: serotonin 5-hydroxytryptophan receptors 2, 3, 6 and 7 (5-HT_2, 5-HT_3, 5-HT_6 and 5-HT_7) have all been suggested to participate in the signalling (Kendig & Grider, 2015; Palmer et al., 2008). The effect of clozapine on bowel movement seems to be related to its anticholinergic and antiserotonergic properties of clozapine (De Hert et al., 2011).

2.3   Evaluation of adverse effects

The UKU rating scale (Udvalg for Kliniske Undersogelser) is currently the most comprehensive method for assessing patients' adverse effects from antipsychotic medication (Lingjaerde, Ahlfors, Bech, Dencker, & Elgen, 1987). The UKU is a structured rating scale administered by a trained investigator such as a psychiatrist. The scale includes 48 possible adverse effects. The administration of the scale takes up to an hour, which limits its usability. For this reason, a self-administered scale

based on UKU called LUNSERS (Liverpool University Neuroleptic Side Effect Rating Scale) has been developed (Day, Wood, Dewey, & Bentall, 1995). LUNSERS contains "red herring" items, which are not de facto adverse effects associated with antipsychotic medications. The purpose of these items is to improve the validity of the scale. The self-administered LUNSERS and clinician-administered UKU total scores have a correlation from 0.48 to 0.58. The correlation is higher when patients scoring high on the "red herring" items are excluded (Lambert, Cock, Alcock, Kelly, & Conley, 2003). The LUNSERS items and their correlation with UKU items are presented in Table 3. For daily clinical use, other simple and easy-to-complete checklists for the adverse effect of antipsychotics have also been developed. These self-administered questionnaires can be completed, for example, while the patient is in the waiting room and they can be later discussed later with the treating physician. Two examples of these rating scales are Systemic Monitoring of Adverse Events Related to Treatments (SMARTS) and the Glasgow Antipsychotic Side-effect scale (GASS) (Haddad et al., 2014; Waddell & Taylor, 2008).

Table 3. Item correlations between UKU and LUNSERS. Red herring items (RH) are not shown. (Lambert et al., 2003, with permission)

LUNSERS item	UKU item	Low RH (n = 24) Spearman rho	All raters (n =29) Spearman rho
1. Rash	4.1 Rash	0.24, p>0.05	0.32, p>0.05
2. Difficulty staying awake during the day	1.3 Sleepiness/Sedation	0.23, p>0.05	0.11, p>0.05
4. Increased dreaming	1.9 Increased dream activity	0.44, p.0.031	0.33, p>0.05
5. Headaches	4.17 Headache intensity	0.52, p.0.011	0.49, p.0.008
6. Dry mouth	3.3 Reduced salivation	0.24, p>0.05	0.28, p>0.05
7. Swollen or tender chest	4.10 Gynaecomastia	—	—
9. Difficulty in concentrating	1.1 Concentration difficulties	0.28, p>0.05	0.32. p>0.05
10. Constipation	3.6 Constipation	0.67, p.0.000	0.64, p.0.000
13. Period problem	4.8 Amenorrhoea	—	0.47, p>0.05
14. Tension	1.6 Tension; Inner unrest	0.64, p.0.001	0.58, p.0.001
15. Dizziness	3.9 Orthostatic dizziness	0.78, p.0.000	0.61, p.0.000
16. Feeling sick	3.4 Nausea; Vomiting	0.45, p.0.026	0.27, p>0.05
17. Increased sex drive	4.11 Increased sexual desire	0.24, p>0.05	0.43, p.0.027
18. Tiredness	1.2 Asthenia/Lassitude/Fatigability	0.47, p.0.020	0.35, p>0.05
19. Muscle stiffness	2.2 Rigidity	0.62, p.0.001	0.51, p.0.005
20. Palpitations	3.10 Palpitations/Tachycardia	0.54, p.0.006	0.48, p.0.008
21. Difficulty in remembering things	1.4 Failing memory	0.82, p.0.000	0.70, p.0.000
22. Losing weight	4.6 Weight loss	0.60, p.0.002	0.48, p.0.008
23. Lack of emotions	1.10 Emotional indifference	0.36, p>0.05	0.34, p>0.05
24. Difficulty in reaching climax	4.15 Orgastic dysfunction	0.42, p>0.05	0.45, p>0.05
26. Depression	1.5 Depression	0.65, p.0.001	0.69, p.0.000
27. Increased sweating	3.11 Increased tendency to sweat	0.40, p>0.05	0.35, p>0.05
29. Slowing of movements	2.3 Hypokinesia/Akinesia	0.52, p.0.009	0.43, p.0.020
31. Sleeping too much	1.7 Increased duration of sleep	0.42, p.0.042	0.36, p>0.05
32. Difficulty passing water	3.7 Micturition disturbances	—	—
34. Muscle spasm	2.1 Dystonia	0.53, p.0.008	0.41, p.0.028
36. Diarrhoea	3.5 Diarrhoea	0.84, p.0.000	0.80, p.0.000
37. Over-wet or drooling	3.2 Increased salivation	0.60, p.0.002	0.48, p.0.009
38. Blurred vision	3.1 Accommodation disturbances	0.17, p>0.05	0.234, p>0.05
39. Putting on weight	4.5 Weight gain	0.59, p.0.002	0.61, p.0.000
40. Restlessness	2.6 Akathisia	0.41, p>0.05	0.48, p.0.008
41. Difficulty getting to sleep	1.8 Reduced duration of sleep	Not asked	—
43. Shakiness	2.5 Tremor	0.24, p>0.05	0.47, p.0.011
44. Pins and needles	2.8 Paraesthesias	0.19, p>0.05	0.06, p>0.05
46. Reduced sex drive	4.12 Diminished sexual desire	0.63, p.0.001	0.45, p.0.019
47. New or unusual skin marks	4.4 Increased pigmentation	—	—
48. Parts of body moving of own accord	2.4 Hyperkinesia	0.16, p>0.05	0.17, p>0.05
49. Itchy skin	4.2 Pruritus	0.48, p.0.017	0.56, p.0.002
50. Periods less frequentb	4.8 Amenorrhoea	—	0.48, p>0.05
51. Passing a lot of water	3.8 Polyuria	0.65, p.0.001	0.42, p.0.023
LUNSERS Total Score	**UKU Total Score**	**0.58, p.0.003**	**0.48, p.0.009**

2.4 Pharmacogenetics and pharmacogenomics in assessing drug efficacy and safety

Pharmacogenomics refers to how the response to drugs is influenced by the genome, either in terms of toxicity or efficacy. The focus of pharmacogenomics is to identify genetic variants that affect the drug's absorption, metabolism, distribution and elimination, or alternatively are involved in biological pathways in such a way that the drug's pharmacological effects vary between genotypes (Relling & Evans, 2015). Compared to pharmacogenomics, the term pharmacogenetics has also been used with the difference that it refers to the effect of one gene instead of the whole genome. (Pirmohamed, 2011)

Personalised medicine has in recent decades become a study topic of wide interest. For example, in the USA, the Precision Medicine Initiative was launched by President Barack Obama in 2014 involving the National Institutes of Health (NIH). The purpose of personalised medicine is to tailor individualised medical treatment on the basis of the biological and socio-economical background of the patient. The fast-developing pharmacogenomics have added a new perspective to personalised medicine (Dickmann & Ware, 2016). The costs of sequencing the genome are continuingly diminishing, and it may not be far in the future, that fully sequenced genomes can be used as an aid in planning treatment in everyday clinical practice (Relling & Evans, 2015). The U.S. Food & Drug Administration (FDA) maintains a table of pharmacogenomic biomarkers in drug labeling (https://www.fda.gov/Drugs/ScienceResearch/ucm572698.htm). Drugs used in oncology have the most biomarkers available, followed by infectious diseases and psychiatry. Variations in the *CYP2D6* are the most prominent biomarkers reported in the FDA drug labeling (Dickmann & Ware, 2016). *CYP2D6* gene variants have an impact on drug safety and efficacy by affecting drug pharmacokinetics. CYP2D6 activity varies widely in the population, from poor metabolizers to ultra-rapid metabolizers (Gaedigk & Leeder, 2014). Approximately 7% of Caucasians are poor metabolizers and 5.5% ultra-rapid metabolizers (Ingelman-Sundberg, 2005; Zanger, Raimundo, & Eichelbaum, 2004). The amount of information in pharmacogenomics is increasing rapidly, but so far there is no widely used formula to apply these discoveries into clinical practice (Dickmann & Ware, 2016). In order for a pharmacogenomic test to reach clinical practice, criteria for analytic validity, clinical validity and clinical utility must be met (Burke, 2009).

The pros and cons of long-term use of antipsychotic medication have recently been discussed. The metabolic and neurological adverse effects can be considerable when antipsychotic medication has been taken in large doses for extended periods of time. (Murray et al., 2016) High doses of antipsychotics have been reported to be associated with a decrease in the volume of cortical and total grey matter (Haijma et al., 2013; Huhtaniska et al., 2017). Pharmacogenetics and pharmacogenomics have provided insights into the genetics of response and adverse effects of antipsychotic medications (Nucifora et al., 2017). Pharmacogenomics is currently still in its infancy. Many pharmacogenetic studies of adverse effects and treatment response to clozapine have been published, but these have yielded mixed results. Further targeted gene studies or GWASs with large samples are needed before the use of pharmacogenomics tools can be implemented in clinical practice (Sriretnakumar, Huang, & Muller, 2015).

3   AIMS OF THE STUDY

Clozapine has a crucial role in the treatment of treatment resistant schizophrenia. In addition to the risk of agranulocytosis, clozapine has several adverse effects that affect compliance with treatment and may prevent the patient from taking the medicine if the adverse effects are intolerable. Special attention should be paid to the incidence and management of the adverse effects. Pharmacogenomics could be one tool to predict possible adverse effects during clozapine treatment before the initiation of medication. The main aim of this study was to find genetic markers, single nucleotide polymorphisms (SNPs), that are associated with different recognised adverse effects in clozapine treatment (Articles I-III). The secondary aim of this study was to investigate the genetic regulation in pharmacokinetics of clozapine (Article IV), which also may affect drug safety, as higher concentrations of clozapine are associated with elevated risk for seizure and a greater total burden of adverse effects.

The specified aims of Articles I-IV were:

I. To study single nucleotide polymorphisms in muscarinic cholinergic receptors M_1 and M_3 (*CHRM1* and *CHRM3*), adrenoceptor α_{2A} (*ADRA2A*) and circadian locomotor output cycles kaput (*CLOCK*) genes and their associations with clozapine-induced sialorrhea (CIS).

II. To investigate whether polymorphisms in genes coding for histamine receptors H_1-H_4 (*HRH1-HRH4*) or histamine metabolism related genes (amidine oxidase copper containing 1 (*AOC1*), histidine decarboxylase (*HDC*) and histamine-*N*-methyltransferase (*HNMT*)) are associated with clozapine-induced sedation.

III. To explore if variations in genes related to regulation of gut motility (*CHRM2*, *CHRM3*, serotonin 5-HT$_2$, 5-HT$_3$, 5-HT$_6$ and 5-HT$_7$ receptors) are associated with clozapine-induced constipation or anticholinergic symptoms. Genes previously reported to be associated with opioid induced constipation (tryptophan hydroxylase 1 (*TPH1*), opiate receptor μ_1 (*OPRM1*), ATP-binding cassette sub-

family B member 1 (*ABCB1*), catechol-O-methyltransferase (*COMT*)) were also included in the study (Laugsand et al., 2015).

IV. To explore if genetic variations in genes related to clozapine metabolism (cytochrome P450 enzyme genes *CYP1A2*, *CYP2C19*, *CYP3A4*, *CYP2C9*, *CYP2D6*, uridine diphosphate glucuronosyltransferase family 1 member A1 (*UGT1A1*) and flavin-containing monooxygenase 3 (*FMO3*)) and thru-membrane transportation (*ABCB1* and ATP-binding cassette sub-family G member 2 (*ABCG2*)) affect the 1) concentration/dose ratios (C/D-ratio), 2) clozapine and norclozapine concentrations, 3) clozapine/norclozapine ratios and 4) prescribed clozapine doses during clozapine treatment.

4 MATERIALS AND METHODS

4.1 Patients

Altogether 256 patients were screened for the study and 19 declined to participate. The study included a total of 237 Caucasian Finnish, adult (18 years or older) patients enrolled from Satakunta, Pirkanmaa and Seinäjoki hospital districts in western Finland. To be included in the study stabilized treatment with clozapine and a diagnosis of a psychotic disorder (F2x.xx) according to ICD-10 were required. Patients with organic psychoses were excluded from the study. Most of the patients had a diagnosis of schizophrenia (n = 223, 94.1%). Some patients with schizoaffective disorder or delusional disorder were also included. The diagnoses were collected from the patients' medical histories. The mean time elapsing from the first hospitalization of the patients due to a psychotic episode was 17.3 years (sd ± 10.0 years). Prior to participation, written and informed consent was obtained from the patients. The study was approved by the Ethics Committee of Satakunta Hospital District on the 23 April 2008. The study was conducted in accordance with the 1964 Helsinki Declaration. Blood samples were drawn from 190 patients, 47 patients made no laboratory visit or some of the planned samples could not be collected. After quality controls, genotyped data was available from 176 patients.

4.2 Controls

A control group was used in the Study I. The control group included 388 healthy controls (180 women, 215 men, mean age 45 years). All controls were Finnish blood donors who completed a health questionnaire with questions about their mental health. Each blood donor was interviewed individually by a nurse about their medication and chronic illnesses.

4.3   Clinical assessments

Patients completed the LUNSERS questionnaire to gather information on adverse effects during the preceding month. Patients rated the severities of 51 adverse effects on a scale from 0 to 4 (0 = not at all, 1 = very little, 2 = a little, 3 = quite a lot and 4 = very much). The patients reported their current height and weight, smoking status (no smoking, occasional smoking, daily smoking and number of daily cigarettes) and whether they had gained weight while on clozapine treatment. Information on whether the medication was taken supervised or unsupervised was also collected. Demographic and clinical data are presented in Table 4. Other medications taken concurrently at the time of the study are presented in Table 5.

Table 4. Clinical and demographic characteristics of the genotyped patients (n = 176)

Sex n (%)	
Men	104 (59.1 %)
Women	72 (40.9 %)
Age mean (sd$_a$) years	43.5 (10.9)
Clozapine treatment duration n (%)	
3 months to 1 year	4 (2.3 %)
1 to 5 years	51 (29.0 %)
Over 5 years	106 (60.2 %)
Unknown	15 (8.5 %)
BMI mean (sd) kg/m2	29.8 (6.38)
Clozapine dose mean (sd) mg	403 (152)
Clozapine dose distribution	
Very low to low (100–300 mg)	57 (32.4 %)
Standard to high (350–800 mg)	111 (63.1 %)
Unknown	8 (4.5 %)
Sum of clozapine and norclozapine concentrations mean (sd) µmol/l	2.40 (1.28)
Regular smoking n (%)	
Non-smoking	85 (48.3 %)
Smoking	89 (50.6 %)
Unknown	2 (1.1 %)

Table 5. Other medications in use among the genotyped patients (n = 176)

Clozapine only, no other medication n (%)	56 (31.8%)
Antipsychotic medication	
Additional antipsychotic medication n (%)	61 (34.7 %)
Clozapine monotherapy n (%)	115 (65.3 %)
Benzodiazepines including hypnotics n (%)	100 (56.8 %)
Mood stabilizers n (%)	51 (28.9 %)
Selective serotonin or serotonin-norepinephrine reuptake inhibitor (SSRI or SNRI) n (%)	65 (36.9 %)
Tricyclic antidepressants n (%)	22 (12.5 %)
Beta blockers n (%)	23 (13.1 %)
Laxatives n (%)	46 (26.1 %)

4.4   Laboratory methods, DNA extraction and genotyping

Liquid chromatography in a commercial laboratory was used for measuring the clozapine and norclozapine serum concentrations. For the genotyping, a separate fasting morning sample of 9.0 ml of EDTA whole blood was drawn from each patient and stored in a freezer at −20°C. QIAamp DNA Blood Midikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany) were used for extracting genomic DNA from peripheral blood leukocytes.

For Study I, genotyping was performed using Taqman©SNP Genotyping assays and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

In Studies II-IV, Illumina Infinium HumanCoreExome-12 DNA Analysis Beadchip, version 1.0., was used for genotyping the patients. The genotyping was performed according to the manufacturer's instructions at Helmholtz Zentrum, Munich, Germany. The quality control filters applied were: GenCall score < 0.15, GenTrain score < 0.20, sample and an SNP call rate < 0.95, Hardy-Weinberg equation p-value < 10^{-6}, excess heterozygosity, cryptic relatedness (pi-hat > 0.2), gender check and multidimensional scaling (MDS). For Studies II-IV, 531,983

SNPs were available, from which the SNPs within the selected genes each study were extracted. SNPs of the promoter region (5,000 base pairs) before the transcription start site of the selected genes, if any available, were also included.

4.5   Selection of the genes

The genes and SNPs were selected for Studies I-IV as follows:

I. Three *CHRM1* SNPs (rs2507821, rs542269 and rs2075748), three *CHRM3* SNPs (rs4620530, rs6429157 and rs6690809), one *ADR2A* SNP (rs1800544) and four *CLOCK* (rs1801260, rs3749474, rs4580704 and rs6850524) were genotyped for the analyses. SNPs from genes coding muscarinic M_1 and M_3 receptors and adrenoceptor alpha-2a receptor were selected based on the hypothesis that these receptors are related with the mechanisms of CIS. *CLOCK* polymorphisms were studied based on the hypothesis of the sialorrhea being affected by circadian mechanisms as it is more severe at night.

II. SNPs in receptor and enzyme genes related to the histaminergic system and their associations with sedation were examined. Fifty-five SNPs were included in the analyses from the *HRH1*, *HRH2*, *HRH3*, *HRH4*, *HNMT*, *AOC1* and *HDC* genes.

III. The study examined SNPs in genes of muscarinic and serotonergic receptors (*CHRM2*, *CHRM3*, *HTR2*, *HTR3*, *HTR4* and *HTR7*) and their association with constipation and anticholinergic symptoms. Further analyses were performed with SNPs in tryptophan 6-hydroxylase 1 *TPH1*, mu-type opioid receptor *OPRM1*, *ABCB1*, *CHRM3* and catechol-O-methyltransferase *COMT* based on previous findings on their contributing to variability in the incidence of constipation in opioid-treated patients (Laugsand et al., 2015). The number of SNPs from the selected genes that were included in the analyses was 192.

IV. Protein genes potentially related to the pharmacokinetics of clozapine were selected. *CYP1A2*, *CYP2C19*, *CYP3A4*, *CYP2C9*, *CYP2D6*, *UGT1A1* and *FMO3* were selected because they are involved in clozapine metabolism and *ABCB1* and

ABCG2 were selected because of their potential effect on drug permeability. In this study, 132 SNPs from the selected genes were included.

In Studies II-IV, the minor allele frequency (MAF) for the SNP to be included in the study was set at ≥ 0.01. In the study IV, no SNPs from *CYP2D6* were analysed, because all the SNPs available from this gene had a MAF lower than 0.01.

4.6 Response variables studied

I. A dichotomous variable was formed based on the LUNSERS question "over-wet drooling mouth" and the information on whether the patients were taking medication for managing the CIS. Patients were divided into two groups, those with 1) salivary flow not increased or little increased and no medication (n = 80) or 2) salivary flow increased from quite a lot to very much or medication for CIS (n = 102).

II. Sedation factor was used as a response variable in Study II as a continuous and as a dichotomized variable with a cut-off point at the median value. Sedation factor contained the following LUNSERS questions: "Difficulty staying awake during the day", "Sleeping too much" and "Tiredness". The factor resulted from a factor analysis performed in an earlier study (Seppälä et al., 2015).

III. LUNSERS question "constipation" was dichotomized into two groups, those with no constipation (n = 49) and those with very little to very much constipation (n = 127). An alternative response variable for constipation was also formed by taking into account the use of laxatives. Two groups in this case were those who had quite a lot to very much constipation (LUNSERS scale 3-4) or were prescribed laxatives (n = 95) and those who had little or no constipation and no laxatives (n = 81). Anticholinergic symptoms were studied with a sum variable of five LUNSERS questions: "Constipation", "Dry mouth", "Palpitations", "Blurred vision" and "Difficulty passing water". Principal components analysis for ordinal data was used for the reliability analysis of the sum variable resulting in a Cronbach's alpha value of 0.60.

IV. Concentration/dose (C/D)-ratio, prescribed clozapine dose and clozapine and norclozapine serum concentrations were used as response variables. To calculate the C/D-ratio, the sum of clozapine and norclozapine concentrations was divided by the weight-corrected dose. In the analyses clozapine dose was used as a linear and as a dichotomous variable divided into very low to low doses (100-300mg, n = 57) and standard to high doses (350-800mg, n = 111). The cut-off point was chosen on the basis of a division used in a Cochrane review on clozapine dosage (Subramanian, Vollm, & Huband, 2017).

4.7   Statistical methods

Pearson's chi-square ($\chi 2$) statistics were used for analysing differences in genotype distributions between patients (I-IV) and between patients and control group (I). Pearson's correlations and t-tests were used in exploratory analyses between response variables such as sialorrhea, sedation, anticholinergic symptoms, constipation and C/D-ratio and potential explanatory variables such as body mass index (BMI), smoking status, gender, age, clozapine monotherapy, total antipsychotic dose and use of other psychiatric medication (mood stabilizers, antidepressants, anxiolytics or hypnotics) (I-IV). Patients with sialorrhea or sialorrhea-related medication were compared to patients without sialorrhea (dichotomous sialorrhea) by $\chi 2$-statistics including odd ratios (I). Logistic regression analysis was used to predict CIS (I). Analysis of the haplotypic effect of the SNPs was performed with haplo.stats package in the R statistical package (I). Linkage disequilibrium (LD) plots and haploblocks were analysed with the confidence intervals method (II). LD test was performed with a threshold of r = 0.2 and 1 Mbp window (II). Basic allelic association tests without adjusting for multiple comparisons were performed with different genotypes and dichotomous sedation ($\chi 2$ test) (II). Gaussian linear univariate models (GLMs) were used with either gaussian distribution for linear response variables or binomial distribution for the dichotomized variables (II-IV). Covariates were chosen according to the literature or according to associations found between variables in the above mentioned exploratory analyses (II-IV). All the SNPs were coded into additional, dominant and recessive models to study various modes of inheritance. In case of additional coding, the trait under investigation is considered to be related to other

of the two alleles. For example with alleles tt, Tt or TT, in additional model the trait studied would be cumulatively linked with the T-allele. In the dominant model, the phenotype studied would be associated with TT or Tt but not with tt and furthermore, in the recessive model associated with tt but not with Tt or TT.

The level of statistical significance was set at $p < 0.05$ (I-IV). Multiple GLMs were corrected with False Discovery Rate (FDR) adjustments (II-IV). The results of the genetic risk score (GRS) analyses were validated with permutation analyses (II-IV). Statistical analyses were performed with SPSS software package versions 19.0-22.0 (IBM Corporation, New York, United states), R statistical analysis software versions 2.15.2 – 3.3.3 (R Core Team 2017, Vienna, Austria, PLINK version 1.07, gPLINK version 2.050 and Haploview analysis software (version 4.2).

4.7.1   Genetic risk score (GRS)

In Studies II-IV, genetic risk scores (GRSs) were used to study the cumulative combined effects of several potential tag SNPs on the phenotype studied. The process of forming the GRSs and using them in the GLM is described in Table 6.

4.7.2   Permutation testing

Combining genetic effects using GLMs may cause inflated type I errors due to overfitting of the data. To validate the result from the GRS analyses, an estimation of the null distribution of the GRS test statistics is required (Hu et al., 2013). A permutation test was performed to minimize the risk of type I error. The data was sampled for each permutation: the patient genotype and phenotype data were separated from each other and the genotype was randomly assigned to a different patient's phenotype. The GLM and GRS analyses were performed with sampled data 999 or 9999 times with different combinations of phenotype and genotype each time. The number of permutations depended on the accuracy needed in the results. P-values for each GRS in the permutations were collected and compared to the actual p-value. If the p-value derived from correct data was less than 5% of the p-values from permutations, the finding was considered statistically significant.

Table 6. GRS analysis described step-by-step

Step	Statistical procedure
1. Selection of the SNPs	Potential tag SNPs that had an unadjusted (not FDR-adjusted) significance level of $p < 0.05$ in the GLM analyses were selected. If any SNP did not reach the significance level, no GRS was formed.
2. AIC	Stepwise analysis with Akaike information criteria (AIC) was used to select best fitting SNPs for the GRS from the SNPs selected in step 1.
3. VIF	A stepwise variance inflation factor (VIF) analysis was used to exclude collinearity bias between the selected SNPs.
4. Formation and weighting of the GRS	The GRS was calculated for each patient with the resulting SNPs. The effect of each SNP was adjusted by multiplying the effect by the corresponding beta estimate value (β) from the primary.
5. GLM analysis with the GRS	The calculated GRS was used as an explanatory variable together with previously used covariates (i.e. age, gender or BMI) in a GLM.
6. Permutation test	The effect of the GRS has a high risk of overfitting the data resulting in a false positive finding, for which a permutation test was used to validate the result.

5 RESULTS

5.1 Clozapine-induced sialorrhea (I)

No differences between patients and controls were found in the genotypes studied. Smoking, gender or various adjuvant medications such as benzodiazepines, mood stabilizers or antidepressants did not account for CIS. CIS was not associated with different *CHRM1*, *CHRM3* and *CLOCK* genotypes ($\chi2$ test), but an association between *ADRA2A* rs1800544 genotype and sialorrhea was found (p=0.029, $\chi2$ test). Particularly CC genotype of *ADRA2A* rs1800544 compared to CC and CG genotypes was associated with sialorrhea or taking medication for sialorrhea (OR 2.13, 95% CI: 1.17-3.88, p = 0.013). When subgroups of clozapine monotherapy and multiple antipsychotics groups were analysed this association was significant only in the monotherapy group. In the haplotype analyses no associations were found between pooled haplotype groups and CIS.

5.2 Sedation and the histaminergic system (II)

Clinical covariates and their relations to different sedation groups are presented in Table 7. BMI correlated positively (r=0.16, p=0.015) and age negatively (r=-0.18, p=0.008) with sedation factor scores. No correlations between sedation and use of benzodiazepines or hypnotics, clozapine concentration or dose and total antipsychotic dose were found. In allelic association tests an unadjusted association between dichotomous sedation and two SNPs in the *AOC1* gene was found (rs6977381, OR = 0.63 (95% CI 0.40–0.98), p = 0.042, and rs41465145, OR = 2.02 (95% CI 1.052–3.89), p = 0.032). These SNPs were not associated with sedation in the FDR-adjusted GLM analysis. GLM analysis revealed eight strongly linked (r^2=1) SNPs (*HNMT* rs1455156, rs2737385, rs1455158, rs1455157 rs1050891, rs4245861, rs4646333 and rs1050900) that were associated with sedation factor score (FDR-adjusted p = 0.013) in a model with age, gender and BMI. No SNP was associated with dichotomous sedation in the logistic regression analysis. GRS analysis resulted in a GRS of four SNPs (recessive *HNMT* rs2737385, recessive

AOC1 rs6977381, additive *HRH1* rs1552498 and dominant *HRH1* rs17034073), which had a trend-wise association with sedation in a GLM together with age and gender as covariates (p = 0.066, permutation test). In this model no considerable collinearity was found between the SNPs or other explanatory variables (VIF < 10). Post hoc analysis was performed without *AOC1* genes because the effect on DAO in the central nervous system is still unclear. The results were similar, except that the resulting GRS contained only three SNPs: recessive *HNMT* rs2737385, additive *HRH1* rs1552498 and dominant *HRH1* rs17034073.

Table 7. Clinical covariates and the differences between dichotomized sedation factor score groups (II, with permission)

	Sedation factor score 0-5 (n=101)	Sedation factor score 6-12 (n=75)	Difference between groups
Age mean (sd) years	45.5 (10.7)	40.8 (10.7)	**p = 0.0052** (t-test)
Gender	32.7% women, 67.3% men	52% women, 48% men	**p = 0.015** (χ2)
Smoking status	48.4% smoked	54.7% smoked	p = 0.51 (χ2)
BMI mean (sd) kg/m²	28.9 (5.98)	30.1 (6.73)	**p = 0.040** (χ2)
Clozapine monotherapy	63.3%	73.3%	p = 0.22 (χ2)
Clozapine dose in cpz* mg, mean (sd) mg	797 (308)	821 (276)	p = 0.59 (t-test)
Total antipsychotic dose in cpz*, mean (sd) mg	899 (400)	924 (391)	p = 0.69 (t-test)

* cpz = chlorpromazine equivalent. Clozapine dose was multiplied by two in conversion to chlorpromazine equivalent. Statistically significant p-values are written in bold text.

5.3　Constipation and anticholinergic symptoms (III)

In the analysis between dichotomized constipation or use of laxatives and potential explanatory variables, older age was associated with more constipation or use of laxatives (44.52 ± 11.6 years of age versus 41.34 ± 10.6 years of age, t-test p = 0.035). No associations were found between anticholinergic factor or dichotomized constipation and BMI, age, smoking status, gender, clozapine and norclozapine concentrations or clozapine dose. Patients with lower BMI were more likely to be using laxatives (28.2 ± 6.3 kg/m2 versus 30.4 ± 6.1 kg/m2, t-test p = 0.032). Use of benzodiazepines was associated with increased use of laxatives (p=0.020, χ2 test) and increased anticholinergic symptoms (5.38 ± 3.36 versus 4.45 ± 3.40, t-test p = 0.042). Use of SSRIs was associated with increased anticholinergic symptoms (5.70 ± 3.13 versus 4.59 ± 3.49, t-test p = 0.015). Benzodiazepine or SSRI use was not associated with dichotomized constipation. In the GLM analyses various explanatory variables were added to the models (age, gender, BMI, use of SSRIs or benzodiazepines). In the GLM and GRS analyses, no associations were found between constipation or use of laxatives and the SNPs studied from genes *CHRM2*, *CHRM3*, *HTR3*, *HTR4*, *HTR7*, *TPH1*, *OPRM1*, *ABCB1* or *COMT*. When associations between the anticholinergic factor and the SNPs in genes *CHRM2*, *CHRM3*, *HTR3*, *HTR4* and *HTR7* were studied, the GLM analysis revealed one SNP, *CHRM3* rs685548, that was associated with anticholinergic factor after FDR-adjustment (additive coding p=0.044, dominant coding p=0.038). When SNPs from the genes *TPH1*, *OPRM1*, *ABCB1* and *COMT* were added to the GLM analysis, the finding became statistically insignificant (additive coding p=0.071, dominant coding p=0.060). When analysing only the genes *CHRM2*, *CHRM3*, *HTR3*, *HTR4* and *HTR7*, no GRS explained anticholinergic symptoms. When all SNPs were included in the GRS analysis, a GRS of 19 SNPs in *CHRM2*, *CHRM3*, *HTR3C*, *HTR7*, *ABCB1*, *OPRM1* and *TPH1* was associated with anticholinergic factor in a GLM model together with BMI and clozapine monotherapy (p=0.017). An additional analysis with the single *CHRM3* SNP rs685548 associated with anticholinergic factor and the genes related to the opiate-related study (*TPH1*, *OPRM1*, *ABCB1* and *COMT*) was performed (Laugsand et al. 2013). This resulted in a GRS of 7 SNPs explaining anticholinergic symptoms in a GLM model with BMI and clozapine monotherapy (p=0.0023). Lists of the rs-codes of the SNPs in the GRS are presented in Table 8. In both previously mentioned statistically significant GRSs, no collinearity was found between the SNPs or other predictor variables (VIF < 10).

Table 8. Table with listing of the SNPs in the two formed GRSs and results of the GLM analysis with BMI, clozapine monotherapy and the GRSs as coefficients explaining anticholinergic factor score

Genes selected for analysis	Formed GRS	GLM model with BMI, clozapine monotherapy and GRS
COMT, TPH1, OPRM1, ABCB1, HTR2, HTR3, HTR4, HTR7, CHRM2 and *CHRM3*	*CHRM2* additive rs10228048, *CHRM2* dominant 12535371, *CHRM3* dominant rs685548, *CHRM3* recessive rs11577797, *CHRM3* additive rs6691263, *CHRM3* recessive rs10925910, *CHRM3* dominant rs2841037, *HTR4* recessive rs7723153, *HTR7* additive rs7074715, *HTR7* dominant rs7074715, *ABCB1* additive rs10276036, *ABCB1* recessive rs2235023, *ABCB1* recessive rs9282564, *ABCB1* dominant rs10260862, *ABCB1* dominant rs10248420, *ABCB1* additive rs2032582, *ABCB1* additive rs3789243, *OPRM1* recessive rs2272381 and *TPH1* additive rs10832876	BMI t=3.3, β=0.20, p=0.00045, clozapine monotherapy t=-3.9, β=-1.7, p=0.00015, GRS t=10.1, β=0.65, p < 2x10^{-16}, adjusted R^2=0.43, permutation test p=0.014
CHRM3 additive rs685548 (single SNP), *ABCB1, OPRM1, TPH1* and *COMT*	*CHRM3* additive rs685548, *OPRM1* recessive rs2272381, *OPRM1* recessive rs34427887, *ABCB1* additive rs1002204, *ABCB1* additive rs10260862, *ABCB1* recessive rs2235023, *ABCB1* recessive rs9282564, and *TPH1* dominant rs10832876	BMI t=1.9, β=0.067, p=0.056, clozapine monotherapy t=-3.8, β=-1.8, p=0.00018, GRS t=8.6, β=0.79, p=6.1x10^{-15}, adjusted R^2=0.36, permutation test p=0.002

5.4   Clozapine pharmacokinetics and dose (IV)

C/D-ratio was associated with BMI (r=0.48, p < 0.001) and regular smoking (smoking 0.49 ± 0.25 μmol/l: mg/kg vs. non-smoking 0.61 ± 0.31 μmol/l: mg/kg, t-test p = 0.0089). The clozapine dose was associated with clozapine monotherapy (monotherapy 370.2 ± 120.9 mg vs. additional antipsychotics in use 466 ± 171 mg, t-test p < 0.001), BMI (r=-0.17, p = 0.031) and regular smoking (smoking, 451.5 ± 151.6 mg vs non-smoking 358.8 ± 125.8 mg, t-test p < 0.001). Gender was associated with the sum of clozapine and norclozapine concentrations (women, 2.67 ± 1.45 μmol/l vs men 2.21 ± 1.12 μmol/l, t-test p = 0.025). Clozapine concentration was associated with the clozapine/norclozapine ratio (r=0.47, p < 0.001). The sum of clozapine and norclozapine concentrations was associated with the clozapine dose (r=0.51, p < 0.001).

In the GLM and GRS analyses the explanatory variables used , in addition to the SNPs studied, were 1) regular smoking and/or BMI for C/D-ratio, 2) gender for clozapine and norclozapine concentrations, 3) clozapine concentration or age for clozapine/norclozapine ratio and 4) regular smoking, BMI and clozapine monotherapy for clozapine dose. In the GLM and GRS analyses, C/D-ratio, clozapine and norclozapine concentrations and clozapine/norclozapine ratio were not associated with single SNPs or GRSs after FDR-adjustment and permutation tests. In the GRS analysis explaining clozapine dose, a GRS of 5 SNPs was associated with dichotomized clozapine dose in a GLM model together with clozapine monotherapy (β = -1.42, z = -2.88, p = 0.004), regular smoking (β = 1.60, z = 3.59, p < 0.001) and BMI (β = -0.010, z = -2.9, p = 0.0035) as other explanatory variables. SNPs in the GRS were (annotation and weight estimate, β): *UGT1A* recessive rs2269352 (promoter region, -1.16), *CYP1A2* additive rs2472304 (intron, -0.74), *CYP2C19* additive rs7916649 (intron, -0.65), *ABCB1* recessive rs3789243 (intron, -0.66) and *ABCB1* additive rs1989830 (intron, -0.97). However, in the permutation analysis the above mentioned GLM with the GRS did not reach statistical significance (p=0.074).

6 DISCUSSION

6.1 Main results

6.1.1 Clozapine-induced sialorrhea (I)

Adrenergic receptor 2A (*ADRA2A*) polymorphism rs1800544 was associated with CIS. The genetic effect observed was additive: the amount of sialorrhea increased with the number of C-alleles. The risk for CIS was approximately doubled (OR 2.13) in patients with the CC genotype compared to those with GG and GC genotypes. It has been reported that alpha(2) adrenoceptor agonists, such as clonidine, reduce salivation due to their effect on the central nervous system. Conversely, yohimbine, which acts as an alpha(2) adrenoceptor antagonist, promotes salivary output. (Phillips, Szabadi, & Bradshaw, 2000) Clozapine has an antagonist effect on alpha(2) adrenoceptor (Kalkman & Loetscher, 2003). Other pharmacological properties may also be involved, as it has been shown that mianserin, which has a high antagonistic affinity at alpha(2) adrenoceptors, does not cause increased salivation as excepted, but quite the reverse, decreases the salivary output (Ogura et al., 1987). Mirtazapin is also an alpha(2) adrenoceptor antagonist and its common adverse effects include dry mouth (Anttila & Leinonen, 2001). *ADRA2A* rs1800544 polymorphism is in the promoter region of *ADRA2A* gene. Rs1800544 SNP has been associated with metylphenidate efficacy in ADHD (Myer, Boland, & Faraone, 2017), but there is at present no hypothesis as to the molecular mechanism by which the SNP alters this response. In one study, rs1800544 SNP did not alter the *ADRA2A* mRNA expression in a bone tissue sample (Mlakar et al., 2015). The number of SNPs analysed in this study was relatively small, representing only small parts of the genes, and the selection of SNPs was limited to the potential tag-SNPs available at the time. The association between rs1800544 and CIS may be also linked to some other SNP not included in this study.

Although the mechanisms of CIS have been studied previously, this is the first study to explore the genetic polymorphisms behind the adverse effect. *CLOCK*, *CHRM2* and *CHRM3* polymorphisms were not associated with CIS in this study. Since Study I was published, one new study has been presented on a similar population (Rajagopal et al., 2014), where an association between a 120-base pair duplication in *DRD4* and CIS was found. The effect of the polymorphism found by Rajagopal et al. was on a level similar to the finding in Study I, with an odds ratio of 2.95 in a log additive model (95% CI 1.51-5.75, p=0.006). Sialorrhea can be a troublesome and disturbing adverse effect and a reason to discontinue clozapine treatment (Legge et al., 2016; Praharaj et al., 2006). The effects of the SNPs found in this study and the other study are clinically rather small, and thus these SNPs alone do not yet suffice for use as markers for predicting sialorrhea in clinical practice. Combined with possible other polymorphisms in the future, these SNPs could be used to predict the risk of CIS.

6.1.2   Sedation (II)

Eight linked SNPs in the *HNMT* gene (rs1455156, rs2737385, rs1455158, rs1455157, rs1050891, rs4245861, rs4646333 and rs1050900) were associated with sedation in the GLM model with age, BMI and gender. In a GRS analysis, a GRS of four SNPs in the genes *HNMT*, *HRH1* and *AOC1* accounted for more than 20% of variance in reported sedation in a GLM with age, BMI and gender, but the result was only trend-like according to the permutation analysis. Associations between sedation and two *AOC1* SNPs were also found in allelic association tests, but their effect was small. No adjustment for multiple testing was used in these analyses and thus the *AOC1* SNPs may be false positive findings.

The results suggest that genetic polymorphisms in the histaminergic system may contribute to the variance of sedation during clozapine treatment. Brain histamine has an important role in regulating alertness and controlling biological rhythms (Panula & Nuutinen, 2013). Alterations in the functioning of the enzymes regulating histamine levels, HNMT and DAO, may affect histamine homeostasis in the CNS. For example, disturbances in the circadian rhythm have been observed in histamine decarboxylase (*HDC*) knockout mice (Abe, Honma, Ohtsu, & Honma, 2004). In a recent *HNMT* knockout study, aggressive behaviour and abnormal sleep-wake cycle were observed in *HNMT* deficient mice (Naganuma et al., 2017).

The study also suggests that HNMT has a crucial role in regulating histamine levels in the CNS and may also regulate the sleep-wake cycle. The eight *HNMT* SNPs associated with sedation in the GLM model are located close to each other in the gene, rs2737385 in the intron region and the other seven in the UTR-3 region. In the *HNMT* gene a functional SNP (rs11558538) has been found to be associated with lower HNMT activity (Preuss et al., 1998). One of the SNPs in the GRS, *HNMT* rs2737385, is in 0.58 r^2 LD with this previously found functional SNP. Functional SNPs in *AOC1* gene have also been found to reduce the activity of DAO in serum (Maintz et al., 2011). *AOC1* rs6977381, which was included in the GRS, is in 0.31 r^2 LD with one of these functional SNPs. The mechanism of how the GRS could affect sedation is that histamine homeostasis is affected by polymorphisms in *AOC1* and *HNMT* genes, which accelerate the activity of HNMT and DAO and decrease histamine levels in the CNS, and in addition, the histamine neurotransmission is altered by changes in the H_1 receptor protein structure caused by SNPs in the *HRH1* gene. An alternative GRS analysis was performed without the *AOC1* gene because it is unclear, whether there is DAO activity in the CNS (Haas et al., 2008). The results for *HNMT* and *HRH1* remained similar without *AOC1* in the analyses. The p-values of the permutation analysis of the GRSs were trend-like and the interpretation of the GRS is debatable. The 8 linked SNPs associated with sedation in GLM analysis, had a skewed distribution (n=8 versus n=168), and this result should also be interpreted cautiously. The findings in this study should be subjected to further investigations: a replication study could confirm or refute the results.

In addition to sedation being the cause of an adverse effect of clozapine, sedation may be linked, for example, to depression or physical conditions such as hypothyroidism or diabetes, which could not be accounted in the analyses. Depression is common among patients with schizophrenia (Bosanac & Castle, 2013). Sedation was more severe in patients with higher BMIs and younger age. Histamine H_1 receptor antagonism is considered one of reasons why many patients gain weight during clozapine treatment. The adverse effects of weight gain and sedation may have some common mechanisms. Polymorphisms in *HRH1* and *HRH3* genes have been studied to ascertain if they are associated with antipsychotic-induced weight gain, but no such relation has been found (Tiwari et al., 2016). To the best of our knowledge, no other study has scrutinised the association between sedation and *HRH1*, *HRH3*, *HNMT* or *AOC1* genes, and no new information on the subject has been published since Study II. Sedation is

clearly the most common adverse effect causing the discontinuation of clozapine medication, by either patient or clinician-led decision. Predicting the severity of sedation in advance with genetic testing would be a useful tool for clinicians. The GLM model with BMI, gender, age and the GRS explained over a fifth of the variation in reported sedation.

6.1.3   Constipation and anticholinergic symptoms (III)

Study III reported a new finding that two GRS (for a full list of the SNPs see Table 8) and one SNP in *CHRM3* (rs685548) were associated with anticholinergic symptoms during clozapine treatment. The observed effect on the variation in anticholinergic symptoms is cumulative from multiple mechanisms, SNPs from serotonergic (*HTR4*, *HTR7* and *TPH1*) and cholinergic (*CHRM2* and *CHRM3*) neurotransmission were included. *ABCB1* and *OPRM1* were included in the analyses due to their association with constipation in an earlier study and they were also included in the formed GRSs. The finding suggests that all these polymorphisms contribute to individual variation in the autonomic nervous system and its reaction to clozapine. The results indicate that the varying degrees of anticholinergic symptoms depend largely on the genotype. The GLM with the 19-SNP GRS, BMI and clozapine monotherapy as coefficients explained as much as 43% of the variance in anticholinergic symptoms. However, the high adjusted R^2 may contain a type I error overestimating the effect of the explanatory variables. The effect size should be confirmed in an independent sample. The SNPs in the GRS are mostly in the intron regions of the genes. In the GRS, *ABCB1* rs9282564 is annotated as a missense frameshift SNP, *ABCB1* rs2032582 a missense SNP and *OPRM1* rs34427887 stop-gain SNP. The effect of the other SNPs in the GRS on the gene expression or protein structure is unclear. The annotations of the SNPs are presented in the original article (Table 3, Article III).

Although associations between the SNPs and anticholinergic symptoms were found, no associations were found between constipation or laxative use and genetic polymorphisms. One aim of Study III was to find genetic polymorphisms that could predict constipation in clozapine treatment. This would be a useful tool for clinicians regarding drug safety. Clozapine-induced constipation is underrecognized and in some cases may lead to severe and even fatal complications (Palmer et al., 2008). Colonic transit times are notably longer with patients on clozapine treatment

than with patients on other antipsychotic medication or healthy controls (Every-Palmer et al., 2016). The LUNSERS questionnaire yielded information on patients' subjective experiences of constipation. The use of laxative medicine was used as a response variable for constipation. It is possible that some patients had been accustomed to slower gut motility since the initiation of the medication and did not report constipation in the survey. More information about the effect of genetic variations could be obtained from information on gut motility, such as measured colonic transit times, reported bowel movement frequencies or stool consistencies with the aid of stool charts. The GRSs explaining anticholinergic symptoms are most likely also linked to gut motility as constipation is a common effect of anticholinergic medication, but in this study GRSs were not associated with patient-reported constipation. Their relation to gastrointestinal hypomotility is a subject for further studies.

6.1.4   Clozapine pharmacokinetics (IV)

No associations between variation in the genes studied related to clozapine metabolism or pharmacokinetics and C/D-ratio, clozapine and norclozapine concentrations, clozapine/norclozapine ratios or clozapine doses were found. A trend-level association between a GRS of 5 SNPs (*UGT1A* rs2269352, *CYP1A2* rs2472304, *CYP2C19* rs7916649, *ABCB1* rs3789243 and *ABCB1* rs1989830) and dichotomized clozapine dose was found. C/D-ratio and clozapine dose were associated strongly with regular smoking, which is consistent with earlier reports (Seppälä et al., 1999). Combined antipsychotic medication was associated with higher clozapine doses. The present results of no association between the genes studied and clozapine pharmacokinetics differed from some earlier findings. In Caucasian population, *ABCB1* rs1045642 was associated with clozapine concentration in one study and in a Japanese population, *ABCG2* rs2231142 was associated with the C/D-ratio (Akamine et al., 2017; Consoli et al., 2009; Jaquenoud Sirot et al., 2009). In the present study, rs1045642 and *ABCG2* rs2231142 were not associated with any of the phenotypes studied. Two SNPs in *ABCB1* gene, rs7787082 and rs10248420, have been reported to be associated with treatment response (Lee et al., 2012). *ABCB1* rs10248420 was not associated with the response variables and *ABCB1* rs7787082 genotype was not available in this study. The differences between earlier findings and the present study could be

related to ethnic differences between study groups, type I errors in the earlier studies or type II errors in this study.

Polymorphisms in the *CYP2D6* gene have been found to affect the rate of functioning of the *CYP2D6* enzyme. Poor, intermediate, efficient and ultra-rapid metabolizing variations of the CYP2D6 enzyme have been reported (Gaedigk & Leeder, 2014), and about 5.5% of Western European population are ultra-rapid metabolizers (Ingelman-Sundberg, 2005). *CYP2D6* gene variations are among the most reported biomarkers in the FDA drug labelling (Dickmann & Ware, 2016). In the prescription information on clozapine by the FDA (http://www.fda.gov), there is a mention that 3-10% of patients have reduced activity of CYP2D6 and these patients may develop higher than excepted plasma concentrations of clozapine. The most common major variant alleles of *CYP2D6* in Caucasian population are *CYP2D6*2xn*, *CYP2D6*4*, *CYP2D6*5* which are, respectively, mutations of gene duplication, defective splicing and gene deletion (Ingelman-Sundberg, 2005). These mutations could not be detected with the genotyping methods used in this study. *CYP2D6* major variant alleles also include missense mutations (P34S, S486T, T107I, R296C and S486T), all of which are rare in Caucasian patients (0-2%), and some of which are more frequent in Asians (51%) and Black Africans (6-35%) (Ingelman-Sundberg, 2005). This also may explain why *CYP2D6* SNPs were not associated with the response variables in this study, as the possible SNPs are rare in Caucasians.

The GRS of 5 SNPs, which had a trend-level association with dichotomized dose, was an interesting finding that constitutes a subject for further studies. Hypothetically, the GRS could help in predicting whether lower doses are sufficient for treatment response or if higher doses are needed. The GRS included two SNPs in *ABCB1* gene. The polymorphisms may affect the functionality of P-glycoprotein coded by *ABCB1* in the blood-brain barrier and as a result the balance between serum and CNS concentrations of clozapine and norclozapine may change. In rats, systemic exposure to clozapine and norclozapine concentrations averages about 10 % compared to the concentrations in the CNS (Cremers, Flik, Hofland, & Stratford, 2012) and similar observations have been reported in human subjects (Nordin, Alme, & Bondesson, 1995). The role of *CYP1A2*, *CYP2C19* and *UGT1A* SNPs in the GRS explaining clozapine dose is unclear, as the other analyses suggested that the SNPs were not associated with clozapine or norclozapine concentrations or C/D-ratio. The effect of *ABCB1* and *ABCG2*

polymorphisms on the CNS levels of clozapine and treatment response would be an interesting topic for further research. The present study did not, unfortunately, have information on treatment response.

6.2   Strengths and limitations

Studies I-IV all present new information on the pharmacogenetics of the adverse effects of clozapine. Studies I-III can be considered as pioneer studies on their respective subjects, since no similar studies have previously been presented. Study IV provides new information on the genetic variance behind the clozapine pharmacokinetics which contradicts existing findings.

The relatively small sample was a limitation in the analyses, although as far as we know the sample used is the largest available on genotyped, clozapine-treated patients with schizophrenia. Small sample size causes limitations when analysing a large number of SNPs and may result in false negative findings (type II errors). Small effects may go unobserved and only moderate to high effects can be found. However, the hypotheses were well-defined and the selection of genes to study reduced the need for a larger sample. The correction for multiple testing was done with FDR-adjustment because Bonferroni correction would have been too strict, as many of the SNPs are correlated with one another. In contrast, the GRS analysis used may lead to overfitting of the SNPs in the sample and result in type I errors. The best way to confirm the results to be free of type I error would be to replicate the study in another sample. This was not an option as no such data is currently available. For this reason, permutation analysis was used in Studies II-IV to generate a null distribution of the GRS test statistics.

All the adverse effects were self-assessed with LUNSERS and no measured objective variables were available in Studies I-III on, for example, patients' intensity of salivation, amount of sleep, bowel movements or consistency of stools. Nonetheless, LUNSERS has been proven to be a validated and reliable method of assessing adverse effects during antipsychotic treatment (Lambert et al., 2003). Study IV had laboratory measurements from a single blood sample, clozapine and norclozapine concentrations used as response variables. More reliable results could have been achieved with mean concentrations from multiple blood samples, as some intra-individual variance in the concentrations has been reported even in

controlled, relatively stable conditions with stable smoking, absence of caffeine and limited co-medication (Diaz et al., 2005).

The study was a cross-sectional study of patients in maintenance treatment. This is both a strength in eliciting information on this particular group of patients, but also a limitation in observing adverse effects especially at the beginning of treatment and the variance on the adverse effects during the treatment. In the sample used in these studies, over 90% of the patients had been taking clozapine for over one year. There may be a group of patients with most severe adverse effects who were not included in this study because their clozapine medication was already stopped already during the first weeks or months of the treatment due to the adverse effects. The use of antipsychotics in addition to clozapine and other psychiatric medications were taken into account in the analyses, but some possibly relevant confounding information was unavailable, such as amount of exercise, alcohol or substance use and chronic somatic illnesses. No information was available on the clinical status and current symptoms of the patients.

The results of the present study are generalizable to Caucasian patients taking clozapine for treatment resistant schizophrenia. When applying the results to other populations, geographical differences in genotype frequencies should be considered. Other antipsychotic medications have receptor affinities partly similar to those of clozapine and cause similar adverse effects. Some of the findings in the present study may be applicable to other antipsychotics having similar adverse effects, such as sedation and anticholinergic symptoms. Before such conclusions can be drawn, the results should be subjected to further research. The AIC used in the GRS was chosen as the model for genetic mapping in this study. It should be emphasized that genetic models are incomplete descriptions of reality, and that these models do not exist as such in real life (Sillanpää & Corander, 2002). A limitation with AIC is that it can lead to the selection of an unnecessarily complex model (Sillanpää & Corander, 2002). The GRSs found in this study should not be regarded as comprehensive genetic models explaining the adverse effects studied, but rather as hypothetical models combining cumulative genetic effects.

7   CONCLUSIONS AND IMPLICATIONS

This study suggests that common and clinically relevant adverse effects during clozapine treatment can be predicted with genetic testing. In this study the main focus was on the adverse effects of sialorrhea, sedation, constipation and anticholinergic symptoms. The main findings of this study were that *ADRA2A* rs1800544 was associated with CIS (I), eight linked single SNPs in the *HNMT* gene were associated with sedation (II), *CHRM3* rs685548 and two GRSs were associated with anticholinergic symptoms (III). No associations were found between the SNPs studied and variations in clozapine pharmacokinetics or clozapine dose (IV). Interesting trend level associations, which constitute for further studies, were found: a GRS of four SNPs explained a high portion of the sedation factor score (II) and a GRS of five SNPs predicted whether the patient received a lower (100-300mg) or higher (350-800mg) dose of clozapine, which could also be related to variation in treatment response (IV).

Clozapine is the drug of choice for treatment resistant schizophrenia, but has several severe adverse effects which may lead to the discontinuation of the clozapine treatment. This study raises awareness of the adverse effects of clozapine and the challenges in treating treatment resistant schizophrenia with clozapine. The findings in this study are not directly applicable as tools for clinical use, but in combination with other findings in the future could aid in the creation of such tools. The data sample sizes in studies on genetic variation in antipsychotic medication related adverse effects have often been limited. More data and studies are needed, and larger samples could possibly be gathered via international consortia or biobanks, for example, the Finnish biobanks. In the future, personalized medicine, pharmacogenetics and pharmacogenomics will be of help in tailoring the treatments to better meet individual needs. One vision for the future is that genetic information will be available if not for all, at least for certain risk groups and the genetic information will be integrated into clinical decision making. With modern data collecting, information about the interactions between genome data and clinical data could possibly be collected systematically via automated processes.

Pharmacogenetics has an important role in promoting drug safety and adherence to drug therapy, and possibly in preventing severe complications. With the available genetic information, antipsychotic medication could be chosen mindful of what would be the most suitable antipsychotic for the patient. In case of elevated risks for certain adverse effects, these could be monitored more carefully or preventive interventions could take place. Treatment response could also possibly be predicted with pharmacogenomic information: efficient antipsychotic medication could be initiated earlier, thereby reducing the duration of the psychotic episode and enabling better treatment for patients with psychotic disorders.

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Polymorphism in alpha 2A adrenergic receptor gene is associated with sialorrhea in schizophrenia patients on clozapine treatment

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Objective   Clozapine-induced sialorrhea (CIS) is a common, inconvenient and socially stigmatizing adverse effect. The pathophysiology of CIS may be related to the effect of clozapine on the muscarinic and adrenergic receptors as well as the disruption of the circadian rhythms. The aim of this study was to find out if polymorphisms in muscarinic M1 and M3 receptor genes (*CHRM1* and *CHRM3*), adrenoceptor alpha 2A gene (*ADRA2A*) or clock circadian regulator gene (*CLOCK*) are associated with CIS.
Methods   Two hundred and thirty-seven clozapine-treated Finnish schizophrenia patients were genotyped for *CHRM1*, *CHRM3*, *CLOCK* and *ADRA2A* polymorphisms, and their salivary dysfunction was assessed with two questions. Twenty-six of these patients had previously been on medication to treat CIS. Comparisons of the genotypes between patients with excessive versus non-excessive salivation were analysed. Genotype distributions between patients and control group and haplotypes were also studied.
Results   *CHRM1*, *CHRM3* and *CLOCK* polymorphisms and haplotypes were not associated with CIS. *ADRA2A* (rs1800544) genotype was associated with CIS ($p = 0.029$). In patients with CIS, CC genotype ($n = 103$) was more common than in G-allele carriers ($n = 79$) ($p = 0.013$, OR 2.13, 95% CI: 1.17–3.88). No differences were found in the distributions of genotypes between patients and controls.
Conclusions   *ADRA2A* genotype was associated with CIS. Copyright © 2014 John Wiley & Sons, Ltd.

KEY WORDS—clozapine; sialorrhea; *ADRA2A*; polymorphism; single nucleotide; drug toxicity

INTRODUCTION

Sialorrhea is a common and inconvenient adverse effect in clozapine treatment. Clozapine-induced sialorrhea (CIS) often develops in an early phase after the initiation of clozapine treatment. (Safferman *et al.*, 1991; Praharaj *et al.*, 2010) CIS occurs in approximately 30% of clozapine-treated patients, although the percentage varies in the literature (Praharaj *et al.*, 2006). Patients may experience CIS throughout the day, but it is most marked during sleep (Safferman *et al.*, 1991; Ben-Aryeh *et al.*, 1996). Patients complaining of sialorrhea often report a wet pillow following a night's sleep (Praharaj *et al.*, 2006), although objective measurements of salivary flow in clozapine-treated patients are few and inconclusive

(Ekström *et al.*, 2010a). Ben-Aryeh *et al.* (1996) found no difference in saliva flow rate or saliva composition in patients complaining of sialorrhea during clozapine treatment compared with healthy controls. As these patients complained of nocturnal sialorrhea, it was suggested that the sialorrhea occurring at night may instead be due to disruptions of the circadian rhythms. Salivary flow follows circadian rhythms as the salivary flow from the major glands, the parotid and submandibular glands, decreases significantly during sleep (Dawes, 1972; Dawes and Ong, 1973; Ferguson *et al.*, 1973; for a review see Thie *et al.*, 2002). The clock mechanisms behind salivary flow still remain unclear. A recent study reports that clock gene RNAs and clock proteins were detected in salivary gland cells, especially in serous acinar and duct cells, indicating that clock genes may contribute to the circadian rhythms of salivary flow. (Zheng *et al.*, 2012)

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Salivary gland secretion is mediated by the autonomic nervous system, particularly the parasympathetic nervous system (Proctor and Carpenter, 2007). The population of muscarinic receptors in the salivary glands, in particular mostly M1 and M3 subtypes, mediates salivation (Tobin *et al*., 2009). Clozapine is an antagonist to M1, M2, M3 and M5 receptors but an agonist to M4 receptor, and the effect on M4 has been previously suspected to cause CIS (Zorn *et al*., 1994). More recent animal studies suggest that clozapine causes salivary secretion by direct action on muscarinic M1-receptors of the acinar cells (Ekström *et al*., 2010a). In another study by Ekström *et al*. (2010b), *N*-desmethylclozapine, the major active metabolite of clozapine, displayed greater excitatory effect on the submandibular and parotid glands than clozapine, mediated by the M1-muscarinic receptor. Ekström *et al*. (2010b) also hypothesized that a prolonged blockade of a fraction of the muscarinic and adrenergic alpha(1) receptors will increase the sensitivity of the non-blocked muscarinic receptors causing an enlarged secretory response by clozapine and *N*-desmethylclozapine. Another hypothesis is that clozapine impedes normal swallowing by blocking target receptors in the pharynx or the muscles involved in the swallowing reflex (Rabinowitz *et al*., 1996). It has been demonstrated that clonidine, an alpha(2) adrenoceptor agonist, decreases ongoing nerve-induced salivary output in the central nervous system, whereas yohimbine, an alpha(2) adrenoceptor antagonist, has an opposite, increasing effect (Phillips *et al*., 2000). The alpha(2) receptors also seem to be involved in swallowing–respiration coordination (Yamanishi *et al*., 2010). Clozapine acts as an alpha(2) adrenoceptor antagonist (Ashby and Wang, 1996; Kalkman and Loetscher, 2003). The theory that sialorrhea is caused by the alpha(2) antagonist effect of clozapine is opposed by the results of a study by Ogura *et al*. (1987) showing that mianserin, a high affinity alpha(2) antagonist, does not induce sialorrhea but on the contrary causes dry mouth.

There is much variation in medications used to treat CIS. Treatment strategies include selective and nonselective anticholinergic medications (such as tricyclic antidepressants (TCAs)), local anticholinergic agents, clonidine (alpha(2) agonist), amisulpride and botulinum toxin (Copp *et al*., 1991; Praharaj and Arora, 2007; Sockalingam *et al*., 2007). Treatment of CIS has not so far been sufficiently studied (Syed *et al*., 2008). There are no generally accepted guidelines for treating this adverse effect due to a lack of randomized placebo-controlled trials. Evidence in the treatment options of CIS is limited to anecdotal reports and open-label series (Sockalingam *et al*., 2007).

In this study, we wanted to explore if polymorphisms in the receptors through which CIS is suspected to be mediated explain the variability in CIS in schizophrenia patients on clozapine treatment. We selected three muscarinic M1 receptor gene (*CHRM1* rs2507821, rs542269 and rs2075748), three muscarinic M3 receptor gene (*CHRM3* rs4620530, rs6429157 and rs6690809), four clock circadian regulator gene (*CLOCK* rs1801260, rs3749474, rs4580704 and rs6850524) and one adrenoceptor alpha 2A gene (*ADRA2A* rs1800544) single nucleotide polymorphisms (SNPs) to be genotyped for the analyses. *CLOCK* SNPs were studied because of the hypothesis of *CLOCK* polymorphisms explaining the disruption of circadian rhythms and thus perhaps nocturnal sialorrhea. Also, comparisons in genotype distributions in the named SNPs between patients and healthy controls were analysed.

MATERIALS AND METHODS

Patients

The study population comprised 237 clozapine-treated patients (136 men, 101 women, mean age 42.5 years and SD 11.0 years) with a diagnosis of schizophrenia, schizoaffective or other nonorganic and non-affective psychoses according to the International Classification of Diseases, Tenth Revision. The mean time elapsing from first hospitalization due to psychotic episode in patients was 17.3 years (SD ± 10.0 years). All patients were adult (≥18 years of age), Caucasian, of Finnish origin and on clozapine treatment. The study was conducted in three hospital districts in Western Finland (Satakunta, Pirkanmaa and Seinäjoki Hospital districts). Inclusion criteria were current F02-group diagnosis according to the International Classification of Diseases, Tenth Revision and stabilized clozapine treatment. Patients with organic brain diseases were excluded.

Patients gave informed consent to participation in this study, and agreed to have blood samples taken for studying adverse effects in clozapine treatment. The study was approved by the Ethics Committee of Satakunta Hospital District.

One hundred and fifty-five patients (65.4 %) were on clozapine monotherapy and 82 (34.6%) on a combination of at least two antipsychotics. All neuroleptic doses were converted to chlorpromazine equivalents (Aronson, 2009). The mean daily dose of clozapine was 403 mg (SD ± 152 mg), 806 mg when converted to chlorpromazine equivalents. The total neuroleptic dose converted to chlorpromazine equivalents was

912 mg (SD ± 304 mg). More than half of the patients had been taking clozapine for more than 5 years, and none less than for three months. Thirty-two patients were on TCA, and in 30 cases, the indication for this medication (amitriptyline, $n = 29$ or doxepin, $n = 1$) was to treat CIS.

Patients completed the Liverpool University Neuroleptic Side Effect Rating Scale (LUNSERS) (Day *et al.*, 1995). LUNSERS contains a list of 51 symptoms. Patients are asked to indicate how much they have experienced each of the symptoms. Two symptoms are related to salivary flow (symptom number 6, 'dry mouth' and symptom number 37, 'over-wet or drooling mouth'). Patients rated the severities of the adverse effects from 0 to 4 (0 = not at all, 1 = very little, 2 = a little, 3 = quite a lot and 4 = very much).

Controls

The control group comprised 388 healthy Finnish blood donors. They completed a health questionnaire including information on their mental health. When donating blood, a nurse interviews each blood donor individually about their medication and possible chronic illnesses.

DNA extraction and genotyping

For DNA extraction, 9.0 ml ethylenediaminetetraacetic acid whole blood was drawn from the participants and stored in a freezer at −20°C. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp©DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany). Genotyping of the rs2507821, rs542269, rs2075748, rs4620530, rs6429157, rs6690809, rs1801260, rs3749474, rs4580704, rs6850524 and rs1800544 was performed using Taqman©SNP Genotyping Assays and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Statistical methods

For the analyses, the patients were divided into two groups according to their answers to question on 'over-wet drooling mouth'. In a separate analysis, patients on low-dose TCA medication (100 mg or less) were added to the excessive salivary flow group, whether or not they had reported increased salivary flow in the LUNSERS questionnaire. Of the total 32 patients on TCA medication, we excluded two patients as it was uncertain whether the indication for this medication was excessive salivation.

Comparisons in genotype distributions between patients and control groups were analysed with chi-square statistics. Comparisons between different genotypes and patients with excessive salivation or excessive salivation and TCA medication combined versus no such side effect or medication were calculated with chi-square statistics including odds ratios. Binary logistic regression analysis was used to predict CIS. In the model, *ADRA2A* G-allele carrying status, smoking, gender and the use of various adjuvant therapies were used as explanatory variables.

Haplotypic effect of the SNPs was studied using the haplo.stats package in the R Statistical package v. 2.15.2 (R Core Team, 2012), all other calculations were made with SPSS software package Version 19.0 (IBM Corporation, New York, United States). The level of statistical significance was set at 0.05.

RESULTS

No differences in sialorrhea were found between the studied *CHRM1*, *CHRM3* and *CLOCK* genotypes (chi-square test). Reported sialorrhea was associated with *ADRA2A* (rs1800544) genotype ($p = 0.029$, chi-square test). Reported sialorrhea and/or the use of TCA was also associated with CC genotype in comparison with CG and GG genotypes (G-allele carriers) combined (Table 1). The associations between *ADRA2A* (rs1800544) G-allele carrying status and CIS for subgroups in clozapine monotherapy or combined antipsychotic treatment were also calculated separately. The association was significant in the monotherapy group ($p = 0.044$) but not in the combined medication group ($p = 0.11$).

In confirming logistic regression analysis, *ADRA2A* G-allele carrying status remained the only significant explanatory variable for CIS, G-allele carriers having less CIS, whereas regular smoking, gender or taking various adjuvant medications (antidepressants, mood stabilizers and benzodiazepines) did not.

In comparisons between patients and controls, there were no differences in the distributions of genotypes in *CHRM1* (rs2507821, rs542269 and rs2075748), *CHRM3* (rs4620530, rs6429157 and rs6690809), *CLOCK* (rs1801260, rs3749474, rs4580704 and rs6850524) and *ADRA2A* (rs1800544) SNPs (chi-square test).

Haplotype analyses resulted in 36 haplotypes in *CHRM1*, 64 haplotypes in *CHRM3* and 16 in *CLOCK*. In *CHRM1*, the two most common haplotypes were compared with other haplotypes in relation to CIS, and in *CHRM3*, the two most common and in *CLOCK* the most common haplotype were likewise compared. In the analyses performed on *CHRM1*, *CHRM3* and *CLOCK* haplotypes, no statistically significant associations were found between CIS and the pooled haplotype groups.

Table 1. Distributions of alpha(2) receptor genotypes (*ADRA2A* rs1800544 CC genotype versus G-allele carriers) in patients with no or little subjective sialorrhea versus quite a lot or much sialorrhea assessed with LUNSERS item 'over-wet or drooling mouth' (or alternatively currently using tricyclic antidepressive medication due sialorrhea)

| | *ADRA2A* rs1800544 polymorphism | | |
| | CC | CG | GG |
Amount of sialorrhea	n (%)	n (%)	n (%)
Salivary flow increased from not at all to little, no TCA medication $n = 80$	37 (35.9%)	35 (52.2%)	8 (66.7%)
Salivary flow increased from quite a lot to very much, or patient on TCA medication $n = 102$	66 (64.1%)	32 (47.8%)	4 (33.3%)

LUNSERS, Liverpool University Neuroleptic Side Effect Rating Scale;
TCA, tricyclic antidepressant.
$p = 0.029$ (chi-square test) between *ADRA2A* genotype and patient group.
$p = 0.013$ (chi-square test) between *ADRA2A* CC versus G-carriers and patient group,
OR 2.13, 95% CI: 1.17–3.88.

DISCUSSION

The main finding of this study is that *ADRA2A* rs180054 CC genotype seems to be associated with CIS, and patients carrying G-allele have less sialorrhea. The likelihood of CIS increased with the number of C-alleles: the CC group experienced more sialorrhea than the CG group, and the CG group had more sialorrhea than the GG group. The CC genotype practically doubled the likelihood for CIS compared with CG and GG genotype groups. As clozapine is an antagonist to adrenergic alpha(2) receptors, the finding may be related to the effect of clozapine on either peripheral or central adrenergic alpha(2) receptors. Alternatively, the studied *ADRA2A* genotype may be linked to another gene polymorphisms not found in this study that could explain the variation in salivary flow.

The finding was specifically associated with clozapine monotherapy. Regular smoking, gender or adjuvant medications did not explain sialorrhea. Patients in the clozapine monotherapy group may have had somatic medications, which were not included in the data but hardly influenced sialorrhea.

There are some studies reporting *CLOCK* 3111C/T (rs1801260) polymorphism to be associated with diurnal preference (Katzenberg *et al.*, 1998; Mishima *et al.*, 2005), but among several other studies Chang *et al.* (2011) could not replicate these findings. Lee *et al.* (2010) reported an association between the polymorphism and preferred circadian phase in bipolar patients. These controversial results may be related to differences in study populations, selection criteria or analytic methods (Chang *et al.*, 2011). In the present study, the *CLOCK* genotype was hypothesized to be involved in diurnal salivary rhythm, but no such association was found. One major limitation was that

the phase of the day the patients experienced sialorrhea was not specified. According to earlier reports, patients mostly experience sialorrhea during the night (Safferman *et al.*, 1991; Ben-Aryeh *et al.*, 1996), and therefore, it might be beneficial to study nocturnal sialorrhea in relation to *CLOCK* polymorphisms. It is possible that the diurnal salivary rhythm is disturbed, but the mechanism behind this is not necessarily related to *CLOCK* polymorphisms.

According to the present results, none of the *CHRM1* and *CHRM3* polymorphisms altered the function of the salivary glands during clozapine treatment, or alternatively, the effects are controversial. Detecting the possible interaction between the effect of clozapine and *CHRM1* and *CHRM3* polymorphisms would necessitate a controlled study design.

CIS is a socially stigmatizing adverse effect (Praharaj *et al.*, 2006). Combined with the sedative effect of clozapine, it may lead to severe medical problems such as aspiration pneumonia (Hinkes *et al.*, 1996). There is no consensus regarding the optimal treatments of sialorrhea. With some medications CIS can be relieved, but these often include adverse effects and add to the toxicity of clozapine. For example, TCAs may increase the anticholinergic, sedative and hypotensive effects of clozapine and lower the seizure threshold (Aronson, 2009).

The burden of adverse effects is a notable reason for abandoning clozapine treatment, especially during the first month (Pai and Vella, 2012). Treatment adherence might be improved if the patient's risk of developing adverse effects could be assessed in advance. Usage of only ADR2A (rs1800544) SNP as a marker of increased risk for sialorrhea is probably not specific enough for clinical use, but in combination with future

SNPs possibly associated with risk of sialorrhea, clinicians might be provided with a tool to predict the risk of CIS.

The statistical power is limited as the study population was relatively small. This limitation was highlighted in the haplotype analyses, as the numbers of haplotypes (16, 36 or 64) were too high for reliable statistical analyses. As haplotypes had to be pooled for analyses, some haplotypes that might have had individual effect on CIS may have gone unnoticed. Regarding the *ADRA2A* rs180054 finding, the number of patients with GG genotype in the *ADRA2A* was also relatively small. However, the separate analyses according to *ADRA2A* genotypes and between CC and G-carriers were in line with each other. In the subgroup analyses for clozapine monotherapy and combined antipsychotic treatment, the small number of subjects in the latter group is a likely reason for the insignificant finding. A further limitation was that the intensity of drooling was only self-assessed by a single question, and no objective or more specific measures of salivation were included. As more than half of the patients had been taking clozapine for more than 5 years, their retrospective assessment of the change in salivary flow may not be entirely reliable. Moreover, pooling the patients on low-dose TCA together with those experiencing CIS was only based on the common clinical practice of using these medications for CIS. The present study has no implications for the choice of treatment in CIS.

CONCLUSION

Clozapine-induced sialorrhea is an inconvenient and socially stigmatizing adverse effect potentially leading to other medical complications. *ADRA2A* rs180054 was associated with CIS in clozapine monotherapy. Further studies**,** preferably with more detailed evaluation of sialorrhea, are needed to confirm the role of this genotype for risk of CIS.

CONFLICTS OF INTEREST

Olli Kampman has consulted or given lectures for Janssen–Cilag, Lundbeck and Pfizer.

Niko Seppälä has participated in international congresses, sponsored by Bristol-Myers Squibb and Janssen–Cilag.

Esa Leinonen has consulted or given lectures for AstraZeneca, Bristol-Myers Squibb, GlaxoSmithKline, Janssen–Cilag, Lilly, Lundbeck, Orion and Servier, participated in international congresses, sponsored by AstraZeneca, Janssen–Cilag, Lundbeck and Servier

and has served on the international adviser board of Servier.

Anssi Solismaa, Merja Viikki, Kari-Matti Mäkelä, Nina Mononen and Terho Lehtimäki have no conflicts of interest to report.

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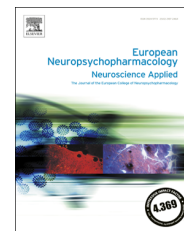
Histaminergic gene polymorphisms associated with sedation in clozapine-treated patients

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Abstract

Sedation is a common adverse effect of clozapine treatment, which may be partly related to clozapine binding to histamine receptors in the central nervous system. The objective of this study was to investigate whether single nucleotide polymorphisms (SNPs) in the histaminergic system are associated with sedation in clozapine-treated patients. The study population comprised 237 clozapine-treated, Finnish, Caucasian patients that were diagnosed with schizophrenia and 176 were genotyped using Illumina HumanCoreExome-12 BeadChip. Sedation levels were assessed using self-rating questions from the Liverpool University Neuroleptic Side Effect Rating Scale (LUNSERS). The relationships between 55 different SNPs in the histaminergic system and adverse sedation effects were examined. SNPs were analyzed separately, and in groups, to formulate a genetic risk score (GRS). A permutation test was performed to avoid type I errors. Eight linked SNPs ($r^2 = 1$) in the *HNMT* gene were also associated with sedation according to the GLM, adjusted for age, gender and BMI (false-discovery-rate-adjusted $p = 0.013$). An association on a trend level between a GRS of four different SNPs (recessive histamine N-methyltransferase *HNMT* rs2737385, additive histamine receptor H_1 rs1552498, dominant *HRH1* rs17034063 and recessive amine oxidase, copper containing 1 *AOC1* rs6977381) and sedation was found (permuted p-value = 0.066) in a generalized linear model (GLM) incorporating age, gender and body mass index (BMI; adjusted $R^2 = 0.22$). Polymorphisms in genes encoding histamine receptors or enzymes related to histamine metabolism may explain individual variation in sedative effects experienced during clozapine treatment.
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1. Introduction

Sedation is a common side effect of clozapine treatment. It is more prominent at the beginning of clozapine treatment, with some tolerance developing during the first days or weeks of treatment (Safferman et al., 1991). The histaminergic network of the central nervous system (CNS) regulates sleep and wakefulness (Panula and Nuutinen, 2013), with blockade of histamine H_1 and activation of histamine H_3 receptors inducing sedation (Lin, 2000). Histamine N-methyltransferase (HNMT) is a histamine-metabolizing enzyme that inactivates histamine in the CNS (Ogasawara et al., 2006), while diamine oxidase (DAO), encoded by the gene amine oxidase, copper containing 1 (*AOC1*, also called *ABP1*), is another histamine degrading enzyme that functions mostly in peripheral tissues. Under normal conditions, DAO has low activity in the CNS (Haas et al., 2008) but may act as a salvage pathway for histamine degradation when HNMT is blocked (Prell et al., 1997). Histidine decarboxylase (HDC) is required to synthesize histamine from histidine, through oxidative decarboxylation (Haas et al., 2008). Therefore, it is proposed that the sedative properties of clozapine may be related to effects on H_1 histamine receptors (Ashby and Wang, 1996; Richelson and Souder, 2000). Clozapine has a very high affinity for CNS histamine H_1 receptors (Ashby and Wang, 1996) and its metabolite *N*-desmethylclozapine acts as a potent and partial H_1-receptor inverse agonist, a weak, full H_2-receptor inverse agonist, a moderate protean H_3-receptor agonist and a moderate, partial H_4-receptor agonist (Humbert-Claude et al., 2012). The metabolic pathway of histamine and functions of the histamine receptors particularly on behalf of regulating wakefulness are presented in Figure 1.

In this study, single nucleotide polymorphisms (SNPs), or combinations of SNPs, in genes encoding receptors and enzymes of the histaminergic system and their potential association with clozapine related sedation in patients were examined. SNPs in histamine receptor genes (*HRH1, HRH2, HRH3* and *HRH4*) and histaminergic system, enzyme genes (*HNMT, HDC* and *AOC1*) were studied.

2. Experimental procedures

2.1. Patients

The sample was screened from 256 patients, of which 19 declined to participate. The study was conducted in three hospital districts in Western Finland (Satakunta, Pirkanmaa and Seinäjoki Hospital districts). For inclusion, patients had to be current F2-group diagnosed, according to the International Classification of Diseases, Tenth Revision (ICD-10), and stabilized on clozapine treatment. Patients with organic brain diseases were excluded. The study population comprised 237 clozapine-treated patients (136 men, 101 women, mean age 42.5, standard deviation (SD) 11.0 years) diagnosed with schizophrenia ($n = 223$, 94.1%), schizoaffective disorder or delusional disorder, according to the ICD-10. The mean elapsed time from first hospitalization due to psychotic episode was 17.3 years (SD $\pm$ 10.0 years). All patients were adults ($\geq$ 18 years of age), Caucasian, of Finnish origin and on clozapine treatment. Patients completed a questionnaire, by which we asked about smoking information, estimate of patient's current weight, height and weight change during clozapine treatment, medication and its dosing method (supervised or unsupervised). Psychiatric diagnoses, medication information including the duration of clozapine treatment and dates of the first hospitalization due to psychosis were collected from the medical history entries. Laboratory samples were obtained from 190 patients, and after quality controls, 176 genotyped patients were eligible to remain in the study. Compliance to medication was accounted by measuring clozapine and norclozapine concentrations for this study. Also, as required by the treatment protocol, patients participated in monthly group sessions, where the white cell blood counts were checked and clozapine prescriptions renewed.

Patients gave written, informed consent for participation in this study and agreed to have blood samples taken for the study of adverse effects of clozapine treatment. The study was performed in accordance
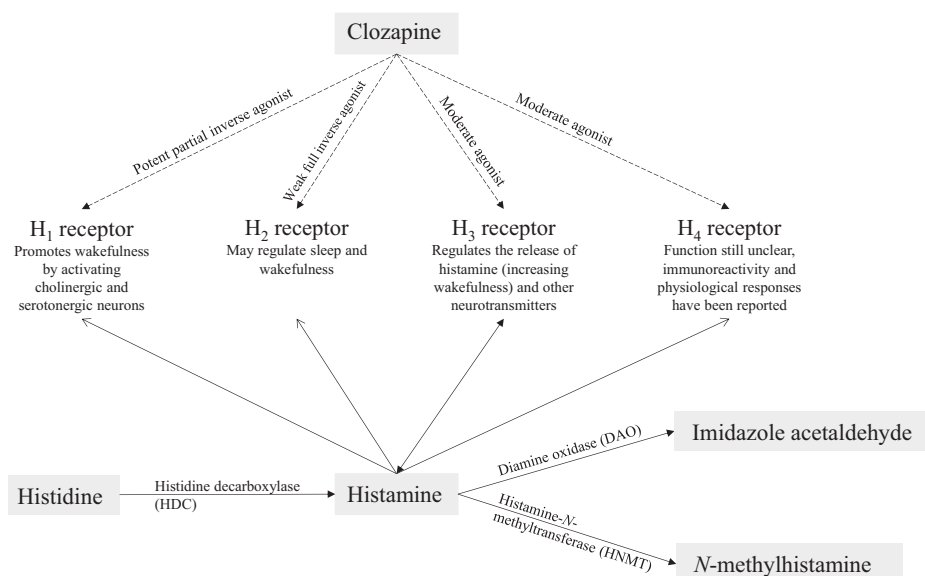
Figure 1 The metabolic pathway of histamine, histamine receptors and their functions particularly on behalf of regulating wakefulness and the affinity of clozapine on histamine receptors.

with the Declaration of Helsinki and was approved by the Ethics Committee of Satakunta Hospital District on 23 April 2008.

Of the 176 genotyped patients, 119 patients (67.6%) were on clozapine monotherapy and 57 (32.4%) were on a combination of at least two antipsychotics. All antipsychotic doses were converted to chlorpromazine equivalents (Aronson, 2009). The mean daily dose of clozapine was 403 mg (SD $\pm$ 152 mg) or 806 mg (SD $\pm$ 304 mg) when converted to chlorpromazine equivalents. The total antipsychotic dose converted to chlorpromazine equivalents was 912 mg (SD $\pm$ 304 mg). More than half of the patients had been taking clozapine for over 5 years and none for less than 3 months.

Patients completed the Liverpool University Neuroleptic Side Effect Rating Scale (LUNSERS) (Day et al., 1995), which contains a list of 51 symptoms. Patients were asked to rate the severities of adverse effects on a scale from 0 to 4 (0 = not at all, 1 = very little, 2 = a little, 3 = quite a lot and 4 = very much). Based on factor analysis on all LUNSERS items (Seppälä et al., 2014), a factor containing items "Difficulty staying awake during the day", "Tiredness" and "Sleeping too much" was used as response variables for sedation, both as a continuous variable (scale 0-12) and as a dichotomized variable. The dichotomized variable had a cutoff point of equal or less vs. greater than the median value, which was 5 (0-5 = not at all or little sedation, 6-12 = moderate or severe sedation).

2.2. DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Midikit and an automated biorobot M48 extraction (Qiagen, Hilden, Germany). Samples were genotyped using Illumina Infinium HumanCoreExome-12 DNA Analysis Beadchip version 1.0., according to the manufacturer's recommendation at Helmholtz Zentrum, München, Germany. The following quality control filters were applied: GenCall score < 0.15, GenTrain score < 0.20, sample and SNP call rate < 0.95, Hardy-Weinberg equation p-value < 10^{-6}, excess heterozygosity, cryptic relatedness (pi-hat > 0.2), gender check and multidimensional scaling (MDS). After quality control, 176 samples and 531,983 SNPs were available, of which 55 SNPs were from the HRH1, HRH2, HRH3, HRH4, HNMT, AOC1 and HDC genes. SNPs with a minor allele frequency (MAF) of less than 0.01 were excluded from the study.

2.3. Statistical methods

Chi square statistics were used for allelic comparisons between patient groups with dichotomous sedation. Pearson correlations and t-tests were used in explanatory analyses between sedation factor scores and potential explanatory variables such as, antipsychotic dose, clozapine concentration, body mass index (BMI) and use of anxiolytics or hypnotics. Covariates were chosen primarily according to literature and secondarily according to known exploratory bivariate associations in this sample. Finally the best fitting model was selected. Generalized linear univariate models (GLMs) and logistic regression were used to analyze the explanatory factors of gender, SNPs encoded separately with additive, dominant and recessive coding and covariates (age and BMI) for sedation factor scores (GLM) and dichotomized sedation factor score (logistic regression). The SNPs were analyzed separately by entering them in the model one at a time. The model used an inverse normal transformation of the sedation factor score to form a symmetrical distribution. The significance levels of explaining variables in the GLMs were adjusted using the false discovery rate (FDR) method for multiple testing, which accounts for the number of SNPs. FDRs in each GLM and logistic regression analysis were calculated separately. Modes of inheritance were accounted in the FDR. Additive, dominant and recessive models were coded as follows. Where a polymorphism contained two alleles, for example G and g, in the additive model the amount of sedation is thought to rise

cumulatively according to the number of G alleles, whereas in the dominant model sedation is thought to be associated with GG and Gg genotypes, and in the recessive model sedation is thought to be associated with gg genotype. SNPs with fewer than three risk alleles and/or one or more missing values were excluded from the data. For all the SNPs in the study, linkage disequilibrium (LD) test was performed with 1 Mbp window and threshold of r2 = 0.2. The model returned by AIC was analyzed with stepwise variance inflation factor (VIF) to examine collinearity between the SNPs in the GRS. In the LD plots, LD and haploblocks were defined using the confidence intervals method (Gabriel et al., 2002). The color schemes of the LD plots are based on D' / LOD score ratio.

In power calculations a sedation factor score difference of ± 1.97 in a SNP with a MAF 0.10 and respectively a difference of ± 1.28 in a SNP with a MAF 0.30 could be detected with a probability of 0.8. The statistical significance level was set at $p < 0.05$.

Statistical analyses were performed using R (version 3.1.2, 2014, The R Foundation for Statistical Computing), SPSS (version 22, IBM inc.), gPLINK (version 2.050), PLINK (version 1.07) and Haploview (version 4.2).

2.4. Genetic risk score (GRS)

Genetic risk scores (GRSs) were calculated for each individual as a weighted sum of the risk alleles in relation to sedation. GRSs were calculated for both GLM analyses and logistic regression analyses. First, all SNPs that had an unadjusted level of significance, $p < 0.05$, for sedation were selected from the GLM. These selected SNPs were inserted into the same model as other covariates and analyzed using Akaike information criteria (AIC) (Akaike, 1981), to avoid overfitting by selecting only tag SNPs among SNPs with high linkage disequilibrium (LD). AIC is used as a model based approach to genetic mapping (Sillanpää and Corander, 2002) to estimate the influential tag SNPs of the SNPs preselected with GLM and logistic regression analyses. SNPs remaining in the model, as indicated by AIC, were used in the GRS. For each SNP, the corresponding beta estimate value (B) from the GLM and logistic regression was used as the adjusting weight for individual effects of a SNP in the GRS. This score was used as an explanatory variable in the final GLM, together with age, gender and BMI. Annotation analysis was performed for the SNPs in the GRS and for SNPs that were in over 0.8 r^2 LD with them in the sample.

2.5. Permutation test

Linear combination of genetic effects that are estimated from univariate models may over fit data and cause inflated type I errors. To avoid this type of error, an estimation of the null distribution of the GRS test statistics is critical. (Hu et al., 2013) Therefore, a permutation test was performed by sampling patient ID numbers from the phenotype data, which resulted in SNP genotypes that were independent of the studied phenotype (sedation). The permutation was conducted 9999 times with the sampled data, using the same method described above. The GRS was calculated for each sampled data set and an association analysis with GLM or logistic regression was performed. If the p-value derived from the actual data analysis was smaller than 5% of the p-values derived from the permutated data, then the result was considered statistically significant.

3. Results

Clinical covariates and the differences between dichotomized sedation factor score groups are presented in Table 1. Fifty-five SNPs were analyzed, and 19 SNPs of HRH1 gene, two of HRH2, two of HRH4, three of HDC, 12 of HNMT and 17 of AOC1

Table 1 Clinical covariates and the differences between dichotomized sedation factor score groups.

	Sedation factor score 0-5 (n = 101)	Sedation factor score 6-12 (n = 75)	Difference between groups
Age mean (sd) years	45.5 (10.7)	40.8 (10.7)	**p = 0.0052** (*t*-test)
Gender	32.7% women, 67.3% men	52% women, 48% men	**p = 0.015** (chi-square)
Smoking status	48,4% smoked	54,7% smoked	p = 0.51 (chi-square)
BMI mean (sd) kg/m^2	28.9 (5.98)	30.1 (6.73)	**p = 0.040** (t-test)
Clozapine monotherapy	63,3% with no other antipsychotic in use	73,3% with no other antipsychotic in use	p = 0.22 (chi-sq)
Clozapine dose in cpz^a mg, mean (sd) mg	797 (308)	821 (276)	p = 0.59 (t-test)
Total antipsychotic dose in cpz^a, mean (sd) mg	899 (400)	924 (391)	p = 0.69 (t-test)

^{a}cpz = chlorpromazine equivalent. Clozapine dose was multiplied by two in conversion to chlorpromazine equivalent.

were included in this study. In unadjusted model for allelic association tests, two SNPs in the *AOC1* gene were associated with dichotomous sedation (rs41465145, p = 0.032, OR = 2.02, CI 95% = 1.052-3.89 and rs6977381, p = 0.042, OR = 0.63, CI 95% = 0.40-0.98). Names of all studied SNPs and the allelic association test results are presented in the Supplementary material. In correlation analyses, between explanatory variables, no significant correlations between sedation and clozapine or total antipsychotic dose, clozapine concentration or use of anxiolytics or hypnotics were found. Both BMI (*r* = 0.16, *p* = 0.015) and age (*r* = -0.18, *p* = 0.008) correlated with sedation factor scores (mean = 4.92, median = 5, sd = 2.78). There was no difference in sedation factor scores between clozapine monotherapy and additional antipsychotic groups (*t*-test, means

4.85 vs. 4.94, *p* = 0.81). In the GLM analysis, a group of eight recessively coded SNPs (rs1455156, rs2737385, rs1050891, rs4245861, rs464633, rs1455158, rs14155157 and rs1050900) in the *HNMT* gene explained sedation (FDR-adjusted *p* = 0.013, Table 2). The eight SNPs were strongly linked (*r*^{2} = 1, see Table 3). In the logistic regression analysis, no SNP explained dichotomous sedation after FDR-adjustment. All GLM and logistic regression analyses and LD plots of the SNPs are presented in the Supplementary material.

GRS analysis of the GLM results found that four of the studied SNPs were involved, recessive *HNMT* rs2737385, additive *HRH1* rs1552498, dominant *HRH1* rs17034063 and recessive *AOC1* rs6977381. This weighted GRS was used as an explanatory variable in the final model, together with

Table 2 Results for the SNPs analyzed using multiple GLMs with sedation factor score as the response variable and age, BMI, gender and each SNP separately as explanatory variables.

Gene	SNP	MAF	Coding	Frequencies (n)	Estimate	Unadjusted p	FDR-adjusted p
HNMT	rs1455156	0.22	Recessive	8 − 166	−0.62	0.00062	0.013
HNMT	**rs2737385**^{a}	0.22	**Recessive**	8 − 168	−0.62	0.00064	0.013
HNMT	rs1050891	0.22	Recessive	8 − 168	−0.62	0.00064	0.013
HNMT	rs4245861	0.22	Recessive	8 − 168	−0.62	0.00064	0.013
HNMT	rs4646333	0.22	Recessive	8 − 168	−0.62	0.00064	0.013
HNMT	rs1455158	0.22	Recessive	8 − 168	−0.62	0.00064	0.013
HNMT	rs1455157	0.22	Recessive	8 − 168	−0.62	0.00064	0.013
HNMT	rs1050900	0.22	Recessive	8 − 167	−0.62	0.00073	0.013
HRH1	**rs1552498**^{a}	0.15	**Additive**	128 − 43 − 5	0.40	0.0070	0.11
HRH1	rs1552498	0.15	Dominant	128 − 48	0.22	0.0083	0.12
AOC1	**rs6977381**^{a}	0.39	**Recessive**	27 − 149	−0.24	0.018	0.24
AOC1	rs6977381	0.39	Additive	66 − 83 − 27	−0.24	0.024	0.29
AOC1	rs10893	0.34	Dominant	76 − 100	0.16	0.037	0.32
HRH1	**rs17034063**^{a}	0.07	**Dominant**	26 − 150	−0.21	0.037	0.32
HRH1	rs17034063	0.07	Additive	150 − 26 − 0	−0.42	0.037	0.32
HNMT	rs1455156	0.22	Additive	106 − 60 − 8	−0.25	0.048	0.32
HNMT	rs1580111	0.39	Recessive	149 − 27	0.15	0.049	0.32

Note: Only results with an unadjusted p of less than 0.05 are presented in this table. A positive estimate value indicates that minor alleles are associated with sedation and a negative estimate value indicates that minor alleles are associated with wakefulness. The effect sizes of the SNPs can be compared with the absolute values of the estimates.
^{a}Marked SNPs were included in the GRS with the estimate value as the weight.

Table 3 SNPs resulting from GRS analyses and SNPs in > 0.8 r^2 LD with them.

Gene	SNPs	SNPs in > 0.8 r^2 LD	Annotation
HNMT	rs2737385		intron
		rs1050891 ($r^2 = 1$)	UTR-3
		rs1050900 ($r^2 = 1$)	UTR-3
		rs4245861 ($r^2 = 1$)	UTR-3
		rs4646333 ($r^2 = 1$)	UTR-3
		rs1455158 ($r^2 = 1$)	UTR-3
		rs1455157 ($r^2 = 1$)	UTR-3
		rs1455156 ($r^2 = 1$)	UTR-3
HRH1	rs1552498	–	intron
	rs17034063	–	intron
HDC	rs2853766	–	intron
HRH4	rs1421125	–	UTR-3
AOC1	rs6977381	–	intron

age, gender and BMI. In this model, age ($p = 0.023$), gender ($p = 0.0094$) and the GRS ($p = 9.2 \times 10^{-8}$, $d = 0.96$) explained sedation ($R^2 = 0.24$, adjusted $R^2 = 0.22$). As a comparison, in a model without the GRS, R^2 was 0.070 and adjusted $R^2 = 0.057$ (age $p = 0.017$, BMI $p = 0.10$, gender $p = 0.013$). There is no considerable collinearity between the SNPs or other predictor variables (VIF < 10). In the LD analysis, no LD was found between the SNPs in the GRS. In the permutation test, the *p*-value of the GRS ranked 662 out of 10000 compared with the other 9999 *p*-values, which were calculated using the permutations ($p = 0.066$). Figure 2 shows a boxplot of the distribution of sedation factor scores in groups with different numbers of the unweighted risk alleles in the GRS SNPs. GRS analysis of the logistic regression results found a GRS of three SNPs (dominant *HRH1* rs1552498, dominant *HDC* rs2853766 and additive *HRH4* rs1421125), but was not statistically significant after permutation analysis ($p = 0.14$).

Since the function of DAO in the CNS is unclear, an alternative post hoc analysis was performed that excluded the *AOC1* SNPs from the data and an alternative GRS was calculated. GLM analysis with age, BMI, gender, individual SNPs or the alternative GRS, as explanatory variables for sedation, were analyzed. The same linked *HNMT* SNPs (FDR-adjusted $p = 0.0091$) were
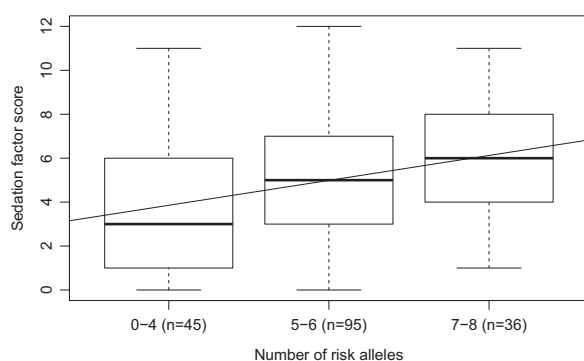
Figure 2 Boxplot and regression line of sedation factor score vs. number of risk alleles in the SNPs included in the GRS.

associated with sedation in the GLM including age, BMI and gender as explanatory variables. The alternative GRS derived from GLM analyses included three SNPs (recessive *HNMT* rs2737385, additive *HRH1* rs1552498, dominant *HRH1* rs17034063) that were also in the GRS with inclusion of *AOC1*. This alternative, weighted GRS was also associated with sedation ($p = 3.13 \times 10^{-6}$) in the GLM ($R^2 = 0.21$, adjusted $R^2 = 0.19$) with age, gender and BMI as other explanatory variables. In the permutation test, the p-value of the GRS ranked 730 out of 10000 p-values ($p = 0.073$). Hence, the effect of the *HNMT* polymorphism and the two *HRH1* polymorphisms remained, even if *AOC1* was removed from the models. The alternative GRS analysis based on the logistic regression found the same three SNPs as the earlier model (dominant *HRH1* rs1552498, dominant *HDC* rs2853766 and additive *HRH4* rs1421125), but the p-value after permutation was closer to statistical significance ($p = 0.072$). This GRS explained sedation ($p = 5.3 \times 10^{-5}$) in a GLM model together with BMI, age and gender.

4. Discussion

Polymorphisms in genes encoding histamine receptors or enzymes related to histamine metabolism and production have not previously been studied in relation to sedation. The main finding is that using the GLM adjusted for age, gender and BMI, eight significantly associated SNPs, after FDR-adjustment, were found. In the GRS analysis, a GLM including age, gender, BMI and a score of four SNPs explained more than 20% of variability in sedation levels reported by clozapine-treated patients. The permutation test of this result remained marginally over the threshold of statistical significance ($p = 0.066$). Permutation test was performed to reduce the chance of type I error. Allelic associations of two *AOC1* SNPs to sedation were also found but these findings were not adjusted for multiple testing and their effects were small (see Supplementary material). These results suggest that variability in sedation levels may be partially caused by functional variation in the enzymes regulating histamine and possible changes in the H$_1$ receptor protein structure.

Pharmacological studies link brain histamine with homeostatic brain functions, such as the control of biological rhythms. Histamine levels are higher during active periods, lower during sleep and show a clear circadian rhythm. HDC, HNMT and DAO are rate limiting enzymes in histamine metabolism that participate in the homeostasis of histamine levels (Haas et al., 2008). Disturbances in the functioning of these enzymes could affect histamine homeostasis; for example, it has been shown that the circadian rhythm of *HDC*-knockout mice is disturbed (Abe et al., 2004). A functional SNP in the *HNMT* gene (rs11558538), resulting in a threonine to isoleucine substitution (Thr105Ile), was previously shown to lower HNMT activity and affect its thermal stability (Preuss et al., 1998). This SNP was not included in the GRS, but the *HNMT* rs2737385, included in the GRS, is in 0.58 r^2 LD and in same haploblock with the aforementioned SNP (rs11558538). SNPs in the *AOC1* gene that increase the risk of reduced serum DAO activity have also been found (Maintz et al., 2011). One of these SNPs (rs2052129) is in 0.31 r^2 LD with the *AOC1* rs6977381 included in the GRS and are also both located in the same

haploblock (LD plots in the Supplementary material). The variation in sedation may be explained by polymorphisms in *HNMT* and *AOC1* genes that affect histamine homeostasis in the brain by increasing the activity of the HNMT and DAO enzymes and resulting in decreased histamine levels.

The sedative properties of clozapine are affiliated with its effects as an H_1 receptor inverse agonist and H_3 receptor agonist. As histamine in the CNS seems to promote alertness and wakefulness, the effect of clozapine on histamine receptors may modulate this influence in the CNS. H_1 receptor binding of clozapine was reported to upregulate H_1 receptors *in vivo* (Humbert-Claude et al., 2012). The *HRH1* SNPs in the GRS that were associated with sedation have not previously been reported, so their possible effects on H_1 receptor protein structure are unknown. *HRH1* rs1552498 and rs17034063 are both intron variants. If the H_1 receptor protein structure was changed, the partial inverse agonistic effect of clozapine on these receptors could alter histamine neurotransmission.

DAO activity in the brain is thought to be low under normal conditions (Haas et al., 2008). *DAO* mRNA expression has been detected in brain tissue samples from mice and pigs but no DAO activity was detected (Kitanaka et al., 2002; Klocker et al., 2005). A vectorial transport system from the brain to the vasculature may help to drain neuronal histamine after excessive surges (Haas et al., 2008) so the amino acid substitution caused by *DAO* polymorphism may affect the metabolism of circulating histamine (García-Martín et al., 2015). However, it remains unclear if this mechanism would affect the histamine homeostasis in the CNS. Therefore, an alternative post hoc analysis was performed that excluded the *AOC1* SNPs and allowed an alternative GRS to be calculated. The alternative GLM analysis with age, BMI, gender, individual SNPs or the GRS as explanatory variables for sedation revealed that the same eight *HNMT* SNPs were associated with sedation. The alternative GRS included three SNPs (recessive *HNMT* rs2737385, additive *HRH1* rs1552498, dominant *HRH1* rs17034063) that were also in the GRS with inclusion of *AOC1*. This alternative, weighted GRS, without *AOC1* SNPs, was also associated with sedation but to a lesser degree. Thus, *AOC1* SNPs and therefore DAO enzyme may contribute to sedation in clozapine-treated patients. The alternative GRS analysis also found a trend level association of a GRS with three SNPs (dominant *HRH1* rs1552498, dominant *HDC* rs2853766 and additive *HRH4* rs1421125) with dichotomized sedation. This finding should be interpreted cautiously as the permutation test p-value was closer to significance level only in the post hoc analysis, not in the analysis including the AOC1 gene data.

Similar statistical approach was used in our earlier report, which included the same patient sample, on genes related with serum neuropeptide Y levels (Klemettilä et al., 2017). The statistical power to detect small or moderate effects, such as the effect of individual SNPS, is limited with relatively small study populations. However, the GRS was normally distributed and the well-defined hypothesis driven testing, of only the selected genes, reduced the need for a larger population. The sample size is too small for genome wide testing, for which it was necessary to reduce the number of SNPs and focus on the histaminergic system on this study. The individual eight linked SNPs that remained significant after adjustment in the GLM analysis all had skewed genotype distributions (8 vs. 168). This particular result should therefore be interpreted cautiously. The

lack of a comparable sedation-assessed control group in this study is a major limitation. However, no other study, with such a large population of clozapine-treated patients, has assessed the associated adverse sedative effects. Thus, confirming the effect of the score in a reference sample against other studies was not possible, so the permutation test was performed, which we consider a valid method based on its properties to generate an empirical distribution and an estimated empirical p-value (Hu et al., 2013). As the permutation test resulted in a significance level marginally above the set level of significance, the association between sedation and GRS should be interpreted as debatable. To study all the most likely ways for biological traits to be inherited, we saw it necessary to include three models: additive, dominant and recessive models. The modes of inheritance differed between the SNPs in the GRSs, which suggests that the genetic effect on sedation is cumulative originating from different mechanisms. In this study FDR (False Discovery Rate) was used in the adjustment of statistical significance. FDR also takes into account LD between SNPs in the correction of *p*-values in multiple testing. As this is a hypothesis based study and our sample size is quite low, we wanted to use less strict multiple test correction method (FDR) to find sufficient number of variants to construct a genetic risk score. After all, the statistical significance of the genetic risk score itself was evaluated using permutation which may be considered as the golden standard of multiple test correction.

Regulation of the sleep-wake system in tuberomammillary nucleus neurons involves many neurotransmitter systems other than histaminergic, such as noradrenergic, serotonergic, cholinergic, hypocretin and GABAergic signaling (Panula and Nuutinen, 2013). Given the complexity of the sleep-wake regulatory system, the findings of this study may be linked to other polymorphisms, not identified here, that may further explain the variability in patient sedation levels.

Analyses in this study were based on self-rated symptoms of patients. Objective assessments of sedation levels, such as the amount of sleep during the day, were not available. LUNSERS is a reliable and validated method of measuring adverse effects associated with antipsychotic treatment (Lambert et al., 2003). The separate assessment of sedation, sleepiness or fatigue using LUNSERS may not be a validated method, but the self-rating scales are commonly used to assess sedation. Sedation can be a symptom of the disorder or a specific adverse effect of the medication in schizophrenia patients. Some patients may have suffered from comorbid depression, which is commonly associated with schizophrenia (Bosanac and Castle, 2013) and can cause fatigue. The GLM, with age, gender and the GRS, explained approximately one fifth of the variation in sedation levels. The remaining variation could be linked to other neurotransmitter systems or phenotypes, such as depression. The sedation levels were greater in young and overweight patients. In addition to sedation, brain histamine also contributes to control of body weight and energy metabolism (Haas et al., 2008) and H_1 receptor antagonism has been identified as a major cause of clozapine related obesity (He et al., 2013). Therefore, similar mechanisms may be involved in causing both sedation and weight gain in these patients. While H_1 receptors seem to be linked to the mechanism of antipsychotic induced weight gain, H_1 and H_3 receptor polymorphisms do not explain the variance in weight gain according to a recent study (Tiwari et al., 2016). In this study, it was not possible to examine whether patients experienced more

sedation at the beginning of the treatment, since most patients had been taking clozapine for over 1 year. Thus, these results apply only to patients on maintenance clozapine treatment. However, other medications used by the patients or the doses of clozapine did not explain the variability in sedation levels of patients.

In conclusion, the model with age, gender, BMI and four SNPs, which are involved in histaminergic neurotransmission, could explain approximately one fifth of sedation experienced by clozapine-treated patients.

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Contributors

Niko Seppälä, Olli Kampman and Esa Leinonen designed the study and wrote the protocol. Niko Seppälä and Merja Viikki have participated in patient recruitment and clinical interviews. Anssi Solismaa managed the literature searches and analyses and wrote the first draft of the manuscript. Terho Lehtimäki and Nina Mononen performed the genotyping. Leo-Pekka Lyytikäinen was responsible of genotyping data management and quality control. Anssi Solismaa, Leo-Pekka Lyytikäinen and Olli Kampman undertook the statistical analysis. All authors contributed to and have approved the final manuscript.

Conflicts of interest

Olli Kampman has consulted for Medivir, received speaker's fees from Janssen and received support from Otsuka and Lundbeck to participate in an international congress.

Merja Viikki has consulted for Astra Zeneca, GlaxoSmithKline, Eli Lilly and Servier; conducted clinical trials for Bristol-Myers Squibb, Eli Lilly, Lundbeck, Minerva Neurosciences, Sanofi-Aventis and Servier; received support from Bristol-Myers Squibb, GlaxoSmithKline, Eli Lilly, Lundbeck and Servier to participate in international congresses; and received a research grant from GlaxoSmithKline, Lundbeck and Pfizer.

Niko Seppälä has received speaker's fees from Orion and Astra Zeneca and received support from Janssen to participate in an international congress.

Esa Leinonen has worked as a lecturer or chairman in symposia sponsored by pharmaceutical companies Astra Zeneca, Eli Lilly, Lundbeck and Servier; served on the national advisory board of Servier and received support from Astra Zeneca, Lundbeck, Otsuka and Servier to participate in international congresses.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2017.03.009.

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ORIGINAL CONTRIBUTION

Genetic Polymorphisms Associated With Constipation and Anticholinergic Symptoms in Patients Receiving Clozapine

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Abstract:
Background: Clozapine impairs gastrointestinal motility owing to its anticholinergic and antiserotonergic properties. This commonly leads to constipation and potentially to more severe complications such as bowel obstruction and ischemia. The aim of this study was to determine whether genetic variations in the genes encoding muscarinic and serotonergic receptors (*CHRM2*, *CHRM3*, *HTR2*, *HTR3*, *HTR4*, and *HTR7*) explain the variations in incidence of constipation and anticholinergic symptoms during clozapine treatment. Genes associated with opiate-induced constipation were also included in this analysis (*TPH1*, *OPRM1*, *ABCB1*, and *COMT*).
Procedures: Blood samples from 176 clozapine-treated, Finnish, white patients with schizophrenia were genotyped. Constipation and anticholinergic symptoms were rated using the Liverpool University Neuroleptic Side Effect Rating Scale self-report questionnaire. In total, 192 single-nucleotide polymorphisms (SNPs) were detected and grouped to formulate a weighted genetic-risk score (GRS).
Results: No significant associations between individual SNPs or GRSs and constipation or laxative use were observed. A GRS of 19 SNPs in *CHRM2*, *CHRM3*, *HTR3C*, *HTR7*, *ABCB1*, *OPRM1*, and *TPH1* was associated with anticholinergic symptoms in a generalized linear univariate model, with body mass index, clozapine monotherapy, and GRS as explaining variables (permuted $P = 0.014$). Generalized linear univariate model analysis performed on the opiate-induced constipation–associated SNPs and a single *CHRM3* SNP revealed an association between anticholinergic symptoms and a score of 8 SNPs (adjusted $P = 0.038$, permuted $P = 0.002$).
Conclusions: Two GRSs are able to predict the risk of anticholinergic symptoms in patients receiving clozapine and possibly an increased risk of gastrointestinal hypomotility.

Key Words: adverse effects, antipsychotics, constipation, clozapine, polymorphism

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Constipation is a common adverse effect of clozapine that is reported to occur in 14% to 60% of patients receiving treatment with this drug,[1,2] often leading to increased use of laxative medication.[3] The impairment of gastrointestinal motility associated with clozapine can also result in severe complications such as dysphagia, ileus, intestinal obstruction, bowel ischemia, and megacolon. Among

clinicians, these risks seem to be less well known than clozapine-induced agranulocytosis, despite clozapine-induced gastrointestinal hypomotility and agranulocytosis having similar prevalence rates (4.0%–8.0% vs 3.8%–8.0%). Furthermore, clozapine-induced gastrointestinal hypomotility is associated with a greater risk of mortality, and careful monitoring of patients receiving clozapine for symptoms of gastrointestinal hypomotility is recommended.[4,5]

Clozapine-induced constipation seems to be associated with the anticholinergic and antiserotonergic properties of this agent.[5,6] In the absence of clozapine, acetylcholine stimulates muscarinic receptors in both smooth muscle cells and the interstitial cells of Cajal of the gastrointestinal tract, the latter of which function as the pacemaker cells of the gastrointestinal tract.[7] Muscarinic receptors (of the M_2 and M_3 subtypes) are expressed in interstitial cells of Cajal and smooth muscle cells of the gastrointestinal tract.[8] However, other anticholinergic agents with an efficacy similar to that of clozapine have much lower risks of gastrointestinal hypomotility. For example, the risk of constipation associated with use of clozapine is 3 times greater than that associated with chlorpromazine.[9] Treatment with clozapine is also associated with a higher risk of ileus than that of other antipsychotics, as demonstrated by data from a large European study.[10] This increase in risk is proposed to be caused by the antiserotonergic effects of clozapine.[5] Clozapine is an antagonist of several 5-hydroxytryptamine receptors (5-HT$_2$, 5-HT$_3$, 5-HT$_6$, and 5-HT$_7$).[11] Serotonin also has a complex and crucial but still controversial role in gut function. Data from many studies have suggested that 5-HT receptors, especially 5-HT$_3$ and 5-HT$_4$, have an important role in the regulation of gut motility.[5,12] 5-HT$_2$ receptors have been suggested to modulate visceral sensation, and 5-HT$_7$ receptors might be involved in mediating relaxation of gastrointestinal smooth muscle. Furthermore, inhibition of 5-HT$_3$ receptors has been reported to reduce the rate of colon transit, inhibit gastrocolic reflexes, increase the level of colonic compliance, and possibly reduce intestinal sensitivity to distension.[5]

The aim of this study was to determine the influence of genetic variations in different subtypes of muscarinic and serotonergic receptors and whether such variations can explain the variable incidence of constipation or anticholinergic symptoms during clozapine treatment. Interactions between such variations and the risk of constipation or anticholinergic symptoms caused by antipsychotic medications have not been studied previously. All muscarinic and serotonergic subtypes that have been previously suggested to have a role in regulating gut motility were included in this study. Single-nucleotide polymorphisms (SNPs) in the genes encoding the M_2 and M_3 muscarinic receptors (*CHRM2*, *CHRM3*) and the 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors (HTR2, HTR3, HTR4, and HTR7) were extracted. Additional analyses were performed, including SNPs in *TPH1*, *OPRM1*, *ABCB1*, *CHRM3*, and *COMT*, which have all previously been suggested to contribute to the variability in incidence of constipation among patients with cancer receiving treatment with opioids.[13] *TPH1* encodes tryptophan

5-hydroxylase 1, which catalyzes the first and rate-limiting step in the biosynthesis of serotonin.[14] TPH1 transcript levels are increased in patients with chronic constipation.[15] *OPRM1* encodes the mu-type opioid receptor. *ABCB1* encodes multidrug resistance protein 1, a protein that transports various molecules across extracellular and intracellular membranes. Furthermore, *ABCB1* is widely expressed in various tissues, including the bowel, blood-brain barrier, liver, and kidneys.[16] *COMT* encodes catechol *O*-methyltransferase, an enzyme that catalyzes the degradation of catecholamines such as dopamine, epinephrine, and norepinephrine.[14]

MATERIALS AND METHODS

Patients

The sample was screened from 256 patients, of which 19 declined to participate in this study. Samples were collected from 3 hospital districts in western Finland (Satakunta, Pirkanmaa, and Seinäjoki). Inclusion criteria included a current F2-group diagnosis, according to the *International Classification of Diseases, 10th Revision*, and a stabilized clozapine treatment. Patients with organic brain diseases were excluded from the analysis. The study population comprised 237 patients receiving clozapine (136 men and 101 women, with a mean age 42.5 ± 11.0 years) who were diagnosed with either schizophrenia (n = 223 [94.1%]), schizoaffective disorder, or delusional disorder. The mean elapsed time from first hospitalization owing to a psychotic episode was 17.3 years (±10.0 years). All patients were adults (≥18 years of age), white, and of Finnish origin. Blood samples were obtained from 190 patients, of which 176 genotyped patients were eligible to remain in the study after quality controls. Patients gave written, informed consent prior to participation. The study was approved by the Ethics Committee of Satakunta Hospital District on April 23, 2008, and was conducted in accordance with the tenets of the Declaration of Helsinki. More detailed information on the data collection process and the clinical and demographic characteristics of the study population is provided in our previous studies.[17,18]

Of the 176 patients whose samples were genotyped, 57 (32.4%) were receiving a combination of at least 2 antipsychotics, including clozapine, and 119 patients (67.6%) were receiving clozapine monotherapy. All doses of antipsychotic medication were converted to chlorpromazine equivalents.[19] The mean daily dose of clozapine received was 403 (SD, 152)mg or 806 (SD, 304) mg expressed as a chlorpromazine equivalent dose. The mean total antipsychotic dose received was 912 (SD, 304) mg as a chlorpromazine equivalent dose. More than half of all patients had received clozapine treatment for more than 5 years and none for less than 3 months at the time of study enrollment. The demographics of the genotyped patients are presented in Table 1. Eleven patients were also receiving anticholinergic medications, of which 5 were for the control of extrapyramidal symptoms (biperiden or similar), and 6 were for bladder functional disorders (oxybutynin or trospium chloride).

Patients completed the Liverpool University Neuroleptic Side Effect Rating Scale (LUNSERS),[20] a self-assessment questionnaire that consists of a list of 51 symptoms. Patients were asked to rate the severity of each listed adverse effect on a scale from 0 to 4 (0 = not at all, 1 = very little, 2 = a little, 3 = quite a lot, and 4 = very much). Question 10 "constipation" was used as a dichotomized variable with 2 groups: those with no constipation at all (LUNSERS score 0, n = 49) versus those who reported very little to very much constipation (LUNSERS score 1–4, n = 127). A more-specific dichotomized variable was also used as a response variable, which included groups of patients who reported

having quite a lot to very much constipation (LUNSERS score 3–4) or were using laxative medications (n = 95), versus those who reported having no constipation at all to a little constipation and were not receiving laxative medications (n = 81). To study patients' anticholinergic symptoms, a factor was formed by summing the severity of the following 5 items from LUNSERS: "dry mouth", "constipation", "palpitations", "difficulty of passing water," and "blurred vision."

DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Blood Midikit and an automated biorobot M48 extraction (Qiagen, Hilden, Germany). Samples were genotyped using an Illumina Infinium HumanCoreExome-12 DNA Analysis Beadchip, version 1.0., according to the manufacturer's instructions at Helmholtz Zentrum, München, Germany. The following quality control filters were applied: GenCall score <0.15, GenTrain score less than 0.20, sample and an SNP call rate less than 0.95, Hardy-Weinberg equation *P* value less than 10^{-6}, excess heterozygosity, cryptic relatedness (pi-hat >0.2), gender check, and multidimensional scaling. After quality control, 176 samples and 531,983 SNPs were available, of which 192 SNPs were in the genes of interest (*CHRM2, CHRM3, HTR2, HTR3, HTR4, HTR7, TPH1, OPRM1, ABCB1*, and *COMT*). Single-nucleotide polymorphisms with a minor allele frequency of less than 0.01 were excluded from the study.

Statistical Methods

Values for comparisons between each group are presented as mean ± SD of the mean, unless otherwise stated. Pearson correlations, χ^2 tests, and *t* tests were used in exploratory analyses of the relationships between constipation, use of laxatives and anticholinergic factor, and potential explanatory variables such as use of clozapine dose, clozapine and its metabolite norclozapine (*N*-desmethylclozapine) concentrations, body mass index (BMI), smoking status, age, and use of benzodiazepines or selective serotonin reuptake inhibitors (SSRIs). Reliability analysis for anticholinergic factor was performed using a principal components analysis for ordinal data, using a 1-component solution in which all 5 items were loaded (>0.5). The Cronbach α value was 0.60. Generalized linear univariate models (GLMs) were used to analyze the explanatory factors: SNPs encoded separately with additive, dominant, and recessive coding and covariates for response variables. The response variables analyzed with GLM were dichotomized constipation (binominal distribution), dichotomized constipation taking laxative use into account (binominal distribution), and the anticholinergic factor (Gaussian distribution). Covariates in the models were chosen primarily according to literature and secondarily according to the results of exploratory bivariate analyses. Finally, the best fitting model was used. In the GLM analysis, the SNPs were analyzed by entering them into the model one at a time. To adjust significance levels for multiple testing, the false discovery rate (FDR) was used. This accounts for the number of SNPs and different modes of inheritance in the GLM. Single-nucleotide polymorphisms were coded with additive, dominant, and recessive models. Details of the coding are described in our previous study.[18] The statistical significance level was set at *P* < 0.05.

Statistical analyses were performed using R statistical analysis software (version 3.1.2, 2014; The R Foundation for Statistical Computing, Vienna, Austria), IBM SPSS Statistics for Windows (version 22.0, IBM Corp, Armonk, NY), gPLINK (version 2.050, Boston, MA), PLINK (version 1.07, Boston, MA), and Haploview analysis software (version 4.2, Cambridge, MA).

TABLE 1. Demographics of the Genotyped Patients (n = 176)

Age, mean (SD), y	43.5 (10.9)
Sex, n (%)	
Men	104 (59.1)
Women	72 (40.9)
BMI, mean (SD), kg/m^2	29.8 (6.38)
Clozapine treatment duration, n (%)	
3 mo to 1 y	4 (2.3)
1–5 y	51 (29.0)
>5 y	106 (60.2)
Unknown	15 (8.5)
Sum of clozapine and norclozapine concentrations, mean (SD), μmol/L	2.40 (1.28)
Regular smoking, n (%)	
Nonsmoking	85 (48.3)
Smoking	89 (50.6)
Unknown	2 (1.1)
Laxative in use, n (%)	46 (26.1)
Quite a lot or very much constipation or laxative in use, n (%)	81 (46.0)

Genetic Risk Score

Genetic-risk scores (GRSs) were calculated for each patient as a weighted sum of the risk alleles in relation to the studied phenotype. Single-nucleotide polymorphisms that had an unadjusted level of statistical significance ($P < 0.05$) in the GLM models were selected for further analysis. These SNPs were inserted into the same model with the other coefficients, and a stepwise analysis was performed using Akaike information criteria (AIC).[21] Akaike information criteria are a method of model-based genetic mapping.[22] Data obtained using AIC are used to identify the influential tag SNPs of the SNPs preselected with GLM by also taking into account the extent of linkage disequilibrium (LD) between the SNPs. Single-nucleotide polymorphisms that remained significant, as indicated by AIC, were used in the GRS. The model returned by AIC was analyzed with stepwise variance inflation factor to examine collinearity between the SNPs in the GRS. The corresponding β estimate value from the GLM for each SNP was used to adjust the weighting of individual effects. The formed GRS was then used as an explanatory variable in the final GLM, together with other explanatory variables.

Permutation Test

Linear combinations of genetic effects estimated using GLMs might overfit data and cause inflated type I errors. An estimation of the null distribution of the GRS test statistics is required to validate the GRS results.[23] A permutation test was performed, in which the patient identification numbers from the phenotype data were sampled. This resulted in SNP genotypes that were independent of the studied phenotypes. The permutation was conducted 999 times using the same statistical methods as described previously but with sampled data. P values were collected for GRSs from each model in each sampled data set. The result was considered statistically significant if the P value derived from the actual data analysis was less than 5% of the P values derived from the permuted data.

RESULTS

In exploratory analyses, patients in the group who had more severe constipation or used laxatives were significantly older compared with those who had less severe constipation and required no laxatives (41.34 ± 10.6 vs 44.52 ± 11.6 years of age, t test

$P = 0.035$). No statistically significant associations were observed using analyses of the correlations between dichotomized constipation or anticholinergic factor and age, BMI, smoking status, sex, clozapine dose, or clozapine and norclozapine concentrations. Use of laxatives was associated with BMI (30.4 ± 6.1 vs 28.2 ± 6.3 kg/m^2, t test $P = 0.032$), with patients with a lower BMI more likely to be using laxatives. Use of laxatives was also associated with use of benzodiazepines (χ^2 test, $P = 0.020$), with patients with benzodiazepine medication also more likely to be using laxatives. Anticholinergic factor was also associated with use of benzodiazepines (4.45 ± 3.40 vs 5.38 ± 3.36, t test $P = 0.042$) and with use of SSRIs (4.59 ± 3.49 vs 5.70 ± 3.13, t test $P = 0.015$). No correlation between anticholinergic medication use and anticholinergic factor was observed ($r = 0.063$). Dichotomized constipation was not associated with use of benzodiazepines or SSRIs.

In the GLM analyses, with constipation or use of laxative as the dependent variable, SNPs in *CHRM2*, *CHRM3*, *HTR2*, *HTR3*, *HTR4*, and *HTR7* with or without the inclusion of additional SNPs in *TPH1*, *OPRM1*, *ABCB1*, and *COMT*, no statistically significant associations were observed level in any model with varying combinations of additional explaining variables (age, BMI, sex, and use of benzodiazepines or SSRIs). With anticholinergic factor as the dependent variable and BMI and SNPs as the explaining variables, following analysis of SNPs in *CHRM2*, *CHRM3*, *HTR2*, *HTR3*, *HTR4*, and *HTR7*, only rs685548 remained statistically significant after FDR adjustment (dominant coding $P = 0.038$, additive coding $P = 0.044$). However, this association became statistically insignificant following FDR adjustment when SNPs associated with opiate-induced constipation were added to this analysis (dominant coding $P = 0.060$, additive coding $P = 0.071$).

In the GRS analysis, no significant associations between GRS and constipation or use of laxatives were observed. When only SNPs in *CHRM2*, *CHRM3*, *HTR2*, *HTR3*, *HTR4*, and *HTR7* were analyzed, associations between the formed GRS and the anticholinergic factor remained statistically insignificant after permutation. When SNPs in genes associated with opiate-induced constipation (*ABCB1*, *OPRM1*, *TPH1*, and *COMT*) were added to the analysis, a score of 19 SNPs in *CHRM2*, *CHRM3*, *HTR3C*, *HTR7*, *ABCB1*, *OPRM1*, and *TPH1* (Table 2) was found to be associated with anticholinergic factor in a GLM model with BMI and GRS as explaining variables ($P = 0.017$). Generalized

TABLE 2. Results (Test Score, β Coefficients, Significance Levels, and Permutation Rankings) of 2 General Linear Models With Genetic Risk Scores, Consisting of Listed SNPs, Explaining the Risks of Anticholinergic Adverse Effects Summarized as "Anticholinergic Factor," Including BMI and Clozapine Monotherapy as Confounding Factors

Genes Selected for GRS Analysis	Formed GRS	BMI			Clozapine Monotherapy			GRS			Adjusted R^2	Permutation Ranking
		t	β	*P*	*t*	β	*P*	*t*	β	*P*		
CHRM2	CHRM2 ADD rs10228048	3.3	0.20	0.00045	−3.9	−1.7	0.00015	10.1	0.65	$<2 \times 10^{-16}$	0.43	14th of 1000 permutations ($P = 0.014$)
CHRM3	CHRM2 DOM rs12535371											
HTR2	CHRM3 DOM rs685548											
HTR3	CHRM3 REC rs11577797											
HTR4	CHRM3 ADD rs6691263											
HTR7	CHRM3 REC rs10925910											
ABCB1	CHRM3 DOM rs2841037											
OPRM1	HTR4 REC rs7723153											
TPH1	HTR7 DOM rs7074715											
COMT	HTR7 ADD rs7074715											
	ABCB1 ADD rs10276036											
	ABCB1 REC rs2235023											
	ABCB1 REC rs9282564											
	ABCB1 DOM rs10260862											
	ABCB1 DOM rs10248420											
	ABCB1 ADD rs2032582											
	ABCB1 ADD rs3789243											
	OPRM1 REC rs2272381											
	TPH1 ADD rs10832876											
CHRM3 ADD rs685548 (single SNP)	CHRM3 ADD rs685548	1.9	0.067	0.056	−3.8	−1.8	0.00018	8.6	0.79	6.1×10^{-15}	0.36	2nd of 1000 permutations ($P = 0.002$)
	OPRM1 REC rs2272381											
	OPRM1 REC rs34427887											
ABCB1	ABCB1 ADD rs1002204											
OPRM1	ABCB1 ADD rs10260862											
TPH1	ABCB1 REC rs2235023											
COMT	ABCB1 REC rs9282564											
TPH1 DOM rs10832876												

ADD indicates additive coding; DOM, dominant coding; REC, recessive coding.

TABLE 3. SNPs in the 2 GRSs Explaining Anticholinergic Symptoms, Including SNPs With a Level of LD of Greater Than 0.8 r^2, Annotations of the SNPs, and the Number of Items Referring to These SNPs in the PubMed Database

Gene	SNP	SNPs in >0.8 LD (r^2)	Annotation	No. Citations of the SNP in Pubmed
CHRM2	rs10228048	—	Intron	—
	rs12535371		Intron, LOC349160 (ncRNA)	—
		rs1364405 (0.83)	Intron	—
CHRM3	rs685548		Intron	—
		rs497576 (0.80)	Intron	—
	rs11577797		Intron	—
		rs6678395 (0.98)	Intron	—
	rs6691263		Intron	—
		rs12090480 (0.86)	Intron	—
	rs10925910	—	Intron	—
	rs2841037		Intron	—
		rs626694 (1)	Intron	—
HTR4	rs7723153		Intron	—
HTR7	rs7074715	—	Intron	—
ABCB1	rs10276036	—	Intron	10
	rs2235023	—	Intron	3
	rs9282564	—	Missense, frameshift	22
	rs10260862	—	Intron	2
	rs10248420	—	Intron	9
	rs2032582	—	Missense	237
	rs3789243	—	Intron	27
	rs1002204		Intron	1
		rs1045642 (0.91)	cds-synon	365
OPRM1	rs2272381	—	Intron	2
	rs34427887	—	STOP-GAIN	—
TPH1	rs10832876		Intron	—
		rs7943884 (0.85)	<5000 base pairs before gene reading region, no annotation	—

linear univariate model analysis performed only on SNPs associated with opiate-induced constipation and a single SNP in *CHRM3* that was associated with anticholinergic factor resulted in a score of 7 SNPs ($P = 0.0023$, Table 2). In both models, no significant collinearity was observed between the SNPs or other predictor variables (variance inflation factor <10).

DISCUSSION

To the best of our knowledge, no previous study has examined the association between clozapine-induced gastric hypomotility or anticholinergic symptoms related to antipsychotic medication and genetic polymorphisms. The main findings of this study are the 2 GRS (Table 2) and 1 single SNP in *CHRM3* (rs685548) that are associated with anticholinergic symptoms in patients with schizophrenia who are receiving clozapine. This effect is a result of SNPs in various genes, including genes linked with cholinergic (*CHRM2* and *CHRM3*) and serotonergic neurotransmission (*HTR3C*, *HTR7*, and *TPH1*), but also with genes associated with opiate-induced constipation (*ABCB1* and *OPRM1*). This finding might reflect individual genetic variations in response to clozapine or in the functionality of the autonomic nervous system. No statistically significant associations between SNPs in serotonergic or muscarinic receptors or SNPs in genes related to opiate-induced constipation and constipation or laxative use were observed in this study.

The model with the 19-SNP score, BMI, and clozapine monotherapy explained 43% of the variability in anticholinergic symptoms in clozapine-treated patients. The effect was confirmed using a permutation test ($P = 0.014$), but the high adjusted R^2 value and very low P value in the GLM ($<2 \times 10^{-16}$) might contain a type I error owing to overfitting in the GRS analysis. The rs685548 SNP located in *CHRM3* is an intron variant with a minor allele frequency of 0.36, with no other SNPs with a level of LD of greater than 0.8 r^2 present in this cohort (Table 3). No significant associations between any phenotype and *CHRM3* rs685548 have been reported previously. Furthermore, no items were found in the PubMed database describing any of the SNPs included in the GRSs or SNPs with a level of LD greater than 0.8 r^2. Single-nucleotide polymorphisms in *ABCB1* have been widely investigated. Single-nucleotide polymorphisms in this gene have been studied in relation to patient's responses to clozapine and plasma clozapine levels[24,25] but not in relation to anticholinergic symptoms. The current study cannot propose a single mechanism explaining the increased risk of anticholinergic symptoms during clozapine treatment, but rather suggests that the genetic effect is cumulative, owing to alterations in muscarinic and serotonergic receptor function (in *CHRM2*, *CHRM3*, *HTR3C*, and *HTR7*), the serotonin-related enzyme *TPH1*, and in *ABCB1* and *OPRM1* function. rs1045642 in *ABCB1*, which was also included in the GRS, might affect the absorption of clozapine from the gut, as previously reported[24]; however, in this study, no significant

association between serum clozapine and norclozapine concentration and anticholinergic symptoms was observed. The variability in anticholinergic symptoms might also be related to the functions of ABCB1 in other tissues. ABCB1 also has an important functional role in maintaining the blood-brain barrier and blood–cerebral spinal fluid barrier.[16]

As Palmer et al[5] state clozapine-induced gastric hypomotility is underrecognized and potentially life-threatening adverse effect of clozapine treatment. Objective evidence of gastrointestinal hypomotility in clozapine-treated patients has been shown by using a colonic transit test.[26] Recent study reported that median colonic transit times are more than 4 times longer than those on other antipsychotics or healthy controls, and four fifths of patients using clozapine had colonic hypomotility.[27] Furthermore, older age, female sex, and treatment with clozapine, tricyclic antidepressants, anticholinergics, and opioids are reported to be associated with an increased risk of ileus.[28] Patients with a greater incidence of anticholinergic symptoms could have an elevated risk of gastric hypomotility and/or bowel complications such as ileus. Thus, the GRSs reported in this study could also be used as markers for the risk of these symptoms. The genes investigated in this study were originally selected based on the hypothesized pharmacological effects of clozapine on the muscarinic and serotonergic receptors that regulate gut motility.

Many confounding factors could not be taken into account by this study, such as variations in diet or amount of exercise, although these can both be expected to influence BMI. In this study cohort, age and sex were both correlated with BMI, which highlights the importance of including BMI in the models used. A total of 192 SNPs were selected for analysis. The ability to detect small or moderate effects associated with these SNPs is limited by the relatively small sample size of the study cohort; however, the well-defined hypothesis and focus only on specific genes reduce the need for a larger sample. A similar statistical approach was used in 3 of our earlier reports, which included the same patient sample.[18,29,30] Limitations of this study include the reliance upon self-assessment of the severity of constipation and anticholinergic symptoms by each patient using the LUNSERS questionnaire. No information on patients' bowel movement frequencies or stool consistencies, with the help of stool charts, for example, was available. LUNSERS is, nevertheless, a reliable and validated method of measuring the adverse effects of antipsychotic treatments.[31] Another limitation is that anticholinergic factor, as used to examine the severity of anticholinergic symptoms, is a sum variable with only a moderate level of internal consistency (Cronbach α = 0.6). However, the symptoms included in the anticholinergic factor (dry mouth, constipation, palpitations, difficulty of passing water, and blurred vision) are all commonly recognized anticholinergic symptoms.[32]

In conclusion, this study suggests that the risk of anticholinergic symptoms during clozapine treatment is associated with a GRS consisting of SNPs in *CHRM2*, *CHRM3*, *HTR3C*, *HTR7*, *TPH1*, *ABCB1*, and *OPRM1* genes, in addition to rs685548 in *CHRM3*. No significant associations between the genes studied in this analysis and constipation or laxative use were found.

AUTHOR DISCLOSURE INFORMATION

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