THE LONG GAME

Towards a better understanding of molecular and genetic aspects of clozapine

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Jytte de With

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The Long Game

Towards a better understanding of molecular and genetic aspects of clozapine

Onderweg naar een beter begrip van de moleculaire en genetische aspecten van clozapine
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 14 mei 2019 des ochtends te 10.30 uur

door

Sera Anne Jytte de With
geboren op 30 december 1985 te Marum

Promotoren: Prof. dr. ir. R.A. Ophoff
Prof. dr. W.W. van Solinge
Prof. dr. R.S. Kahn

“The way to get started is to quit talking and begin doing”
Walt Disney

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Chapter I

Introduction

Paul[1], 36 years, diagnosed with schizophrenia when he was 18:

"Imagine, all of a sudden, ordinary things you see and hear have a special meaning to you, making you believe you are being followed by the FBI or that thoughts are being implanted in your head. That happened to me; during these times I was often scared and confused. I took different kinds of antipsychotics but they did not work. The last drug I tried made me gain ten pounds within ten weeks, but the voices finally faded. Since a couple of months things are back to normal and I have been feeling good. I talked to my psychiatrist about stopping with the drug, since I don't like the blood draws every month and I want to lose weight. She was against it, but I think I will try it anyway."

This example of the difficulties treating psychosis is not an exception, unfortunately. Antipsychotic drugs are successful in 50-70% of patients and it is impossible beforehand to know which drug is going to work for which patient, or which adverse effects will occur. This thesis aims to expand our knowledge on the genomic and molecular effects of clozapine; the most effective antipsychotic drug in history, in the hopes to facilitate targeted or personalized treatment in the years to come.

"A short history…"

Doctors, shamans and priests have observed and reported psychotic symptoms or psychosis for centuries. There is even evidence from ancient Egypt of people experiencing psychotic symptoms. It would take until the turn of the 19th century, for a new way of thinking regarding mental illness, with more attention paid to the biological background of psychiatric symptoms and disorders.

[1] Fictive patient derived from my own experiences and experiences of colleagues.

Textbox 1. What is schizophrenia?

- Schizophrenia (SCZ) is a neuropsychiatric disorder characterized by hallucinations, delusions, negative symptoms (social withdrawal, anhedonia), disorganized speech and/ or disorganized behavior[295];
- Lifetime prevalence of SCZ is approximately 0.48%[296,297]
- First symptoms typically occur in late adolescence;
- 85% of patients experiences more than one psychotic episode[298];
- On average 22% of patients have a paid job[299];
- Schizophrenia ranks among the top 15 causes for disability worldwide[300];
- Approximately 30% of SCZ patients are treatment resistant[9,95];
- SCZ is a multifactorial disorder, with both environmental and genetic factors playing a role;
- There are 136 genetic loci known to be associated to SCZ, but it is likely that there are many hundreds more that have not been discovered yet[99,301];
- Brain imaging studies show, next to a general decrease in brain volume[302], a decrease in hippocampus, amygdala, thalamus, accumbens and intracranial volume and an increase in pallidum and lateral ventricle volumes[303].

Physicians started to hypothesize about the etiology of psychiatric syndromes. Emil Kraepelin and Eugene Bleuler introduced the first concepts of schizophrenia, and the latter coined the term[1]. For the first time there was a described set of symptoms to treat.

In the beginning of the 20th century, treatment consisted mainly of sedating the patient with medication such as opioids and barbiturates[2]. Popularity of different methods of treatment came and went, almost like fashions changing with the seasons. Fever inducing drugs were used for a short period, thought to drive out the symptoms. Treatments inducing epileptic insults were also popular (insulin, electro shock treatment); they were thought to reset the brain. If the behavior of the patient proved to be uncontrollable, sometimes a lobotomy was performed, a brain surgery in which the frontal lobe is (partially) removed. Unfortunately, none of these treatments led to recovery or rehabilitation of the patient.

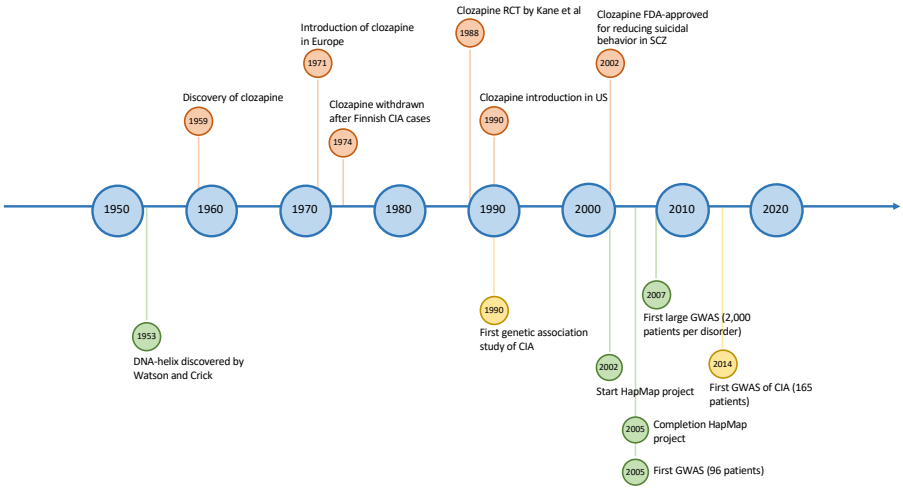
A new wind

In 1951, chlorpromazine inaugurated the era of antipsychotic drugs in psychiatry. A drug originally developed as a sedative and body temperature-lowering drug. Around this time, lowering body temperature during heart surgery was thought to be essential. A surgeon, Henri Laborit, noticed that chlorpromazine calmed his patients considerably before and after surgery, without severely sedating them[3]. He recommended the drug to psychiatrists, who noticed great improvements in patients receiving the drug. Chlorpromazine was introduced to the market in 1952[4,5], revolutionizing treatment options in the psychiatric field.

In the following decade a number of other, similar, antipsychotics were developed. Although the drugs were effective in treating psychotic symptoms, physicians noticed that almost all patients developed movement disorders such as akathasia, parkinsonism and dystonia. The prevalence of these symptoms, also called extrapyramidal symptoms (EPS), was so high that doctors were convinced these symptoms were part of the beneficial mechanism[6]. It became a common notion that if the drug was effective, the patient would also experience EPS. In other words; a drug not causing EPS probably would have little antipsychotic properties.

This dogma greatly hindered the introduction of a new type of antipsychotic: clozapine. Clozapine was originally developed as a tricyclic antidepressant in 1959[6] (Figure 1), but its antipsychotic properties were, in fact, much stronger. The drug was met with great skepticism, as it did not induce extrapyramidal symptoms. However, it did make its way to the market and slowly, physicians began to warm to the idea of an antipsychotic drug without EPS.

Figure 1. A timeline of clozapine development and use.



Just as clozapine was about to be introduced to the United States (US) market, a report was published on clozapine-induced agranulocytosis in cases from Finland[7]. In a period of 12 months, 18 patients had developed agranulocytosis while taking clozapine; 8 of these patients had died. Agranulocytosis is a very low neutrophil (a type of white blood cell) count, and it predisposes the individual to potentially severe infections that the weakened immune system cannot fight. Clozapine was quickly withdrawn from the market in Europe and became only available on request by psychiatrists.

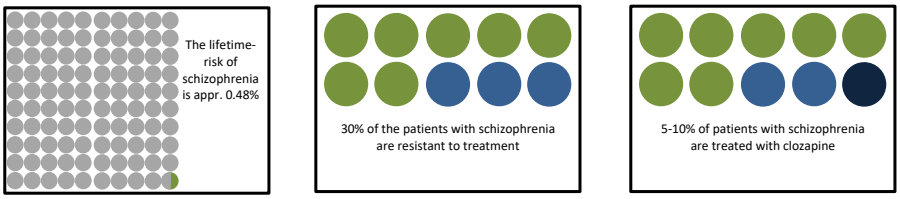
Psychiatrists kept requesting clozapine and patients and families lobbied for the re-introduction of the drug. For the introduction in the US, the US Food and Drug Administration (FDA) demanded a trial demonstrating superior efficacy of clozapine over other antipsychotics[8], in addition to a follow-up system for patients. Kane published said trial in 1988, showing superior efficacy of clozapine in reducing psychotic symptoms in comparison with chlorpromazine[9]. This landmark study launched the clozapine era in the US. The introduction (and re-introduction in Europe) of clozapine was accompanied with the introduction of a rigorous follow-up system: weekly blood draws for the first 18

weeks of treatment, and monthly thereafter to prevent clozapine-induced agranulocytosis (CIA). This follow-up system has decreased the incidence of CIA from 1% in 1990-1995 to a current prevalence of 0.3-0.7%[10,11]. Since the introduction of clozapine, other atypical antipsychotic drugs have been developed and introduced. None of these, however, have equaled the potency and efficacy of clozapine. It remains the only antipsychotic effective in treatment resistant schizophrenia to date[12].

Most effective, most prescribed?

With proven superior efficacy in treatment resistant schizophrenia and a rigorous follow-up system, nothing should prevent clozapine from becoming the most prescribed antipsychotic drug. Yet the contrary is, in fact, true (Figure 2). Physicians are hesitant to prescribe clozapine and patients are equally hesitant to try the drug[13–15]. The possibility of CIA and the extensive follow-up system to prevent it are not the only limiting factors[16]; other adverse effects play a role as well. Clozapine can induce serious metabolic adverse effects such as weight gain, Type 2 Diabetes and dyslipidemia. In fact, it is the drug that induces (on average) the most weight gain compared to other antipsychotics. Those prescribed clozapine gain *on average* 4kg in the first 10 weeks of treatment[17]. Additional adverse outcomes include drowsiness and hyper salivation.

Figure 2. Prescription of clozapine.



Mechanisms of action

The mechanism of action of clozapine is still unknown. In fact, the precise mechanism of action of antipsychotic medication as a group is still unknown. It is thought that in schizophrenia the dopamine metabolism in the brain is

dysregulated, with hyperactivity of dopamine in the mesolimbic part of the brain and hypoactive dopamine activity in cortical areas [5,18]. The first is thought to be responsible for the 'positive' symptoms such as hallucinations and delusions, whereas the latter would play a role in 'negative' symptoms and cognitive functioning. Antipsychotic drugs antagonize de dopamine receptor, thereby reducing dopamine activity (and positive symptoms). Dopamine, however, is not the only neurotransmitter that is being targeted by antipsychotic drugs. Depending on the specific drug, other neurotransmitters such as serotonin, histamine, etc. are also targeted. This is also true for clozapine, targeting a wide variety of different neurotransmitters, with dopamine and serotonin being the most important ones. Additionally, it is possible that clozapine also plays a role in pathways or cellular processes that have yet to be discovered. To even further complicate the understanding of clozapine's mechanism, the drug is metabolized to a number of different metabolites, each with different pharmacological characteristics and different target receptors. The many ways in which clozapine and its metabolites affect pathways and processes are undoubtedly highly complex.

Not only is the mechanism of action of antipsychotic drugs unclear, the mechanisms in which they trigger adverse effects are not yet understood as well. As discussed above, clozapine targets a wide variety of neurotransmitter receptors in the central and peripheral nervous system. Most of these receptors also play a role in satiety, energy homeostasis and feeding behavior[19–21], complicating the matter. In addition, their is evidence that clozapine affects insulin metabolism, this could also be one of the mechanisms of action (and adverse effects)[22,23].

Where metabolic adverse effects could be a direct consequence of clozapine acting on neurotransmitters, the etiology of CIA is an even larger mystery. Some researchers hypothesize CIA is the result of a (faulty) immune response to clozapine or one of its metabolites. This hypothesis has been fueled by the fact that autoantibodies against neutrophils have been identified in the serum

of CIA patients[24,25]. This finding has, however, never been replicated. A review has concluded that challenging patients with a history of CIA with clozapine more often and more rapidly leads to CIA[26], suggesting sensitization of some kind. Other serious adverse effects, such as Stevens-Johnsons syndrome, have also been shown to have an immunological background[27]. Thus, a number of arguments can be made to say CIA has an immunological component.

Other researchers hypothesize clozapine or one of its metabolites has a direct toxic effect on the neutrophil. When clozapine is exposed to myeloperoxidase (MPO), a product that is released by neutrophils, it is oxidized to a reactive nitrenium ion[28–30]. There is evidence that this nitrenium clozapine ion has a direct toxic effect on the neutrophil[31,32], this is, however, only been shown in *in vitro* experiments. It is very well possible that both hypotheses are 'true' and contribute in the origin of CIA. The neutrophil, for instance, could be damaged by the nitrenium clozapine ion, making it more susceptible to improperly-triggered autoimmune processes[33].

Recently, an additional and striking observation was made, that neutrophils of clozapine users have fluorescent properties[34]. This was a serendipitous finding after analyses of a large database of blood count measurements. It appeared that neutrophils from clozapine users are auto-fluorescent, whereas those of healthy individuals are not[34]. The origin of this fluorescence signal is not clear, it is clear however, that it is unique to patients taking clozapine. This finding could provide a piece of the CIA puzzle. Additionally, it could serve as a therapeutic marker, since adherence to medication is low in patients with schizophrenia[35].

On the prediction of effect and adverse effects

It is likely that genetic factors play a role in the development of clozapine-induced adverse effects. Serious adverse effects caused by other drugs have been found to be genetically determined[36]. Additionally, the heritability of antipsychotic induced weight gain is estimated to be 60%[37]. Thus far, it has

been difficult, though, to pinpoint exact genetic markers determining risk for clozapine (or antipsychotic drugs) induced adverse effects. There have been numerous studies to investigate possibly associated genetic variants with clozapine-induced adverse effects[38–41]. Two previous genome wide association studies (GWAS) investigating metabolic adverse effects of antipsychotic drugs failed to find any genome-wide significant findings ($p<5x10^{-8}$), but sample sizes were modest, so this approach is still promising for future analyses with larger samples[42,43]. GWAS investigating CIA have shown promising results, but sample sizes are small and replication is needed [44–46]. Although it is commonly believed that clozapine-induced (adverse) effects have a genetic component, it has proven difficult to identify specific genetic markers conferring risk.

In summary

Clozapine is a highly effective drug; its use is, however, complicated by the possibility of serious adverse effects such as clozapine-induced agranulocytosis and metabolic syndrome. Although clozapine has been on the market for nearly 60 years, it remains unclear how clozapine induces these adverse effects. Research is complicated by different factors including the complexity of the metabolism of clozapine in the human body and the collection of patient cohorts large enough to perform large-scale genetic studies. In this thesis we try to shed light on the genomic and molecular actions of clozapine.

In **part one** of this thesis we describe the effects of clozapine at the DNA level. We start with a review of genetic association studies investigating CIA performed to date (chapter II). In Chapter III we describe clozapine-induced reduced viability in a cell model as a model for clozapine-induced agranulocytosis and perform a GWAS.

In **part two** studies toward the effect of clozapine at the RNA level are outlined. Effects of clozapine on gene expression and methylation in a cell model were studied in chapter IV, after which the gene expression results were used to search for genetic enrichment for schizophrenia susceptibility. In chapter V we further explored fluorescence in neutrophils from patients using clozapine.

Neutrophils from patients taking clozapine and healthy controls were collected to search for differences in gene expression through RNA sequencing.
Part three is directed towards proteins; in chapter VI the auto-fluorescence of neutrophils from patients taking clozapine is further studied by determining the origin of the fluorescence inside the cell.

1

DNA

Chapter II

More than 25 years of genetic studies of clozapine-induced agranulocytosis.

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Abstract

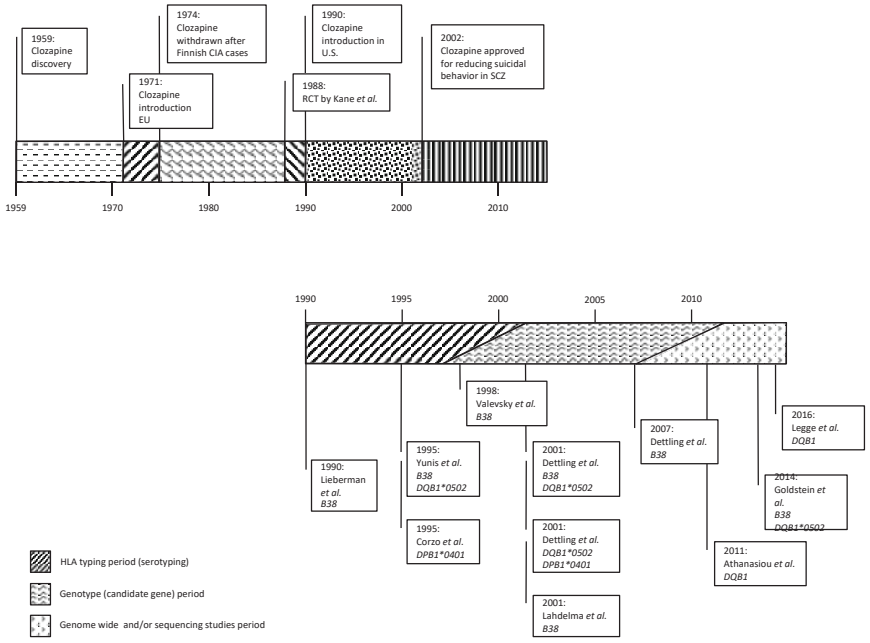
Clozapine is one of the most effective atypical antipsychotic drugs prescribed to patients with treatment-resistant schizophrenia. Approximately 1% of patients experience potential life-threatening adverse effects in the form of agranulocytosis, greatly hindering its applicability in clinical practice. The etiology of clozapine-induced agranulocytosis (CIA) remains unclear, but is thought to be a heritable trait. We review the genetic studies of CIA published thus far. One recurrent finding from early candidate gene study to more recent genome-wide analysis is that of involvement the HLA locus. We conclude that CIA is most likely a complex, polygenic trait, which may hamper efforts to the development of a genetic predictor test with clinical relevance. To decipher the genetic architecture of CIA, it is necessary to apply more rigorous standards of phenotyping and study much larger sample sizes.

Introduction

Before the discovery of antipsychotic medications in the 1950s, treatment options for psychosis were limited. Treatment included inducing seizures through insulin treatment or electroshock therapy, and lobotomy, an invasive procedure in which connections of the prefrontal cortex were removed. Additionally, the available medication to treat psychotic symptoms consisted mainly of sedating drugs. When clozapine was first introduced in 1959 (Figure 1), it was a revolutionary time for the development of antipsychotic therapies, as the first antipsychotics and antidepressants were just being developed. The development of antipsychotic drugs meant that, for the first time, it was possible to more effectively treat psychotic disorders with reduced risk of adverse effects for the patient. The breakthrough spiked a wave of optimism in the field. The success of the first drugs fueled drug development, and soon manufacturers were testing a wide array of compounds for future use in the treatment of psychiatric disease.

Though originally developed as a tricyclic antidepressant, clozapine had a much more pronounced effect as an antipsychotic. The first antipsychotics developed could induce extrapyramidal symptoms (EPS), depending on dosage. For some time it was thought that antipsychotic drugs did not work unless it induced EPS. Clozapine, which did not induce EPS, was therefore met with some skepticism, and several years passed before the drug was used in clinical practice.

Figure 1. Timeline of clozapine discovery, introduction to the pharmaceutical market, and genetic studies of CIA.



The upper left panel depicts a time-line the introduction of clozapine to the market, beginning by its discovery in 1955 and ending with the FDA registration for decreasing suicidal ideations. The lower right panel shows genetics studies of CIA with findings in the *HLA* region over time, starting with the first study by Lieberman et al in 1990 and ending with Legge et al. in 2016. The squares show the different studies (by year and first author) and the HLA alleles (in *Italic*) they found to be significantly associated with CIA.

Clozapine became available in Europe as an antipsychotic and preparations were made to introduce it to the market in the United States (U.S.). However, these preparations were unexpectedly interrupted when, in 1974, the first reports of clozapine-induced agranulocytosis emerged. Within 12 months in Finland, eighteen patients suffered from the condition and 8 died as a result.[47] Agranulocytosis, a very low neutrophil count (absolute neutrophil count < 500), had been reported as a very rare adverse effect of clozapine, however never before at such a high rate; in Finland, the incidence of clozapine-induced

agranulocytosis was 20 times higher than expected. As a precaution, clozapine was withdrawn from the European market so that the deaths in Finland could be more thoroughly investigated. The drug remained available only upon request as a courtesy to psychiatrists.

Despite the apparent risks, demand for clozapine remained. In 1988, a long overdue randomized clinical trial was published, indicating that clozapine was more effective than chlorpromazine in reducing psychotic symptoms of treatment-resistant schizophrenia patients.[9] Finally, 30 years after its creation, clozapine was introduced in the U.S., but with a rigorous follow-up system to allow rapid identification of adverse response: patients prescribed clozapine required mandatory white blood cell counts weekly for the first 18 weeks of treatment and monthly thereafter.

A few years after the introduction of clozapine, the incidence of clozapine-induced agranulocytosis (CIA) was estimated at approximately 1%;[10,11,48] an additional 2-3% of patients developed neutropenia, abnormally low neutrophil counts that results in a severely weakened immune system. The etiology of these blood dyscrasias remain unclear. One hypothesis was that CIA is immune mediated. Patients who had developed agranulocytosis would, after stopping treatment, often develop CIA again if exposed to clozapine for a second time, oftentimes faster and more severely than before. Such a response indicated that those prescribed the medication were potentially developing a sensitization to the drug.[26,49,50] Additionally, rare serious adverse effects from other psychotropic prescription drugs, such as the development of Stevens Johnsons syndrome during carbamazepine treatment, seem to have an immunological origin.[51] Consequently, some speculated that CIA, like adverse responses to similar drugs, could also have an immunological component. Antibodies against myeloperoxidase, an enzyme excreted by neutrophils, have been described (though inconsistently) in patients suffering from CIA,[52] further indicating that CIA may be the result of an inadequate immune response.

Another hypothesis has been that clozapine is toxic to neutrophils, and consequently causes CIA. Clozapine is metabolized by different cell types, such as liver cells and neutrophils, to a number of different metabolites. Some have speculated that one of these metabolites may play a key role in the development of CIA. This metabolite is produced after clozapine is oxidized to a nitrenium ion when H_2O_2 is added; this nitrenium clozapine ion has been shown to be toxic to both neutrophils and its precursor cells.[29,31,53,54] The hypotheses of toxicity and of immune regulation are not mutually exclusive; possibly, clozapine is oxidized to a toxic nitrenium ion, which could bind to neutrophils and induce cell death, or induce a pseudo-autoimmune response by coercing the immune system to act on the neutrophils.

It has been suggested that CIA is a genetic trait but no large twin or family studies estimating heritability of CIA have been published. Two case studies have described a single twin pair, both of whom suffered from CIA.[55,56] We reported an estimated heritability of 76% of decreased viability in lymphoblasts exposed to clozapine, an experimental *in vitro* setting that may or may not be reflective of CIA in patients.[57] Other serious adverse psychotropic drug responses, such as carbamazepine-induced Stevens Johnsons syndrome, appear to have a genetic basis.[27] This phenomenon is not limited to psychotropic drugs; serious adverse effects induced by other drugs such as abacavir, allopurinol and flucloxacillin have also been shown to be associated with common genetic markers.[58–60]

Identifying the genetic components of CIA remains vital, as a genetic test identifying one or more risk variants would be crucial in a clinical setting, and would improve diagnostics and prevent future incidence of CIA. The search for a genetic variant or variants conferring risk to CIA traces back more than 25 years (Figure 1), to the first study of genetic associations in CIA, published in 1990.[61] At the time, CIA was thought to be primarily immunologically mediated and other serious adverse effects had already been associated with certain human leukocyte antigen (HLA) alleles in the major histocompatibility complex

(MHC),[62] a region on chromosome 6 of the human genome known to contain a host of variations associated to immune-related diseases.[36,63–66] Thus, for this first candidate gene study, the HLA locus was an obvious choice to begin searching for risk alleles. Numerous studies followed in the footsteps of the first CIA study and focused exclusively on this locus. Recently, with the advent of new technologies and improved methods for identifying risk alleles more comprehensively, a whole-exome sequencing study and two genome-wide association studies (GWAS) were performed,[44,45,67] the first efforts to search for genetic variants conferring risk to CIA across the whole genome.

Given the importance of clozapine as a “last resort” form of treatment for psychosis, and the genetic data assembled over the last 26 years, we sought to examine the studies that laid the groundwork for current genetic studies of CIA, discuss recent work that has expanded the candidate gene approach, and look forward to future efforts that will help reveal the genetic underpinnings of CIA and potentially improve the treatment of drug-resistant schizophrenia.

Materials and methods

Literature

We identified all articles describing genetic associations studies about clozapine-induced agranulocytosis (CIA) and/or neutropenia, published up to October 1st 2016. A PubMed search was performed with the following keyword: “clozapine”, “agranulocytosis”, and “genetics”. Articles were gathered in PubMed and references of the articles were further checked for additional studies published previously. In total, we identified 23 articles that examined genetic associations in CIA; 20 of the studies utilized the candidate gene approach, two studies performed genome-wide association studies (GWAS) of common variants, and one study utilized whole-exome sequencing that focused on rare coding variants in the genome.

Scoring method

We adapted an existing scoring method for genetic association studies to evaluate the selected articles based on study design, methods, and evaluation of results.[68,69,70] Specifically, points were given to each manuscript for quality of clinical information, quality control of genotyping data, consideration of population stratification, power considerations, methods regarding multiple testing correction, and replication (Table 1). Articles received a grade based on the quality scoring system from ‘poor’ to ‘excellent’ (Table 2).

Table 1. Quality measure system.

Quality measure system	
Quality of clinical information (0 – 3 points)	Adequate description of inclusion criteria (i.e. definition of agranulocytosis and agranulocytosis only or also neutropenia patients)
	Description of baseline characteristics (age, sex and other clinical variables)
	Mentioning of clozapine dose in patients with agranulocytosis (and/or neutropenia) and in patients without
Quality of genotyping (0 – 3 points)	Mentioning of the Hardy Weinberg equation (regardless of the conclusions of the authors)
	Assessment of genotype quality, either by reporting the number of failed genotypes or genotype validation with a different technique
	Genotyping (not only serotyping)
Quality of report of study population (0 – 2 points)	Mentioning of the geographical point of collection of the sample
	Any method of correction for (possible) population confounders (i.e. stratification or exclusion of samples)
Quality of study sample size and correction for multiple testing (0 – 2 points)	Mentioning of power (regardless of the conclusions of the authors)
	Any form of correction for multiple testing
Quality of replication (0 – 2 points)	Replication described in the same article
	Replication of results in different article with an independent cohort

Table 2. Grades based on quality measure system.

Total of scored points	**Grade**
0 - 2	Poor
3 – 5	Average
6 – 8	Good
9 – 12	Excellent

Results

We evaluated a total of twenty-three different genetic studies in CIA (Supplemental Table 1). Two studies were excluded: one[71] because it presented already published data to illustrate a novel statistical method, and another[72] because the article failed to report association testing p-values. We will first briefly describe the different studies and then score the remaining 21 studies.

1990 – 2000: HLA-serotyping

The first study of CIA genetics was published in 1990 by Lieberman *et al.* [61] They studied a small population (31 patients) of Jewish and non-Jewish individuals of whom 6 developed CIA. At that time it was only possible to determine the HLA-alleles by serotyping. The design of the study was influenced by studies of other serious adverse effects and the possible immune mechanisms mediating these reactions. Although the study comprised only 6 CIA patients, it yielded nominally significant results at the HLA-B38 allele. Other research groups were inspired by this study and, two years later, Claas *et al.* published a larger study on CIA response.[73] They had collected a little over 100 CIA patients and 95 controls of white European descent but could not replicate the earlier results reported by Lieberman et al. In fact, they did not report any significant results.

The research group from Hillside Hospital, New York pushed on and published 3 papers on the subject the following years.[74–76] Their patient cohort had risen to 30 patients and 40 controls and they reported increased significance for the earlier finding at the HLA-B38 allele. They described inconsistent results at the HLA-DQB1*05:02 allele; two of the studies presented a significant association[74,76] and one study did not replicate this.[75] One year later, Amar *et al.* published a small study (6 CIA-patients and 13 controls) reporting no significant association at HLA-DQB1*05:02.[77] The studies up to then had collected a homogeneous patient and control sample. However, Amar *et al.*

reported a study cohort with mixed ethnicities, possibly confounding their results. Valevski *et al.* replicated the first finding at HLA-B38 in their small sample (11 CIA patients and 50 controls), but did not report any other significant findings.[78]

2000 – 2010: Genotyping and candidate gene analyses

Around the year 2000, the studies mostly stopped using serotyping and started using genotyping methods to determine HLA alleles. In 2000, Dettling *et al.* published the first of what would be 7 articles from the research group at the University Hospital Benjamin Franklin and University Hospital Charite in Berlin, Germany. They made use of a nationwide program registering adverse drug reactions and through this system collected a study cohort of approximately 30 CIA patients and 75 controls. Their first study in 2000 focused on *MPO* and *CYP2D6* and yielded no significant results.[79] In 2001, two articles were published from the same group, also targeting the HLA region.[80,81] They did not replicate the earlier finding at HLA-B38 from the research group from Hillside Hospital, New York. Dettling *et al.* also found a significant association with CIA and HLA-DQB1*05:02, thereby replicating findings from the New York research group. Little discussion was given to possible differences in population ancestry in these studies.

In 2001 a Finnish research group publishes their findings on the association within the HLA region and CIA.[82] Lahdelma *et al.* collected a cohort of 26 patients and 19 controls and focused on the HLA-A and B genes. They reported one finding (at HLA-A1) which survived correction for multiple testing.

Beginning in 2003, a period commenced in which the focus shifts towards other genes that are part of the MHC region or genes that play a role in pharmacokinetics. Ostrousky *et al.* (from the same research group as Valevski *et al.*) report a significant association between CIA and the *NQO2* (dihydronicotinamide riboside (NRH) quinone oxidoreductase 2) gene.[83] In 2004 and 2005, Mosyagin *et al.* reported their results on association between

the *MPO, NADPH* and *FcγR* genes and CIA.[84,85] None of the results were significant after correction for multiple testing, however. In 2007, Dettling *et al.* returned to the HLA region after having collected a larger patient cohort (42 patients).[86] They tested a variety of loci within the HLA-B, Cw and DRB regions. However, none of the p-values survived correction for multiple testing.

2010 – present: Large scale common-variant and rare variant association studies

In 2011, Athanasiou *et al.* published the first sequencing study of CIA.[87] They sequenced 74 candidate genes in the search of variants contributing to CIA risk. They were also the first study that made use of a replication cohort. This study used two cohorts: one had not been described earlier and the other cohort was from the research group in Berlin, Germany. They used the first cohort to select target genes from the 74 selected genes and attempted to replicate these results in the second independent cohort. The first analyses showed 5 genes associated with CIA, from these 5, only one was replicated in the second cohort: a variant in the HLA-DQB1 region.

Tiwari and colleagues published the first whole-exome sequencing paper in 2014.[67] They collected a small cohort of 24 CIA patients and 26 controls in Finland. It was not surprising that with this small cohort, they did not report any significant findings. It was, however, a methodologically sound first attempt at a whole-exome sequencing study of CIA.

In the same year, Goldstein *et al.* published the first GWAS of CIA.[45] It was a study with one of the largest sample sizes, with 162 CIA and CIN (clozapine-induced neutropenia) cases, of whom more than 50% had not been studied before. They first performed a GWAS on 161 cases and 1,196 (population) controls and found no genome-wide significant ($p < 5 \times 10^{-8}$) hits. They continued by combining whole-exome sequencing data with exome SNP array data to perform an association study, also interrogating rare variants. However, no genome-wide significant results were observed. To further investigate the

previously implicated HLA region, they imputed classical HLA alleles and performed an association analysis with the imputed data, showing 2 independently genome-wide significantly associated loci (HLA-DQB1*0502 and HLA-B38/39/67).

In 2016 Legge *et al*. published a second GWAS of CIN [44]. They made use of the CLOZUK sample (a large sample of treatment resistant schizophrenia patients prescribed clozapine) and the CardiffCOGS cohort. In total their study sample comprised of 66 CIA and CIN cases and 5583 controls. The previously mentioned cohort (Goldstein *et al.*)[45] was used as a replication cohort and for meta-analysis. A GWAS was performed, showing two independent loci reaching genome-wide significant evidence for association to the trait. These results, however, did not replicate in the second cohort. In the meta-analysis, a common intronic variant *in SLCO1B3/7* was observed at genome-wide significance. Further HLA imputation, however, yielded no additional significant results. Genotyping of the previously implicated variant in the HLA region[87] in 60 cases and 305 controls provided nominal evidence for association. A gene-by-gene analysis of rare coding variants implicated two genes (*UBAP2* and *STARD9*) at gene-level significance ($p < 2.5 \times 10^{-6}$).

Most recently, two candidate gene studies were published by van der Weide *et al.*[88] and Yagcioglu *et al.*[89]. The first one genotyped a number of SNP's in genes involved in clozapine metabolism, the latter focusing on the *ABCB1* gene. There were no significant findings after correction for multiple testing.

Methodological quality of publications

One study, the exome-wide analysis[67], was given the highest possible score (Table 3). The three publications with the highest scores (9, 8 and 8 points, respectively) were the exome-wide study[67] and the two genome-wide association studies.[44,45] The majority of the studies examined a small set of samples (17 of 18 studies with $N < 80$), used a liberal nominal p-value threshold of $p < 0.05$ without correction for multiple testing (19/21 studies), or failed to

replicate their findings in independent samples (19/21 studies), all prevalent flaws in candidate gene studies interrogating complex traits.[90]

Table 3. Quality scores of articles

Quality	Articles
Poor (0-2)	Corzo *et al.* (1995)[$76]
	Amar *et al.* (1998)[77]
	Ostrousky *et al.* (2003)[@83]
	Mosyagin *et al.* (2004)[$85]
Average (3-5)	Lieberman *et al.* (1990)[$61]
	Claas *et al.* (1992)[73]
	Yunis *et al.* (1995)[$74]
	Turbay *et al.* (1997)[$75]
	Valevski *et al.* (1998)[@78]
	Dettling *et al.* (2000)[&79]
	Dettling *et al.* (2001a)[&80]
	Dettling *et al.* (2001b)[&81]
	Lahdelma *et al.* (2001)[&82]
	Yagcioglu *et al.* (2016)[89]
Good (6-8)	Mosyagin *et al.* (2005)[&84]
	Dettling *et al.* (2007)[&86]
	Athanasiou *et al.* (2011)[&87]
	Goldstein *et al.* (2014)[&$45]
	Legge *et al.* (2016)[44]
	Van der Weide *et al.* (2016)[88]
Excellent (9-12)	Tiwari *et al.* (2014)[67]

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To identify repeated findings across the studies, many of which focused on an array of HLA alleles, alleles that had been implicated more than once in independent samples across the 21 publications were identified. Here we show the alleles that were reported more than once or were reported in a genome-wide study (Table 4, complete information in Supplemental Table 2). In addition, the main findings from the Goldstein *et al.* GWAS[45], implicating HLA-B38/B39/B67 (OR = 3.3, $p = 6.4x10^{-10}$) and HLA-DQB1*05:02 (OR = 0.19, $p = 4.7x10^{-14}$) in 163 cases and 4,319 controls, were traced back and searched for in older studies with more limited samples sizes (Table 5). We show that the direction of effect is consistent throughout the different studies.

The Legge *et al.* genome-wide study[44] performed a myriad of analyses providing suggestive evidence for involvement of three genes: *SLCO1B3/7, UBAP, STARD9*. They also replicated the finding of Athanasiou *et al.*[87].

Table 4. Overview of genetic association studies of CIA and CIN reviewed in this article

Study	N: cases/ controls	Pheno-type	Population	MT correction	*HLA-B*	*HLA-Cw*	*HLA-DRB*	*HLA-DQB*	*HLA other*	Other
Lieberman *et al.* (1990)[$61]	6/25	CIA	Jewish and non-Jewish	Uncorrected	***B38*** p=0.005	-	-	-		-
Claas *et al.*. (1992)[73]	103/95	CIA and CIN	White European	Uncorrected	-	-	-	-	-	-
Yunis *et al.*. (1995)[$74]	31/52	CIA	Jewish and non-Jewish	Uncorrected	***B38*** p=0.0001*	-	*DRB1*0402* p=0.002*	***DQB1*0502*** p=0.02	-	-
Corzo *et al.*. (1995)[$76]	32/43	CIA	Jewish and non-Jewish	Uncorrected	-	-	*DRB1*0402* p=0.02*	***DQB1*0502*** p=0.018	*DPB1*0401* p=0.02*	-
Turbay *et al.*. (1997)[$75]	33/33	CIA	Jewish and non-Jewish	Corrected	-	-	*DRB1*0402* NS*	***DQB1*0502*** NS	-	
Amar *et al.*. (1998)[77]	6/13	CIA and CIN	Jewish, Moroccan, unknown	Uncorrected	-	-	*DRB1*0402* NS	***DQB1*0502*** NS	-	-
Valevski *et al.*. (1998)[@78]	11/50	CIA and CIN	Jewish and non-Jewish	Uncorrected	***B38*** p=0.0001	-	-	-	-	-

Dettling *et al.*. (2000)[&79]	31/77	CIA	German whites	Uncorrected	-	-	-	-	-	*MPO* NS, *CYP2D6* NS
Dettling *et al.*. (2001a, Pharmacogenetics)[&80]	31/77	CIA	German whites	Uncorrected	*B35*/***B38*** NS	-	*DRB1*0402* NS	***DQB1*0502*** p=0.006	-	-
Dettling *et al.*. (2001b, Arch Gen Psych)[&81]	30/77	CIA	German whites	Uncorrected	*B7* NS	*Cw12* NS	-	***DQB1*0502*** p=0.007	*DPB*0401* p=0.034	-
Lahdelma *et al.*. (2001)[82]	26/19	CIA and CIN	Finnish	Uncorrected	*B7/B8/B13/B18/B35/B40* NS	-	-	-	-	-
Ostrousky *et al.*. (2003)[@83]	18/80	CIA and CIN	Jewish and non-Jewish	Uncorrected	-	-	-	-	-	*NQO2* p=0.001
Mosyagin *et al.*. (2004)[&85]	49/78	CIA	German whites	Uncorrected	-	-	-	-	-	*MPO* p=0.04
Mosyagin *et al.*. (2005)[&84]	48/75	CIA	German whites	Uncorrected	-	-	-	-	-	
Dettling *et al.*. (2007)[&86]	42/75	CIA	German whites	Uncorrected	*B7/B8/B18/B35/**B38**/B40* NS	*Cw12* NS		-	-	-

Athanasiou *et al..* (2011)[87]	Cohort 1: 33/54 Cohort 2: 49/78	CIA	Cohort 1: US, Russian and South African people Cohort 2: German Whites	Corrected	-	-	-	Cohort 1: *DQB1* p=0.0040, cohort 2: *DQB1* p = 0.001 (uncorrected)	-	Cohort 1: *CYP2D6* /*MPO*/ *TNF* NS,
Tiwari *et al.* (2014)[67]	24/26	CIA and CIN	Finnish	Corrected	-	-	-	-	-	-
Goldstein *et al.* (2014)[&$45]	162/4319[#]	CIA and CIN	Israeli (Jewish and non-Jewish), German whites, US, Russian and South African people	Uncorrected	GWAS: ***B38,B39*** or *B67* p=1.54* 10^-8	-	-	GWAS: ***HLA-DQB1*05:02*** p=4.7* 10^-14	-	-
Legge *et al.* (2016)[44]	Cohort 1: 66/5583[#] Cohort 2: 162/4319[#]	CIA and CIN	European ancestry	Uncorrected	-	-	-	GWAS cohort 1: *HLA-DQB1* 6672G>C p=0.015	-	GWAS: 2 GWS hits, GWAS meta-analysis: *SLCO1B3/7* p=1.79*10^-8, Exome-SKAT: *UBAP2* p=1.02*10^-7,

										STARD9 p=2.85*10^-7
Van der Weide *et al.*(2016)[88]	38(CIN)/ 31(CIA)/ 241	CIA and CIN	Unknown	Uncorrected	-	-	-	*HLA-DQB1* NS	-	*MPO/TNF* NS, *NQO2* p=0.004
Yagcioglu *et al.* (2016)[89]	10/91	CIA	Unknown	Uncorrected	-	-	-	-	-	*ABCB1* NS

[*]P-values tested in a population of Ashkenazi Jews only

[#]Control cohort (also) consisting of healthy population controls

NS = not significant

MT = Multiple testing

Bold = Alleles found in both candidate gene studies and GWAS

Underlined = Alleles tested multiple times in different cohorts

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Research groups were specified to provide insight in the number of publications per cohort.

Table 5. GWAS results and candidate gene results

HLA allele	Author	P-value	CIA or CIN
HLA – B (B38/B39/B67)	Goldstein *et al.* (2014) [&$45]	$6.4*10^{-10}$	CIA & CIN
	Lieberman *et al.* (1990) [$61]	0.005	CIA
	Yunis *et al.* (1995) [$74]	0.0001	CIA
	Valevsky *et al.* (1998)[78]	0.0001	CIA
	Dettling *et al.* (2001) [&80]	Not significant	CIA
	Dettling *et al.* (2007) [&86]	Not significant	CIA
HLA – DQB1 (DQB1*05:02)	Goldstein *et al.* (2014)[45]	$4.7*10^{-14}$	CIA & CIN
	Yunis *et al.* (1995)[74]	0.02	CIA
	Amar *et al.* (1998)[77]	Not significant	CIA & CIN
	Dettling *et al.* (2001) [&80]	0.007	CIA

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Discussion

We evaluated 21 genetic association studies of a cumulative number of almost 500 independent patients (and matched controls) with clozapine-induced agranulocytosis. After evaluation, the whole-exome[67] and GWAS studies[44,45] received an 'excellent' to 'good' score, respectively, while the candidate gene studies scored poorly. We did not observe variants unambiguously associated with CIA in any of the 18 candidate gene studies, but the two findings from the Goldstein *et al.* GWAS[45] are the same as described before by five of these candidate gene studies (HLA-DQB1*0502 and HLA-B38/39/6).

We know now that candidate gene studies must be performed with great care, as they can be quite limited in power and claims of association are rarely replicated.[90,91] This said, the field of genetics is one of numerous transitions: twenty-five years ago, candidate gene studies were the only method available, and remained so until the HapMap project was finished and developing technology allowed for GWAS approaches (around 2008). The first CIA candidate gene study was published 26 years ago and one of the significant findings from that study was replicated in a GWAS last year, an indication that even the earliest studies were detecting true signals of genetic risk. As technology and methodology has evolved, studies of CIA have become more rigorous and robust and this evolution is reflected in the quality of the studies, as the scores improve with time.

The results from the most recent candidate gene study[87] and the Goldstein *et al.* GWAS[45] have shown that there are variants within the HLA region associated to CIA with substantial effect size (OR 0.19 and 3.11). The subtle effect that these variants confer on risk, however, make them unsuitable to use in clinical practice as predictors. The variants discovered so far could be used for a test of CIA prediction with a high specificity but extremely low sensitivity; that is, such a test would likely yield a large number of false positive results. A recent study explored the necessary characteristics of a pharmacogenetic test

for CIA.[92] For a test to be clinically relevant, it should be able to differentiate between patients with low and high risk of CIA. For another psychotropic prescription drug, zuclopentixol, we accept a 0.1% risk of drug-induced agranulocytosis without a rigorous follow-up system with weekly blood draws. Applying the same risk levels for CIA, an extreme high sensitivity is required. The test reported by Athanasiou *et al.*[87] with a sensitivity of 21.5% and a specificity of 98.4%, for instance, is therefore insufficient.

The effect size of the associated alleles discovered in the GWAS by Goldstein[45] (OR = 0.19 – 3.11) or the GWAS by Legge[44] (OR = 4 – 15) are not nearly as penetrant as effects found for other adverse drug responses, such as the effect of HLA-B*1502 on the risk of carbamazepine-induced Stevens Johnsons syndrome (OR = 2,504[27]). Consequently, it is reasonable to hypothesize that risk for CIA is genetically complex, involving a large number of genes throughout the human genome and an additional environmental component. Such a polygenetic architecture complicates the development of a genetic risk prediction test, as it is unlikely that a small number of alleles will explain the full heritability of CIA. Once we know more about the true genetic basis of CIA, and it proves to be a polygenic trait, it may be possible to apply polygenic risk scores (i.e., risk as determined by an aggregation of polymorphisms) as a pharmagenomic predictor test, identifying low-risk patients that do not require the rigorous follow-up system while maintaining the system for the subjects that are considered as higher-risk.

If CIA is a heritable trait with a number of moderate-effect loci involved, it remains possible that a study with sample sizes of N<1,000 is sufficient to uncover additional risk alleles. For 80% power to detect a genome-wide significant association that explains 10% of the variance, a sample size of approximately 450-500 subjects is needed.[93] None of the CIA studies thus far has even remotely approached including this number of subjects, and even combining the almost 500 independent patients that were part of the studies considered in our review would not yield sufficient power to detect such a

variant. We estimate the number of subjects with CIA in Europe and the USA to be approximately 650 and 1,500, respectively. This estimate is based on the assumption of a 1% prevalence of schizophrenia in the general population[94], a 30% prevalence of treatment resistance among schizophrenia patients[95], a 20% clozapine treatment rate of these patients[96], a CIA prevalence of 0.7%[10,11] and a study participation rate of 50%.[97,98] The collection of these patients would require a concerted international endeavor, similar to other ongoing large efforts with the Psychiatric Genomics Consortium (PGC).[99,100] If the genetic basis is similar to other polygenic traits such as schizophrenia or adult height[99,101], however, the necessary number of cases to be included in a successful study needs to increase at least ten-fold.

Further complicating genetic analyses, CIA may represent two possible phenotypes instead of one. The first possible phenotype is clozapine-induced neutropenia (CIN), defined as ANC < 1500. It is typically benign and has a prevalence of 2-3%. The second possible phenotype is clozapine-induced agranulocytosis, defined as ANC < 500. CIA can be fatal if not identified early and has a prevalence of 1%, reduced more recently to ~0.7% as a result of the strict monitoring system of people being treated with clozapine.[10,11] CIN does not always lead to CIA and patients who develop CIN can be more successfully "rechallenged" with clozapine than CIA patients.[26] Future studies including more subjects with both CIN and CIA will be able to further dissect potential genetic overlap or differences between the two phenotypes.[102] This has been a successful approach in the past for other psychiatric traits, providing evidence that schizophrenia and bipolar disorder share genetic risk factors.[102]

An additional limiting factor is that most studies of complex traits performed to date have focused exclusively on European-ancestry samples. Inclusion of non-European populations in future studies may yield more information about the underlying etiology of CIA.[103] For example, clozapine is a frequently prescribed drug in China, where 30-60% of schizophrenia patients is treated with the drug.[104,105] Clozapine has been available to Chinese patients since the

1970s and was never withdrawn from the market. The reported prevalence of clozapine-induced agranulocytosis in China is 0.21%,[104] markedly lower than the reported prevalence in Western countries, from which all of the genetic studies in CIA performed to date have drawn their samples. Though the estimate of CIA in the Chinese population may be biased due to a suboptimal and/or lack of follow-up system, genetic association studies in Chinese, population isolates (such as in Finland, where the first cases of CIA were reported), and other non-European samples may be particularly revealing, as there could be population-based differences in risk alleles, and the lower prevalence may be in part due to an enrichment of ancestry-specific protective alleles.

In conclusion, sixty-six years after its introduction to the pharmaceutical market, clozapine remains the only drug registered for treatment-resistant schizophrenia. Its use is complicated by the risk of developing agranulocytosis. After more than 25 years of research into the genetic components that contribute to this adverse response, evidence indicates that the HLA region is associated with CIA. The GWAS of CIA has provided novel and invaluable insight into the genetic underpinnings of CIA, but it is likely only the beginning. A clinical predictor test based on genetics alone is at this time not within reach. To decipher the genetic architecture of CIA, it is necessary to apply consistent standards of phenotype definition and to perform genome-wide analysis in much larger sample sizes. Such discoveries will yield biological insight into CIA, improve diagnostics, and enhance the treatment of the 7 million individuals around the globe with treatment-resistant schizophrenia.

Chapter III

Genome-wide association study of lymphoblast cell viability after clozapine exposure.

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Abstract

Background

Clozapine is an antipsychotic drug with proven efficacy in treatment-resistant schizophrenia but also known to induce potentially lethal agranulocytosis (CIA) in 1% of patients. Genetic factors are likely to play a role in the molecular basis of CIA. We explored an *in vitro* system to study the genetic susceptibility of CIA.

Methods

Cell viability was measured in 90 lymphoblast cell lines exposed to a series of increasing concentrations of clozapine for 48 hours. Quantitative trait measures of cell viability as well as area under the survival curve were used in a linear mixed model for genome-wide association analyses.

Results

The estimated heritability of clozapine-induced cell viability reduction in these cell lines is $h^2 = 0.76$. No genome-wide significant association was observed after correction for multiple testing. Two independent loci with nominal evidence of association were observed at 30x clinical clozapine concentration: rs2709505 ($p=1.41x10^{-8}$) in an intron of *MDFIC* and rs10457252 ($p=1.79x10^{-8}$) located in a gene desert at chromosome 6q21. We identified one locus (rs1293970) near *PRG4* that was consistently associated for all separate concentration analyses at $p<5x10^{-5}$. *PRG4* encodes hemangiopoietin, a growth stimulator for hematopoietic stem cells. No evidence was observed for involvement of the MHC region.

Conclusions

Our results demonstrate that clozapine-induced viability reduction in lymphoblast cell lines is a heritable, polygenic trait. Thus, *in vitro* models of CIA might be a useful tool for future discovery of genetic risk factors, although larger sample sizes will be required to unambiguously identify these loci.

Introduction

Clozapine is an atypical antipsychotic drug first developed almost sixty years ago. The introduction of clozapine in the US was complicated by the occurrence of agranulocytosis in a number of Finnish patients, resulting in the withdrawal of clozapine [8]. The drug was reintroduced after persistent demand of psychiatrists and proven efficacy in treatment-resistant schizophrenia [8]. Despite a tumultuous history, clozapine has never been equaled in efficacy and remains the only antipsychotic approved by the Federal Drug Administration for treatment-resistant schizophrenia [9,106,107].

Clozapine has a number of serious adverse effects. Approximately 50% of patients taking clozapine suffer from adverse metabolic effects [108]. The average weight gain in the first 10 weeks of treatment is about 4 kg [109] and patients may also suffer from lipid level disturbances and type 2 diabetes [108]. Although adverse metabolic effects are common, they are not the main reason for clinicians to be hesitant in prescribing clozapine. Approximately 1% of patients treated with clozapine develop agranulocytosis, which can potentially be lethal. Agranulocytosis is typically defined as an absolute neutrophil count (ANC) below 500 cells/μL [110,111] and can result in susceptibility to (severe) infection. To prevent agranulocytosis, an intensive follow-up system has been developed, with weekly visits during the first 18 weeks of treatment and every four weeks thereafter. The possibility of agranulocytosis and the extensive follow up have led to marked underuse and under-prescription of clozapine [112]. Development of a prediction biomarker for identification of patients susceptible to developing CIA prior to prescribing the drug will enormously benefit care of schizophrenia patients.

The mechanisms underlying clozapine-induced agranulocytosis (CIA) are not well understood. Drug-induced neutropenia and agranulocytosis are typically thought to have an immunological basis or to be the result of toxicity of the drug [113]. The fact that some patients experience a faster and more severe onset of agranulocytosis after re-

challenge, *i.e.* the reoccurrence of prescribing the drug after the occurrence of an adverse event with clozapine [26,50], supports the hypothesis of an immunological background. However, this does not occur in every patient re-challenged with clozapine, suggesting that other factors must also play a role in susceptibility to CIA [26,114,115]. The fact that clozapine and some of its metabolites induce toxicity towards neutrophils and bone marrow cells in vitro [31,32,53,116,117] indicates that CIA could also be the result of direct toxicity of clozapine or one of its metabolites.

The genetic background of CIA remains unclear. Given that CIA is thought to have an immunologic component, genetic studies have mainly focused on the major histocompatibility complex (MHC) region [41,61,76,86,87,118,119].There have been numerous candidate gene studies, but sample sizes have typically been small and findings are rarely replicated [41,118,119] (Supplemental Table 2). A recent exome sequencing study in a Finnish sample of 24 cases and fifty controls yielded no further insight into sequence variants causally involved in CIA [67].

The relatively small number of schizophrenia patients using clozapine as well as the low prevalence of the trait itself has hampered study of the genetic underpinnings of CIA. The introduction of a patient follow-up system, monitoring blood cell counts, made it possible to quickly identify patients in danger of developing neutropenia, which is considered a pre-stage of agranulocytosis. This systematic approach has successfully decreased the prevalence of agranulocytosis [120] thereby making it even more difficult to recruit large cohorts of patients diagnosed with CIA. A cellular model could therefore provide a viable alternative to study the molecular mechanisms underlying CIA.

Lymphoblast cell lines have successfully been used before in studies of the genetic underpinnings of the cytotoxicity of a drug [121]. Additionally, previous studies have shown reduced viability in lymphoblasts after exposure to supratherapeutic concentrations of clozapine [122,123]. Genetic studies of this clozapine-induced viability reduction could give us insight in the (genetic) background of CIA, as this apparent toxicity in lymphoblasts could share molecular mechanisms with the etiology of agranulocytosis. This study therefore aims to investigate the genetic background of

clozapine-induced viability reduction in lymphoblast cell lines. We used this *in vitro* model of CIA to perform a genome-wide association study while examining the MHC region in detail in a separate analysis.

Materials and methods

Cell culture

Ninety lymphoblast cell lines (30 parent-offspring trios) were used for drug exposure experiments. All samples were part of the CEPH family collection of Utah residents of Northern and Western European ancestry (HapMap, CEU phase 1). Cell lines were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (VWR, West Chester, PA, USA), supplemented with 15% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% penicillin/streptomycin (all Fisher Scientific, Fairlawn, NJ, USA) and incubated in 5% CO_2, at 37 degrees Celsius. Cells were counted with the TC10 automated cell counter (Biorad, Hercules, CA, USA), according to manufacturer's instructions.

Drug exposure

Clozapine was ordered from Sigma Aldrich (St. Louis, MO, USA). We determined clinical concentration to be 2μM[124]. Clozapine is marginally soluble in water, therefore it was dissolved in dimethyl sulfoxide (DMSO) with a final DMSO concentration of 0.05% in the culture medium. To enhance the effects of clozapine-exposure on cell vitality, we chose to expose the lymphoblast cells with supratherapeutical concentrations, as was done in previous studies[122,123]. To create a survival curve we exposed cells to a fixed set of 7 different concentrations, ranging from 10 times clinical concentration to 70 times (10x, 20x, 30x, 40x, 50x, 60x, and 70x clinical concentration), as well as exposure to vehicle (0.05% DMSO) only. Cells were exposed for 48 hours.

Viability measurements

Cells were cultured for one week before starting the experiment (Supplemental Figure 1). After one week cells were counted and viability was assessed with the TC10 cell counter. Cells were diluted to $1x10^5$ live cells/mL and 100 µL of the cell mixture was transferred to 96 well round-bottom plates (Corning, Inc., Corning, NY, USA). After 24 hours, different concentrations of clozapine were added (see above). Viability of the

cells after 48 hours was measured with AlamarBlue® dye. AlamarBlue® (Invitrogen, Carlsbad, CA, USA) was added and absorbance readings were performed after 24 hours (48 hours of exposure) at wavelengths of 570 and 600nm using the Tecan Infinite f200 Pro plate-reader (Tecan, San Jose, CA, USA). Experiments were performed in quadruplicate to enhance measurement accuracy.

Viability assessment with AlamarBlue® took place after 48 hours of exposure to clozapine, according to manufacturer's instructions. Measurements were evaluated for differences across batches as well as normality. Quality control showed that the results from 40x clinical concentration suffered from severe batch effects, an increased variance, and consistently violated the assumption of a normal distribution of cell vitality (data not shown). For these reasons we included only measurements from vehicle to 30x clinical concentration for subsequent genetic analyses (Supplemental Figure 2). Viability measurements were plotted against concentration, thereby creating a survival curve. The area under this (survival) curve (AUC) was calculated with the PK [125] package in R (a free software environment for statistical computing) [126]. The AUC as well as the viability measurements at concentration points vehicle, 0x, 10x, 20x and 30x clinical concentration were used as quantitative trait in subsequent genetic analysis.

Statistical analysis

All cell lines used for the experiment are part of the HapMap Consortium, which has made genome-wide genotype data of all samples freely available [127,128]. Quality control of the genotype data was performed with PLINK[129]. Before filtering there were 3 967 651 single nucleotide polymorphisms (SNPs). SNPs with a minor allele frequency < 5%, > 10% missingness, Hardy Weinberg equilibrium violations in the independent founders ($p < 0.001$) and Mendelian errors were excluded from the data, leaving 2 092 490 SNPs for analysis. Narrow heritability was calculated with GCTA [130], including sex and batch as covariates.

Genome-wide association analysis was performed with factored spectrally transformed linear mixed models (FaST-LMM) [131], using log-transformed AUCs for the analysis. FaST-LMM is a linear mixed model that addresses population structure and family relatedness, without the need to correct for these possible confounders by including them as covariates [132]. We performed genome-wide association analysis using five different trait measures: the AUC and viability measurements taken from the four different concentration exposures (vehicle, 10x, 20x and 30x clinical concentration). Accordingly, we used a genome-wide significance threshold of $p \leq 1x10^{-8}$ after Bonferroni correction for multiple testing. Independently-associated SNPs were determined with the 'clump' option in PLINK [129].

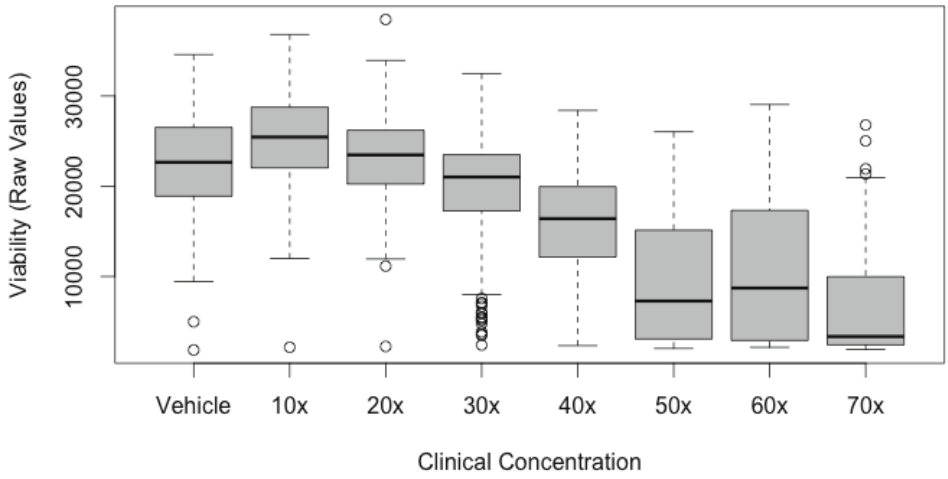
The MHC region was imputed for all samples as described before [133]. After imputation there were 8 048 SNPs for association analysis. We performed a genetic association study with FaST-LMM for the different trait measures mentioned above (AUC and vehicle, 10x, 20x and 30x clinical concentration).

Results

Viability

Cell viability was markedly affected by increasing clozapine concentration (Figure 1). We estimated the narrow heritability of the AUC measurement to be $h^2 = 0.76$ (95% confidence interval 0.59-0.93). Respective viability measures from vehicle to 30x clinical concentration were highly correlated with r-square values between 0.58 and 0.84 ($p<2.2x10^{-16}$).

Figure 1. Boxplot of viability measures at different concentration points.



The y-axis depicts fluorescence values, higher values mean higher viability.

Genome-wide association analyses

We set out to perform a GWAS using the area under the survival curve (AUC) as quantitative trait measure. Correction for population stratification was not necessary because of the relatively homogeneous ancestry of the HapMap CEU samples as well as internal correction of relatedness and substructure by the FaST-LMM method using the available genotype data. The regression results did not show evidence for an inflated type 1 error with a $\lambda = 1.044$ (Supplemental Figure 3). There were 60 independent SNPs reaching $p < 5x10^{-5}$, but none reached genome-wide significance ($p < 1x10^{-8}$) (Supplemental Table 1).

In addition to using the combined AUC survival measure we also used the separate survival measures at four different clozapine concentrations (vehicle, 10x, 20x, and 30x clinical concentration) as quantitative traits for genome-wide analysis. None of these showed evidence for statistical inflation of the type 1 error with lambda values (λ) ranging from 1.034 to 1.049 (Supplemental Figure 4-7). The separate phenotypes were highly correlated and the p-values showed similar distributions, with r-squares between 0.16 and 0.54 ($p<2.2x10^{-16}$). We did not observe any locus with genome-wide significant evidence ($p<1x10^{-8}$) for association with cell viability after clozapine exposure in lymphoblast cell lines.

We identified one SNP (rs1293970) with consistent association signal across all separate concentration analyses at $p < 5x10^{-5}$ (Table 1). The locus resides 6 kb upstream from the proteoglycan 4 (*PRG4*) gene, a gene coding for the hemangiopoietin protein, a growth stimulator for bone marrow stem cells. Two independent loci yielded nominal significant evidence ($p<5x10^{-8}$) for involvement in cell viability after exposure of 30x clinical clozapine concentration. The first of these SNPs is rs2709505 (with $p=1.4x10^{-8}$) located within an intron of the MyoD Family Inhibitor Domain Containing (*MDFIC*) gene on chromosome 7. The second SNP with nominal significance evidence of involvement is rs10457252 ($p=1.79x10^{-8}$), located in a 1.5Mb gene desert flanking laminin alpha 4 (*LAMA4)* on chromosome 6q21.

Table 1. Findings quantitative trait analyses with p < 1x10-6, or with consistent suggestive evidence (p-value < 5x10-5) throughout different analyses.

Trait	Chr.	SNP	Position	MAF	HWE*	P - value	Gene
AUC	20	rs6011368	62356948	0.492	1	8.07x10-7	2kb upstream of PCMTD2
Vehicle	1	rs1293970	186258229	0.497	0.074	1.91x10-6	6kb upstream of PRG4
10x	1	rs1293970	186258229	0.497	0.074	3.37x10-6	6kb upstream of PRG4
20x	1	rs1293970	186258229	0.497	0.074	7.43x10-7	6kb upstream of PRG4
30x	1	rs1293970	186258229	0.497	0.074	2.71x10-5	6kb upstream of PRG4
30x	7	rs2709505	114384077	0.05	1	1.41x10-8	MDFIC
30x	6	rs10457252	113251477	0.31	0.38	1.79x10-8	-

Abbreviations: Chr. = chromosome, MAF = minor allele frequency, HWE = Hardy Weinberg equation

* P values for Hardy Weinberg equation.

MHC region

Given the prior suspicion of an immunological component involved in CIA, we performed a separate analysis of the MHC region and the different trait measures of cell vitality using the comprehensive set of genotyped and imputed SNP data of this chromosomal region. Bonferroni correction for multiple testing for this separate analysis with 8 048 SNPs included was estimated at $p < 1.2 \times 10^{-6}$. Even with a more lenient threshold of $p < 5x10^{-5}$ we did not observe any evidence of association between the MHC region and clozapine-induced viability reduction in lymphoblasts cell lines.

Discussion

The aim of this study was to investigate the genetic underpinnings of clozapine-induced viability reduction in lymphoblasts as a possible *in vitro* model for CIA observed in patients. We exposed lymphoblast cell lines to different concentrations of clozapine and determined cell viability after 48 hours, thereby creating a survival curve. This survival curve showed markedly decreased viability with increasing clozapine concentration.

The viability measurements at different, fixed concentration points and the deducted AUC were used for genome-wide association analysis and targeted MHC region association analyses. We found two SNPs with nominal significant evidence of association with cell viability after 30x clinical clozapine concentration and identified one locus with consistent evidence of association with cell viability for all levels of clozapine exposure but these findings do not survive significance thresholds after correction for multiple testing. One of these nominally significant findings is with rs2709505 ($p = 1.41x10^{-8}$), located in an intron of the myoD family inhibitor domain-containing (*MDFIC*) gene, a gene first described in regulation of viral gene expression [134].. The second best finding is rs10457252, located in a gene desert at chromosome 6q21 ($p = 1.79x10^{-8}$).

We observed one locus (rs1293970) with consistent suggestive evidence (p-value < $5x10^{-5}$) of involvement in cell viability at all separate clozapine concentrations. This SNP resides on chromosome 1, 6KB upstream from the *PRG4* (proteoglycan 4) gene. The *PRG4* gene codes for different proteins, the most important being lubricin, megakaryocyte-stimulating factor (MSF), and hemangiopoietin. Lubricin is produced by chondrocytes and is a large proteoglycan, important for lubrication of joints [135]. Hemangiopoietin has been shown to stimulate hematopoietic stem cells and progenitor endothelial cells [136] and it accelerates hematopoietic reconstitution in mice after chemotherapeutic or radiation treatment [137]. The latter could play a role in susceptibility to clozapine-induced viability in lymphoblasts and other cells.

Although some of the genes mentioned above may play a role in clozapine-induced viability reduction and in CIA susceptibility based on their functionality, none of these findings are significant after correction for multiple testing.

Clozapine induced agranulocytosis is a serious condition with little known about its underlying genetic architecture. Low prevalence has made the implementation of GWAS for understanding this architecture quite challenging, but the use of a cellular model can be a useful step in the process of unraveling the background of CIA. Cellular models are, of course, not without limitations. For example, clozapine is metabolized in the human body to numerous metabolites. Our cell system was not designed to test these metabolites, though they may play an important role in CIA [29,31,53].

The duration of exposure is another important difference between our in vitro model and the in vivo occurrence in patients. CIA occurs most often after 3 to 6 months of clozapine use [11,110], whereas cellular models in the past have used different exposure times [31,116,122,138], but typically do not extend beyond 24 hours. We have chosen exposure time of 48 hours to focus on the immediate effects of clozapine on viability of lymphoblasts.

In this study we have exposed lymphoblast cell lines to supratherapeutical concentrations of clozapine, as decrease in viability has been reported for these concentrations [122,123]. Although these concentrations are not described to occur in blood, there have been reports suggesting clozapine concentrations up to 30 times clinical concentration in brain and fat cells [139,140].

Additionally it is important to consider which type of cells will be used in the model. CIA typically affects neutrophils, but these cells tend to show decreasing viability in vitro after more than 24 hours [141], possibly confounding an experiment towards viability. This study has used lymphoblasts, as these cells originate from the same hematopoietic stem cell as neutrophils and do not show reduced viability with prolonged culture times. Expanding this study to neutrophils could give further insight into the biological mechanisms underlying CIA.

Genetic association studies towards CIA until now have focused on the MHC region (Supplemental Table 2), as its background might be immunologic. These studies have been relatively small and replication of findings has been difficult [41,118,119]. A recent exome sequencing study of CIA did not find any significant association within the MHC region either [67]. As of yet a GWAS has not been performed, this could give more insight in the genetics background of CIA. We were able to study at the MHC region in greater detail by imputing the MHC region and performing association testing. However, we did not find any evidence of involvement of this region in cell vitality after clozapine exposure in our analyses. This could be the result of the use of a unicellular model, not being able to test the complete immunological system. These results, however, could also indicate, together with findings of direct toxicity of clozapine and its metabolites, that the immunological component of CIA is smaller than previously implicated.

None of the SNPs in our study reached genome-wide significance after multiple test correction. This is likely due in part to lack of power. Our results, combined with results from literature, suggest that clozapine-induced viability in lymphoblasts is a highly heritable, complex polygenic trait. Susceptibility to CIA is likely conferred by many loci of modest effect rather than a small number of loci with large effects. Future international efforts to assemble large-scale samples will be necessary to improve power to detect polymorphisms that contribute to CIA susceptibility.

Despite this study's limitations, we demonstrate that cellular models can aid in revealing genetic architecture of complex traits in the absence of large sample sizes. First, we show that clozapine-induced viability reduction is highly heritable ($h^2 = 0.76$). Additionally we have identified *PRG4* and *MDFIC* as possible new candidate genes that might play a role in reduced cell viability induced by clozapine. Future studies with larger patient or cell line numbers are needed to successfully and unambiguously identify the loci involved [142]. Such efforts will expand our understanding of the genetics of CIA and improve patient care in treatment-resistant schizophrenia.

2

RNA

Chapter IV

Clozapine-induced *in vitro* gene expression profiles are linked to cholesterol and cell cycle pathways without similar evidence for DNA methylation changes.

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Abstract

Background

Clozapine is an important antipsychotic drug, however, it's use is often accompanied by metabolic adverse effects and by agranulocytosis. The molecular mechanisms underlying these adverse events are unclear. We therefore sought to investigate gene expression and DNA methylation changes resulting from clozapine exposure in lymphoblastoid cell lines (LCLs).

Methods

LCLs were exposed to increasing concentrations of clozapine after which RNA and DNA were extracted for genome-wide gene expression and DNA methylation profiling. Using linear regression and gene network analyses, we examined the molecular signatures derived from clozapine-dependent changes in gene expression and DNA methylation. Lastly, we performed gene-set enrichment analyses to test for a genetic link between molecular targets of clozapine and schizophrenia disease risk, as well as with metabolic traits.

Results

We observed that clozapine exposure results in significant changes in expression levels of 463 genes implicated in cholesterol and cell cycle pathways. Gene-set analyses showed that differentially expressed genes were not enriched for genetic risk associated with schizophrenia or known psychotropic drug targets. We did observe evidence for enrichment of genetic signal with total cholesterol and low-density lipoprotein levels.

Conclusion

These results shed light on the biological mechanisms through which clozapine functions. Differentially expressed genes did not show genetic enrichment for schizophrenia risk loci, suggesting that the genetic architecture of disease susceptibility may be independent from effects of clozapine exposure in LCLs. The

observed association with the genetic architecture of cholesterol may be indicative of the metabolic adverse effects observed in clozapine users.

Introduction

Antipsychotic drugs (APs) play an important role in the treatment of psychotic disorders such as schizophrenia. Clozapine is the most effective antipsychotic drugs [9]. However, the decision to prescribe clozapine is complicated by its potential to induce severe adverse effects. The most severe adverse effect is clozapine-induced agranulocytosis, a dramatic reduction of white blood cells with a prevalence of <1%. Clozapine is also known to induce adverse metabolic effects such as weight gain, dyslipidemia and type 2 diabetes. These adverse metabolic effects are, in addition to the chance of developing agranulocytosis, one of the primary reasons for patient noncompliance and discontinuation of treatment [143,144].

The biological mechanisms underpinning the effect of clozapine, as well as its adverse effects, remain elusive. A twin study estimated that the heritability of APs-induced weight gain is approximately 60% [37], suggesting a substantial role for genetic factors in antipsychotic-induced adverse metabolic effects. A number of genetic studies have focused on candidate genes that potentially associate to adverse effects of clozapine, but results have been ambiguous and lack consistent replication (reviewed by [19,20,38,40,145]). Finally, two genome-wide association studies (GWAS) have investigated antipsychotic-induced metabolic adverse effects [43,146]. These large-scale genetic studies have the potential to discover genes and pathways that associate with adverse effects of antipsychotic medication. However, results up to now have been limited due to insufficient sample sizes and the largely unexplained heritability of clozapine-induced side effects.

Intermediate molecular phenotypes, such as gene expression studies in specific cell lines, may help in improving our understanding of the phenotype. Gene expression studies in multiple cell types show that atypical antipsychotic drugs in general induce cholesterol metabolism through transcription factors such as sterol regulatory element binding proteins (*SREBP1* and *2*) (reviewed by Ferno and colleagues [22]). These studies suggest that drug-induced cholesterol metabolism may lead to adverse metabolic effects seen under antipsychotic drug treatment, although replication of

these results in whole blood showed mixed results [147,148]. Effects of antipsychotic drugs on DNA methylation could mediate the changes in gene expression, and a small number of studies have investigated the effect of antipsychotic drugs on DNA methylation in humans [149–157]. Methods and types of analyses between these efforts differ greatly, as do the results. None of the outcomes has been replicated, except for an association between hypomethylation of the *FAR2* gene and insulin resistance after AP treatment [155].

A major obstacle in investigating the biological mechanisms of clozapine-induced metabolic effects is the availability of a large and controlled prospective cohort of patients treated with clozapine; only 6% of schizophrenia patients are treated with clozapine [96], and of those, only 1% develop CIA. We therefore implemented an *in vitro* cell model for studying the effects of drug exposure at the molecular level, allowing us to perform many replicates and exposures, without the need to assemble a large-scale patient cohort. Since lymphoblast cell lines (LCLs) have successfully been employed before to study the genetic contribution to (adverse) effects [57,121,158,159], we decided to use LCLs to study the effect of clozapine.

Material and Methods

Lymphoblast cell lines

We used lymphoblast cell lines (LCLs) from four unrelated samples, all part of the collection of Utah residents of Northern and Western European ancestry (HapMap CEPH/CEU phase 1)[160]. We obtained LCLs from the Coriell Institute for Medical Research (Camden, NJ, USA) and maintained the cell lines as previously described [57].

To study methylation changes after exposure to clozapine, we performed a separate experiment using six LCLs (HapMap, CEPH/CEU phase 1) consisting of two parent-offspring.

Clozapine exposure and in vitro experimental design

We performed drug exposure experiments in 6-well plates (Genesee, San Diego, CA, USA) and assessed cell viability using the TC10 automated cell counter (Bio-Rad, Hercules, CA, USA), according to manufacturer's instructions. Clozapine was purchased from Sigma Aldrich (St. Louis, MO, USA). Previous work has suggested that clinical concentrations of antipsychotics may not induce significant gene expression changes *in vitro* [161]. There is evidence that *in vivo* concentrations of antipsychotic drugs are higher in brain tissue than in peripheral blood [139,140]. We therefore chose to expose cell lines to different clozapine concentrations, with clinical concentration set at 2μM [124]. Clozapine was dissolved in culture medium with dimethyl sulfoxide (DMSO), with a maximum concentration of 0.1%. Cell lines were exposed for 24 hours to clinical concentration, 10x, 50x and 100x clinical concentration (20μM-100 μM-200 μM clozapine) and vehicle (DMSO); each concentration was measured in 4 cell lines, after which RNA was obtained for gene expression analysis.

To study DNA methylation changes in response to clozapine, we performed an experiment similar to the gene expression study; LCLs were exposed to different concentrations of clozapine (vehicle (DMSO), 1x, 20x, 40x and 60 times clinical concentration) and exposure times were 24h and 96h (Supplemental Figure 1B).

Sample processing and gene expression data

We performed RNA extraction with Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions. RNA quantity and quality were measured with T2100 BioAnalyzer (Agilent, Santa Clara, CA, USA) and verified with a NanoDrop Spectrophotometer (NanoDrop products, Wilmington, DE, USA). Gene expression profiling was carried out using Illumina® HumanHT-12 v4 Expression BeadChip technology (Illumina, San Diego, CA, USA).

Sample processing and DNA methylation data

After desired exposure time, cells were lysed and DNA was extracted with DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA), according to the instructions of the manufacturer. DNA quality and quantity were assessed with the picogreen® assay (VWR, West Chester, PA, USA) and Nanodrop (ThermoScientific, Wilmington, DE, USA). DNA methylation assays were performed with Illumina® Infinium HumanMethylation450 Beadchip arrays (Illumina, San Diego, CA, USA), assaying approximately 450,000 CpG sites.

Data preprocessing and normalization

We processed raw gene expression values using the "Lumi" R-package [162]. We log2 transformed and quantile normalized the raw data, keeping only expressed gene transcripts (detection $p < 0.01$) for further analysis (22,926 probes).

We preprocessed the raw methylation data in R using the "WateRmelon" Bioconductor package [163], removing probes that were known to cross-hybridize, probes containing SNPs in target CpG regions, probes with detection p-value greater than 0.01 in 5% of samples, and probes with beadcounts > 3 (n=86,068 probes in total) [164,165]. We then normalized the data using the *dasen* function (WateRmelon package). LCL culture cell composition was assessed using methylation-derived estimation of cell proportion with the estimateCellCount function in the minfi package [166]. Methylation is expressed as β-values, with β ranging from 0 (no methylation) to 1 (completely methylated). In general, probes with a mean β-value < 0.2 are considered hypo-methylated and β-value > 0.8 are considered hyper-methylated, within these regions very little variation

is observed [167]. For analysis, we selected only variable probes with a mean β-value between 0.2 and 0.8 (165,014 probes) (preserving most of the variance, data not shown) [167,168]. Finally, we performed gene ontology enrichment analysis using R-package "missMethyl" [169]

Statistical analyses

To detect clozapine-induced molecular changes, for each probe, we tested for association between measured level of gene expression or DNA methylation and increasing clozapine concentrations in a linear model using the "Limma" package in R [170]. Each model was first run within an individual and for each probe subsequently meta-analyzed across individuals by combining p-values using Stouffer's method with directionality of the effect sizes taken into account. We included RNA-integrity number (RIN) as a covariate in the gene expression model and applied a Bonferroni correction to correct for multiple testing, resulting in a significance threshold of $p < 2.18x10^{-6}$ for the gene expression model (n = 22,926 probes) and $p < 3.03x10^{-7}$ for the DNA methylation model (n = 165,014 probes).

Gene ontology analysis

We performed functional gene ontology analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery, version 6.8, interrogated February 2018) [171,172].

Weighted gene co-expression network analysis

We performed a gene expression network analysis. To avoid bias due to a different number of probes per gene, we collapsed probe-level to genes prior to analyses. We then analyzed gene transcripts with nominal significant evidence ($p<0.05$) of differential expression linked to clozapine exposure (5,708 probes, residing in 4,897 genes) using weighted gene co-expression network analysis (WGCNA) [173–175]. WGCNA is a method for discerning correlation patterns among genes across different samples using gene expression data [174,176]. Gene expression data is clustered in modules (networks) of co-expressed genes. To define a representative module expression profile, the (standardized) gene expression profile of the module is

summarized by its first principal component, i.e., the module "eigengene." The eigengenes of the different gene co-expression modules were analyzed for association with clozapine exposure. Gene ontology analysis of the modules was also performed with DAVID.

Additional DNA methylation analyses

We used the *comb-p* tool to search for evidence of regional enrichment of differential DNA methylation by analyzing spatially correlated p-values [177]. Additionally, we ran a candidate gene study for CpG sites within close proximity of genes with evidence of clozapine-induced differential gene expression. Methylation probes within the gene body, in the 3`/5` prime region and up to 1,500 nucleotides upstream of the transcription start site of the 463 'topgenes' ($p<0.05$) were selected for the posthoc analysis (n = 1,004). A list of genes can be found in Table 1 of the Supplemental Material.

MAGMA gene-set analysis

To investigate if genes identified to be differentially expressed after clozapine exposure were enriched for genetic association signal of schizophrenia or cardiovascular-related traits, we performed gene-set analysis using MAGMA (multimarker analysis of genomic annotation), a tool for generalized gene set analysis of GWAS data [178]. For the purpose of this analysis, probes of the same gene were collapsed by taking the average Z-score per gene. A total of 11,533 genes were available for analysis. We created the following three gene-sets, each with three different significance thresholds;

1. All genes differentially expressed;
2. Upregulated differentially expressed genes;
3. Downregulated differentially expressed genes.

GWAS summary statistics were downloaded for schizophrenia [99], body mass index [179], coronary artery disease [180], type 2 diabetes [181], total cholesterol, triglycerides, high-density-lipoprotein, low-density-lipoprotein [182]. For each summary statistic, aggregate GWAS gene-level test statistics were computed and subsequently associated with the annotation of interest using Entrez ID of overlapping genes.

Linkage Disequilibrium (LD) was estimated externally using the 1000 Genomes European reference panel [183]. MAGMA was run using a two-sided competitive test while correcting for gene size, SNP density, minor allele count and LD.

Antipsychotic drug gene-sets

To assess whether the clozapine associated differential gene expression profiles could be linked to known drug targets, we performed an enrichment analysis of antipsychotic drug gene-sets. Drug gene-sets were downloaded from supplementary materials from a previous study [184] that obtained drug targets from drug-gene interaction databases [185,186]. We identified 50 sets of drugs with ATC (Anatomical Therapeutic Chemical) code N05A, a drug class to which all antipsychotic drugs belong, including clozapine. We ran gene-set analysis per N05A antipsychotic drug, for all N05A drug target genes combined, and for all N05A drug target genes combined excluding clozapine.

LD Score Regression estimation of heritability and genetic correlations

SNP-based heritability (SNP-h^2) was estimated using LD Score Regression (LDSR) [187]. Pre-computed LD scores from the 1000 Genomes European reference panel provided through the LDSR *github* and GWAS summary statistics file processed and reformatted to s*umstats* format were used as input to the software. Genetic correlations between traits were estimated using cross-trait LDSR via the *--rg flag* [188].

Results

Clozapine exposure induces widespread gene expression changes

We exposed lymphoblast cell lines to different concentrations of clozapine and tested for gene expression changes (Supplemental Figure 1A). First, we performed a linear regression analysis per individual, and then meta-analyzed the results across all individuals. In total, we tested 22,926 gene expression probes, of which 5,708 showed nominal significance ($p<0.05$) and 518 probes exceeded a Bonferroni-corrected $p < 2.18^{*}10^{-6}$ ($p = 0.05/22{,}926$ probes). These 518 probes consisted of 234 up-regulated probes and 284 down-regulated probes, representing a total of 463 unique genes. The top 10 up-regulated and down-regulated probes are shown in Table 1. See Supplement for the complete list of genes.

Table 1. Top 10 up- and downregulated genes after clozapine exposure.

Down-regulated genes		**Up-regulated genes**	
Gene	P-value*	Gene	P-value*
STMN1 *(Stathmin 1)*	$5.16^{*}10^{-16}$	*LSS* *(Lanosterol synthase)*	$6.14^{*}10^{-17}$
HIST2H2AC *(Histone cluster 2 H2A family member C)*	$1.98^{*}10^{-15}$	*MAL* *(Mal, T-cell differentiation protein)*	$1.10^{*}10^{-16}$
RPS7 *(Ribosomal protein S7)*	$2.52^{*}10^{-15}$	*MIR1974* *(MicroRNA 1974)*	$5.56^{*}10^{-16}$
HIST1H2BJ *(Histone cluster 1 H2B family member J)*	$4.42^{*}10^{-15}$	*LDLR* *(Low density lipoprotein receptor)*	$3.97^{*}10^{-14}$
HIST2H2AA3 *(Histone cluster 1 H2A family member A3)*	$1.97^{*}10^{-14}$	*DHCR7* *(7-dehydrocholesterol reductase)*	$4.26^{*}10^{-14}$
RPS15 *(Ribosomal protein S15)*	$2.17^{*}10^{-14}$	*PASK* *(PAS Domain Containing Serine/Threonine Kinase)*	$5.09^{*}10^{-14}$
HIST2H2AA4 *(Histone cluster 2 H2A family member A4)*	$2.78^{*}10^{-14}$	*RGS1* *(Regular Of G Protein Signaling 1)*	$2.31^{*}10^{-13}$
HIST1H2AC *(Histone cluster 1 H2A family member C)*	$2.79^{*}10^{-14}$	*LYPD6B* *(LY6/PLAUR Domain Containing 6B)*	$4.38^{*}10^{-13}$

AURKA *(Aurora Kinase A)*	$5.19^{*}10^{-14}$	*TPP1* *(Tripeptidyl Peptidase 1)*	$6.77^{*}10^{-13}$
SGOL1 *(Shugoshin 1)*	$2.09^{*}10^{-13}$	*RENBP* *(Renin Binding Protein)*	$7.54^{*}10^{-13}$

* P-values are uncorrected

See supplemental table 1 for full list of significant genes.

GO analysis of up-regulated transcripts showed significant functional enrichment for cholesterol metabolism (13 genes, $p = 4.15^{*}10^{-15}$) and steroid biosynthesis (11 genes, $p = 1.01^{*}10^{-8}$) (Table 2), and enrichment for down-regulated transcripts for genes involved in cellular processes such as mitosis (44 genes, $p = 1.87^{*}10^{-39}$), chromosome (49 genes $p = 3.03^{*}10^{-35}$) and nucleosome (16 genes, $p = 3.12^{*}10^{-14}$) and other cell cycle pathways (Table 2).

Table 2. Top up- and downregulated pathway 'cluster' after clozapine exposure.

Up-regulated pathways						
Functional annotation cluster	Enrichment score*	Best p-value	Best fold enrichment#	Average p-value	Average fold enrichment	Number of pathways in cluster
Cholesterol/lipid/steroid biosynthesis	8.44	$4.15^{*}10^{-15}$	46.92	$1.19^{*}10^{-3}$	18.46	19
Cholesterol/Steroid biosynthesis	4.77	$1.01^{*}10^{-8}$	68.45	$4.66^{*}10^{-4}$	54.32	3
Down-regulated pathways						
Functional annotation cluster	Enrichment score	Best p-value	Best fold enrichment	Average p-value	Average fold enrichment	Number of pathways in cluster
Mitosis/cell division	31.26	$1.87^{*}10^{-37}$	14.77	$1.12^{*}10^{-21}$	10.69	5
Chromosome/centromere	16.47	$3.03^{*}10^{-35}$	21.32	$7.76^{*}10^{-8}$	15.79	9
Histone/nucleosome	6.93	$3.12^{*}10^{-14}$	20.72	$1.73^{*}10^{-3}$	9.13	18
Spindle	6.02	$4.95^{*}10^{-12}$	12.89	$7.45^{*}10^{-4}$	10.13	6
Nucleotide/ATP-binding	5.72	$1.46^{*}10^{-13}$	2.71	$7.78^{*}10^{-5}$	2.47	5
Microtubule/kinesin	4.60	$1.90^{*}10^{-4}$	16.92	$4.99^{*}10^{-2}$	8.56	19

* Enrichment score is used as an overall importance marker of annotation groups. It is defined as the geometric mean of all enrichment p-values of each annotation term within the group. It is expressed as the minus log of the p-value, an enrichment score of 1.3 is nominally significant. This table shows clusters with an enrichment score > 4.6, corresponding to a p-value < 0.01. Fold enrichment is a measure to express the enrichment of this particular group of genes in comparison with the genes in the human genome.

Fold enrichment is a measure to express the enrichment of this particular group of genes in comparison with the genes in the human genome.

A list of all pathways is available in supplemental information.

Gene expression network analysis after clozapine exposure

WGCNA network analysis of clozapine-induced differential expressed genes (N=4,987) yielded 15 co-expression modules, ranging in size from n=61 to 1,791 genes. No gene co-expression module was significantly associated with clozapine exposure after multiple test correction. Five gene co-expression modules were nominally significantly associated with clozapine exposure: M14 (upregulated, 61 genes), M10 (upregulated, 155 genes), M9 (upregulated, 158 genes), M3 (upregulated, 579) and M1 (downregulated, 1,791 genes). Gene ontology analysis showed that the M14 co-expression module was enriched for genes involved in cholesterol metabolism (13 genes, $p = 4.8^{*}10^{-15}$), the M1 co-expression module was enriched for genes involved in cell cycle (133 genes, $p = 7.3^{*}10^{-32}$) and the M9 and M3 co-expression module was enriched for mitochondrion genes (15 genes, $p = 6.7^{*}10^{-6}$ and 46 genes $p = 3.8^{*}10^{-7}$ respectively). The M10 co-expression module was enriched for genes involved in the nucleosome (9 genes, $p = 1.4^{*}10^{-7}$).

DNA methylation patterns are unaffected by clozapine exposure

To assess if clozapine exposure has similar strong epigenetic effects, we performed a DNA methylation experiment. For statistical analysis, we adapted the method that we also used for the gene expression experiment. We first performed an analysis with a linear regression model at the individual level, after which a meta-analysis was performed. This was done twice, first for the 24h clozapine exposure data, and again for the 96h clozapine exposure data. After both 24h and 96h of clozapine exposure, we did not observe significant changes in methylation, after correction for multiple testing with Bonferroni ($p < 3.03^{*}10^{-8}$) (Table 3).

Secondly, we performed a candidate gene analysis based on a list of 463 genes and 1,004 CpG sites that showed significantly different expression patterns after clozapine exposure. After 24h exposure, 3 probes showed significant changes in DNA methylation ($p < 1.08^{*}10^{-4}$); after 96h, 1 probe showed significant changes in DNA methylation (Table 4).

Table 3. Top 10 methylation probes after 24h and 96h of clozapine exposure.

24h clozapine exposure					**96h clozapine exposure**				
Probe ID	Chr#	Coordinate	Annotation	P-value*	Probe ID	Chr#	Coordinate	Annotation	P-value*
cg09495017	16	57,124,763	*CNOT1*	$5.40^{*}10^{-7}$	cg21293934	18	14,738,230	*ANKRD30B*	$8.87^{*}10^{-7}$
cg16258062	2	234,048,887	-	$5.60^{*}10^{-7}$	cg19182557	2	130,061,825	-	$9.21^{*}10^{-7}$
cg16258062	1	47,654,324	*FOXE3*	$1.15^{*}10^{-6}$	cg15463280	11	95,955,596	-	$2.36^{*}10^{-6}$
cg15066636	6	33,187,127	*HLA-DPB2*	$1.42^{*}10^{-6}$	cg12564567	11	115,876,398	-	$5.87^{*}10^{-6}$
cg17488052	1	77,993,925	*USP33*	$1.48^{*}10^{-6}$	cg26647200	16	2,422,776	*CCNF*	$6.87^{*}10^{-6}$
cg25181236	4	56,082,032	*CLOCK*	$1.48^{*}10^{-6}$	cg09333631	3	44,777,608	*KIF15, KIAA1143*	$7.99^{*}10^{-6}$
cg01531409	14	59,781,022	*PPM1A*	$2.07^{*}10^{-6}$	cg16924010	3	195,500,852	-	$8.51^{*}10^{-6}$
cg09840472	7	22,730,922	-	$2.27^{*}10^{-6}$	cg01842314	10	106,102,325	*CCDC147*	$1.07^{*}10^{-5}$
cg24207009	17	73,549,157	*TNRC6C*	$2.37^{*}10^{-6}$	cg23898204	2	724,927	-	$1.11^{*}10^{-5}$
cg27170003	17	3,713,677	*CAMKK1*	$2.39^{*}10^{-6}$	cg15000279	19	33,976,849	-	$1.28^{*}10^{-5}$

* P-values are uncorrected

Table 4A & 4B. Candidate gene analysis: Significant methylation probes after 24h and 96h of clozapine exposure.

24h clozapine exposure				
Probe ID	Annotation	P-value*	Gene expression Probe ID	Gene expression P-value
cg05455234	*PCNT* *(Pericentrin)*	$1.98^{*}10^{-5}$	ILMN_1810922	$5.68^{*}10^{-8}$
cg22971501	*LDLR* *(Low Density Lipoprotein Receptor)*	$4.75^{*}10^{-5}$	ILMN_2053415	$3.97^{*}10^{-14}$
cg01233620	*CLEC16A* *(C-Type Lectin Domain Containing 16A)*	$6.30^{*}10^{-5}$	ILMN_1781752	$1.04^{*}10^{-7}$

* P-values are uncorrected

96h clozapine exposure				
Probe ID	Annotation	P-value*	Gene expression Probe ID	Gene expression P-value
cg26647200	*CCNF* *(Cyclin F)*	$6.87^{*}10^{-6}$	ILMN_1773119	$4.41^{*}10^{-11}$

Clozapine transcriptome signatures and schizophrenia disease risk

To investigate whether the identified *in vitro* gene expression signatures were associated with genetic susceptibility of schizophrenia, we performed gene-set analysis. We first performed an analysis using all differentially expressed genes as a gene-set (n=463); this did not show a significant association (p = 0.91). Additionally, we did not observe significant associations for up-regulated genes (p = 0.74), nor for down-regulated genes (p = 0.64). Decreasing the significance threshold of differentially expressed genes to FDR 1% and 5% did not change these findings (Table 5).

We investigated whether SCZ genetic susceptibility aggregates to antipsychotic drug target genes with and without clozapine targets. The drug target gene list was defined by drug-gene interaction databases [185,186]. We found no evidence for SCZ enrichment in antipsychotic drug target genes overall (n = 96 genes, p = 0.96) nor with clozapine targets excluded (n = 52, genes, p = 0.60). We did observe a strong concordance between the p-values of individual drug gene-sets from our analysis and the p-values reported by the previous study [184] (n = 50 drug gene sets, rho = 0.85, $p = 1.49^{*}10^{-15}$), indicating our analysis framework was able to reproduce previous results.

Table 5. Geneset analyses with schizophrenia and different significance levels.

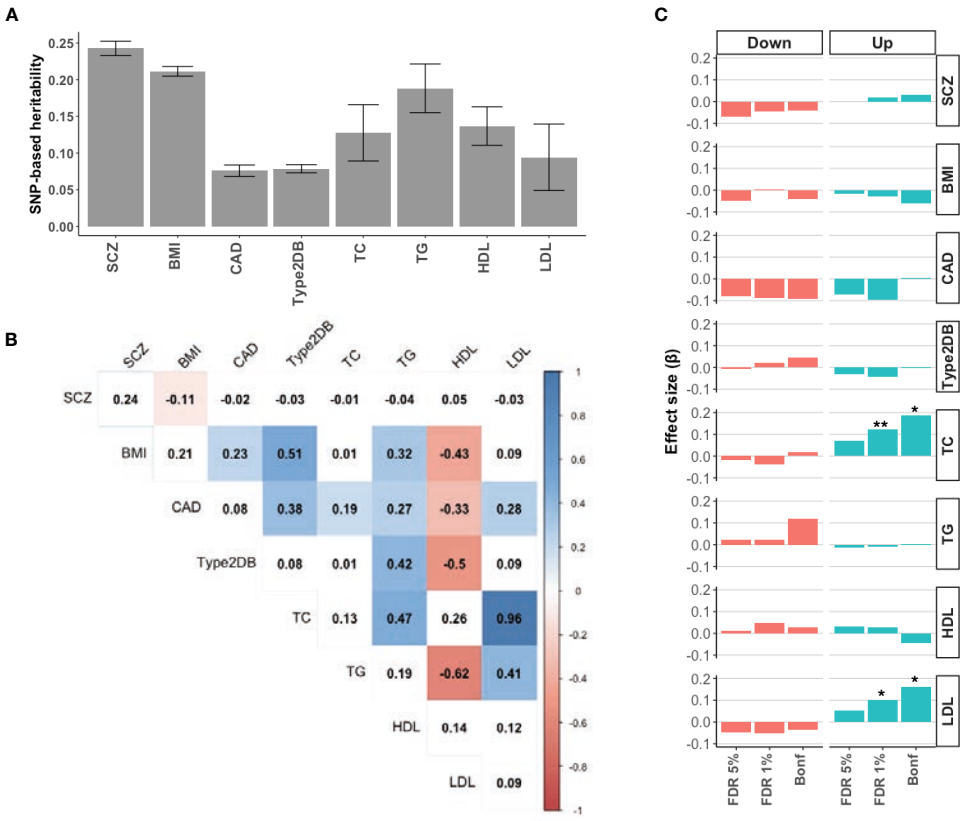
Set	**Bonferroni**	**FDR < 1% (q<0.01)**	**FDR<5% (q<0.05)**
Differentially expressed genes	p = 0.92 (311 genes)	p = 0.74 (919 genes)	p = 0.22 (1543 genes)
Upregulated genes	p = 0.75 (138 genes)	p = 0.71 (457 genes)	p = 0.99 (772 genes)
Downregulated genes	p = 0.64 (173 genes)	p = 0.39 (462 genes)	p = 0.09 (771 genes)

Genes upregulated after clozapine exposure show an association with GWAS signal of total cholesterol and low-density lipoprotein.

Next, we explored whether differentially expressed genes were enriched for genetic signal of cholesterol- and cardiovascular disease-related traits. We included seven traits for which summary statistics of large GWASs were available and significant SNP-

h^2 was captured (Figure 1A). These traits are in part genetically correlated as has been previously reported (Figure 1B) [188]. While no association remained significant after correction of multiple testing (72 tests, $p < 6.9^{*}10^{-4}$), we did observe an increasing association across thresholds of <FDR 5%, <FDR 1%, and Bonferroni correction for total cholesterol and LDL respectively (Figure 1C). We did not find such a pattern for HDL or any of the other traits tested, including SCZ (Figure 1C).

Figure 1. Clozapine gene-set analyses across cardiovascular traits.



(A) SNP-based heritability estimates on the observed scale. (B) Genetic correlations between traits used in the analysis. The magnitude of the genetic correlation is only color-coded for estimates with P-value < 0.01. (C) MAGMA effect sizes (β) of GWAS association with clozapine target genes are shown for down- and upregulated genes identified to be differentially expressed at FDR 5%, FDR 1%, and Bonferroni correction thresholds. Asterisks denote nominal significance of *P < 0.05 **P< 0.01. SCZ = schizophrenia, BMI = body mass index, CAD = coronary artery disease, Type2DB = type 2 diabetes, TC = total cholesterol, TG = triglycerides, HDL = high-density-lipoprotein, LDL = low-density-lipoprotein.

Discussion

We used an *in vitro* cell system of LCLs to study molecular effects of clozapine exposure aiming to better understand mechanisms involved in adverse effects of psychotropic drug use. Genome-wide gene expression profiling of cells exposed to clozapine showed strong activation of cholesterol metabolism and deactivation of genes involved in cell cycle processes. Genome-wide methylation profiling, however, showed limited evidence of altered methylation in response to clozapine exposure. To investigate whether the results found in gene expression analysis were associated with genetic susceptibility of schizophrenia and cholesterol and cardiovascular disease-related traits, we performed different gene-set analyses. Overall, the genetic evidence points to clozapine-response being independent to schizophrenia disease risk, depleted from known drug targets of antipsychotic drugs, while it is likely linked to the genetic architecture involved in cholesterol and low-density lipoprotein levels.

The central role of cholesterol metabolism in the response to clozapine in lymphoblast cell lines is concordant with previously reported studies performed in other cell types and for different antipsychotic drugs [22,189]. Even though most of these were based on candidate genes rather than by genome-wide analyses, the results consistently implicate cholesterol metabolism in response to antipsychotic drugs [190,191,200,201,192–199]. Here, using an unbiased, genome-wide array-based analysis for gene expression, we again show activation of cholesterol metabolism, for the first time in lymphoblast cell lines. The fact that the network analysis did not show a similarly large effect (although the direction of effect was the same) as in the single-gene analysis may be due to the widespread effects on gene expression by clozapine or by our relatively small sample size and therefore limited power.

Up-regulation of cholesterol metabolism throughout the body could play an important role in metabolic adverse effects of clozapine. A small number of studies have identified a link between genetic markers in genes involved in cholesterol metabolism and adverse effects of antipsychotic treatment, helping to further substantiate these findings [202–204].

Additional studies suggest that alterations in cholesterol metabolism by antipsychotic drugs may contribute to the beneficial effects in treating psychosis. Cholesterol is extremely important in brain development and in sustaining neuronal connections and myelination [205]. Furthermore, Le Hellard et al. described an association between genes important in cholesterol metabolism (*SREBP1* and *SREBP2)* and schizophrenia [206], which was confirmed in a schizophrenia GWAS [99].

In addition to significantly up-regulated genes, we also identified 284 genes that are downregulated in the presence of clozapine, with significant enrichment for genes involved in cell cycle pathways. A small number of studies have shown differing gene expression levels in cell cycle genes in patients with schizophrenia, compared to healthy controls [207–211], this link has not reported before for antipsychotic drug effects. It is possible that genes involved in cell cycle are important in the etiology of schizophrenia. Clozapine could possibly affect these genes and thereby exert therapeutic qualities. Conversely, the described effect may be a direct consequence of clozapine toxicity. We have shown before that clozapine, in high concentrations, has a direct effect on viability of lymphoblastoid cell lines [57]. Down-regulation of processes involved in cell cycle could thus be a direct (toxic) effect of clozapine. The toxic effects of clozapine and its metabolites have been associated with clozapine-induced agranulocytosis [30,117,212]. More research is necessary to investigate whether the changes found in cell cycle genes associate with clozapine-induced agranulocytosis, which affects neutrophils. We note that for our experiments, we used LCLs, which are blood-derived cells, but from a different progenitor cell and with different cellular functions than neutrophils. The question remains whether findings from *in vitro* LCL can be directly extrapolated to *in vivo* neutrophils.

While we found strong effects of clozapine exposure at the gene expression level, we did not observe similarly large effects at the level of DNA methylation. We did find 3 differently methylated CpGs located within genes implicated by our gene expression analysis; these effects, however, were not observed after 96h of clozapine exposure. Although previous studies have indicated that antipsychotic drugs may induce changes in DNA methylation [154–157], we did not find evidence for immediate effects on

DNA methylation. Possibly, subtle changes in DNA methylation patterns play a regulatory role in the observed gene expression changes but a larger sample size is needed to decipher these changes [213].

To further explore our gene expression findings, we set out to investigate these effects and their correlation with genetic susceptibility of schizophrenia. We did not observe significant evidence of enrichment of genetic signal in recent schizophrenia GWAS.

Two recent studies did report an enrichment in genetic signal in schizophrenia GWAS from antipsychotic target genes [214,215]. Their approach, however, differed greatly from ours. They have used lists of antipsychotic target genes originating from pharmacological databases, whereas we have used a list of differentially expressed genes after exposure to an antipsychotic. Although these databases draw their associations from a number of different sources, they can be biased towards genes that are thought to be targeted by a drug. The genes we found to be differentially expressed were not enriched for genes associated with clozapine from these two previous studies. It is therefore not surprising that we present different results, since we only tested for genes with altered gene expression. Analyses in larger samples may help elucidate signal that we failed to observe here.

If we assume that the clozapine-induced gene expression differences are a proxy for adverse effects, the lack of evidence of the enrichment analysis suggests that the genetic architecture of disease susceptibility is likely independent from susceptibility to adverse effects. We explored this further by examining the enrichment of genetic signal linked to cholesterol and cardiovascular disease-related traits such as body mass index, coronary artery disease, type 2 diabetes, and triglyceride levels. We observed an increased enrichment for total cholesterol and LDL genetic signal in upregulated genes in response to clozapine exposure. The increase of signal linked with increased significant thresholds strongly suggest that this enrichment is biologically meaningful and could be linked to the observed metabolic adverse effects that are oftentimes observed in patients using clozapine. Substantially larger pharmacogenetics GWAS of adverse effects are necessary, however, to fully disentangle these potential relationships.

The great advantage of using cell models is the controlled environment, which is likely to decrease heterogeneity and improve signal-to-noise ratio. We do not know, however, if LCLs are appropriate cell types to capture the molecular changes that are most relevant for studying adverse effects of APs in patients. Our current results are consistent with previous findings in other cell types, providing opportunity for larger studies with LCLs. The issue of limited power for human studies of understanding drug response remains. Statistical power of human studies in the pharmacogenomic field is often complicated by the lack of large patient cohorts. While sample collection remains a challenge, our efforts to integrate molecular signatures of an *in vitro* cell model with existing data of large-scale genetic studies could have additional value and lead to new insights.

Conclusion

We used LCLs as an *in vitro* model for studying molecular effects of clozapine exposure in an effort to improve our understanding of antipsychotic-induced adverse effects. Genome-wide gene expression profiling demonstrated a robust up-regulation of cholesterol metabolism and down-regulation of cell cycle pathways, with only limited changes in DNA methylation profiles. We did not find evidence that genes up- or down-regulated during clozapine exposure were enriched for genetic variation associated with schizophrenia but the observed enrichment signal with the genetic basis of total cholesterol and LDL levels may provide important leads linked to antipsychotic drug induced metabolic adverse effects. To advance this work, future studies should focus on prospective pharmacological studies and large-scale collaborative efforts to gather patient cohorts of considerable size and generate innovative ways to combine these large-scale studies with *in-vitro* and *in silico* studies.

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Chapter V

No clear evidence for transcriptome differences associated with clozapine-induced fluorescence in neutrophils.

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Submitted.

Abstract

Clozapine is considered the most effective antipsychotic drug and it is the only antipsychotic drug registered for treatment resistant schizophrenia. Its use, however, is complicated by clozapine-induced agranulocytosis, a potentially lethal adverse effect with an incidence of roughly 1%. Recently, neutrophils of clozapine users were found to be auto-fluorescent, a phenomenon that is absent in non-users. The molecular mechanism behind the clozapine-induced autofluorescence is unknown but could be informative for understanding the therapeutic point of action of clozapine or its adverse effects. We examined clozapine-induced auto-fluorescence at the level of transcriptome differences. Neutrophils from 10 clozapine users and 10 healthy controls were collected for transcriptome analysis by RNA sequencing. Despite the strong and distinctive nature of auto-fluorescence in neutrophils of all clozapine users differential gene expression analysis did not show apparent differences between clozapine users and healthy controls. Neither did our sequencing-based examination of immune and microbial related signatures. We did, however, observe limited evidence for decreased expression of *SIRT1* in clozapine users but without further evidence this finding remains elusive. The absence of significant transcriptome differences suggests that the auto-fluorescence in neutrophils may be caused by post-translational modification at the protein level. Alternatively, transcriptome effects might only be detectable at earlier maturation stages of neutrophil development, for example in the bone marrow. Deciphering the origin and nature of auto-fluorescence in clozapine users remains a challenge and requires further study.

Introduction

Clozapine is considered the most effective antipsychotic drug and is the only antipsychotic drug registered for treatment resistant schizophrenia [106]. Its use, however, can induce a potentially lethal adverse effect: clozapine-induced agranulocytosis, a condition in which the neutrophil count in blood drops significantly (absolute neutrophil count <500/μl) with an incidence of approximately 1% of patients [10,11]. The diminished white blood cell count greatly increases the risk of severe and possibly fatal infections in the patient. To prevent clozapine-induced agranulocytosis, a rigorous follow-up system has been designed with weekly blood draws the first 16-18 weeks and monthly thereafter for the duration of treatment. This set-up has decreased the incidence of clozapine-induced agranulocytosis significantly [48]. The possibility of development of clozapine-induced agranulocytosis and the burden of the frequent follow-ups, however, have made clinicians hesitant to prescribe clozapine [144,216].

The etiology of clozapine-induced agranulocytosis remains unclear, although toxic effects of clozapine and its metabolites and immune mechanisms are thought to play a role [24,30,31,117,217,218]. It is likely that there are genetic factors in play, and recent findings have implicated two loci within the major histocompatibility complex (MHC) locus with small effect sizes[45], leaving the question whether clozapine-induced agranulocytosis is a heritable trait yet unanswered.

We recently showed that neutrophils from clozapine users have *auto*-fluorescent properties [34]. The clozapine-induced fluorescence occurred in all patients treated with at least 100mg of clozapine per day for at least 2-3 weeks, in an apparently time and dose dependent manner. After the fluorescent signal in neutrophils reaches a plateau, it remains stable for the duration of clozapine use. It decreases slowly when patients stop taking the drug and fully diminishes 2-3 weeks afterwards, which is slower than turnover of neutrophils in circulation [219], but might represent the turnover of the neutrophil progenitor compartment in the bone marrow. There is no evidence of any other antipsychotic drug to

induce this auto-fluorescent effect. This finding could be used as biomarker for clozapine treatment compliance, since the auto-fluorescence can be tested in a standard complete blood count assay. As a biomarker it provides information about clozapine use in the past weeks, whereas the currently used direct measurements of clozapine in blood gives information about compliance in the past few days. It remains a question whether the clozapine-induced autofluorescence of neutrophils can be used as a biomarker for the development of adverse effects of the drug, especially clozapine-induced agranulocytosis. Understanding the mechanism underlying the fluorescence signal may benefit the prescription rate of this highly effective antipsychotic drug. In a separate study, we recently observed evidence that the clozapine-induced fluorescence originates from the azurophilic granules in the neutrophils and is emitted by a protein(fragment) of 14 kDa (de With et al., submitted).

We set out to further examine clozapine-induced fluorescence by transcriptome analysis using RNA sequencing of neutrophils obtained from clozapine users and non-clozapine-exposed healthy controls. We hypothesized that the unique feature of autofluorescence in neutrophils when exposed to clozapine would be linked to differential gene expression and leading to the presence of one or more specific RNA transcripts detectable by RNA sequencing. The differentially expressed RNA transcripts could provide a link between the fluorescence and the origin of clozapine-induced agranulocytosis.

Materials and Methods

Reagents and antibodies

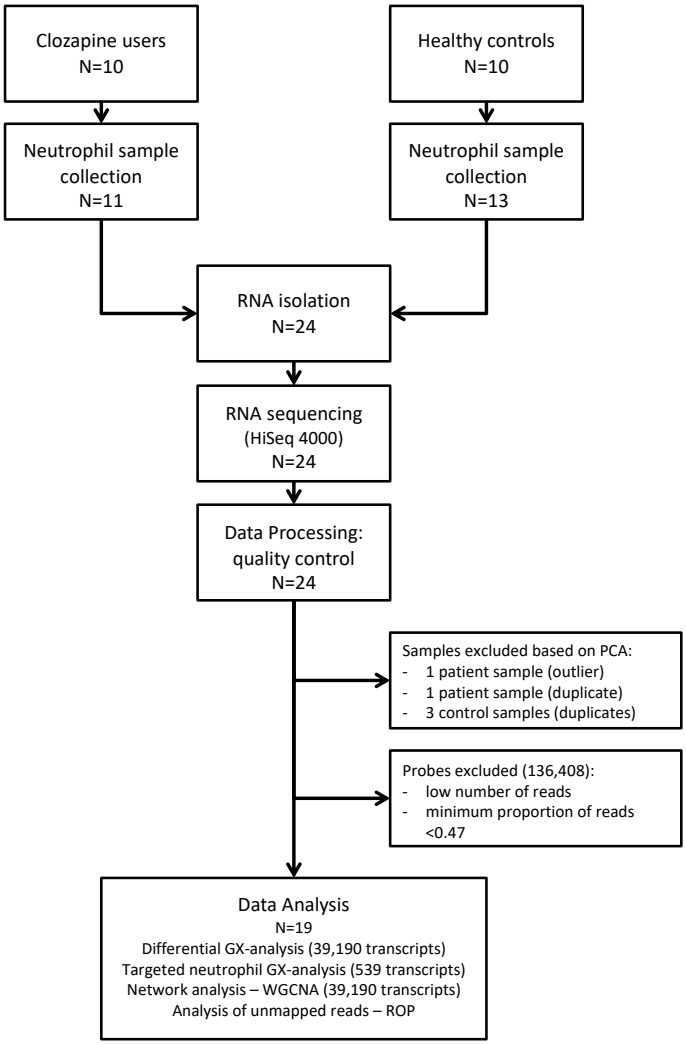
Shock buffer consisted of 155 mM Ammonium Chloride, 10 mM Potassium Bicarbonate and 0.1 mM EDTA. Phosphate buffered saline (PBS) was supplemented with 0.32% trisodium citrate and 10% pasteurized plasma solution (Sanquin, Amsterdam, the Netherlands) to create PBS2+. Antibodies for CD14 (PE-Cy™7, clone M5E2, dilution 1:50) and CD16 (APC H7, clone 3G8, dilution 1:50) were obtained from BD Biosciences (San Diego, CA, USA). Lysis reagent was obtained from Qiazol (Qiagen, Valencia, CA, USA).

Subjects and study design

Blood samples were collected from patients taking clozapine at the psychiatry outpatient clinic at the University Medical Center (UMC) Utrecht. For the purpose of this study, the surplus of routine blood samples were collected and anonymised. The study was exempted from obtaining written informed consent from patients according to Dutch law. The local Medical Research and Ethics Committee and local Biobank Committee approved the study. Blood samples were collected of 10 patients taking at least 100mg clozapine for at least 1 month, thereby ensuring clozapine induced fluorescence had reached detectable and stable levels [34]. In total 11 samples were collected from 10 patients (one patient was sampled twice, Figure 1).

Control samples of subjects who are not using clozapine were collected from healthy volunteers via the blood donor bank of the UMC Utrecht after written informed consent was obtained in accordance to the declaration of Helsinki [220]. Controls (N = 10) were matched on sex and age group (20-30yrs, 30-40yrs, 40-50yrs, etc.). In total 13 samples were collected from 10 healthy controls.

Figure 1. Flowchart of experiments.



Isolation of neutrophils

Whole blood samples were treated with erythrocyte shock buffer and stained with antibodies CD14 and CD16. Neutrophils were sorted based on FSC/SSC (forward-scattered light; FSC and side-scattered light; SSC) and CD16high/CD14neg with a BD FACSAria III™ (BD Biosciences, San Jose, CA, USA). Samples were typically >99% pure as determined by re-analysis with the BD FACSAria III™ and microscopic evaluation of May-Grünwald Giemsa

stained cytospins. We confirmed the presence of autofluorescent signal of neutrophils for all clozapine users and the absence of this signal in the neutrophils of control subjects. Sorted neutrophils were lysed with Qiazol Lysis reagent (Qiagen, Venlo, The Netherlands), according to manufacturer's instructions, and stored at -80°C. When sample collection was complete, samples were shipped to the University of California, Los Angeles (UCLA) for further processing.

RNA Isolation and sequencing

RNA was isolated with the miRNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. RNA quantity and quality was measured with a T2100 BioAnalyzer (Agilent, Santa Clara, CA, USA) and verified with a NanoDrop Spectrophotometer (NanoDrop products, Wilmington, DE, USA). RNA was amplified with the Ovation® RNA amplification system (Nugen, San Carlos, CA, USA) and the preparation of the library was done with the TruSeq Nano DNA library prep kit (Illumina, San Diego, CA, USA). Paired-end sequencing (of 69 nucleotides) was performed on a HiSeq 4000 (Illumina, San Diego, CA, USA) at the UCLA Neuroscience Genomics Core facility.

Data processing

RNA sequencing reads were aligned and quantified using *Kallisto*[221], a program based on the principal of pseudo-alignment, to rapidly determine compatibility of reads and their targets, without the need for actual alignment. We used human genome build 38 as reference, used 100 bootstrap samples of paired-end reads. In total, 24 RNA-samples were collected. Principal component analysis (PCA) was used to detect outliers, after which one sample was excluded; an additional four samples were excluded because of duplicate status, leaving 19 samples available for analysis; 9 patient and 10 control samples. Transcripts with low number of reads (mean <5 reads) and transcripts with a minimum proportion of reads <0.47 were excluded, resulting in a total of 39,190 transcripts for further analyses.

Differences in gene expression between patients prescribed clozapine and healthy controls (not prescribed clozapine) were investigated with *Sleuth,* a companion program of *Kallisto* for analysis of transcript abundances[222]. Patients and healthy controls differed significantly in RNA concentration and sample handling time (Table 1, Supplemental Figure 1). Since both of these factors could affect the outcome of RNAseq analysis, they were taken in to account as covariates in the analysis. We also performed an analysis without considering these covariates, to assess a possible masking of the clozapine effects, since sample handling time and RNA concentration were also correlated with case-control status. The false discovery rate (FDR) was used to correct for multiple testing [223]. FDR values below 0.05 were deemed statistically significant.

In addition to testing all detected transcripts, we also performed a targeted analysis of neutrophil-specific genes only using a transcript list (50 of 67 genes present in data) from Westra *et al.* [224].

Weighted gene co-expression network analysis (WGCNA) was performed to gain further insight in the correlation patterns among genes across different samples [173,225,226]. Genes were clustered into different co-expression modules (or networks), based on their gene expression signatures, using a hierarchical clustering tree. The gene expression signature of the module was then summarized by its first principal component, i.e. the Module Eigengene, to define a representative module expression profile for further analyses. The Module Eigengene is calculated for each module per sample, providing a summary of gene expression values for all probes in a certain module per sample This Module Eigengene can used to test for associations with clinical traits, such as clozapine exposure, sex or RIN value. We constructed a signed weighted gene co-expression network, based on the matrix of pairwise Pearson correlation coefficients. This matrix was raised to a power of 9, as described by Zhang and Horvath [227]. The minimal module size was 50.

Analysis of unmapped sequence reads

Additional analyses were performed to further investigate the unmapped RNAseq reads. Since *Kallisto* does not create an actual alignment for all reads and therefore analyses of unmapped reads is not possible, we used *hisat2* (version 2.0.6) [228] to align the reads to the human genome (hg38), unmapped reads were extracted with *samtools* [229]. After which we performed read original profiling (ROP) analysis with *ROP* (version 1.0.6) [230], to investigate the unmapped reads. This tool maps unmapped reads to complex RNA molecules (such as circular RNA and repeat regions), B- and T-cell receptors and non-human microbial RNA.

Results

General

For the purpose of this study 24 samples were collected from 20 individuals. Samples were processed and after quality control nineteen samples were selected for analysis (9 patients and 10 healthy controls); sample characteristics are depicted in Table 1.

Table 1. Sample characteristics.

	Healthy controls	**Patients taking clozapine**
Age (Yrs.)	35.3 (8.58)	36.56 (5.83)
Sex (M/F)	7/3	6/3
Sample handling time (Hrs)[#]*	2.50 (0.32)	1.95 (0.19)
RNA concentration (ng/uL)[*]	4.01 (2.07)	2.18 (1.76)

Mean and standard deviation (between brackets)

[#] Sample handling time is time between receiving blood sample and sorting neutrophils.

* $P < 0.05$ with Mann-Whitney U test.

Differential gene expression analysis

RNA sequencing was performed and for the 19 samples 175,598 transcripts were detected; after quality control 39,190 transcripts (22%) were available for further analysis. As noted earlier, two possible confounders (RNA concentration and sample handling time) were significantly associated with patient-status (Supplementary information). We therefore performed the gene expression analysis with and without correcting for these variables. We considered that correction for variables that are linked with case status may remove true clozapine-linked gene expression effects. When taking covariates RNA concentration and sample handling time into account, no significant changes in gene expression between patients taking clozapine and healthy controls were found (after correction for multiple testing at $q < 0.05$, Figure 2). The two genes with the lowest q-value (FDR corrected p-value) were *SIRT1* (Sirtuin 1) and *INTS8* (Integrator complex subunit 8) with $q < 0.076$ (Table 2), with a *beta* of -

0.84 and 3.34 respectively. For the same analysis without considering these covariates, *SIRT1* yielded a highly significant q-value of $2.75*10^{-7}$ (with beta of -0.75). while the *INTS8* signal became non-significant (q = 0.49 with beta -0.26). We found that RNA concentration was slightly correlated with *SIRT1* expression (r = -0.27, Supplemental Figure 2A.), whereas sample handling time and *SIRT1* expression did not show a clear correlation (r = -0.04, Supplemental Figure 2B). RNA concentration and sample handling time were also correlated (r = 0.424, Supplemental Figure 3.). The other 8 transcripts mentioned in Table 2. have gene expression values that are either both associated with sample handling time and RNA concentration or associated with one of these (data not shown).

Figure 2. Volcanoplot of differential gene expression analysis.

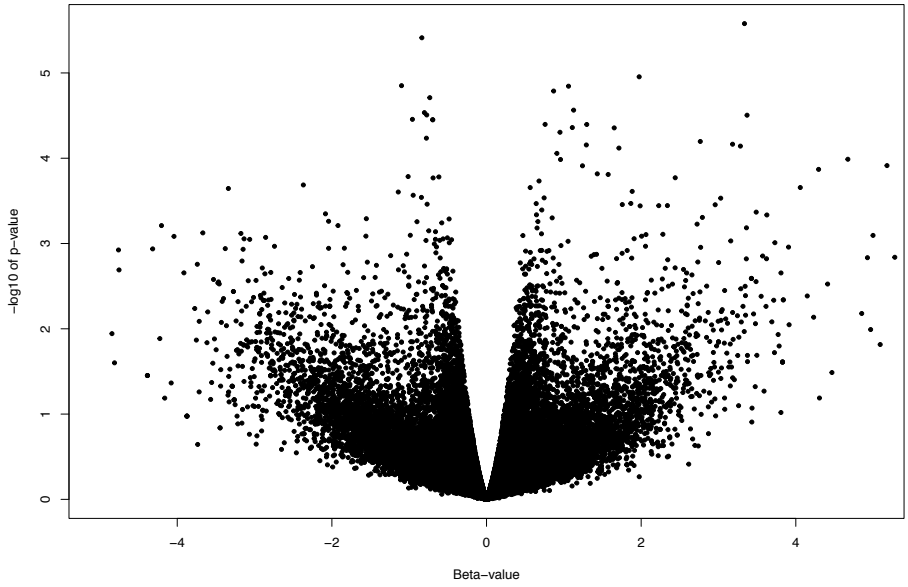


Table 2. Top 10 transcripts associated with clozapine use at FDR p<0.10.

Transcript-ID	**Annotation**	**FDR p-value**	**FDR p-value***	**Function / Association**
ENST-00000212015	*SIRT1* *Sirtuin 1*	0.076	$2.75*10^{-7}$	Dopamine and cholesterol metabolism, neutrophil maturation, tumorigenesis[231–233]

ENST-00000523321	*INTS8* *Integrator complex subunit 8*	0.076	0.493	RNA processing[234]
ENST-00000379350	*PHACTR1* *Phosphate and actin regulator 1*	0.096	0.011	Vascular disease[235]
ENST-00000369091	*PRDM1* *PR/SET domain 1*	0.096	0.018	B- and T-cell lymphoma[236]
ENST-00000334062	*RASA3* *Ras P21 protein activator 3*	0.096	0.003	Thrombocytosis and blood clotting[237]
ENST-00000309575	*CHST2* *Carbohydrate sulfotransferase 2*	0.096	$2.08*10^{-4}$	Microglia, upregulated in patients with AD[$,238]
ENST-00000485341	*LSP1* *Lymphocyte specific protein 1*	0.096	$8.00*10^{-4}$	Neutrophil motility, adhesion and transendothelial migration[239,240]
ENST-00000367497	*IVNS1ABP* *Influenza virus NS1A binding protein*	0.096	0.094	Processing Influenza virus[241]
ENST-00000540006	*CARS2* *Cysteinyl-TRNA synthetase 2, mitochondrial*	0.096	0.002	Epilepsy[242]
ENST-00000586802	*HDAC5* *Histone deacetylate 5*	0.096	0.008	Axon regeneration, muscle differentiation, angiogenesis, T-cell function, cancer, energy homeostasis[243,244]

Supplemental Table 1 provides a list of transcripts with FDR p-value <0.20

* Without correction for RNA concentration and handling time.

[$] Alzheimer's disease

Analysis of transcripts specific to neutrophils

We selected 58 neutrophil-specific genes[224] and matched 539 transcripts from our results to these. Of the 539 transcripts, 361 passed the filtering step (67%). The neutrophil transcripts were higher expressed compared to the remaining transcripts. We did not find significant differences in neutrophil specific genes between patients using clozapine and healthy controls

Network analysis

Analysis with WGCNA with all 39,190 expressed transcripts was performed. Sixty-five modules were created, ranging in size from 56 to 6062, two of which were nominally significant with clozapine use; gene co-expression module 45 (74 transcripts) with a p-value of 0.013 and module 41 (, 80 transcripts) with a p-value of 0.041. Both gene co-expression modules, however were not significantly associated with clozapine use after correction for multiple testing, nor were they significantly enriched for specific biological processes.

Analyses of unmapped reads

No significant difference was found in the overall number of unmapped reads in clozapine users versus healthy controls. Analyses of specific microbial patterns, circular RNA load and repeat sequences did not show significant differences between clozapine users and healthy controls.

Discussion

The goal of this study was to further investigate the origin of clozapine-induced fluorescence and by extension its possible role in clozapine-induced agranulocytosis. We collected neutrophils from clozapine users and from non-exposed healthy controls, extracted RNA and performed RNA sequencing to search for differences in gene expression as well as of unmapped reads. Given the strong fluorescence signal that is uniquely present in neutrophils of clozapine drug users, we expected that, if the phenomenon is directly linked to RNA transcription levels, aberrant neutrophil behavior or immune activation, even a small sample of cases and controls could yield significant new insights[34].

Potentially differentially expressed genes

We did, however, not observe any genome-wide significant evidence of a specific transcript that was differentially expressed between clozapine users and control subjects. We found two genes with nominal significant evidence, namely *SIRT1* and *INTS8*. In a subsequent analysis, in which we did not correct for RNA concentration and sample handling time that were significantly different between the user and non-user groups, the *SIRT1* but not *INTS8* finding became highly significant. This may imply that *SIRT1* expression is closely linked with clozapine use or neutrophil autofluorescence, but current evidence is limited. We observed that Sirtuin 1 (*SIRT1*) is down-regulated in patients taking clozapine. The sirtuin protein family plays an important role in NAD-dependent histone deacetylating. *SIRT1* is involved in different cellular processes such as energy metabolism, tumorigenesis, longevity and cellular stress resistance [231–233]. A recent paper suggests that up-regulation *SIRT1* may hamper development and maturation of neutrophils [245]. A different study suggests that *SIRT1* knockout cells have impaired terminal differentiation [246]. This might be relevant for understanding the effect of clozapine on neutrophil maturation. We have shown before that the fluorescence is likely originating from a 14kDa protein (de With et al., submitted), *SIRT1,* however, is a protein

with a mass between 81.7 and 61.1 kDa. Downregulation of *SIRT1* has also been associated with advanced stages of Alzheimer's disease and Parkinson's disease. It is thought to play a role in dopamine metabolism in the central nervous system, but the exact mechanism is not known [247]. What this means for our study of neutrophils in schizophrenia patients remains hypothetical. Lastly, *SIRT1* is thought to play a role in cholesterol biosynthesis in the liver, activation of *SIRT1* leads to lower serum lipid levels and it is a target in studies towards reducing metabolic syndrome symptoms [248,249]. Clozapine causes metabolic adverse effects such as weight gain in lipid disturbances and *in vitro* gene expression studies also show up-regulation of cholesterol metabolism [190] (de With et al., submitted).

The up-regulated Integrator complex subunit 8 (*INTS8*) plays a role as part of the integrator complex in RNA processing and transcription regulation, residing on chromosome 8 [234]. With a molecular mass of 113 – 111 kDa, this protein too would be too large, although it is possible that the fluorescence is caused by a small part of the (degraded) protein. Although recently mutations in this gene have been associated with a severe neurodevelopmental syndrome [250], *INTS8* has, however, not been associated with antipsychotic use or schizophrenia before.

Unmapped reads

Analysis of unmapped reads did not reveal significant differences between clozapine users and healthy controls. We did observe, however, a trend towards slightly less T-cell receptor alpha chain diversity in clozapine users, when compared with healthy people not taking clozapine. The overall activity of the TCR was not different between clozapine users and control subjects. Larger sample sizes are needed to establish a link between clozapine use and the decreased T-cell alpha chain diversity.

Considerations

Despite the specificity and intensity of the fluorescence signal in neutrophils of clozapine users, we did not find differences in gene expression between

patients taking clozapine and control subjects. Power calculations show that given our sample size we had 80% power to detect differential expression with a minimum fold change of 3 or higher [251]. Admittedly, smaller fold changes at transcript level of a stable protein might still be sufficient to explain the observed fluorescence in neutrophils. There may also have been technical confounders affecting the results. The time span between receiving a blood sample and performing cell sorting is a window that could induce stress in the neutrophil, thereby altering gene expression. We observed a significant difference in sample handling time and RNA concentration between healthy controls and patients taking clozapine, which could have impacted our results. In our analyses we corrected for these two possible confounders, however, it is possible that this has made it difficult to detect a (true) signal. Although we did not observe a correlation between handling time and *SIRT1* in controls, we remain conservative in drawing strong conclusion. Future studies of neutrophils in clozapine users should take this finding into account.

We expected to find differences in gene expression between patients taking clozapine and healthy controls. We hypothesized that the fluorescence originates from a protein more abundant when taking clozapine, assuming this would also translate in differences in transcript levels. However, the absence of differences in transcription levels suggests that this protein becomes fluorescent without an effect on its RNA-abundance in the mature neutrophil. It is possible that because of limited statistical power of our relatively small study we were not able to find a difference between the two groups. We hypothesize, however, that the effect of clozapine in neutrophils leading to the fluorescent signal may primarily lie in earlier stages of neutrophil maturation. The fluorescence signal was shown to be detectable in patients after 14 to 21 days of clozapine prescription [34]. The maturation of neutrophils from their most primitive committed progenitor to mature neutrophils in the circulation takes approximately 14 days [252]. The neutrophil goes through different maturation stages inside the bone marrow, during which, amongst other things, the granules are formed. We recently showed that clozapine-induced fluorescence in neutrophils most likely originates from the azurophilic granules in neutrophils

(de With et al., submitted). Since these granules have been formed earlier in the maturation process, it is possible that gene expression changes at that stage are the culprit, but that no apparent differences of transcript levels are detectable afterwards. The fluorescence could be originating from a protein, translated and subsequently processed in granules in an early maturation stage. It would be interesting to study this in (unmatured) neutrophils taken from the bone marrow, in humans, or in animal models. It is also possible that clozapine or its metabolites may affect the post-translational modification of some proteins, which in turn results in the fluorescent signal, without impacting RNA transcript levels significantly.

Conclusion

We explored the molecular background of clozapine-induced fluorescence by examining differential gene expression in neutrophils from schizophrenia patients taking clozapine and control subjects not exposed to clozapine. Despite the strong fluorescence finding, significant differences between the two groups were not observed. This could be due to a lack of power, due to post-translational modifications without RNA transcript differences, but it could also be that differences in gene expression may have been detectable in an earlier stage in neutrophil maturation, but that the differences in this stage are only measurable at protein level. Future research should focus on determining gene expression at different stages of neutrophil maturation in relation to clozapine-induced fluorescence.

3

Protein

Chapter VI

Neutrophil fluorescence in clozapine users is attributable to a 14kDa secretable protein

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Submitted.

Abstract

Clozapine is the only antipsychotic agent with demonstrated efficacy in refractory schizophrenia. However, use of clozapine is hampered by its adverse effects, including potentially fatal agranulocytosis. Recently, we showed an association between neutrophil autofluorescence and clozapine use. In this study, we evaluated the subcellular localization of clozapine-associated fluorescence and tried to elucidate its source.

Neutrophils of clozapine users were analyzed with fluorescence microscopy to determine the emission spectrum and localization of the fluorescence signal. Next, these neutrophils were stimulated with different degranulation agents to determine the localization of the fluorescence. Lastly, isolated neutrophil lysates of clozapine users were separated by SDS-PAGE and evaluated.

Clozapine-associated fluorescence ranged from 420nm to 720nm, peaking at 500-550nm. Fluorescence was localized in a large number of small loci, suggesting granular localization of the signal. Neutrophil degranulation induced by Cytochalasin B/fMLF reduced fluorescence, whereas platelet activating factor (PAF)/fMLF induced degranulation did not, indicating that the fluorescence originates from a secretable substance in azurophilic granules. SDS-PAGE of isolated neutrophil lysates revealed a fluorescent 14kDa band, suggesting that neutrophil fluorescence is likely to be originated from a 14kDa protein or peptide fragment.

We conclude that clozapine-associated fluorescence in neutrophils is originating from a 14kDa soluble protein (fragment) present in azurophilic granules of neutrophils. This protein could be an autofluorescent protein already present in the cell and upregulated by clozapine, or a protein altered by clozapine to express fluorescence. Future studies should further explore the identity of this protein and its potential role in the pathophysiology of clozapine-induced agranulocytosis.

Introduction

Clozapine is an effective antipsychotic drug, developed in the 1950s. It is the only antipsychotic drug with proven superior efficacy in treatment-resistant schizophrenia [9] and FDA approval for treatment of suicidal ideation and behavior in schizophrenia patients [106,253]. However, despite its effectiveness, physicians are hesitant to prescribe the drug due to the risk of development of severe adverse effects [112], most notably being clozapine induced agranulocytosis (CIA) occurring in approximately 0.7% of patients [10,11]. Agranulocytosis is hallmarked by a low absolute neutrophil count, (ANC < $500x10^9$/L), severely weakening the immune system and if left untreated, can be fatal [7,47]. To prevent this serious adverse effect, a rigorous monitoring program was established. Patients are required to undergo weekly blood draws to monitor neutrophil count for the first 18 weeks of treatment and every 4 weeks thereafter for the remainder of the treatment duration. When the neutrophil count decreases to neutropenia (ANC < $1500x10^9$/L), treatment with clozapine is discontinued. The monitoring program has successfully lowered the prevalence of clozapine-induced agranulocytosis [48].

To date, the etiology of CIA remains unknown. There are two hypotheses involving the etiology of CIA. First, CIA is thought to have an immunological component [254,255]; when patients with a history of CIA are challenged with clozapine a second time, they develop CIA more often and faster, suggesting sensitization of the immune system [26]. Additionally, there are some (inconsistent) findings of antibodies against myeloperoxidase (MPO) – an enzyme secreted by neutrophils -, also suggesting immune mediation of the response. Second, clozapine can be oxidized by a combination of MPO and H_2O_2 to a reactive nitrenium ion that is thought have a direct toxic effect on the neutrophil, though this has only been shown *in vitro* [28–31].

The two hypotheses are not mutually exclusive; it is possible that the reactive clozapine nitrenium ion irreversibly binds to neutrophils, resulting in altered membrane structure and therefore can act as a hapten in the production of antibodies [256].

Still, it remains unknown how CIA would affect only some patients and not all. An explanation for this might lay in the identification of a genetic component to CIA: a recent genome-wide association study (GWAS) has identified two alleles with genome-wide significance associated with CIA [45]. These two alleles (HLA-DQB1 and HLA-B) reside in the major histocompatibility complex (MHC), a region coding for the human leucocyte antigens (HLA), proteins essential for antigen presentation to the adaptive immune system and subsequent clearance of pathogens. Both proteins can probably be expressed on neutrophils [257,258], however, the mechanism by which these alleles predispose for CIA remains unknown. In addition, the specificity and sensitivity of the genetic markers associated with CIA up to now do not make them suitable to use for a clinical predictor test. Such a (genetic) predictor test would be of great clinical significance.

Understanding of the mechanisms involved in the pathogenesis of CIA is of obvious clinical importance. Recently, our group found a significant association between neutrophil fluorescence and clozapine use. In this study we showed that neutrophil fluorescence was significantly higher in clozapine users than in non-users [34]. As this fluorescence was specifically observed in neutrophils, studying the origin of this fluorescence could give insight in the way clozapine affects the neutrophil and induces CIA.

The present study aimed to explore clozapine-associated fluorescence of neutrophils in further detail by evaluating its subcellular localization and elucidating its source. We show that the fluorescence spectrum of clozapine-associated autofluorescence of neutrophils ranges from 420 to 720nm with a peak near 520 nm and that the fluorescence follows a granular pattern. Additionally, stimulation and degranulation of the neutrophils induced a significant decrease in fluorescence signal, also suggesting a granular localization of the fluorescence. Lysates of these neutrophils show a fluorescent signal at 14kDa indicating the signal is originating from a protein or protein fragment of 14kDa.

Materials and Methods

Reagents and antibodies

Shock buffer consisted of 155 mM Ammonium Chloride, 10 mM Potassium Bicarbonate and 0.1 mM EDTA. The SDS lysis buffer contained 0.5% (w/v) SDS, 0.05 M Tris·Cl and 1 mM fresh dithiothreitol (DTT). Phosphate buffered saline (PBS) was supplemented with 0.32% trisodium citrate and 10% pasteurized plasma solution (Sanquin, Amsterdam, the Netherlands) to create PBS2+. Functional assays were performed in HEPES buffer (20mM HEPES, 132mM NaCl, 6mM KCl, 1mM MgSO4, 1.2mM KH2PO4), supplemented with 5mM glucose, 1.0mM CaCl2 and 0.5% Human Serum Albumin. Antibodies for CD14 (PE-Cy™7, clone M5E2, dilution 1:50), CD16 (APC H7, clone 3G8, dilution 1:50) and CD63 (PE, clone H5C6, 1:50) were obtained from BD Biosciences (San Diego, CA, USA). Platelet activating factor-16 (PAF) was from Calbiochem (Darmstadt, Germany), formyl-methionyl-leucyl-phenylalanine (fMLF) and cytochalasin B were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Subjects and study design

Remnant blood samples of psychiatric patients using clozapine (N=7) were collected at the psychiatry outpatient clinic of the University Medical Center Utrecht (UMC Utrecht). Samples consisted of residuals from monthly check-ups and were anonymized for the purpose of the study. Therefore, the study was exempted from obtaining written informed consent according to Dutch law. The study was approved by the local Medical Research and Ethics Committee and Biobank. Patients using clozapine with a dose higher than 100mg/day for longer than one month were included in the study, insuring clozapine induced fluorescence had reached detectable and stable levels [34].

Healthy volunteer samples were obtained from the bloodbank Mini Donor Dienst of the UMC Utrecht after written informed consent was obtained in accordance to the declaration of Helsinki.

Confocal microscopy of neutrophils

Blood samples of patients and healthy volunteers were used to prepare blood films for evaluation with confocal microscopy. First, the samples were fixed with 4% paraformaldehyde in PBS. The fixed cells were transferred and attached to a glass slide by Cytospin centrifugation (Thermo Scientific, Waltham, MA, USA) for evaluation with a Zeiss LSM710 (Carl Zeiss, Sliedrecht, the Netherlands) confocal laser scanning microscope. Using a multi-laser flow cytometer, we observed clozapine-associated fluorescence to be stronger when excited with a 405nm laser instead of a 488nm laser as published previously [34] (Supplementary Figure S1). Therefore, clozapine-associated fluorescence emission spectrum was examined using lambda stacks and violet laser light exciting at 405nm, measuring fluorescence emission intensity from 420 to 720nm in 3.2nm steps. Fluorescence was imaged using excitation of 405 nm violet laser light and the standard DAPI (4',6-diamidino-2-phenylindole) filter set.

Neutrophil sorting, immunostaining and degranulation assays

Whole blood samples were treated with erythrocyte shock buffer and stained with CD14 and CD16. Neutrophils were sorted based on FSC/SSC (forward-scattered light; FSC and side-scattered light; SSC) and $CD16^{high}/CD14^{neg}$ with a BD FACSAria III™ (BD Biosciences, San Jose, CA, USA). Samples were typically >99% pure as determined by re-analysis with the BD FACSAria III™ and microscopic evaluation of May-Grünwald Giemsa stained cytospins. Sorted neutrophils were kept on ice in HEPES buffer or pre-incubated for 5 minutes with either cytochalasin B (5 µg/mL) or PAF (10^{-6} M) followed by stimulation with 10^{-6} M fMLF for 15 minutes to determine localization of the fluorescence signal.

The remainder of the sample was immunostained for CD63 in PBS2+ for 20 minutes to confirm successful neutrophil degranulation.

After fixation with 1% formaldehyde, samples were analyzed on a Beckman Coulter Gallios™ within 24 hours. Fluorochromes were chosen for minimal bleed-through from clozapine signal.

Isolation of neutrophils (for use in SDS-PAGE assay)

Mononuclear cells were depleted from whole blood by centrifugation (1500 rpm for 5 min (450G)) over isotonic Ficoll (GE Healthcare Life Sciences) to obtain a neutrophil-containing erythrocytes layer. Isotonic lysis of remaining erythrocytes took place for 5 minutes at room temperature with shock buffer. Neutrophils were washed and resuspended in shock buffer, followed by boiling the samples for 5 minutes.

SDS-PAGE assays

Neutrophil lysates were prepared from the isolated neutrophils using sodium dodecyl sulfate (SDS) lysis buffer. Samples prepared with identical neutrophil numbers were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bolt™ 4-12% Bis-Tris Plus gel (Thermo Scientific, Waltham, MA, USA) following manufacturer's instructions. Gels were scanned at different wavelengths using a Typhoon 9400 Scanner (GE Healthcare, UK).

Data and FACS analyses

Images were analyzed with ImageJ 1.47T (National Institutes of Health, Bethesda, MD, USA) and FACS data were analyzed with FCS Express 5.01 (De Novo Software, Glendale, CA, USA). Statistical analysis was performed with the free software environment R [126]. A Wilcoxon signed-rank test was used to compare groups, a p-value of 0.05 or lower was considered statistically significant.

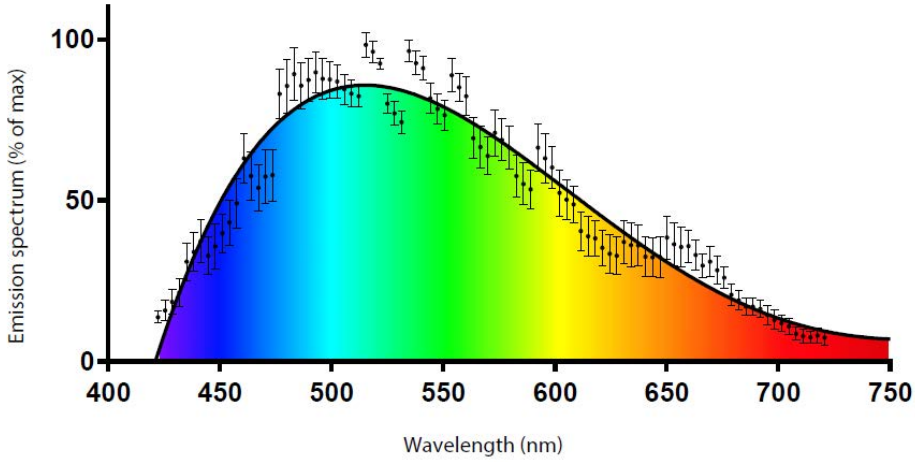
Results

Localization of clozapine-associated fluorescence

To confirm previous findings of autofluorescence, specifically observed in neutrophils of clozapine users [34], we started by determining the emission spectrum of clozapine-associated neutrophil fluorescence. Clozapine-associated fluorescence showed a broad emission spectrum, with peak emission around 520nm (Figure 1). Therefore, we chose to use FL-10 fluorescence channel of the FACS (excitation 405nm, emission window ranging from 530-570nm) in our current study. It should be noted that the emission spectrum of most molecules is independent of the excitation wavelength and excitation with either 405 or 488nm will most probably result in a similar emission spectrum [259].

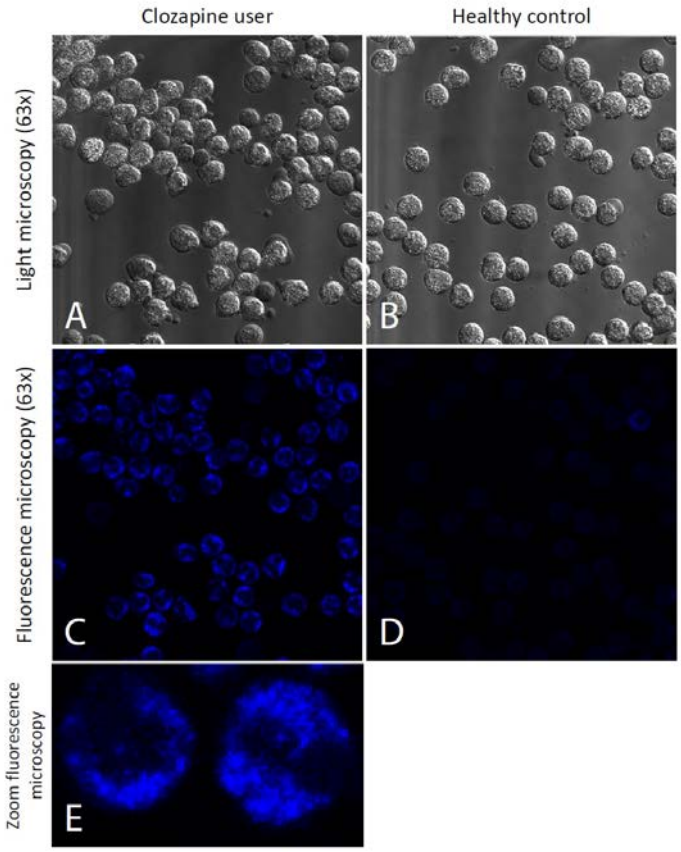
In accordance with our previous findings, we observed a high fluorescence intensity in patient samples signal when compared to controls (Figure 2). Moreover, microscopy images showed that fluorescence was specifically located in the neutrophils of the patient samples. The fluorescence followed a perinuclear uneven staining pattern, reminiscent of granular staining.

Figure 1. Fluorescence spectrum of clozapine-associated autofluorescence in neutrophils.



The emission spectrum of clozapine-associated autofluorescence in neutrophils was determined with a confocal scanning microscope in lambda mode, (Zeiss LSM710) measuring emission from 420 to 720nm in 3.2nm steps. The (unstained) sample was excited with laser light at 405nm. An emission peak was found near 520 nm indicating green fluorescence. Depicted dots and error bars within the figure are averages and 95% standard deviations, respectively (n = 3).

Figure 2. Confocal fluorescence microscopy images of unstained blood samples from a clozapine user and a healthy control.



Blood samples of a clozapine user (A,C,E) and a healthy control (B,D) were fixed with 4% paraformaldehyde in PBS and attached to a glass slide for confocal microscopy imaging. Samples were excited at 405 nm violet laser light and fluorescence was detected using the standard DAPI (4',6-diamidino-2-phenylindole) filter set (C,D,E). Neutrophils of clozapine user showed high fluorescence intensity, whereas this signal was absent in the control sample. The fluorescent neutrophils show a perinuclear-staining pattern (E).

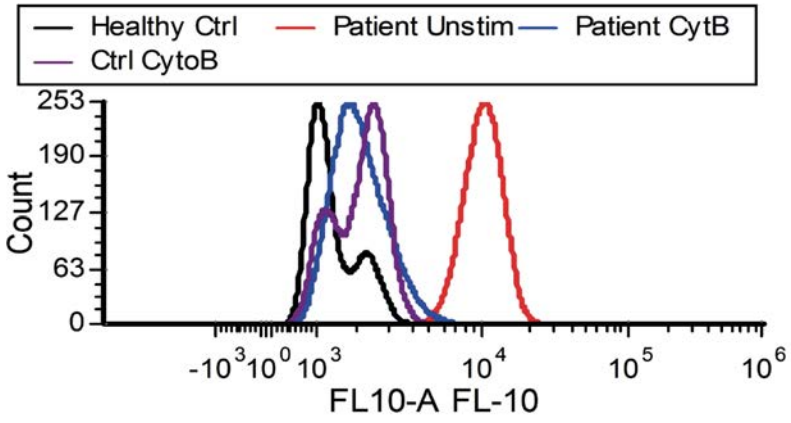
Fluorescence intensity decreases after neutrophil degranulation

To demonstrate that the fluorescence signal originates from granules isolated neutrophils from patient samples were stimulated with degranulation-inducing agents PAF/fMLF and cytochalasin B/fMLF. The strong degranulation-inducing agent cytochalasin B/fMLF stimulates the neutrophils to release all types of granules (secretory, gelatinase containing, specific and azurophilic), achieving complete degranulation, while the moderately degranulation-inducing agent PAF/fMLF induces release of most types of granules, except azurophilic granules [260].

Before and after stimulation, neutrophil autofluorescence (Figure 3 and 4) and a protein marker (CD63, a lysosomal protein residing in the azurophilic granules [261]) for degranulation of the neutrophil azurophilic granules were monitored. A significant increase in the degranulation marker ($p<0.015$) was found, indicating successful stimulation (Figure 4A).

A significant decrease in clozapine associated fluorescent signal after stimulation with cytochalasin B/fMLF ($p = 0.015$) was observed, but not after stimulation with PAF/fMLF ($p = 0.81$) (Figure 4B). This finding suggests that the fluorescent signal originates from the azurophilic granules.

Figure 3. Flowcytometry histograms of neutrophil fluorescence before and after neutrophil stimulation using Cytochalasin B/fMLF



Neutrophil autofluorescence was measured in a patient and a control sample in the FL10 channel (excitation: 405nm, emission: 550/40nm). Among the unstimulated samples (black and red) an increased fluorescence is seen only in the patient sample (red). Histograms show a decrease in neutrophil fluorescence after stimulation of the patient sample (blue).

Figure 4. Effects of neutrophil stimulation on neutrophil fluorescence and CD63 expression in clozapine users

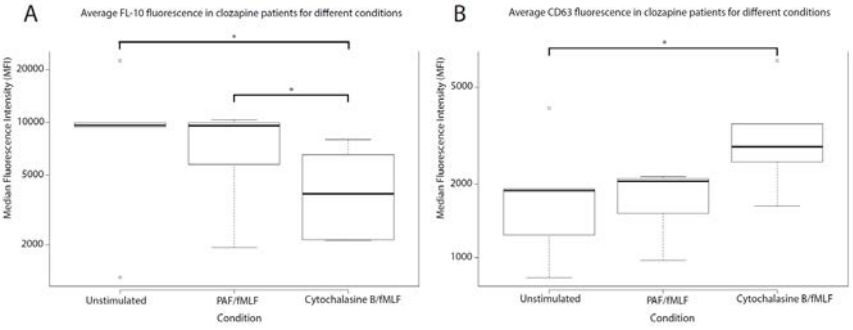
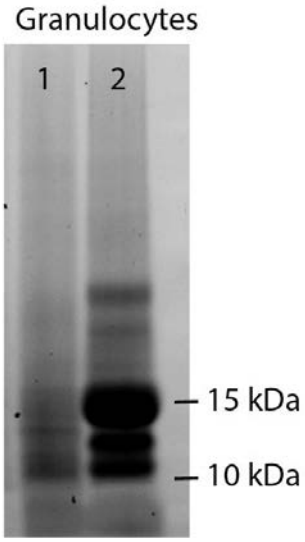


Figure 4A. Neutrophil stimulation of clozapine users (N=7) with cytochalasin B/fMLF significantly reduced fluorescence intensity ($p = 0.015$), while stimulation with PAF/fMLF did not ($p = 0.81$). Figure 4B. shows a significant increase in CD63 ($p = 0.015$) – the degranulation marker of azurophilic granules after incubation with degranulation agents (Cytochalasin B and PAF), indicating successful neutrophil stimulation and degranulation.

Neutrophil fluorescence originates from a 14kDa protein or peptide fragment

To identify the source of the observed fluorescence, isolated neutrophil lysates were separated by SDS-PAGE and were evaluated under a laser scanner (Figure 5). The gels were excited at 532nm and emission was detected at 555nm. A fluorescent 14kDa band was detected in the patient samples, whereas this band was absent in the healthy control samples. Neutrophil fluorescence is likely to be originated from a 14kDa protein or protein fragment.

Figure 5. SDS-PAGE of isolated peripheral blood mononuclear cells and neutrophil lysates from samples of clozapine users and healthy controls.



Isolated granulocytes of a healthy control (sample 1) and a clozapine user (sample 2) were separated by SDS-PAGE and evaluated under a laser scanner (excited at 532nm, emission at 555nm). A fluorescent protein band of nearly 14 kDa was observed in clozapine sample (sample 2), whereas this band was absent in the healthy control (sample 1).

Discussion

With this study we have further explored clozapine-associated neutrophil fluorescence in clozapine users to gain more insight into the source and localization of the signal. We have confirmed previous findings of enhanced fluorescence following clozapine exposition [34] and further defined the fluorescent emission spectrum of clozapine-associated neutrophil autofluorescence. Microscopy showed that the fluorescence follows a granular staining pattern. Stimulating neutrophils from clozapine users to release their granules showed a significant decrease in signal with the release of azurophilic granules. Lastly, with SDS-PAGE of the neutrophil lysates we detected a 14kDa fluorescent band, suggesting that the fluorescent peptide originates from a 14kDa protein or protein fragment.

Clozapine-associated fluorescence originates from the granules

The granular pattern of clozapine-associated fluorescence and the signal reduction after release of the granules, suggest that the fluorescence arises from the granules in the neutrophil. The fact that the fluorescence signal decreases significantly after cytochalasin B exposure and not after PAF indicates that this signal mainly arises from the azurophilic granules. This azurophilic granule contains a number of oxidizing agents, including myeloperoxidase (MPO) [262]. MPO is thought to play a role in the formation of the clozapine nitrenium ion, [31,32], an ion possibly toxic to neutrophils [28,30]. Interestingly, MPO is most abundantly present in azurophilic granules of neutrophils – the same type of granules involved in observed clozapine-associated fluorescence-. Therefore, clozapine-associated fluorescence might be linked with the formation of the clozapine nitrenium ion and indirectly with CIA.

The fluorescent molecule is most-likely (bound to) a 14 kDa granular protein

The results from the performed SDS-PAGE analyses showed that clozapine-associated fluorescence originates from a protein (or protein fragment) of

approximately 14kDa. As clozapine itself is a non-peptide, small molecule, it is not the direct origin of the fluorescent band. The signal must therefore be originating from a protein with added clozapine (or metabolite), from increased expression of an auto-fluorescent protein, or a combination of the two. As mentioned before, clozapine can be oxidized and catalyzed by peroxidases such as MPO, located in the azurophilic granule, into a reactive nitrenium metabolite potentially causing cell damage. Hence, an explanation for the fluorescence could be the alteration of an endogenous protein into a fluorescent protein, induced by intracellular oxidized clozapine.

Moreover, the decrease in fluorescence signal after neutrophil stimulation suggests that the signal originates from a soluble protein, as a membrane bound protein would show an increased fluorescence on the outer membrane of the cell (which we did not observe).

It remains difficult, however, to pinpoint one protein responsible for the fluorescence. It is possible that the fluorescence arises from a protein of 14kDa, residing in the granules. However, it is also possible that the protein is altered by a reaction with clozapine (or one of its metabolites) additionally, the protein could be degraded by the enzymes in the granule.

We cannot exclude the possibility that neutrophil fluorescence results from oxidized flavoproteins. These are proteins containing a flavin adenine dinucleotide (FAD) group, making the protein auto-fluorescent when oxidized [263]. There are approximately 90 flavoproteins known in humans [264]. It is possible that one of these oxidized flavoproteins is responsible for the clozapine-associated fluorescence observed by our group, since the emission/excitation spectrum (measured at 488/530nm) is very similar to the spectrum we have observed. Additionally, it has been shown that certain flavoproteins are involved in clozapine oxidation such as NADPH, mediated by NADPH oxidase, an enzyme that together with MPO forms the major oxidation system in neutrophils and can generate a superoxide (O^{-}_{2}) that is converted to H_2O_2 [29,265]. Consequently, MPO catalyzes the formation of hypochlorous acid (HOCl) from

H_2O_2 and chloride [29,265]. Eventually, hypochlorous acid oxidizes clozapine to the reactive nitrenium metabolite that is irreversibly bound to cells [28–31].

The fluorescence could also be originating from (a part of) MPO. MPO is built up from two light chains and two heavy chains that are linked by sulfur bridges. These sulfur bridges are cleaved by DTT in the lysis buffer during SDS-PAGE analysis, leading to the availability of the light and heavy chains in the SDS gel. Interestingly, the light chain is approximately 14kDa [266]. Given that MPO is secreted during neutrophil degranulation [267], the observed fluorescent 14kDa band could be originating from MPO.

It is also possible that clozapine (or one of its metabolites) alters an existing protein to exhibit a fluorescent signal. Since our results indicate the signal originates from the granules, it must be a protein present in the granules. There have been lists of granular proteins published [268]. We have used one of these lists to select proteins of approximately 14kDa. If we assume that the fluorescence is arising from the azurophilic granules and the protein is not degraded by enzymes, the list of candidate proteins is reduced to *Brain Protein I3 and NADH dehydrogenase (ubiquinone).* The first is a protein (*BPI3*) expressed in mouse brain [269], but its function in humans remains unknown. The latter, *NADH dehydrogenase (ubiquinone)* is an enzyme complex involved in the respiratory chain [270]. Moreover, *NADH dehydrogenase* may have a role in triggering apoptosis and appears to be a potent source of reactive oxygen species by transferring one electron from reduced flavin mononucleotide (*FMNH2)* to oxygen [271–273]. In this regard, observed clozapine-associated fluorescence, if indeed appears to be originating from (upregulated) ubiquinone, could be involved as a part in the CIA puzzle since ubiquinone plays a role in triggering apoptosis. This is, however highly speculative and future studies have to reveal the nature of this protein.

Implications of the fluorescence finding

The finding of clozapine-associated fluorescence of neutrophils from clozapine users could be a mere coincidence without clinical meaning. It is, however, likely that this finding could be exploited in clinical practice. The signal could be

used as a biomarker for long term adherence, as the half-life of enhanced fluorescence in clozapine users was estimated on 230 hours [274]. A marker to evaluate adherence would be highly beneficial in this specific patient population, as medication non-adherence is the major cause of treatment failure in schizophrenia patients [35].

More importantly, it is possible that the clozapine-associated fluorescence in neutrophils provides a piece of the CIA puzzle. Considering the half-life of FL3-fluorescence, the fluorescence could be indication of an early effect on the maturation process of a neutrophil in the bone marrow. It is possible that clozapine affects the maturation process of the neutrophil, resulting in slightly damaged or activated neutrophils, or in neutrophils that survive for a shorter period in the bloodstream, thus requiring an increased production. Previous studies showing direct toxicity of the clozapine nitrenium ion to neutrophils and bone marrow precursors are in agreement with this hypothesis [29,30].

We hypothesize that clozapine alters the balance between production and degradation of neutrophils in a subtle way. This fragile balance in turn might be more easily disturbed, possibly by intracellular superoxide production, leading to clozapine-induced agranulocytosis in only a small number of patients. This hypothesis is supported by Fehsel *et al*. who reported that blood of CIA patients who underwent an episode of agranulocytosis showed enhanced apoptosis expression markers and intracellular superoxide production compared to clozapine users without agranulocytosis [275].

Conclusion

This study has further explored clozapine-associated neutrophil autofluorescence by investigating the location and origin of this fluorescence. We conclude that the fluorescence originates from neutrophil azurophilic granules. Additionally, this fluorescence originates from a protein or protein fragment of approximately 14kDa. These findings could provide a piece of the clozapine-induced agranulocytosis puzzle, however, future studies are necessary to further explore the clinical importance of clozapine-associated neutrophil fluorescence in clozapine-induced agranulocytosis and leukopenia.

Acknowledgements

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Chapter VII

Discussion

Clozapine is an antipsychotic drug with an important place in the treatment of psychosis, especially since it has been and still is the only drug registered for treatment resistant schizophrenia. Although clozapine is highly effective, the exact mechanism of action remains elusive. This is not only true for beneficial effects of it, but also for the adverse effects of clozapine. It is not possible to know beforehand if a patient will respond to clozapine, whether he or she will develop adverse effects. The need for genetic testing, therefor, is great. The aim of this thesis was to gain more insights in the black box that are the working mechanisms of clozapine. To do so a number of different techniques were used at various levels of the genomic spectrum. We have used a human cell model and collected data from a patient cohort. In this section I will summarize our findings, discuss why it is and has been difficult to study clozapine-induced effects and shed some light on directions for future research.

Summary

Part One: DNA

The first part of this thesis consists of studies that have studied clozapine in the context of DNA. First, I present a review of studies that have researched DNA variants that might increase or decrease the risk of CIA. I then discuss a study of our own of DNA variants contributing to clozapine-induced decreased viability in lymphoblast cell lines.

Chapter II reviews 23 genetic association studies of clozapine-induced agranulocytosis published to date, describing a large number of candidate gene studies, as well as a few genome wide studies. It was interesting to discover that almost all candidate gene studies of CIA have focused on the major histocompatibility complex and that only with the advent of genome-wide technology this focus has shifted towards other parts as well. Other genetic studies towards serious drug-induced adverse effects (carbamazepine, abacavir) have shown genetic markers with very large effect sizes. Therefore, the expectations for clozapine and CIA have been high. We concluded, however, that CIA is most likely a complex, polygenic trait, meaning that not one or two (or maybe even a few) genetic markers contain great risk. This may

hamper efforts to the development of a genetic predictor test with clinical relevance. To decipher the genetic architecture of CIA it will be necessary to apply more rigorous standards of phenotyping and study much larger sample sizes.

In **Chapter III** the findings of a genome wide study of clozapine-induced viability reduction in human lymphoblast cell lines as a model for CIA are presented. We exposed lymphoblast cells to a series of increasing concentrations of clozapine, after which a survival curve was calculated and used for genome wide association testing. We showed a surprisingly high heritability of clozapine-induced viability reduction (h2 = 0.76) and one genetic locus near the *PRG4* gene that showed consistent association with reduced viability. Although *in vitro* models have disadvantages, they can be useful in the discovery of genetic risk factors. Especially when the phenotype (CIA) is quite rare, making it difficult to collect substantial patient cohorts, it can be helpful to research possible intermediate phenotypes in a cellular model.

Part Two: RNA

The second part of this thesis encompassed studies towards the effects of clozapine on gene expression and regulation of gene expression.

Chapter IV describes the results of experiments in which lymphoblast cell lines were exposed to different concentrations of clozapine, after which gene expression and DNA methylation analyses were performed. Gene expression in lymphoblast cell lines was significantly altered after exposure to clozapine; up-regulated genes were enriched for cholesterol metabolism and down-regulated genes were enriched for cell cycle pathway genes. We did not find differentially methylated regions after exposure to clozapine. There was no evidence that genetic variation in genes associated with changes in gene expression is involved in schizophrenia susceptibility. Our results indicate, together with other studies, that clozapine significantly alters the cholesterol metabolism. This could be (one of the) mechanisms behind metabolic adverse effects clozapine induces. It would be interesting to study genetic variation in genes involved in cholesterol metabolism in relation to clozapine (and maybe

even antipsychotic-induced) induced metabolic effects. Additionally this study shows that *in* vitro data can be combined with data from large-scale (patient based) studies. I think that this is one of many ways to intelligently make use of different data types that are available and it is important to keep thinking of ways to combine data.

For **Chapter V** we set out to further study clozapine use associated auto-fluorescence in neutrophils by looking at differences in gene expression. The origin of the fluorescence is unknown, the differences in fluorescence between patients taking clozapine and healthy controls is surprisingly large, however. We collected neutrophils from patients using clozapine and healthy controls and determined gene expression values by performing RNA sequencing. Since the we found the difference in fluorescence between patients and controls substantial, we expected significant differences in gene expression. This was, however, not what we found; we did not detect significant gene expression changes after correcting for multiple testing. We hypothesized that the auto-fluorescence might be originating from a difference in protein in neutrophils, the fact that we did not observe differences in gene expression do not exclude this hypothesis. It is very well possible that the fluorescent protein is formed earlier in the neutrophil maturation process and stored in the neutrophil (granules) for later use. Consequently, the expression difference would not be detectable in the mature neutrophil at the gene expression level.

Part Three: Protein

The last part of this thesis focuses further on clozapine-induced auto-fluorescence in neutrophils, as we attempted to narrow down which protein causes the fluorescence.

In **Chapter VI** we described the search for the exact location and origin of the auto-fluorescence in neutrophils from patients taking clozapine. We collected neutrophils from patients and healthy control subjects. Fluorescence microscopy was then used to determine the exact fluorescence emission spectrum and the localization of the fluorescent signal. Matures neutrophils contain different type of granules that have specific functions and that can be

released. Our findings showed a pattern consistent with granular localization of the signal. Second, we performed different neutrophil assays, determining that the auto-fluorescence originated from a specific type of granules; the azurophilic granules. Lastly, SDS-PAGE was used to determine the size of the auto-fluorescent protein and we found a protein or protein fragment of 14 kDa to be auto-fluorescent. Although, from our findings, it is difficult to pinpoint an exact protein, it brings us one step closer to identifying the origin of the auto-fluorescent signal in neutrophils from clozapine users and possibly the mechanism behind clozapine-induced agranulocytosis.

With this thesis we set out to gain more knowledge about the way clozapine affects processes in the human body, in the hope that this would help us understand its effects (and adverse effects) and maybe even predict these. I think the work described in this thesis represents a small step towards more knowledge about the effects of clozapine. We have tried the use of intermediate phenotypes of (adverse) effects of clozapine, with success. We have shown activation of cholesterol metabolism and cell cycle pathways after exposure of clozapine and found a gene linked to clozapine-induced reduced viability in lymphoblasts. Additionally, we gained more insight in the origin of clozapine induced auto-fluorescence in neutrophils. We know now that the fluorescence originates from the azurophilic granules in the neutrophils, and that it is likely that the protein causing the fluorescence is produced in the early phases of the neutrophil maturation process.
In the next part of this discussion, I will explain what complicates research towards the effects of clozapine (and other drugs), and I will share my thoughts on how we should continue down this path.

Why is it so difficult to study the genomic and molecular effects of clozapine?

1. A polygenic nature
This thesis set out to study genomic and molecular effects of clozapine in a broad sense. Although we know substantially more than 60 years ago, when

clozapine was first introduced, a large number of questions remain. The past 15 years or so have brought both feelings of hope and disappointment when it comes to genetic research. The first half was filled with hope. After the completion of the HapMap project[160] and the first genome wide association studies, the scientific community expected great things from GWAS. This was also true for the psychiatric field: with GWAS we thought we would certainly uncover the genes responsible for schizophrenia, bipolar disorder and autism spectrum disorder. Disappointment came when the first GWAS did not show an abundance of significant results and the hypothesis that a small number of genes was responsible for susceptibility for psychiatric disease had to be abandoned. It became clear that a polygenic architecture, with a large number of variants, each with a small effect on susceptibility, would determine psychiatric disease. This did not only hold true for psychiatric disorders, in the past years it has become clear that this is also true for the pharmacogenomic background of clozapine (or any other antipsychotic). Since 2011 a number of GWAS have focused on the (adverse) effects of clozapine and other antipsychotic drugs [42–46,276–279]. It has been difficult, however, to replicate results and to achieve genome-wide significant results. The review in this thesis (**Chapter II**) concludes that clozapine-induced agranulocytosis (CIA) is (also) most likely a polygenic trait. Additionally, there is mounting evidence that this is true for other adverse effects (such as weight gain) and the therapeutic effect of antipsychotics altogether[42–46,276–279]. With the acceptance of the polygenetic nature of CIA, we have to come to terms with the fact that chance of discovery and use of a simple genetic predictor marker is very slim. Should we then abandon the idea of a genetic predictor test altogether? Maybe in the sense of a single gene test predicting the chances of adverse effects. In my opinion, we should focus on research helping to unravel the (genetic) mechanisms leading to CIA (and other effects of antipsychotic drugs). With the results it might be possible to create a risk predictor, consisting of different types of data (genetic markers, biomarkers, etc.). With these insights it might also be possible to develop new drugs, without the serious adverse effects of the antipsychotics used now.

2. The collection of a patient cohort

The collection of a patient cohort is a time-consuming, difficult and expensive matter. The importance of large sample sizes became even more clear with the advent of GWAS and the discovery that large numbers of genetic variants with each a small effect are the essence of this research field. The only way to obtain large enough patient cohorts is through collaboration. In the Psychiatry field, a large consortium was founded in 2007: the Psychiatric Genomic Consortium (PGC), with the aim of conducting large scale genetic analyses. It has been a very successful collaboration with more than 800 investigators from 38 countries and over 900 000 individuals in analyses [280]. The results of this effort have greatly increased our current knowledge of the genetic basis of psychiatric diseases[99,281–284]. Although it has been and is a huge effort to collect and combine patient cohorts (like the PGC has done), in comparison with collecting a pharmacological cohort, it is fairly straightforward: the 'only' patient parameter necessary is diagnosis (and maybe age and sex). For a patient cohort suitable for pharmacogenomics analyses it is necessary for a patient to be taking a certain drug, with a certain follow-up time and a certain outcome measurement, complicating collaboration and collection substantially. Oftentimes studies used in the pharmacogenomic field were not necessarily designed for a pharmacogenomic study, but for a case-control or optimization of treatment study. The consequence of this is that even though the sample can be used, the conditions to do so are suboptimal. For instance, patients have used different antipsychotic drugs, or different dosages were used, or multiple drugs were used simultaneously, or follow-up times and outcome measurements were different. Additionally, when conducting a genetic study there are other things important, such patient ancestry, making analyses more difficult. These difficulties are part of the reason why the patient cohorts used in **chapters V** and **VI** are relatively small. They also made us think about creative ways to circumvent the collection of a patient cohort and revert to a cell model.

3. The cell model

In a perfect research world there would be an unlimited number of patients willing to participate in research and an infinite amount of time and money to design and conduct these studies as well as possible. The limitations of the real world, however, force us to come up with creative options and alternatives. In this thesis, a lymphoblast cell model was used in several chapters (**chapter III** and **IV**). The use of lymphoblast cell lines has considerable advantages, such as relatively easy maintainable cells, the possibility of choosing specific features or designs (such as a homogenous ancestry or family design) and the availability of different types of genomic data for a large number of cells[158,285]. We have made use of these advantages, by choosing cell lines from the same population ancestry, partly in a trio (parents and offspring) design, and by choosing cell lines from which SNP data was available (**chapter III** and **IV)**. The extent of these studies has been relatively small due to the fact that they were pilot studies, however, they would be easily up scalable. A cell model has limitations; it will never be a true or perfect surrogate for the complexity of the human body. This leads to disadvantages, in our case we were not able to study metabolites of clozapine, since lymphoblast cell lines do not metabolize clozapine. It would be interesting to repeat the experiments with the metabolites of clozapine, or, better yet, with a cell system capable of metabolizing clozapine to all (!) of its metabolites. Additionally, we were not able to study the specific cells (neutrophils) that are affected in CIA. Neutrophils have a short lifespan (especially when cultured), making it difficult to study drug exposure in the context of agranulocytosis.

There has also been concern about artificially induced mutations in LCL's, recent studies have showed, however, that differences between whole blood DNA and LCL DNA are small [286–289]. Differences in gene expression profiles have been described, although these changes were limited to genes involved in cell proliferation[290]. It is possible that this has affected our results in **Chapter III**, since the changes we observed, however, are mainly in genes involved in

cell cycle and cholesterol metabolism, the effects of artificially induced mutations (if there are any) would be small.

Findings in a broader (patient) perspective

I wish I could write here that we have changed the lives of patients with the outcomes of this thesis, unfortunately, this is not the case. We might change lives in the long run and for now give hope that we will unravel the origin of CIA, albeit in the next 50 years. There is still too much we don't know, but I am convinced we will be successful in our search. How should we continue?

Through the past 10 years we have learned that in the field of genomics, bigger is better. The psychiatric field has adapted to this motto very well and has successfully created large collaboration consortia. The most recent GWAS of CIA have showed the fruits of collaboration as well, giving rise to large(r) cohorts and new findings [44,45]. I think it is important to continue down this path, and make sure that new trials are designed in a way that they can also add to the pharmacogenomics field.

Next to increasing cohort size, we should search for creative alternatives. In this thesis we have used a cell model, which comes with obvious limitations (as discussed earlier). The advent of organoids can fill (part) of the gap between *in vitro* and *in vivo*. An organoid is a small and simplified version of an organ, produced in 3D and shows relatively realistic microanatomy[291]. This provides a model that is a better representation of the processes inside the human body, broadening opportunities for *in vitro* testing. For clozapine, several organoids could be interesting. For one, it would be interesting to monitor clozapine exposure in a bone marrow organoid[292]. In **chapter V**, we hypothesize that exposure to clozapine in early stages of hematopoiesis could give rise to fluorescence in neutrophils. To be able to test this, we would have to study bone marrow from patients, this is a very invasive procedure, however. With the use of a bone marrow organoid, we can first test our hypothesis *in vitro*, before performing invasive testing in patients. Second and maybe more obvious, creating a brain organoid, exposing it to clozapine and studying the effects

would be very interesting. Even though it is not entirely clear yet how an organoid model of the brain translates to the adult human brain [293,294], it would give us an alternative to postmortem research.

Thus, we have to go aim at collecting (even) larger cohorts and we have to consider alternatives *in vitro*, but how do we translate these 'large cohort' and *in vitro* findings to our clinical practice? To our psychiatrists and patients? Most physicians (let alone the layman and patient) have little knowledge of genetic research, cell models or organoids. And I am not sure that they should. Nowadays, research is being performed by a large number of people with an even larger variety of educational backgrounds (medicine, biomedical, bio-informatic, mathematics, statistics, etc). While this is vital for quality of research, we must assure that findings in fundamental research translate to physicians and patients. It is therefore paramount that we educate *some* of our doctors in these fields, so that they are able to educate and advocate fundamental research to their peers. I will do so with all my heart.

"Sometimes it's the journey that teaches you about your destination."

Drake

Chapter VIII

Nederlandse samenvatting

(Summary in Dutch)

Schizofrenie is een psychiatrische ziekte die gekenmerkt wordt door periodes van psychose, het vóórkomen is ongeveer 1% in de algemene samenleving. De behandeling is, naast voorlichting en leefstijladviezen, vooral medicamenteus van aard, met antipsychotica (middelen die de psychose bestrijden). Er bestaat een groot aantal verschillende antipsychotica, allemaal effectief, allemaal met een verschillend bijwerkingenprofiel. Welk middel het beste past bij welke patiënt, is (tot op heden) een kwestie van 'trial and error'.

Hoe antipsychotica precies werkzaam zijn in het lichaam en het brein is nog niet precies duidelijk. Het doel van dit proefschrift was om meer kennis te vergaren in de precieze werkingsmechanismen van één van de meest bekende en besproken antipsychotica, clozapine. Clozapine is een interessant medicijn omdat het één van de meest effectieve antipsychotica is die we kennen, ondanks dat het middel al sinds de jaren 50 van de vorige eeuw op de markt is. Het is een berucht middel, omdat het een ernstige, zeldzame bijwerking heeft: agranulocytose; het nauwelijks meer aanwezig zijn van een specifiek type witte bloedcellen; neutrofielen, welke essentieel zijn voor afweer. Agranulocytose geeft een verhoogd risico op (ernstige) infecties, met potentieel fatale afloop. Om dit te voorkómen is er een follow-up systeem ontwikkeld. Als mensen clozapine krijgen voorgeschreven moeten ze de eerste 18 weken iedere week bloedcontroles ondergaan, de rest van de behandelingsduur (soms jaren) maandelijks. Op deze manier wordt iedere week het neutrofielen aantal gecontroleerd en kan een ernstige daling vroeg ontdekt worden en soms zelfs voorkomen. Naast agranulocytose kan clozapine-gebruik gepaard gaan met gewichtstoename, cholesterol- en triglyceridenverhoging, speekselvloed en sufheid. Vanwege de ernstige bijwerkingen is het middel, ondanks de effectiviteit, niet het middel van eerste keus bij schizofrenie. Een andere bijwerking, voor zover bekend zonder klinisch effect, is autofluorescentie (het uitzenden van licht zonder het bijvoegen van een andere stof) van de neutrofiel bij het gebruik van clozapine. De ontstaanswijze hiervan is onbekend en het zou kunnen dat deze autofluorescentie van neutrofielen verband houdt met agranulocytose, maar dat is (nog) onvoldoende onderzocht.

De precieze werkingsmechanismen van clozapine zijn nog niet duidelijk. Het is waarschijnlijk dat er verschillende signaalstoffen in de hersenen (neurotransmitters) reageren op behandeling met clozapine, waaronder bijvoorbeeld dopamine. De precieze ontstaanswijze van clozapine geïnduceerde agranulocytose (en van andere bijwerkingen, overigens) is ook onduidelijk. Mogelijk spelen genetische factoren een rol bij de gevoeligheid voor de werking en bijwerkingen van clozapine. Zo heeft een tweelingstudie laten zien dat eeneiige tweelingen een grotere overeenkomst hebben in vóórkomen van gewichtstoename bij antipsychotica gebruik dan niet eeneiige tweelingen, wat duidt op een genetische bijdrage aan het risico op gewichtstoename. Een genetische test om de kans op effectieve werking en de kans op bijwerkingen te bepalen zou een grote stap voorwaarts zijn, maar is tot op heden nog niet ontwikkeld.

Het onderzoek beschreven in dit proefschrift had tot doel om de genetische en moleculaire achtergronden van de effecten van clozapine verder te onderzoeken.

Deel 1: DNA

Het eerste deel van dit proefschrift heeft als thema DNA, het onderzoek wat wordt beschreven in dit deel is verricht op het niveau van DNA. Om een overzicht te krijgen van de verschillende genetische onderzoeken naar clozapine geïnduceerde agranulocytose hebben we een review van de literatuur uitgevoerd. De resultaten hiervan staan beschreven in **hoofdstuk II** van dit proefschrift. We concluderen dat het onwaarschijnlijk is dat het risico op agranulocytose bij clozapine gebruik wordt bepaald door één of een klein aantal genen. Dit gegeven maakt het ingewikkelder om een genetische test te ontwikkelen met voldoende voorspellende waarde.

Daarnaast blijkt dat er een groot aantal patiënten nodig is om conclusies te trekken, wat wordt gecompliceerd door het verzamelen van een groot patiënten cohort. Een alternatief voor een patiënten cohort is onderzoek in een cel model.

Een cel model heeft verschillende voordelen; zo zijn cellen over het algemeen makkelijk te onderhouden, de basiscondities tussen verschillende experimentlijnen (wel of geen blootstelling aan een middel bijvoorbeeld) hetzelfde en is schaalvergroting relatief gemakkelijk. Vanwege deze redenen hebben wij ervoor gekozen om een aantal experimenten uit te voeren in lymfoblasten, een type witte bloedcel dat relatief makkelijk te kweken en te onderhouden is.

In **hoofdstuk III** presenteren we de resultaten van een onderzoek naar de genetische factoren geassocieerd met verminderde levensvatbaarheid in lymfoblasten na blootstelling aan verschillende concentraties clozapine. Lymfoblasten werden blootgesteld aan oplopende concentraties clozapine, waarna de levensvatbaarheid van de cellen werd gemeten. Vervolgens werd door middel van een genoom wijde associatie studie gekeken of varianten in het DNA een associatie vertoonden met de levensvatbaarheid. De uitkomsten lieten een verrassend hoge erfelijkheid zien van clozapine geïnduceerde verminderde levensvatbaarheid in lymfoblasten van 0.72 (schaal van 0-1) en het *PRG4* gen dat een consistente associatie liet zien. Mogelijk geeft dit gen nieuwe aanknopingspunten in de zoektocht naar genetische factoren die van invloed zijn op het ontstaan van clozapine geïnduceerde agranulocytose.

Deel 2: RNA

DNA wordt, in de cel, omgezet naar RNA, wat uiteindelijk leidt tot genexpressie. Allerlei factoren kunnen van invloed zijn op genexpressie, zoals blootstelling aan medicatie, maar ook zonlicht, voedsel, etc. In **hoofdstuk IV** hebben we gepoogd informatie van verschillende experimenttypen (cel model, groot patiënt cohort) te combineren met als doel zo intelligent mogelijk gebruik te maken van informatie die beschikbaar is. Allereerst hebben we lymfoblasten blootgesteld aan verschillende concentraties clozapine, waarna we hebben gekeken naar de effecten hiervan op genexpressie niveau en naar de effecten op DNA methylatie. DNA methylatie zegt iets over de mate waarin het DNA beschikbaar is om afgelezen te worden en omgezet te worden naar RNA om uiteindelijk tot expressie te komen in de cel. Na blootstelling aan clozapine

veranderde de genexpressie significant, waarbij genen die meer tot expressie kwamen, verrijkt waren voor processen in de cholesterol metabolisme en genen die minder tot expressie kwamen, verrijkte waren voor processen in de levenscyclus van de cel. Er werden echter geen verschillen gezien in DNA methylatie. Daarnaast hebben we de data van de genexpressie experimenten gebruikt om te onderzoeken of de genen die differentieel tot expressie komen ook genetische variatie bevatten die geassocieerd is met het risico op schizofrenie. Hier zijn geen aanwijzingen voor gevonden. We concluderen op basis van deze en andere studies dat clozapine het cholesterol metabolisme significant beïnvloedt, wat een van de manieren zou kunnen zijn waarop clozapine metabole bijwerkingen induceert.

Een cel model is een goede manier om processen in de mens te onderzoeken, het blijft echter altijd een gesimplificeerde weergave van het complexe menselijk lichaam. Het zal dan ook niet patiëntgebonden onderzoek kunnen vervangen. In **hoofdstuk V** doen we onderzoek naar genexpressie in neutrofielen van mensen die clozapine gebruiken, die we vergelijken met mensen die geen clozapine gebruiken. Onderzoek hiernaar is niet alleen interessant vanwege clozapine geïnduceerde agranulocytose, maar ook vanwege de eerdergenoemde autofluorescentie in juist deze cellen. Vanwege deze autofluorescentie hadden wij de verwachting dat er ook significante genexpressie verschillen te zien zouden zijn. Dit was echter niet het geval, er waren geen verschillen in genexpressie. Onze hypothese voorafgaand aan het onderzoek was dat de fluorescentie voort zou komen uit een eiwit dat meer tot expressie komt door blootstelling aan clozapine. Ons onderzoek laat zien dat dit eiwit niet op het moment van bloedafname wordt geproduceerd, het is goed mogelijk dat het wel eerder in de maturatiefase van de neutrofiel in grote(re) hoeveelheden wordt geproduceerd, maar dat hebben wij niet kunnen onderzoeken.

Deel 3: Eiwit

Na genexpressie, meetbaar in de vorm van RNA, volgen uit het RNA, eiwitten. In **hoofdstuk VI** wordt de zoektocht naar de origine van het eiwit dat

fluorescentie veroorzaakt in neutrofielen van clozapine gebruikers beschreven. Net als in hoofdstuk V werden neutrofielen verzameld van mensen die clozapine gebruiken en mensen die dat niet gebruiken. Vervolgens is met behulp van fluorescentie microscopie het exacte emissie spectrum bepaald van het autofluorescentie signaal uit de neutrofiel. Het bleek dat het patroon van het signaal het best past bij fluorescentie afkomstig uit granulae (kleine blaasjes) in de neutrofiel. De neutrofiel bevat verschillende typen granulae, waarbij de uitkomsten van het onderzoek erop wijzen dat het signaal zich bevindt in de azurofiele granulae. Ten slotte is met behulp van een methode waarbij moleculen op basis van moleculairgewicht worden gescheiden, gepoogd het precieze eiwit te achterhalen. Hierbij werd duidelijk dat een eiwit of eiwitfragment van 14kDa een fluorescent signaal had, het was echter niet mogelijk een specifiek eiwit aan te wijzen.

Dit proefschrift had tot doel meer inzicht te verkrijgen in de manier waarop clozapine processen in het lichaam beïnvloedt, met de hoop dat dit ons zou helpen de effecten en bijwerkingen beter te begrijpen en misschien zelfs te voorspellen. De onderzoeken hier beschreven brengen de wetenschap een kleine stap dichterbij dit doel. Het gebruik van cel modellen als tussenstap naar patiëntgebonden onderzoek heeft enig succes opgeleverd, waarbij we activatie van cholesterol metabolisme na clozapine hebben laten zien en er een gen gevonden is dat gelinkt is aan clozapine geïnduceerde levensvatbaarheid reductie in lymfoblasten. Daarnaast is nu duidelijk waar in de neutrofiel van clozapine gebruikers de autofluorescentie zich bevindt en kunnen we hypothetiseren dat het eiwit dat de fluorescentie produceert, in de vroege fase van neutrofiel ontwikkeling geproduceerd wordt.
Dit proefschrift heeft ons een kleine stap dichterbij een beter begrip van de werkingsmechanismen van clozapine gebracht. Er is echter nog een grote behoefte aan meer kennis. Toekomstig onderzoek zou zich aan de ene kant moeten richten op cel-/organoïd-/diermodelonderzoek en aan de andere kant op het verzamelen van een zo groot mogelijk patiënten cohort om de resultaten uit fundamenteel onderzoek te valideren en complementeren.

4

Appendices

Chapter IX

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Chapter X

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Chapter XI

List of publications and presentations

de With SAJ, Tak T, de Jong S, Olde Loohuis L, Wu T, ten Berg MJ, Cahn W, van Solinge WW, Ophof RA. *No clear evidence for transcriptome differences associated with clozapine-induced fluorescence in neutrophils.* Submitted.

de With SAJ, Ori APS, Pulit SL, Wang T, Strengman E, Glennon JC, Buitelaar JK, Viana J, Staal WG, Mill J, de Jong S, Ophoff RA. *Clozapine-induced in vitro gene expression profiles are linked to cholesterol and cell cycle pathways without similar evidence for DNA methylation changes.* Submitted.

de With SAJ, Man WH, Maas C, Koekman CA, van Solinge WW, Tak T. *Neutrophil fluorescence in clozapine users is attributable to a 14kDa secretable protein.* Submitted.

Pulit SL, **de With SAJ,** de Bakker PIW (2017). *Resetting the bar: Statistical significance in whole genome sequencing-based association studies of global populations.* Genetic epidemiology (41): 145-151.

de With SAJ, Pulit SL, Staal WG, RS Kahn, Ophoff RA (2017). *More than 25 years of genetic studies of clozapine-induced agranulocytosis.* The Pharmacogenomics Journal (17); 304-311.

de With SAJ, Pulit SL, Wang T, Staal WG, van Solinge WW, de Bakker PIW, Ophoff RA (2015). *Genome-wide association study of lymphoblast cell viability after clozapine exposure.* American Journal of Medical Genetics Part B: Neuropsychiatric Genetics(168); 116-122.

de With SAJ, Staal WG (2013). *Genetica van autisme. Snel veranderende inzichten en toenemende complexiteit.* Wetenschappelijk Tijdschrift voor Autisme.

de Bildt A, Mulder EJ, van Lang ND, **de With SAJ**, Minderaa RB, Stahl SS, Anderson GM (2012). *The visual rooting reflex in individuals with autism spectrum disorders and co-occurring intellectual disability.* Autism Research (5); 67-72.

List of presentations

2014 Poster presentation at the **World Congress of Psychiatric Genetics** (Copenhagen), title: “Genome-wide association study of lymphoblast cell viability after clozapine exposure.”

2013 Poster presentation at the **American Society of Human Genetics** conference (Boston), title: “Primary action of clozapine exposure on activation of SREBP-controlled lipogenic gene expression may explain benefit and detriment.

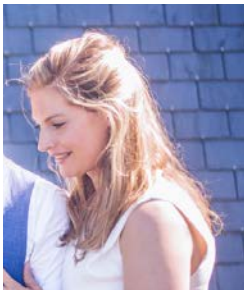
2013 Speaker at the **World Congress of Psychiatric Genetics** (Boston), title: “Primary Action of Clozapine Exposure on Activation of SREBP-controlled Lipogenic Gene Expression May Explain Benefit and Detriment.”

2013 Speaker at the 65-year celebratory meeting of the **Dutch Child and Adolescent psychiatry association**.

Chapter XII

Curriculum Vitae

Jytte Sera Anne de With



Jytte was born in the winter of 1985 in a very small town in the province of Groningen (the Netherlands). After graduating from the Praedinius Gymnasium in Groningen in 2004, she was able to pursue her childhood dream: medical school. She stayed in Groningen to start her medical career at the Rijksuniversiteit Groningen/University Medical Center Groningen. In her fourth year she was involved in a research project at Accare Child and Youth Psychiatry, leading to her first scientific publication. She graduated from medical school in November 2010, spending the last year as a research and clinical intern at the psychiatry department at the University Medical Center Utrecht. She postponed the start of her Psychiatry Residency program, inspired by her research internship and was given the opportunity for a PhD at the University Medical Center Nijmegen (UMCN) psychiatry department in collaboration with the University of California, Los Angeles (UCLA) and the UMCU. During this time she spent almost a year and a half at the Psychiatric Genetics lab of professor Ophoff at UCLA to work on cell model systems and extend her knowledge in the field of genetics. In the spring of 2015 she started her Psychiatric Residencies at the UMCU, while continuing to work on her PhD thesis. She is currently in her 4th year as a Resident in Psychiatry and she works at the Reiner van Arkel PsychoTrauma Center. Jytte lives in Amsterdam and Den Bosch with her fiancé Maarten and her daughter Vesper.

Chapter XIII

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‘*it takes a village’*

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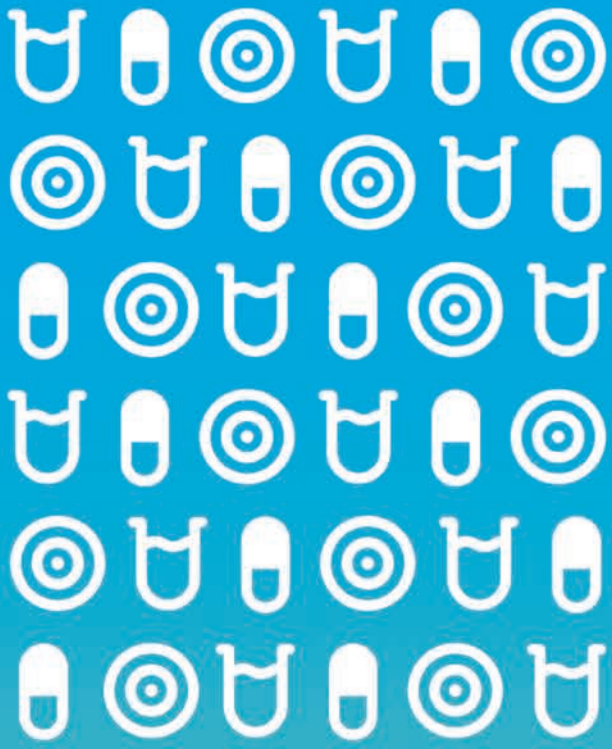
Vesper, iets mooiers dan jij bestaat er niet.



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