Examining Treatment Response and Adverse Effects of Clozapine

Thesis submitted for the degree of Doctor of Philosophy at

Cardiff University by

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This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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Thesis Summary

The antipsychotic clozapine is uniquely effective in the management of treatment-resistant schizophrenia (TRS), but its use is limited by its potential to induce agranulocytosis. A substantial proportion of patients discontinue clozapine treatment, which carries a poor prognosis, and only 30-60% of patients with TRS will respond to clozapine.

The causes of clozapine-associated agranulocytosis, and of its precursor neutropenia, are largely unknown although genetic factors contribute. To examine the genetic susceptibility to clozapine-associated neutropenia, I conducted a multifaceted genetic analysis in the largest combined sample studied to date. Using GWAS, I identified a novel genome-wide significant association with rs149104283 (OR = 4.32, P = 1.79×10^{-8}), a SNP intronic to transcripts of *SLCO1B3* and *SLCO1B7*, members of a family of hepatic transporter genes involved in drug uptake. Furthermore, I replicated a previously reported association between neutropenia and a variant in *HLA-DQB1* (OR = 15.6, P = 0.015).

I investigated clozapine discontinuation and clinical response in a two-year retrospective cohort study of 316 patients with TRS receiving their first course of clozapine. By studying the reasons for discontinuations due to a patient decision, I found that adverse drug reactions accounted for over half of clozapine discontinuations. High levels of deprivation in the neighbourhood where the patient lived were associated with increased risk of clozapine discontinuation (HR = 2.12, 95% CI 1.30-3.47). Female gender (HR = 0.63, 95% CI 0.41-0.96) and clinical improvement after one month of treatment (HR = 0.56, 95% CI 0.44-0.71) were significantly associated with a good response to clozapine. However, I found that up to six months of treatment may be required to determine non-response.

This thesis implicates variants that may increase susceptibility to clozapine-associated neutropenia and contributes to our current understanding of the causes of clozapine discontinuation and treatment response.

Structure of Thesis

This thesis is divided into six chapters. Chapter 1 provides an overview of schizophrenia and the use of clozapine in its management. The focus then turns to the literature base of clozapine-associated agranulocytosis and neutropenia, all-cause discontinuation, and clinical response to clozapine. Chapters 2, 4 and 5 are presented as results chapters, each with their own introduction, method, results and discussion sections. Chapter 3 provides details of methods relevant for both Chapters 4 and 5. Finally, Chapter 6 provides an overall discussion of the principal findings of the thesis in the context of the existing literature, the strengths and weaknesses of the studies, and provides indications for future research.

Publications resulting from work in this thesis

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Chapter 1

Background Literature

1.1. Introduction

The aim of this thesis is to investigate treatment response and the adverse effects of clozapine, a uniquely effective antipsychotic medication in the management of treatment-resistant schizophrenia. Specifically, the thesis aims are to (i) identify genetic risk variants associated with clozapine-induced agranulocytosis and neutropenia, (ii) investigate the clinical risk factors, reasons and timing of clozapine discontinuation, and (iii) identify clinical predictors of clozapine response. The identification of predictor variables associated with these different outcomes of clozapine treatment may be valuable in assisting clinicians in determining if clozapine is likely to be beneficial. This introductory chapter will provide an overview of schizophrenia, treatment-resistant schizophrenia, and the use of clozapine in the management of these conditions. The focus will then turn to the literature base of clozapine-associated agranulocytosis and neutropenia, all-cause discontinuation, and clinical response to clozapine. Finally, the limitations of the current research will be discussed and the aims of the thesis outlined.

1.2. Schizophrenia

Schizophrenia is a severe psychiatric syndrome characterised by positive symptoms, such as delusions and hallucinations, negative symptoms and cognitive deficits. Although the illness course of schizophrenia is highly heterogeneous, it is frequently life-long and debilitating (Tandon et al., 2008). Consequently, schizophrenia is one of the leading causes

of disability worldwide (Rossler et al., 2005; World Health Organisation, 2008). The associated economic burden to society is extremely high, from both direct healthcare and non-healthcare related costs such as unemployment, reduced workplace productivity, family care-giving and premature mortality (Knapp et al., 2004; Wu et al., 2005). In England the estimated cost of schizophrenia per year to society and the public sector is £11.8 billion and £7.2 billion, respectively (The Schizophrenia Commission, 2012). People with schizophrenia have a reduced life expectancy of approximately 15-20 years compared to the general population, driven by both increased rates of suicide and a wide range of comorbid somatic conditions (Saha et al., 2007; McGrath et al., 2008). Worryingly, this mortality gap is widening over time (Saha et al., 2007; Walker et al., 2015).

Schizophrenia occurs across diverse cultures and populations worldwide. A large systematic meta-analysis of epidemiological studies indicates that the lifetime prevalence of schizophrenia is 4.0 per 1,000 people and the median incidence is 15.2 per 100,000 people per year (McGrath et al., 2008). Although there are no sex differences in the prevalence (Perala et al., 2007; McGrath et al., 2008), the incidence is higher in males, with a male:female incidence rate ratio of 1.4:1 (Aleman et al., 2003; McGrath et al., 2008; Abel et al., 2010). Prevalence and incidence rates also vary based on ethnicity, seasonality of birth, urbanicity and latitude (McGrath et al., 2008; Tandon et al., 2008). The onset of schizophrenia is usually during adolescence or early adulthood and the onset in males is three to four years earlier than females, who have a second peak around age 50 (Hafner et al., 1993).

Schizophrenia has only been considered as a unique syndrome or disorder for a little over a century and its classification has been based on the observation that certain symptoms occur together. Our current conceptualisation of schizophrenia has derived principally

from the clinical concepts described by Kraepelin, Bleuler and Schneider (Tandon et al., 2009). In 1899, German psychiatrist Emil Kraepelin described a discrete mental illness called 'dementia praecox' (early dementia), characterised by an onset in early adulthood, a chronic and deteriorating course, and the lack of depression or mania, which he called manic depressive insanity (Kraepelin, 1971). The term schizophrenia was coined in 1911 by a Swiss psychiatrist called Eugen Bleuler (Bleuler, 1950), derived from the Greek words 'schizo' (split) and 'phren' (mind), intended to describe a loosening of association rather than a split personality, which is a common misconception. He proposed an alternative term to dementia praecox to reflect the fact that the disease can occur in later life and mental deterioration is not always present (Moskowitz & Heim, 2011). Bleuler was the first to distinguish the positive and negative symptoms of schizophrenia (Bleuler, 1950). In 1959, German psychiatrist Kurt Schneider described 11 'first-rank' symptoms that were particularly characteristic of schizophrenia, for example bizarre delusions or two or more voices conversing with each other (Schneider, 1959). However, it has become apparent that the diagnostic value of first-rank symptoms in schizophrenia is unclear and thus they are no longer solely sufficient for a diagnosis in the latest classifications (Moskowitz & Heim, 2011; Soares-Weiser et al., 2015). Aspects of the clinical concepts described by Kraepelin, Bleuler and Schneider are incorporated in the International Statistical Classification of Diseases and Related Health Problems (ICD-10) (World Health Organization, 1992) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013) classifications of schizophrenia. **Table 1.1** details the DSM-V criteria for schizophrenia (American Psychiatric Association, 2013). The classification of schizophrenia has changed over time and has been the subject of significant debate, particularly regarding whether schizophrenia and bipolar disorder are distributed across a dimensional spectrum.

DSM-V diagnostic criteria for schizophrenia

Criterion A. Characteristic symptoms

Two or more of the following, each present for a significant portion of time during a one-month period (or less if successfully treated). At least one of these should include 1-3.

1. Delusions
2. Hallucinations
3. Disorganised speech
4. Grossly disorganised or catatonic behaviour
5. Negative symptoms (affective flattening, alogia, or avolition)

Criterion B. Social/occupational dysfunction

For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning, such as work, interpersonal relations, or self-care, are markedly below the level achieved prior to the onset (or when onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

Criterion C. Duration

Continuous signs of the disturbance persist for at least six months. This six-month period must include at least one month of symptoms (or less so if successfully treated) that meet Criterion A and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or by two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

Criterion D. Schizoaffective and major mood disorder exclusion

Schizoaffective disorder and depressive or bipolar disorder with psychotic features have been ruled out because either (1) no major depressive or manic episodes have occurred concurrently with the active phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration is brief relative to the active and residual periods.

Criterion E. Substance/general mood condition exclusion

The disturbance is not attributed to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or other medical condition.

Criterion F. Relationship to Global Developmental Delay or Autism Spectrum Disorder

If there is a history of autism spectrum disorder, the additional diagnoses of schizophrenia is made only if prominent delusions or hallucinations are also present for at least one-month (or less if successfully treated).

Table 1.1. *Diagnostic and Statistical Manual of Mental Disorders (DSM-V) classification for schizophrenia (American Psychiatric Association, 2013).*

1.2.1 Symptoms of schizophrenia

The clinical features of schizophrenia are variable between affected individuals, but are likely to be a mixture of positive, negative and cognitive symptoms.

Positive symptoms

The positive symptoms of schizophrenia are characterised by distortions of thinking and perception, named 'positive' because they are 'in addition' to normal experiences. Hallucinations are false perceptions in the absence of an appropriate stimulus and can take any sensory modality including auditory, visual, tactile, gustatory and olfactory, although auditory hallucinations, usually in the form of hearing voices, are the most common (Tandon et al., 2009). These voices may talk directly to the individual, perhaps stating derogatory remarks or issue commands, but can also talk in third person (American Psychiatric Association, 2013).

Delusions are firmly held beliefs that cannot be substantiated with evidence and are not part of the individual's culture. The specific content of delusions can vary widely, but delusions of persecution are the most common whereby the individual may believe that they are being followed, spied on, or conspired against. Other delusions may include beliefs that comments, gestures or text is specifically directed at them (delusions of reference), that they are someone of great importance (delusions of grandiose), or may be related to religion. Bizarre delusions are delusions that are clearly implausible, such as delusions of control (Tandon et al., 2009).

Disorganised thinking or formal thought disorder, relates to unusual or dysfunctional ways of thinking, primarily detected through speech (Tandon et al., 2009). The person may slip off track (derailment), give obliquely related answers to questions (tangentiality), or in extreme examples be incomprehensible (American Psychiatric Association, 2013).

Disorganised behaviour frequently co-occurs with formal thought disorder and may manifest as unpredictable agitation, inappropriate sexual behaviour or dressing in a very unusual way (for example winter clothing on a hot day).

Negative symptoms

The negative symptoms of schizophrenia are characterised by a loss of affective or behavioural functions that are present among healthy individuals. Common negative symptoms include a blunting of emotional expressiveness either facially or through body language (affective flattening), a decreased production or fluency of speech (alogia), and the reduced motivation or ability to carry out goal-directed activities (avolition) (American Psychiatric Association, 2013). These symptoms can be harder to recognise as they are part of a continuum of normal behaviour and can occur as a result of other factors, such as medication side effects or depression (Kirkpatrick et al., 2006). Nonetheless, negative symptoms are resistant to current treatments and are a debilitating component of schizophrenia (Tandon et al., 2009).

Motor symptoms

Patients with schizophrenia may show abnormalities in psychomotor activity, manifested as either excessive unstimulated motor activity or as a marked decrease in reactivity to the environment, in its extreme form catatonia (Tandon et al., 2009). Psychomotor slowing is common in schizophrenia (Morrens et al., 2007) and neuromotor disturbances have been found in up to a quarter of first episode treatment-naive patients with schizophrenia (Cortese et al., 2005). Catatonic symptoms are now more common in mood disorders than schizophrenia and are thus no longer considered a core feature of schizophrenia (Taylor & Fink, 2003).

Cognitive deficits

Although cognitive deficits are a core feature of schizophrenia, they are not currently part of the diagnostic classification due to the lack of diagnostic specificity (Tandon et al., 2009). Cognitive deficits contribute significantly to the functional disability in schizophrenia, impacting employment, independent living and general quality of life (Bowie et al., 2008). People with schizophrenia perform on average up to two standard deviations below the general population on cognitive tasks (Keefe & Harvey, 2012) and nearly all patients with schizophrenia have cognitive deficits to some degree (Heinrichs & Zakzanis, 1998). Even if the cognitive ability of an individual with schizophrenia is not severe enough to be 'impaired', studies have shown that their performance is below what would be expected based on their premorbid functioning (Keefe et al., 2005). The cognitive deficit in schizophrenia is of a generalised nature and thus includes many important aspects of cognition (Dickinson et al., 2008).

The clinical course of cognition in schizophrenia is still unclear. Many studies have consistently shown that cognitive impairment precedes the onset of schizophrenia (Bilder et al., 2006). There appears to be a cognitive decline around the onset of psychotic symptoms, relative stability thereafter, and there is mixed evidence regarding the impact of pharmacologic treatment on cognition in schizophrenia (Keefe & Harvey, 2012). A considerable challenge in this field has been the wide variability of assessments, making comparisons between studies difficult. To combat this issue the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) consensus cognitive battery was developed to provide a standardised outcome measure for the key cognitive domains related to schizophrenia: speed of processing, attention, working memory, verbal learning, visual learning, reasoning and problem solving, and social cognition (Nuechterlein

et al., 2008). The significant interest in cognition over the last 10 years has led to valuable insights, such as evidence indicating that the deficits are related to genetic susceptibility to schizophrenia (Lencz et al., 2014).

Comorbid symptoms

There is an increased prevalence of anxiety, depression and substance-abuse disorders in people with schizophrenia (Buckley et al., 2009). The overall lifetime prevalence of anxiety disorders in schizophrenia is between 30-85%, with post-traumatic stress disorder, panic disorder, and obsessive-compulsive disorder being the most common (Pokos & Castle, 2006). Approximately 50% of patients with schizophrenia will manifest affective symptoms at some point (Siris, 2000), and 47% will have a lifetime diagnosis of comorbid substance abuse (Buckley et al., 2009). These comorbid psychiatric disorders are associated with poorer outcomes and complicate the clinical picture in schizophrenia (Buckley et al., 2009). Their relationship to schizophrenia (whether they are related or separate conditions) and the underlying causes are still poorly understood.

People with schizophrenia are also at high risk of multiple physical comorbidities, which contribute to premature mortality. In particular, there is an increased prevalence of cardiovascular disease, type II diabetes, obesity, HIV infection, hepatitis, and smoking-related lung disease compared to the general population (Dixon et al., 2000; Leucht et al., 2007a; Smith et al., 2013). Numerous factors are likely to contribute to these increased rates. Large proportions of people with schizophrenia smoke cigarettes and have substance abuse disorders (Leucht et al., 2007a). Negative symptoms and cognitive deficits can lead to a lack of exercise and poor diet (McCreadie & Scottish Schizophrenia Lifestyle Group, 2003), and may mean that individuals are less motivated to seek treatment for

physical illness. Lastly, side effects of antipsychotic medication such as weight gain are likely to have a negative impact on physical health (Leucht et al., 2007a).

1.2.2 Aetiology of schizophrenia

The exact causes of schizophrenia are still unclear. We know that schizophrenia is highly heritable and heritability has been estimated at approximately 80% (Sullivan et al., 2003). Family, twin and adoption studies have demonstrated that the degree of risk of schizophrenia increases with greater genetic relatedness (Lichtenstein et al., 2009; Plomin et al., 2013). However, the aetiology of schizophrenia is complex and multifactorial, likely a result of numerous environmental and genetic causes.

Environmental

Epidemiological studies have implicated a number of environmental influences that increase the risk of schizophrenia: obstetric complications, prenatal nutritional deficiency, winter births, childhood trauma, urbanicity, immigration and cannabis use. There is strong evidence that obstetric complications increase the risk of schizophrenia in later life. In a Finnish general population birth cohort of 11,017 people, being born early (<37 weeks) and a low birth weight were associated with an increased risk of schizophrenia (Jones et al., 1998). In a similar Swedish study, schizophrenia was associated with multiparity, maternal bleeding during pregnancy, and birth in late winter (Hultman et al., 1999). A systematic meta-analysis concluded that the well-replicated obstetric risks for schizophrenia include (i) complications in pregnancy (preeclampsia, diabetes or bleeding in pregnancy), (ii) abnormal foetal growth and development (low birth weight, congenital malformation) and (iii) complications with delivery (asphyxia, uterine atony and emergency caesarean section) (Cannon et al., 2002).

Two key studies have shown that prenatal exposure to famine increased rates of schizophrenia in later life, indicating that prenatal nutrition may play a role in schizophrenia. Being conceived during the height of the Dutch Hunger Winter famine of 1944/1945, a result of a Nazi blockade, resulted in a 2-fold increased risk for schizophrenia (Susser et al., 1996). Furthermore, births that occurred during the Chinese famine of 1959-1961 were also associated with a 2-fold risk of developing schizophrenia in later life compared to births before or after the famine (St Clair et al., 2005). The mechanism by which famine increases risk of schizophrenia is currently unclear, but hypothesis include prenatal folate deficiency, which is related to neural tube defects (St Clair et al., 2005), or epigenetic changes (Heijmans et al., 2008).

Adversity and trauma in childhood is associated with a 3-fold increased risk for schizophrenia in later life (Varese et al., 2012). Childhood adversity is a heterogeneous concept for physical, sexual or emotional abuse, neglect, death or separation from a parent, and bullying. Childhood adversity may also affect the persistence of psychotic symptoms (Trotta et al., 2015). However, it is possible that the association between childhood adversity and schizophrenia is mediated by child-related cognitive and affective difficulties (Fisher et al., 2013).

Individuals with schizophrenia are more likely to be born during the winter or spring compared to the general population (Davies et al., 2003). A large meta-analysis of studies from both Northern and Southern hemisphere countries found a 5-15% excess of births in winter months in schizophrenia (Torrey et al., 1997), which could be a result of increased rates of infections, vitamin D deficiencies, or other winter-related factors (Davies et al., 2003; McGrath et al., 2010). The incidence of schizophrenia is 2-fold higher in urban compared to rural environments, although this difference is not reflected in prevalence

estimates (McGrath et al., 2008; Vassos et al., 2012). Explanations for this association include an increased exposure to infections, pollutants, poor diet, social environment and selected migration (Vassos et al., 2012).

Increased rates of schizophrenia have been reported for migrant and ethnic minority groups (McGrath et al., 2008). A systematic meta-analysis reported that the relative risk of developing schizophrenia among first- and second-generation migrants was 2.7 and 4.5, respectively, and that the effect was greater for migrants from developing countries (Cantor-Graae & Selten, 2005). The Aetiology and Ethnicity in Schizophrenia and Other Psychoses (AESOP) study found that the incidence of first-episode psychosis in the African-Caribbean (IRR = 9.1) and Black African (IRR = 5.8) populations in the UK was remarkably high (Fearon et al., 2006) and African Americans born in the US were found to have a 3-fold increased risk of schizophrenia (Bresnahan et al., 2007). There are additional factors to migration *per se* that may account for the increased risk of schizophrenia in ethnic minority groups. For example, the association has been partly explained by factors related to socioeconomic disadvantage: unemployment, lone parent status, low perceived social support and poverty (indicated by lack of car ownership) (Brugha et al., 2004). Further investigations regarding the nature of these relationships may provide valuable insights into the aetiology of schizophrenia.

Cannabis use has been associated with an increased risk of psychosis (Andreasson et al., 1987; Zammit et al., 2002) and this risk is particularly evident for those who use cannabis during adolescence (Arseneault et al., 2002). Furthermore, a recent meta-analysis identified a dose related effect; regular and ever users were 2.1 and 1.4 times more likely to develop schizophrenia, respectively (Moore et al., 2007). This association is not explained by the use of other psychoactive drugs, personality traits or pre-existing

psychotic symptoms (Andreasson et al., 1987; Arseneault et al., 2002; Moore et al., 2007). People with schizophrenia are more likely to smoke tobacco compared to the rest of the population (de Leon & Diaz, 2005), thought to be because of self-medication, or to relieve distress. Although it is difficult to determine the direction of causality, a recent meta-analysis found that daily tobacco use was associated with an increased risk of psychosis and an earlier age at onset of psychotic illness, suggesting that there may a causal link (Gurillo et al., 2015).

Genetic

It has become clear that the genetic architecture of schizophrenia involves cumulative effects of common alleles of small effect and rare variants of relatively large effect, both of which are distributed across many genes at numerous loci (Rees et al., 2015). Due to a number of well powered studies and consortia there have recently been major advances in the understanding of the genetic aetiology of schizophrenia (Ripke et al., 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Rees et al., 2015). The most recent contributions have come from common single nucleotide polymorphisms (SNPs), rare copy number variation (CNV), rare single nucleotide variants (SNVs), and *de novo* mutations.

GWAS studies investigate the contribution of common (present in more than 1% of the population) single nucleotide polymorphisms (SNPs) to disease. The SNPs used in GWAS are an informative backbone of tag SNPs that capture most of the common genetic variation in the genome and have made large-scale whole-genome genotyping affordable (Visscher et al., 2012). GWAS is a hypothesis-free approach and has been successful in identifying numerous risk loci for schizophrenia. Individually, these SNPs have a small effect but they have been demonstrated to have cumulative effects, a polygenic

component involving thousands of common alleles (International Schizophrenia Consortium et al., 2009). Recent advances have come from analyses conducted by the Schizophrenia Working Group of the Psychiatric Genomics Consortium (PGC) (Ripke et al., 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The PGC was created to combine data from multiple international research groups and thus maximise sample size. The latest GWAS analysis of 36,989 schizophrenia cases and 113,075 controls identified 128 independent associations from 108 distinct loci that were associated at the genome-wide significance level (GWS) of $P < 5 \times 10^{-8}$, which corresponds to a Bonferroni multiple testing correction for 1 million SNPs in linkage equilibrium (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The most significant associations were in the major histocompatibility complex (MHC) on the short arm of chromosome 6 (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Additional GWS loci included genes previously hypothesised to be involved in schizophrenia, such as the dopamine D_2 receptor gene *DRD2*, the target of antipsychotic drugs. Other findings included genes involved in glutamatergic neurotransmission and synaptic plasticity (*GRM2*, *GRIN2A*, *SRR*, *GRIA1*) and voltage-gated calcium channels (*CACNA1C*, *CACNB2* and *CACNA1I*) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). It has become apparent that many of these common risk alleles for schizophrenia also convey risk for other neuropsychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013a, 2013b). Specifically, there is a high genetic overlap with bipolar disorder, a moderate overlap with major depressive disorder and a small but significant overlap with autism spectrum disorders (ASD) (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013a). Furthermore, polygenic risk scores for schizophrenia have also been associated with lower cognitive ability (Lencz et al., 2014).

The SNPs identified by GWAS are likely to be associated with other variants in the genome through linkage disequilibrium (LD). Thus, complimentary studies to GWAS are required to identify causal variants and to determine the possible direct or indirect functional effect variants may have on the risk of schizophrenia (Visscher et al., 2012). Liability threshold models, in comparison to observed models, are used to show that once genetic risk surpasses a threshold, schizophrenia will develop. One estimate using all imputed SNPs explains approximately a quarter to a third of the variation in liability to schizophrenia (Lee et al., 2012c; Rees et al., 2015). When restricted to a subset of SNPs in relative linkage equilibrium only 7% of the variation on the liability scale to schizophrenia is explained (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Differences in liability across methods are likely explained by the different number of SNPs that are included within the model, making direct comparisons challenging. A small amount of additional variation can be explained by rare (present in less than 1% of the population) mutations, taking the form of large copy number variation (CNVs) or single nucleotide variation (SNVs), both of which may either be inherited or occur as *de novo* mutations.

CNVs are chromosomal deletions and duplications that range in size from kilobases to megabases of DNA. The first CNV associated with an increased risk for schizophrenia was a ~2.3 Mb deletion on 22q11.2, noted after high rates of schizophrenia were found in adults with velocardiofacial syndrome, a syndrome characterised by deletions at 22q11.2 (Murphy et al., 1999). Collectively, individuals with schizophrenia have an increased genome-wide burden of rare CNVs compared with the general population (International Schizophrenia Consortium, 2008; Stefansson et al., 2008). Up to 15 specific CNVs have so far been implicated in schizophrenia risk and cumulatively they are found in approximately 2.5% of schizophrenia cases and 0.9% of controls (Kirov et al., 2014; Rees et al., 2014). However, only 11 of these 15 CNVs have been robustly shown to increase the risk of

schizophrenia; deletions at 1q21.1, *NRXN1*, 3q29, 15q11.2, 15q13.3 and 22q11.2, and duplications at 1q21.1, 7q11.23, 15q11.2-q13.1, 16p13.1 and 16p11.2 (Kirov, 2015). These CNVs have a large effect on the risk for schizophrenia, with odds ratios varying between 2 and over 50, and generally the rarer the CNV the higher the risk it poses for schizophrenia (Kirov, 2015). All of the CNVs robustly associated with schizophrenia also increase the risk for developmental delay (DD), ASD and congenital malformations (CM), but not for bipolar disorder (Kirov et al., 2014; Kirov, 2015). The penetrance of CNVs is higher for these developmental conditions than for schizophrenia (Vassos et al., 2010; Kirov et al., 2014). Furthermore, they are also associated with cognitive deficits in the general population (Stefansson et al., 2014).

Strong selection pressures remove CNVs from the population in a few generations and thus the low frequencies of CNVs are maintained by a balance between negative selection from reduced fecundity (Bundy et al., 2011) and new *de novo* mutations (Kirov, 2015). In fact, a large proportion of the 11 replicated CNVs are not inherited and instead caused by *de novo* mutations (Kirov et al., 2012). *De novo* CNVs in general have been shown to occur more frequently and are larger in patients with schizophrenia compared to controls, indicating at least some of them are pathogenic (Kirov et al., 2012). There is some evidence to suggest that the *de novo* CNV mutation rate is higher among individuals with schizophrenia that do not have any family history of the disorder (Kirov et al., 2012). Furthermore, advancing paternal age at conception, which is correlated with the number of *de novo* mutations, has been associated with increased schizophrenia risk, supporting the hypothesis that *de novo* mutations contribute to risk for non-familial schizophrenia (Malaspina et al., 2001; Malaspina et al., 2002).

Many of the CNVs associated with schizophrenia span numerous genes making biological insights difficult. However, the genes disrupted by *de novo* CNVs are enriched for synaptic proteins, an association largely explained by N-methyl-D-aspartate receptor (NMDAR) and neuronal activity-regulated cytoskeleton-associated protein (ARC) complexes (Kirov et al., 2012). A recent functional analysis of CNVs in 11,355 schizophrenia cases and 16,416 controls found CNV enrichment among genes involved in synaptic plasticity and glutamatergic signalling; NMDAR, post synaptic density-95 (PSD-95, a major postsynaptic scaffolding protein at glutamatergic synapses) and ARC complexes (Pocklington et al., 2015). They also found enrichment for GABAergic neurotransmission, an inhibitory modulator of synaptic signalling hypothesised to contribute to schizophrenia pathophysiology (Pocklington et al., 2015).

Recently the contribution of rare single nucleotide variants (SNVs) to the risk of schizophrenia has been assessed. A large exome sequencing study did not find a general increased burden of rare SNVs in people with schizophrenia but did note a polygenic burden of very rare (less than 1 in 10,000), damaging mutations in approximately 2000 genes that had prior evidence for being associated with schizophrenia (Purcell et al., 2014). At the level of gene-sets, these rare damaging mutations were enriched for genes involved in NMDAR, ARC, fragile X mental retardation protein (FMRP)-targets and voltage-gated calcium channels. A further study examining the exome-wide mutation rate of *de novo* SNVs and indels also did not find increased rates in schizophrenia cases (Fromer et al., 2014). However, the *de novo* SNV mutation rate was associated with low educational attainment and there was significant enrichment for nonsynonymous and loss-of-function mutations among synaptic gene sets, as well as specifically for NMDAR, ARC and FMRP-targets. (Fromer et al., 2014). Studies of rare variants are likely to be underpowered and larger samples are needed to implicate single genes with rare variants.

In conclusion, genetic studies of different mutation classes and allele frequencies in schizophrenia have converged on common biological pathways, such as post-synaptic proteins, providing robust support that these genes are disrupted in schizophrenia.

1.2.3 Neurochemical pathophysiology of schizophrenia

Dysregulation of the neurotransmitters dopamine and glutamate are the two primary neurochemical hypotheses of schizophrenia. Neurotransmitters enable transmission of signals from one neuron to another across synapses in the brain. The dopamine hypothesis of schizophrenia originated from the observation that antipsychotic drugs such as chlorpromazine were highly correlated with dopamine D_2 receptor potency (Seeman et al., 1976). In contrast, repeated use of dopamine agonists, such as amphetamine, can induce a psychosis that is similar to schizophrenia (Lieberman et al., 1987). Thus, it was hypothesised that individuals with schizophrenia had altered dopaminergic neurotransmission (Howes & Kapur, 2009). Post-mortem studies have found higher dopamine concentrations and dopamine receptor densities in the brains of people with schizophrenia compared to healthy controls (Davis et al., 1991). However, post-mortem studies may have been biased by the use of antipsychotics in study participants and were unable to measure other aspects of dopamine function, such as dopamine release. The investigation of dopamine in schizophrenia has been significantly advanced by the use of positron emission tomography (PET) and single-photon emission computed tomographic (SPECT) imaging in treatment-naive patients. A recent meta-analysis of these imaging studies confirmed that presynaptic dopaminergic function is significantly elevated in individuals with schizophrenia (Howes et al., 2012a).

The glutamate hypothesis of schizophrenia was based on the observation in the 1950s that the dissociative anaesthetic phencyclidine (PCP) could induce schizophrenia-like symptoms

(Moghaddam & Javitt, 2012). PCP is an antagonist of N-methyl-D-aspartate receptors (NMDAR) (Javitt & Zukin, 1991), glutamate receptors that play an important role in excitatory neurotransmission and synaptic plasticity (Marsman et al., 2013). In contrast to amphetamine, PCP can induce the negative and cognitive as well as the positive symptoms of schizophrenia. Thus, it was hypothesised that glutamate dysfunction may be particularly relevant to chronic schizophrenia (Moghaddam & Javitt, 2012). Post-mortem studies have identified lower concentrations of glutamate in the cerebrospinal fluid and abnormalities of glutamate receptor density in the prefrontal cortex, thalamus and temporal lobe in patients with schizophrenia (Goff & Coyle, 2001). A recent meta-analysis of magnetic resonance spectroscopy (MRS) studies found lower levels of glutamate and higher levels of glutamine (glutamate's metabolite) in the frontal regions of the brains of people with schizophrenia compared to healthy controls (Marsman et al., 2013). Furthermore, glutamate and glutamine levels in the frontal region decreased progressively with age, indicating there could be a progressive loss of synaptic activity in patients with schizophrenia (Marsman et al., 2013).

1.2.4 Treatment of schizophrenia

There is currently no cure for schizophrenia and its treatment is focused on alleviating symptoms and improving the individual's quality of life. Successful treatment will most likely include a combination of antipsychotic drug therapy (Zhang & Malhotra, 2011; Lally & MacCabe, 2015), psychological interventions such as cognitive behavioural therapy (National Institute for Health and Care Excellence, 2009), and psychosocial and family support. Typical or first-generation antipsychotics (FGA) are effective in improving the positive symptoms of schizophrenia but often cause extrapyramidal symptoms (EPS). Newer atypical or second-generation antipsychotics (SGA) are less likely to cause EPS

(Zhang et al., 2013a). However, SGAs are associated with increased rates of weight gain, metabolic changes and cardiovascular related adverse effects (Leucht et al., 2009). Unfortunately, there have been very few new antipsychotic drug advancements in the last 40 years. The mechanism of action of antipsychotic drugs is mediated by the dopamine neurotransmitter system and antagonism of the dopamine D_2 receptor appears to be necessary for antipsychotic drug action (Kapur et al., 1999). Many patients will discontinue and switch antipsychotic treatments, primarily due to intolerable side effects or lack of efficacy (Lieberman et al., 2005). It is currently unclear which patients will respond to which antipsychotic and clinicians have little empirical evidence to guide drug selection. Furthermore, 20-30% of patients will remain symptomatic and significantly impaired despite antipsychotic treatment (Kane et al., 1988; Meltzer, 1997; Suzuki et al., 2012). This group of patients are referred to as treatment-resistant or treatment-refractory.

1.3. Treatment-resistant schizophrenia

Treatment-resistant schizophrenia (TRS) is one of the most disabling forms of mental illness (Kane et al., 1988; McEvoy et al., 2006; Leucht et al., 2009) and may manifest itself either as a failure to achieve remission from the initial onset of psychosis, failure to maintain remission, or as a gradual deterioration from successive relapses (Sheitman & Lieberman, 1998). It is yet to be determined whether TRS should be considered simply as a chronic form of schizophrenia, at the severe end of the illness spectrum, or as a biologically and genetically distinct subgroup (Barnes & Dursun, 2008). Patients with TRS present a major clinical challenge as most remain highly symptomatic, often require multiple hospitalisations, and experience extremely poor outcomes (Conley & Kelly, 2001; Kennedy et al., 2014). Consequently, care for TRS patients consumes a disproportionally high share of the total cost of treating schizophrenia (Conley & Kelly, 2001). Davies and

Drummond found that care for the 10% of patients with a long-term disabling course of schizophrenia accounted for 80% of total lifetime direct costs of schizophrenia within the UK (Davies & Drummond, 1994). Furthermore, a recent systematic review by Kennedy and colleagues reported that annual costs for treating TRS patients were 3-11-fold higher than for patients with non-TRS schizophrenia and that this cost was driven by increased rates of hospitalisations (Kennedy et al., 2014).

1.3.1 Defining TRS

The definition of TRS has varied widely, making comparisons between studies difficult (Suzuki et al., 2011b). **Table 1.2** details the four most frequently used criteria. Kane and colleagues proposed the first, and most stringent, criteria in their landmark clozapine trial (Kane et al., 1988). Although subsequent definitions have tended to be less conservative (Conley & Kelly, 2001; Barnes & Dursun, 2008; Suzuki et al., 2012), they have had consistent themes. In general, criteria have reflected (i) a failure to respond to at least two antipsychotic trials of adequate duration and therapeutic dose, (ii) the current presence of at least moderate positive psychotic symptoms, and (iii) persistent disability in functional and psychosocial aspects (Barnes & Dursun, 2008). Some studies have additionally included a further prospective trial to confirm resistance (Suzuki et al., 2011b). Importantly, TRS should be distinguished from intolerance (sensitivity to pharmacological side-effects of a drug) or non-adherence (Conley & Kelly, 2001; Caspi et al., 2004). Because the NICE guidelines (National Institute for Health and Care Excellence, 2009) represent eligibility for clozapine, several studies have used a history of clozapine treatment as a proxy for TRS (Frank et al., 2015).

Definitions of treatment-resistant schizophrenia
Kane et al (1988)
Drug refractory condition: At least three periods within the preceding five years with conventional antipsychotics (from at least two chemical classes) at doses ≥ 1000 mg/day of chlorpromazine equivalents for six weeks, each without significant symptom relief, and failure to improve by at least 20% in total BPRS score or intolerance to a six-week prospective trial of haloperidol at 10 to 60 mg per day.
Persistence of illness: No stable period of good social and/or occupational functioning within the last five years.
Persistent psychotic symptoms: Item score ≥ 4 (moderate) on at least two of four positive symptom items on the Brief Psychiatric Rating Scale (BPRS).
Current presence of at least moderately severe illness: Total BPRS score ≥ 45 on the 18-item scale and a score ≥ 4 (moderate) on Clinical Global Impression (CGI).
Conley and Kelly (2001)
Drug refractory condition: at least two prior drug trials of 4-6 weeks duration at 400-600mg of chlorpromazine (or equivalent) with no clinical improvement.
Persistence of illness: more than five years with no period of good social or occupational functioning.
Persistent psychotic symptoms: BPRS total score > 45 (on 18 item scale) and item score > 4 (moderate) on at least two of four positive symptom items.
National Institute for Health and Care Excellence (2009)
Lack of satisfactory clinical improvement despite the sequential use of adequate doses of at least two different antipsychotic drugs for 6-8 weeks. At least one of the drugs should be a non-clozapine second-generation antipsychotic.
Suzuki et al (2012)
Drug refractory condition: At least two failed antipsychotic trials with different antipsychotics (at chlorpromazine-equivalent doses of ≥ 600 mg/day for ≥ six consecutive weeks) that could be retrospective or preferably include prospective failure to respond to one or more antipsychotic trials.
Persistence of illness: Both a score of ≥ 4 on the CGI-Severity and a score of ≤ 49 on the Functional Assessment for Comprehensive Treatment of Schizophrenia (FACT-Sz) or ≤ 50 on the Global Assessment of Functioning (GAF) scales.

Table 1.2. *Definitions of treatment-resistant schizophrenia.*

1.3.2 Mechanisms of TRS

Given the considerable heterogeneity in the illness course of schizophrenia, there has long been a search for outcome predictors and to understand the mechanisms behind poor clinical outcomes such as TRS. If reliable clinical, neurological or genetic predictors could be identified, there is the potential to tailor treatment strategies for particular patient subgroups (Carbon & Correll, 2014).

Clinical

In 1980, Huber and colleagues followed 502 patients longitudinally and concluded that it was not possible to predict the outcome of schizophrenia from clinical factors (Huber et al., 1980). However, there is now a substantial amount of research in this area and with particularly good evidence from first-episode studies, several reliable clinical predictors have been identified (Carbon & Correll, 2014). The most replicated clinical predictors of poor outcome in schizophrenia include; male gender, a longer duration of untreated psychosis, poor premorbid functioning, comorbid substance-use disorders and non-adherence. However, very few studies have assessed TRS directly and there has been a wide variability in outcome measures.

Male gender has been associated with a decreased likelihood of recovery in schizophrenia as well as a poor response to antipsychotic treatment, independent of confounding factors such as age of onset and illness severity (Robinson et al., 1999b; Usall et al., 2007; Rabinowitz et al., 2014; Thorup et al., 2014; Frank et al., 2015). However, this is still a relatively controversial finding as many studies have not replicated the association (Carbon & Correll, 2014). It is unclear why males are more likely to have a chronic course of illness, but it may be driven, at least in part, by the overrepresentation of other risk factors in

males such as substance abuse, non-adherence, prominent negative symptoms and poor social functioning (Thorup et al., 2014).

A longer duration of untreated psychosis, the period of psychosis experienced prior to antipsychotic treatment, is one of the most replicated predictors of poor clinical outcome in schizophrenia (Marshall et al., 2005; Perkins et al., 2005). Poor premorbid functioning has also been consistently associated with multiple poor outcomes measures, including a lack of response to antipsychotics (Levine & Rabinowitz, 2010; Rabinowitz et al., 2011), rate of relapse (Robinson et al., 1999a), and clozapine treatment (proxy for TRS) (Caspi et al., 2007; Frank et al., 2015). Poor premorbid function may be a marker for greater neurodevelopment disturbance (Carbon & Correll, 2014). An earlier age at onset of schizophrenia has been associated with a poor antipsychotic response (Levine & Rabinowitz, 2010; Carbon & Correll, 2014) and a recent study found that patients taking clozapine had a significantly earlier age and insidious disease onset (Frank et al., 2015). However, this has not been replicated in other large studies (Amminger et al., 2011; Rabinowitz et al., 2014) and the effect may be mediated by a longer illness duration (Carbon & Correll, 2014).

Comorbidities, especially substance-use disorders have been associated with poor clinical outcomes in schizophrenia and increased rates of mortality (Volkow, 2009) but it is currently unclear what drives this association. For example, substance use is associated with non-adherence, which also impacts functional outcome in schizophrenia (Ascher-Svanum et al., 2006). Additional factors that may be associated with poor outcome in schizophrenia include prominent negative symptoms (Foussias & Remington, 2010; Ucok et al., 2011), a family history of schizophrenia (Frank et al., 2015), and a lack of early

antipsychotic response (Agid et al., 2003), poor cognitive functioning (Levine & Rabinowitz, 2010).

Neurological

It is hypothesised that glutamate dysfunction may be particularly relevant for TRS (Moghaddam & Javitt, 2012), as discussed in section 1.2.2. There have been very few neuroimaging studies focusing specifically on patients with TRS and there are currently no reliable neuroimaging correlates of TRS in comparison to healthy controls (Nakajima et al., 2015). In comparison to schizophrenia patients responsive to treatment, TRS has been associated with decreased thickness of dorsolateral prefrontal cortex (Zugman et al., 2013), relatively normal striatal dopamine synthesis and elevated anterior cingulate cortex glutamate levels (Demjaha et al., 2014). A recent study found increased rates of minor physical anomalies and craniofacial features in patients with TRS, implicating a neurodevelopmental mechanism (Lin et al., 2015).

Genetic

There have been relatively few studies directly investigating the genetic aetiology of TRS. However, pharmacogenetic studies of the genetic variability associated with individual responses to drug treatments have offered insights. There have been many pharmacogenetic candidate studies focusing on either the neurotransmitters impacted by antipsychotics (dopamine and serotonin) or genes encoding the enzymes responsible for drug metabolism (cytochrome P450 family). There is evidence for a role in antipsychotic response for the -141C Ins/Del polymorphism in *DRD2* (Zhang et al., 2010), the -1438G polymorphism in *HRT2A* (Ellingrod et al., 2003), and 5-HTTLPR, a degenerate repeat polymorphism in *SLC6A4* (Dolzan et al., 2008). *SLC6A4* encodes the serotonin transporter and 5-HTTLPR has been demonstrated to affect the rate of serotonin uptake (Dolzan et al.,

2008; Zhang & Malhotra, 2011). The Val108Met polymorphism in *COMT,* which affects dopamine clearance, has also been associated with antipsychotic response (Zhang & Malhotra, 2011). A study of 74 candidate genes from the Clinical Antipsychotic Trials for Intervention Effectiveness (CATIE) trial in the US of 738 individuals failed to identify any variants that were significantly associated with discontinuation of olanzapine, quetiapine, risperidone, ziprasidone or perphenazine after correction for multiple testing (Need et al., 2009). A GWAS of the same sample identified an intergenic variant on chromosome 4p15 that predicted the effect of ziprasidone on positive symptoms only (McClay et al., 2011). Candidate pharmacogenetic studies of antipsychotic response have produced a number of false positive and false negative findings with few consistent replications. This is likely due to inadequate statistical power and the considerable variability in treatment response definitions. It has become clear from the field of schizophrenia genetics that biology-driven candidate gene studies are unlikely to yield robust insights into disease aetiology (Farrell et al., 2015). Thus, large, well-powered GWAS studies are required to validate and extend these findings.

More recent studies have directly investigated the genetic aetiology of TRS. In 2012, a study of 384 candidate markers from 46 genes failed to identify any variants significantly associated with TRS (Teo et al., 2012). However, a study of 74 candidate genes in 89 patients treated with clozapine (and thus TRS) and 190 schizophrenia patients, found that variants within *BDNF*, including the Val66Met (rs6265) polymorphism, were associated with TRS (Zhang et al., 2013b). *BDNF* interacts with multiple neurotransmitters, including dopamine and serotonin, may lead to reduced synaptic plasticity, and has been previously associated with therapeutic response in schizophrenia (Krebs et al., 2000). A significant association between TRS and *DISC1* was reported in a further candidate study (Mouaffak et al., 2011), although this was not replicated in a study of Japanese patients (Hotta et al.,

2011). No significant associations have been reported from candidate gene studies specifically relating to serotoninergic genes and TRS (Ji et al., 2008a; Ji et al., 2008b). A GWAS in 79 TRS patients and 95 non-TRS schizophrenia patients failed to identify any variants significantly associated with TRS (Li & Meltzer, 2014). Furthermore, a GWAS in 795 Han Chinese TRS patients and 806 healthy controls also failed to identify any significant variants (Liou et al., 2012). However, suggestive variants from these two GWAS included the 7p12.2 region (Li & Meltzer, 2014) and intronic variants in *NFKB1*, *RIPK4* and *SLAMF1* (Liou et al., 2012). A study by Frank and colleagues found that the polygenic risk score for schizophrenia was increased in 434 patients treated with clozapine in comparison to 370 patients with no history of clozapine treatment (Frank et al., 2015) but this finding was not replicated in another study (Martin & Mowry, 2015), which found an increased burden of genome-wide rare copy number duplications in 277 patients with TRS compared to 385 individuals with schizophrenia (Martin & Mowry, 2015). In conclusion, there are no robustly replicated risk loci for TRS. Studies to date have been underpowered as a result of small sample sizes and the variability in TRS definition has limited the comparability between studies.

1.3.3 Treatment of TRS

Clozapine is the only medication with robust effectiveness for patients with TRS (Kane et al., 1988; McEvoy et al., 2006; Leucht et al., 2009). Given its potential to induce severe adverse effects, particularly agranulocytosis, it is recommended that the drug is offered after failure to respond to two adequate antipsychotic trials (National Institute for Health and Care Excellence, 2009). Other pharmacological interventions for TRS include the use of high dose antipsychotic medication, although there is little empirical evidence to suggest that this has any advantage over standard dosage (Royal College of Psychiatrists, 2014).

There is a lack of convincing evidence of efficacy for other strategies such as the concomitant use of two or more antipsychotics, which is used despite the increased risk of side effects (Caspi et al., 2004; Correll et al., 2009). There is some evidence for adjunctive use of lithium and anticonvulsants for the treatment of TRS (Conley & Kelly, 2001). Psychological and psychosocial interventions such as cognitive behavioural therapy (CBT), a good therapeutic alliance with the patient, and providing support to patients and carers are also critical to the treatment of TRS (Barnes & Dursun, 2008; Burns et al., 2014).

1.4. Clozapine

The Swiss pharmaceutical company Sandoz identified clozapine in 1959. Other antipsychotics in use at the time, such as chlorpromazine, caused extrapyramidal symptoms (EPS) (Crilly, 2007). Because clozapine did not cause EPS, it was labelled as having an 'atypical' profile. Ironically, this limited clozapine's acceptance because of the assumption that antipsychotics needed to cause EPS in order to effectively treat psychotic symptoms (Hippius, 1999). Following encouraging clinical trials in the early 1970s (Stille & Hippius, 1971), it was introduced in Switzerland and Austria in 1972, in West Germany in 1974 and in Finland in 1975. Within four months of clozapine being marketed in Finland, a report was published of 18 patients developing severe haematological disorders, 9 of whom died (Idanpaan-Heikkila et al., 1975). In addition, there were 12 other cases reported from other countries (Griffith & Saameli, 1975). The majority of cases had developed agranulocytosis, a deficiency of infection-fighting white blood cells called neutrophils that renders the person vulnerable to infections (Amsler et al., 1977). This alarming accumulation of severe adverse reactions led to clozapine being voluntarily withdrawn by Sandoz and, for the most part, it fell out of favour for more than a decade. However, some countries continued to use clozapine under the condition of weekly blood

monitoring to aid early detection of agranulocytosis (Hippius, 1999). A turning point in clozapine's history came from two clinical trials in the United States of America (USA). The first demonstrated its therapeutic superiority over chlorpromazine for the treatment of schizophrenia (Claghorn et al., 1987) and the second demonstrated its therapeutic superiority over chlorpromazine for the treatment of TRS (Kane et al., 1988). Consequently, in 1990, the Food and Drug Administration (FDA) in the USA approved clozapine for use in patients with TRS, under the condition of mandatory blood monitoring via a safety system, and it was reintroduced in other countries around the same time. Clozapine is now well proven to have superior beneficial effects in patients with TRS. This section discusses the pharmacology, metabolism, efficacy and current use of clozapine.

1.4.1 Pharmacology

Clozapine is a dibenzodiazepine with a unique pharmacological profile. It has affinities toward dopamine, serotonin, α adrenergic, muscarinic and histamine receptors (Selent et al., 2008). Clozapine is classified as an atypical antipsychotic because it has a lower affinity for D_2 receptors than seen with conventional antipsychotics and a higher affinity for 5-HT_{2A} receptors than for D_2 receptors (Meltzer, 1989; Meltzer et al., 1989b). However, the mechanism that causes clozapine's superior efficacy is still unclear.

Dopamine

Consistent with all antipsychotics, clozapine is a D_2 antagonist. At therapeutic doses clozapine has a lower affinity for D_2 receptor occupancy (20-67%) than conventional antipsychotics (70-90%) (Nordstrom et al., 1995) or for several other atypicals such as risperidone and olanzapine (Kapur et al., 1999). Clozapine's low D_2 occupancy is responsible for the absence of EPS (Kapur & Seeman, 2001), which are caused by an excess of D_2 receptor occupancies. Clozapine has a rapid dissociation from D_2 receptors and is

thus called a 'fast-Off-D_2' antipsychotic (Seeman, 2014). Clozapine was found to occupy 72% of the D_2 receptors in the human striatum two hours after administration, followed by a quick reduction to less than 30% by 24 hours (Seeman, 2002). Similar to quetiapine, clozapine binds briefly to the D_2 receptor, triggering a suppression of psychotic symptoms, while allowing endogenous dopamine to displace the loosely bound drug (Seeman, 2014). After dissociating, clozapine allows normal dopamine neurotransmission, which keeps prolactin levels normal and also spares cognition (Seeman, 2002). This was thought to explain clozapine's reduced effects on EPS, but this does not explain the same effects in other antipsychotics lacking the fast-Off-D_2 profile (e.g. aripiprazole, ziprasidone) (Horacek et al., 2006).

Clozapine shows a higher affinity for D_4 compared to D_2 receptors (Guan & Sunahara, 1991; Horacek et al., 2006). However, because pure D_4 antagonists have not shown any independent antipsychotic effect (Kramer et al., 1997), D_4 is mainly considered to have a supplementary effect to D_2 receptor antagonism. Although clozapine is a D_1 antagonist, there is limited support the role for D_1 receptors in antipsychotic drug efficacy (Karlsson et al., 1995).

Serotonin

Clozapine has a high affinity for the serotonin 5-HT_{2A} receptor (Meltzer, 1999) and studies indicate that 96% of 5-HT_{2A} receptors are occupied at daily doses of 300-600 mg (Kapur et al., 1999). It was thought that this action was responsible for the absence of EPS, but this has since been refuted (Kapur & Seeman, 2001; Seeman, 2014). Clozapine is also a 5-HT_{1A} agonist, and 5-HT_{2B}, 5-HT_{2C}, 5-HT_6 and 5-HT_7 antagonists. It is currently thought that rather than having an independent effect, it is likely that the effect on serotonergic receptors

works in combination with D_2 receptors to cause the therapeutic effect of clozapine (Horacek et al., 2006).

Other neurotransmitters

Clozapine is an antagonist of α_1- and α_2-adrenoceptors (Brosda et al., 2014). Many antipsychotic drugs also possess α_1-adrenoceptor blocking properties. Although a clinical trial of the pure α_1-adrenoceptor antagonist prazosin found no direct antipsychotic effect (Hommer et al., 1984), one study reported that prazosin enhanced the effect of antipsychotic drugs (Svensson, 2003). In contrast to other antipsychotic drugs, clozapine has a high affinity for α_2-adrenoceptor antagonism. α_2-adrenoceptor blocking is hypothesised to act by augmenting prefrontal dopaminergic functioning, and has received some recent interest (Svensson, 2003; Brosda et al., 2014). Clozapine also impacts histamine receptors (Humbert-Claude et al., 2012) and is a marked agonist of muscarinic acetylcholine M_1 – M_5 receptors (Bymaster et al., 2003).

1.4.2 Metabolism

Orally administered drugs such as clozapine are absorbed by the digestive system and carried via the hepatic portal vein into the liver where it is metabolised before reaching the rest of the body. Clozapine is 90-95% absorbed when administered orally but due to the first pass metabolism effect, it has only moderate (60-70%) bioavailability, which is not affected by food (Wenthur & Lindsley, 2013). The peak concentration of clozapine occurs approximately 2.5 hours after oral dosing. The mean elimination half-life is 8 hours after a single 75mg dose and 12 hours after achieving steady state dose of 100mg twice daily. Clozapine is approximately 97% bound to serum proteins. Clozapine is extensively metabolised in the liver by the cytochrome P450 (CYP450) system and only trace amount of unchanged drug are detected in the urine and faeces. Clozapine is metabolised into two

major stable metabolites norclozapine (*N*-desmethylclozapine, NDMC) and clozapine *N*-oxide, and is bioactivated into a reactive metabolite (Pirmohamed et al., 1995). Norclozapine is the major metabolite and is pharmacologically active (Li et al., 2005). The reactive metabolite, thought to be a nitrenium ion, has been linked to agranulocytosis (Liu & Uetrecht, 1995; Maggs et al., 1995; Pirmohamed & Park, 1997). The CYP450 enzyme 1A2 (*CYP1A2*) is primarily responsible for clozapine metabolism, and agents that induce *CYP1A2* such as cigarettes reduce clozapine levels (van der Weide et al., 2003) whereas agents that inhibit *CYP1A2* such as caffeine increase clozapine levels (Bertilsson et al., 1994). There are also additional contributions of *CYP2D6*, *CYP3A4* and *CYP2C19* to clozapine metabolism (Urichuk et al., 2008). Approximately 50% of the administered dose is excreted as metabolites in the urine and 30% in the faeces. Excretion of N-oxide and norclozapine is dependant on the activity of P-glycoprotein, a transmembrane transporter expressed in the liver and kidneys, coded by the *ABCB1*/*MDR1* gene. There is evidence that *ABCB1* polymorphisms affect serum clozapine levels (Consoli et al., 2009; Krivoy et al., 2015). These factors contribute to wide inter-individual differences in metabolism and thus monitoring of plasma clozapine levels during treatment can be used to assess metabolism and adjust dosage (Wenthur & Lindsley, 2013).

1.4.3 Efficacy of clozapine

The efficacy of clozapine on positive, negative and cognitive symptoms of schizophrenia, as well as other outcomes such as mortality, suicide and aggression has been extensively studied.

Positive and negative symptoms

An early landmark clinical trial indicated the superior efficacy of clozapine over chlorpromazine, a typical antipsychotic, for patients that had an inadequate response to

conventional antipsychotics (Claghorn et al., 1987; Kane et al., 1988). Subsequent to the reintroduction of clozapine, there have been a significant number of randomised controlled trials investigating the efficacy of clozapine treatment. Several meta-analyses of these studies have demonstrated clozapine's superiority over typical antipsychotics in terms of improvement in overall psychopathology for patients with TRS and reduced extrapyramidal side effects (Chakos et al., 2001; Davis et al., 2003; Essali et al., 2009). Furthermore, clozapine has increased efficacy over atypical antipsychotics in patients with TRS (Wahlbeck et al., 2000; Davis et al., 2003). Considerable support was provided by three large non-commercial trials. The Clinical Antipsychotic Trials for Intervention Effectiveness (CATIE) in the USA assigned patients that had discontinued a previous antipsychotic, primarily due to inadequate response, to either clozapine or another atypical antipsychotic (olanzapine, quetiapine or risperidone) (McEvoy et al., 2006). The primary outcome of the CATIE study was time to discontinuation, chosen to encapsulate multiple kinds of drug failure and also to represent real-world practice. The decision to discontinue a treatment represents a synthesis of clinician and patient judgements balancing efficaciousness against tolerability of adverse effects. The CATIE trial found that time to discontinuation of clozapine was significantly longer than other atypical antipsychotics; the median time to clozapine discontinuation was 10 months, twice the time from olanzapine, the next best (McEvoy et al., 2006). Clozapine has been consistently associated with a prolonged time to discontinuation in other studies (Rosenheck et al., 2000; Kane et al., 2001; Kroken et al., 2014). The UK Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS) found clozapine to be superior in a clinical trial of clozapine vs. other atypical antipsychotics in 136 patients with TRS (Lewis et al., 2006). Lastly, the Schizophrenia Outpatient Health Outcome (SOHO) study found that clozapine and olanzapine were associated with a lower risk of relapse compared to other atypical

antipsychotics (Haro & Salvador-Carulla, 2006). Although there is substantial evidence regarding clozapine's superiority in overall psychopathology, it has generally not been associated with greater improvements in quality of life scales compared to other antipsychotics (Rosenheck et al., 1997; Lewis et al., 2006).

Early clinical trials suggested that clozapine could be more effective for treating negative symptoms than chlorpromazine (Kane et al., 1988). However, no superior effects were found in trials comparing clozapine with haloperidol (Buchanan et al., 1998; Kane et al., 2001) and other studies have found no effect after controlling for the impact on positive symptoms (Lieberman et al., 1994b; Rosenheck et al., 1999a). A recent review concluded that clozapine does not have an independent effect on negative symptoms (Arango et al., 2013).

There is some evidence to suggest that clozapine is also more effective than haloperidol (Kane et al., 2001) and risperidone (Azorin et al., 2001) in patients with non-TRS. A recent meta-analysis by Leucht and colleagues of 15 antipsychotic drugs, which excluded patients with TRS, found that clozapine was significantly more effective than placebo, and had the largest effect size of all the antipsychotics (Leucht et al., 2013). However, there is no evidence for the increased efficacy of clozapine in first-episode patients (Girgis et al., 2011).

Cognitive symptoms

Although initial studies suggested clozapine had superior efficacy in improving cognition (Buchanan et al., 1994), there does not appear to be any clear advantage over other atypical antipsychotics (Bilder et al., 2002). There have been mixed results for specific effects of antipsychotics on cognition and no one drug appears to have a superior profile (Woodward et al., 2005; Nielsen et al., 2015). Meta-analyses indicate that there may be a

superior effect of clozapine specifically on verbal fluency (Meltzer & McGurk, 1999; Woodward et al., 2005; Nielsen et al., 2015). Furthermore, a recent study demonstrated that the ratio of serum clozapine and norclozapine (its major metabolite) was significantly correlated with working memory (Rajji et al., 2015).

Mortality and suicide

Clozapine has been associated with decreased rates of all-cause mortality and suicidality. Meltzer & Okayli reported markedly reduced suicidality among 88 neuroleptic-resistant patients treated with clozapine for six months to seven years (Meltzer & Okayli, 1995) and further support came from the International Suicide Prevention Trial (InterSePT), a large prospective randomised study in patients with schizophrenia at high risk of suicide, which found that clozapine was superior to olanzapine in reducing key measures of suicidality (Meltzer et al., 2003a). Clozapine has also been associated with a reduced risk of mortality from suicide (Reid et al., 1998; Tiihonen et al., 2009; Ringback Weitoft et al., 2014). Walker and colleagues, using the Clozaril National Registry to identify 67,000 current and former clozapine users in the United States, found that mortality from suicide was markedly decreased in current compared to past clozapine users (Walker et al., 1997). Although another study failed to replicate this association in veterans (Sernyak et al., 2001), a large meta-analysis concluded that clozapine substantially lowered the overall risk of suicidal behaviours and mortality from suicide in comparison to other treatments (Hennen & Baldessarini, 2005).

There is evidence indicating that clozapine decreases rates of mortality in general, not solely those attributable to suicide. A nationwide register study in Finland (FIN11) with an 11-year follow-up found that long-term exposure to any antipsychotic treatment was associated with lower mortality and that clozapine had the lowest mortality risk (Tiihonen

et al., 2009). Although this study has received some methodological criticism (De Hert et al., 2010), the results have been replicated by another Finnish registry study (Kiviniemi et al., 2013). It is possible that the reduction in suicidality and mortality in clozapine patients was due to increased clinical contact from the mandatory haematological monitoring. However, Hayes and colleagues, in a large naturalistic cohort study, found a strong association between clozapine and lower mortality rates, which persisted after controlling for the frequency of clinical contact (Hayes et al., 2015). Furthermore, there was evidence that clozapine reduced the risk of mortality due to natural as well as unnatural causes (Hayes et al., 2015).

Aggression

There is emerging evidence that clozapine is effective in reducing violent and aggressive behaviour in patients with schizophrenia, and particularly in those with TRS (Frogley et al., 2012; Victoroff et al., 2014). Evidence has come from randomised-controlled trials, crossover trials, retrospective and prospective studies, and these effects have been found to be independent of general antipsychotic and sedative effects (Frogley et al., 2012).

Economics

Many studies investigating the cost-effectiveness of clozapine treatment in patients with TRS have used mirror-image designs; comparing the period of time prior to and after the onset of clozapine. These studies have consistently found reduced costs in the two years after the onset of clozapine compared to the two years before (Honigfeld & Patin, 1990; Meltzer et al., 1993; Aitchison & Kerwin, 1997; Hayhurst et al., 2002). This cost saving appeared to be driven by reduced hospitalisations (Meltzer et al., 1993). There are only a limited number of studies comparing the benefits of clozapine with other atypical antipsychotics (Rosenheck et al., 1997). The CUtLASS study found that clozapine treatment

was associated with increased costs after one year in comparison to other atypical antipsychotics, although the difference was not statistically significant (Lewis et al., 2006). Davis and colleagues also found that clozapine was more expensive than other atypical antipsychotics within the first year (Davies et al., 2008). However, these increased costs appear to be driven by the inpatient admission required for commencing clozapine. Long-term studies are required to assess the cost-effectiveness of clozapine, as the long-term benefits may offset the cost of initial hospitalisation and this has yet to be studied.

1.4.4 Side effects of clozapine

Although clozapine is the most effective antipsychotic in the management of TRS, its use has been limited by the potential for adverse effects (Meltzer, 2012). The FDA requires clozapine to carry serious warnings for agranulocytosis, seizures, myocarditis, other adverse cardiovascular and respiratory effects, and increased mortality in elderly patients with dementia-related psychosis. These adverse effects are rare but potentially fatal. The risk of agranulocytosis is discussed in section 1.5. The risk of potentially fatal myocarditis or cardiomyopathy is between 0.015% and 0.188% of those treated with clozapine (Merrill et al., 2005). Another serious risk is clozapine's potential to cause marked weight gain, type II diabetes and diabetes ketoacidosis (Tang et al., 2008; Meltzer, 2012). Clozapine and olanzapine are the most likely antipsychotics to cause clinically significant weight gain and metabolic dysfunction (Newcomer, 2005; Leucht et al., 2013). Other common side effects of clozapine include drowsiness and sedation, dizziness, tachycardia, constipation and hypersalivation (Waserman & Criollo, 2000; Essali et al., 2009).

1.4.5 Clozapine utilisation

The National Institute of Clinical Excellence (NICE) guidance states that clinicians should 'offer clozapine to people with schizophrenia whose illness has not responded adequately

to treatment despite the sequential use of adequate doses of at least two different antipsychotic drugs' (National Institute for Health and Care Excellence, 2009). Despite this guidance, and considering clozapine is the only licenced drug for TRS, clozapine is widely underutilised (Stroup et al., 2009; Joober & Boksa, 2010; Royal College of Psychiatrists, 2012). The UK National Audit of Schizophrenia in 2012 found that 43% of patients eligible for clozapine in the UK have not yet been considered for a trial (Royal College of Psychiatrists, 2012). The under prescription of clozapine has also been documented in other countries (Koen et al., 2008), particularly in the US (Stroup et al., 2009), although its use is very high in China (Tang et al., 2008). In patients that do receive clozapine, there is a delay to treatment. In New Zealand, Wheeler and colleagues reported an average duration of illness of 9.7 years before patients were started on clozapine (Wheeler, 2008). In 2003, Taylor and colleagues found a theoretical delay of five years from eligibility to first clozapine treatment in the UK (Taylor et al., 2003). An update found this delay to still be an average of four years (Howes et al., 2012b).

There are many reasons for the underutilisation of clozapine. Of particular importance is the attitude and experience of the prescribing psychiatrist (Patel, 2012; Tungaraza & Farooq, 2015). In a survey of psychiatrist's attitudes, Nielsen and colleagues found that 64% of psychiatrists in Denmark would rather prescribe two antipsychotics than use clozapine and 66% thought that patients treated with clozapine were less satisfied compared to those treated with other atypical antipsychotics (Nielsen et al., 2010). Wheeler and colleagues found that audit and feedback improved clozapine prescription in New Zealand (Wheeler et al., 2009a), suggesting targeting clinician attitudes may be an effective intervention in improving clozapine prescription. In a recent UK online survey of 243 consultant psychiatrists, Tungaraza and colleagues found that the major concerns of psychiatrists that limited clozapine prescription were in regards to side effects and

concerns over patient's reluctance for blood monitoring (Tungaraza & Farooq, 2015). However, a survey of patient's attitudes found that although patients disliked the blood monitoring, 87% felt that the advantages of clozapine outweighed the disadvantages and only 1.6% wanted to discontinue due to this reason (Taylor et al., 2000). Other studies have also found that concerns over side effects are a major contributor to the widespread underutilisation of clozapine (Atkin et al., 1996; Henderson et al., 2000), particularly in regards to the risk of agranulocytosis.

1.5. Clozapine-induced agranulocytosis and neutropenia

Neutropenia is the reduction of white blood cells called neutrophils to levels below 1500 cells/mm^3 of blood. Agranulocytosis is a more severe form where the neutrophil levels fall below 500 cells/mm^3. Neutrophils are the most abundant type (50-70%) of white blood cells (leukocytes) and play a fundamental role in the immune system. Thus, the decrease or disappearance of neutrophils renders the person susceptible to infection due to a suppressed immune system. Neutrophils are formed from stem cells in the bone marrow over a period of approximately five days and have a short circulating half-life in the blood of 6-8 hours, after which cells die by apoptosis (Summers et al., 2010). The principal regulator of neutrophil production is granulocyte colony stimulating factor (G-CSF). Agranulocytosis and neutropenia can be caused by a number of factors, including exogenous agents such as chemotherapy, but also vitamin deficiency, leukaemia, autoimmune diseases, and bacterial and viral infections such as human immunodeficiency virus (HIV) (Flanagan & Dunk, 2008). The main clinical manifestations of agranulocytosis are secondary to infection and may include fever, fatigue, weakness, cough, sore throat and pneumonia (Flanagan & Dunk, 2008). Neutropenia was first recognised as a side effect of antipsychotic medication when it was observed in association with chlorpromazine in

the 1950s and has now been associated with many antipsychotics, although clozapine is the only one with mandatory haematological monitoring.

To facilitate the early detection of agranulocytosis, clozapine treatment is accompanied by regular and, in many countries including the UK, mandatory haematological monitoring. This burden of monitoring limits the acceptability of the drug to patients, and poses an additional obstacle to clinician recommendation and use in clinical practice (Patel, 2012). If neutropenia or agranulocytosis is identified, clozapine treatment is discontinued and usually haematological parameters will subsequently normalise. However, some may require treatment with G-CSF to stimulate neutrophil production.

The monitoring systems put in place for clozapine have been successful in reducing the prevalence of clozapine-induced agranulocytosis (CIA) and associated fatality rates (Schulte, 2006). Studies utilising the centralised clozapine monitoring system in the UK and US have found the cumulative prevalence of CIA and neutropenia in those taking clozapine to be approximately 0.8% and 2.9%, respectively (Alvir et al., 1993; Atkin et al., 1996; Munro et al., 1999). Furthermore, the prevalence of fatal CIA is now very low. In the UK, fatal CIA occurred in 0.016% to 0.03% of patients treated with clozapine (Atkin et al., 1996; Munro et al., 1999). In the US, the case fatality rate of CIA was estimated between 4.2-16%, depending on whether the individual was treated with G-CSF (Schulte, 2006).

The peak incidence of both CIA and neutropenia is in the first 6-18 weeks of clozapine treatment (Atkin et al., 1996; Munro et al., 1999). There is a reduced incidence after six months of treatment (Alvir et al., 1993). After the first year, the incidence of agranulocytosis decreases to rates similar to other medications, such as chlorpromazine, that do not require haematological monitoring (Atkin et al., 1996; Schulte, 2006). These findings led to a relaxation in haematological monitoring and current European guidelines

are for weekly blood tests in first 18 weeks of treatment, fortnightly up to one year and then monthly thereafter.

1.5.1 Aetiology

The mechanism by which clozapine causes agranulocytosis and neutropenia has been extensively studied but is still not well understood (Pirmohamed & Park, 1997; Flanagan & Dunk, 2008). CIA is not due to an extension of known pharmacological effects of clozapine. Clozapine or its stable metabolites (norclozapine or clozapine *N*-oxide) are not directly cytotoxic to neutrophils and do not interfere with the turnover of bone marrow precursor cells at therapeutic drug concentrations (Gerson et al., 1994; Pirmohamed & Park, 1997). However, there is evidence indicating that CIA and neutropenia are caused by the activation of clozapine or a stable metabolite of clozapine to a chemically reactive nitrenium ion (Liu & Uetrecht, 1995; Maggs et al., 1995; Pirmohamed & Park, 1997). The nitrenium ion has been shown to cause dose-dependent apoptosis to neutrophils at therapeutic levels of clozapine (Williams et al., 2000; Pessina et al., 2006) as well as toxicity to stromal cells, the precursors of neutrophils in bone marrow (Pereira & Dean, 2006). Neutrophils themselves can generate hypochlorous acid via the nicotinamide adenine dinucleotide phosphate (NADPH)/ myeloperoxidase (MPO) pathway, which is capable of oxidizing clozapine to reactive metabolites that covalently bind to neutrophils (Uetrecht, 1989; Liu & Uetrecht, 1995; Gardner et al., 1998). The covalent binding of these reactive metabolites could in turn lead to agranulocytosis or neutropenia via direct toxicity or by initiating an immune mechanism, or both. An immune mechanism, perhaps initiated by presence of nitrenium ions, has been suggested since CIA occurs more quickly and is more severe upon rechallenge of clozapine; features typical of Type B (idiosyncratic) adverse drug reactions (Dunk et al., 2006; Flanagan & Dunk, 2008). Genetic association studies

have implicated variants in *HLA-B* and *HLA-DQB1* with CIA (Goldstein et al., 2014), supporting the likelihood of an immune-mediated mechanism.

The reason why only 0.8% of individuals treated with clozapine are affected by agranulocytosis has not been fully elucidated (Pirmohamed & Park, 1997). There has been substantial interest in the identification of factors predictive of an increased susceptibility for CIA that in turn have provided aetiological insights. Conceivably, a sensitive and reliable predictor could be used as a screening procedure to identify patients vulnerable to CIA. For those at low risk, there could be a reduced need of regular monitoring, thereby broadening those that could be treated with clozapine. This section describes the demographic, pharmacokinetic and genetic factors that have been associated with CIA and neutropenia.

Demographic

Large epidemiological studies utilising data from clozapine registers in the UK and US have indicated that there may be different aetiological mechanisms for neutropenia and agranulocytosis (Flanagan & Dunk, 2008). Evidence from these studies suggest that the risk of CIA increases with age, by approximately 53% per decade, whereas the risk of neutropenia decreases with age (Alvir et al., 1993; Munro et al., 1999). Alvir and colleagues reported higher rates of CIA among women in the US (Alvir et al., 1993). In the UK, patients of Asian ancestry were 2.4 times more likely to develop CIA in comparison to those of European ancestry whereas risk of neutropenia was 77% higher in patients of African-Caribbean ethnicity (Munro et al., 1999). The increased risk of neutropenia in patients of African-Caribbean ethnicity may have been driven by an average lower baseline white blood cell (WBC) count, which independently predicted neutropenia but not agranulocytosis (Munro et al., 1999). By utilising clozapine registry data, these studies

have benefited from large sample sizes. However, the information recorded on drug registries is limited, and not subjected to verification. Furthermore, the sample will be biased towards patients that have remained on the treatment long-term and thus may not be generalisable to a cohort of patients starting clozapine.

Dosage and pharmacokinetics

Clozapine dosage has not been associated with agranulocytosis or neutropenia in observational studies (Atkin et al., 1996; Munro et al., 1999). Furthermore, there is no evidence to suggest an association with plasma concentrations of clozapine and/or its major metabolites (Hasegawa et al., 1994; Centorrino et al., 1995; Combs et al., 1997; Mauri et al., 1998; Oyewumi et al., 2002). However, previous studies have either failed to include patients with abnormally low neutrophil levels (Centorrino et al., 1995; Combs et al., 1997; Mauri et al., 1998; Oyewumi et al., 2002) or control for treatment duration or dosage (Hasegawa et al., 1994; Centorrino et al., 1995). Lastly, considering the marked inter-individual variation in clozapine plasma levels (Olesen et al., 1995), studies have been conducted in small samples (Hasegawa et al., 1994).

Genetic

The possibility of a genetic component to CIA was implicated from an early stage because the incidence in Finland was 20 times higher than other countries such as Austria, Switzerland and West Germany where the drug was widely used (Griffith & Saameli, 1975). Furthermore, there have been reports of concordant manifestations of CIA in monozygotic twins (Horacek et al., 2001; Anil Yagcioglu et al., 2011). Two early studies suggested that a direct familial gene was unlikely to be responsible for CIA. In 1977, De La Chapelle and colleagues found no evidence of significant parental consanguinity or genetic kinship in a six-generation pedigree analysis (de la Chapelle et al., 1977) and Amsler and colleagues

also failed to identify a genetic factor that could have been responsible for agranulocytosis in the Finnish patients (Amsler et al., 1977).

The majority of genetic association studies have focused on candidate genes involved in immune response from the human leukocyte antigen (HLA) region, although some studies have investigated the role of non-HLA genes (Opgen-Rhein & Dettling, 2008; Chowdhury et al., 2011). The first genome-wide association study (GWAS) study was recently reported, providing considerable support for the role of HLA genes (Goldstein et al., 2014). This section describes previous studies implicating (i) HLA genes, (ii) non-HLA genes in the MHC, and (iii) non-MHC genes in CIA.

HLA genes

HLA genes are located in the major histocompatibility complex (MHC) on chromosome 6 (6p21.31-32). MHC genes are divided into three groups: class I, class II and class III. HLA genes *HLA-A*, *-B* and *–C* are MHC-class I genes and *HLA-DR*, *-DP*, and *–DQ* are class II genes. Given HLA genes encode proteins that are responsible for regulating the immune system, it was reasonable to investigate associations between polymorphisms in these genes and CIA (Chowdhury et al., 2011). In 1990, Lieberman and colleagues conducted the first case control study of CIA in 6 cases and 25 clozapine-treated controls. They performed HLA typing because of an observed association in their sample between Ashkenazi Jewish ancestry and CIA and found that *HLA-B38* was present in 86% of cases versus 20% of controls. Furthermore, a three-allele haplotype known to occur frequently in the Ashkenazi Jewish population, *HLA-B38/DR4/DQw3,* was present in all 5 of the Ashkenazi Jewish cases and only 12% of controls of the same ancestry (Lieberman et al., 1990). This is a marked overrepresentation of this haplotype, which is present in approximately 10-12% of people with Ashkenazi Jewish ancestry in the US and in 0.4-0.8% of people with

European ancestry. The association of *HLA-B38* and the *HLA-B38/DR4/DQw3* haplotype was also significant in further studies from the same research group after the inclusion of 31 additional Jewish and non-Jewish CIA cases, although the original cases were not excluded (Yunis et al., 1992; Yunis et al., 1995). Furthermore, typing of class II alleles revealed a significant overrepresentation of *HLA-DRB1*0402*, *-DQB1*0302* and *-DQA1*0301* and an underrepresentation of *-DRB1*011* and *-DQB1*0301* antigens in CIA patients of Ashkenazi Jewish ancestry (Yunis et al., 1995). Significant associations of *HLA-DR*02*, *-DQB1*0502* and *–DQA1*0102* were observed in patients with non-Jewish European ancestry (Yunis et al., 1995).

Valevski and colleagues provided independent replication for the association of *HLA-B38*, which was present in 72% (8/11) of CIA cases and 12% (6/50) of controls, both of Israeli Jewish ancestry (Valevski et al., 1998). However, none of the 5 CIA cases of Jewish ancestry in a study by Amar and colleagues carried the *HLA-B38* antigen (Amar et al., 1998) and Dettling and colleagues failed to find an association in 31 German CIA cases and 77 controls (Dettling & Cascorbi, 2001; Dettling et al., 2001).

Amar and colleagues found that *HLA-DQB1*0201* was present in all 5 CIA cases and 54% (7/13) of controls (Amar et al., 1998). Dettling and Cascorbi also found that *HLA-DQB1*0201* was more common in CIA cases (43%, 13/30) than controls (26%, 25/77), although the difference was not statistically significant (Dettling & Cascorbi, 2001). In addition, significant associations at *HLA-DQB1*0502*, *-DRB1*0202*, *-DRB3*0202* and *-Cw7* were found (Dettling & Cascorbi, 2001; Dettling et al., 2001). In an extended sample size of 42 CIA cases, Dettling and colleagues found significant associations at two *Cw7*-related haplotypes (*HLA-Cw7/B18* and *-Cw7/B39*), a further two-locus haplotype (*HLA-*

*DRB5*0201/DRB4*000*) and three three-locus haplotypes (*HLA-Cw7/B18/DRB5*000*, *-Cw7/B39/DRB5*000* and *-Cw7/B44/DRB5*000*) (Dettling et al., 2007).

In a Finnish sample of 26 CIA cases and 19 controls, Lahdelma and colleagues found that *HLA-A1* was present in 12% of CIA cases, 58% of clozapine-treated controls and 20% of healthy controls and thus could be considered a marker for successful treatment and low risk of agranulocytosis (Lahdelma et al., 2001). A large study conducted by Claas and colleagues in 1992 failed to find any association of *HLA-A*, *-B*, *-C*, *-DR* or *–DQ* markers in 103 CIA cases and 95 controls (Claas et al., 1992). However, this study has been criticised on methodological grounds because very little clinical information was given about the samples and cases were pooled across different European populations (Dettling et al., 2001).

In 2011, Athanasiou and colleagues looked for association with 74 candidate genes in 33 CIA cases and 54 controls from the US, Russia and South Africa and found significant associations for variants within five genes: *HLA-DQB1*, *HLA-C*, *HLA-DRD1*, *NTSR1* and *CSF2RB* (Athanasiou et al., 2011). Using a previously reported replication sample of 49 CIA cases and 79 controls (Dettling & Cascorbi, 2001; Dettling et al., 2001; Dettling et al., 2007), they found a significant association with *HLA-DQB1* 6672G>C (OR=16.9), that was present in 21.5% of the combined cases and 1.6% of combined controls (Athanasiou et al., 2011). The sensitivity and specificity of *HLA-DQB1* 6672G>C in the combined sample was 21.5 and 98.4, respectively. On the basis of this study, *HLA-DQB1* 6672G>C was marketed as a genetic predictive test but the low sensitivity limited its clinical utility and it has now been withdrawn due to the low uptake (Chowdhury et al., 2011).

Considerable support for the role of HLA genes in CIA came from a recently published study by the Clozapine-Induced Agranulocytosis Consortium (CIAC) (Goldstein et al., 2014).

They conducted a comprehensive genetic study including a genome-wide association study (GWAS), whole exome sequencing, copy number variation, and imputed HLA alleles in up to 163 cases with clozapine-induced neutropenia, 249 clozapine-exposed controls without neutropenia and 7970 unexposed controls. They found that two amino acid polymorphisms in the MHC were independently and significantly associated with CIA; *HLA-DQB1* (126Q) and *HLA-B* (158T) (Goldstein et al., 2014). However, it should be noted this sample is not independent of earlier studies.

Non-HLA genes in MHC

One research group investigated whether non-HLA genes in the MHC region contributed to the genetic risk of CIA. They hypothesised that the association of different HLA types in Ashkenazi Jewish and non-Jewish CIA patients could be caused by the linkage disequilibrium of a common genetic marker (Turbay et al., 1997). The role of heat shock protein 70 (*HSP-70*) genes, located within the class III region of the MHC, were examined due to their role in immune system regulation (Corzo et al., 1995). Variants within *HSP70-1* and *HSP70-2* were found to be in linkage disequilibrium with HLA class I and class II markers that had been previously associated with CIA in both Ashkenazi Jewish and non-Jewish patients (Corzo et al., 1994). The clozapine control group had an excess number of *HSP70-1* C and *HSP70-2* 8.5kb variants compared to CIA patients, suggesting they may have a protective role (Corzo et al., 1995). Furthermore, tumor necrosis factor (*TNF*) microsatellites are in linkage disequilibrium with *HLA-B* and *–DR* variants (GarciaMerino et al., 1996), and they found that *TNF b4* and *d3* microsatellite alleles were overrepresented in both Ashkenazi Jewish and non-Jewish patients with CIA, whereas the *d5* microsatellite was underrepresented (Turbay et al., 1997). However, these findings are yet to be

replicated in an independent sample and there is no evidence to suggest that these genes account for the association of *HLA* genes with CIA.

Non-MHC genes

Non-immune mediated hypotheses have prompted other candidate gene studies. Based on the hypothesis that a defective oxidative mechanism is the cause of CIA, Ostrousky and colleagues investigated the candidate gene dihydronicotinamide riboside (NRH) quinone oxidoreductase 2 (*NQO2*), located on chromosome 6p25 (Ostrousky et al., 2003). They found a significant association of polymorphisms in *NQO2* in 18 CIA cases and 80 clozapine-treated controls. *NQO2* has been implicated in the detoxification of chemicals and protection of cells against drug-induced oxidative and electrophilic stress (Long & Jaiswal, 2000). Ostrousky and colleagues suggested that *NQO2* is involved in the detoxification of clozapine metabolites and if this detoxification is not sufficiently effective, the reactive metabolites of clozapine could accumulate in neutrophils leading to apoptosis (Ostrousky et al., 2003). However, these findings are yet to be replicated in an independent sample.

Because neutrophils can generate hypochlorous acid via the NADPH/MPO pathway, which is capable of oxidizing clozapine to reactive metabolites, there have been studies investigating the role of polymorphisms that alter MPO and NADPH activity (Dettling et al., 2000; Mosyagin et al., 2004). Dettling and colleagues found no association of G-463A, a polymorphism in *MPO* that has been shown to decrease MPO activity, in 31 CIA cases and 77 controls (Dettling et al., 2000). However, Mosyagin and colleagues found that AA carriers of this variant were significantly overrepresented in CIA cases (Mosyagin et al., 2004). No significant associations were found for C242T and A640G polymorphisms within NADPH subunit *CYBA* (Mosyagin et al., 2004). Recently, Lobach and Uetrecht

demonstrated that the binding of clozapine to neutrophils was decreased 2-fold in MPO knockout mice and 6-7 fold in gp91 knockout (NADPH oxidase null) mice (Lobach & Uetrecht, 2014).

To assess the hypothesis that genetic variants of leukocyte *Fcγ* receptors modulate the risk for CIA, Mosyagin and colleagues compared the frequency of polymorphisms in *FcγRIIa*, *FcγRIIIa FcγRIIIb* in 48 patients with CIA and 75 controls (Mosyagin et al., 2005). However, the lack of association between CIA and *Fcγ* receptor polymorphisms indicates that these receptors are unlikely to play a major role in CIA (Mosyagin et al., 2005). Lastly, Dettling and colleagues found no evidence of a role of polymorphisms in *CYP2D6*, a gene involved in clozapine metabolism (Dettling et al., 2000).

In 2013, a whole exome sequencing study of 24 Finnish cases with neutropenia and 24 age and sex matched controls did not identify any variants that were significantly associated after correction for multiple testing, although the sample size was small (Tiwari et al., 2014).

In conclusion, previous studies indicate that the genetic aetiology of CIA and neutropenia is complex and is likely to involve several genes. The rare incidence of CIA has limited the availability of suitable patients and thus studies have small sample sizes. Furthermore, most studies have been conducted by four or five research groups and there is a considerable overlap between study samples (Zhang & Malhotra, 2013). Nevertheless, HLA markers in association with CIA have emerged as a consistent finding across studies. A comprehensive genetic analysis in a well-powered independent sample is required to further investigate the role of genetics in CIA and neutropenia.

1.6. Discontinuation of clozapine

There are numerous reasons why clozapine treatment is discontinued, other than the occurrence of agranulocytosis or neutropenia. The rates of discontinuation of clozapine are lower than for other antipsychotics; the SOHO study of 7186 adult outpatients found that clozapine (20.5%) and olanzapine (23%) had the lowest rates of discontinuation after 12 months of treatment and quetiapine (48.6) and amisulpride (41.8) had the highest (Haro et al., 2006). However, a substantial number of patients will still cease clozapine treatment, often causing a rapid deterioration in psychotic symptoms (Seppala et al., 2005; Atkinson et al., 2007). Clozapine cessation has been associated with increased rates of compulsory treatment, hospitalisation, and poorer functioning in living and vocational activities in comparison to those that continue (Wheeler et al., 2009b). Atkinson and colleagues found that global functioning significantly worsened after clozapine discontinuation and the number of inpatient days increased by an average of 45.7 in the year following clozapine discontinuation compared to the previous year (Atkinson et al., 2007).

The rates of clozapine discontinuation reported by previous studies range from 20-54% in study durations from 6 months to 15 years (Moeller et al., 1995; Laker et al., 1998; Ciapparelli et al., 2000; Hayhurst et al., 2002; Ciapparelli et al., 2003; MacGillivray et al., 2003; Whiskey, 2003; Krivoy et al., 2011; Davis et al., 2014). In a 15-year naturalistic retrospective study, 24% of all patients starting clozapine had discontinued after 1 year, 32% by 2 years and 50% by 7 years of treatment (Davis et al., 2014). Studies indicate that 20-34% of patients will discontinue within the first year of treatment (Munro et al., 1999; Haro et al., 2006) and 35-44% will discontinue within two years (Laker et al., 1998; Ciapparelli et al., 2000; Hayhurst et al., 2002). Rates reported in studies with longer

durations suggest that 45% will discontinue with 3 years (MacGillivray et al., 2003) and 54% within 4 years of clozapine onset (Ciapparelli et al., 2003). Discontinuations are most likely within the first year of treatment; two studies reported that 66% (Pai & Vella, 2012) and 78% (Krivoy et al., 2011) of total discontinuation events occurred during the first year. However, Ciapparelli and colleagues in a four-year naturalistic study found that only 33% of discontinuation events occurred during the first year of treatment and 65% within 18 months (Ciapparelli et al., 2003). Davis and colleagues concluded that the highest risk for discontinuation was between 3 and 6 months from the start of treatment (Davis et al., 2014).

Considering the favourable outcomes of clozapine treatment and poor prognosis for those that discontinue, efforts have been made to understand the causes of discontinuation and to identify patients that may be at increased risk.

1.6.1 Reasons for discontinuation

Several retrospective studies have identified *non-adherence*, *patient decision*, *adverse effects*, *death* and *inadequate response* as reasons for discontinuation of clozapine (Ciapparelli et al., 2000; Ciapparelli et al., 2003; Atkinson et al., 2007; Taylor et al., 2009; Krivoy et al., 2011; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015).

Non-adherence and patient decision

There have been inconsistent approaches to the way that *patient decision* and *non-adherence* have been classified in previous studies. Some studies have assigned *patient decision* and *non-adherence* as mutually exclusive causes (Pai & Vella, 2012) and others have assigned them together (Taylor et al., 2009; Krivoy et al., 2011; Davis et al., 2014). In a study of 151 discontinued patients, Pai and Vella found that *patient decision* accounted

for 40% of discontinuations and *non-compliance* for 36% (Pai & Vella, 2012). Other studies that have considered these reasons together (called *non-adherence* or *non-compliance*) have consistently found *non-adherence* to be the single most common reason for discontinuation (Leppig et al., 1989; Krivoy et al., 2011). In 2009, Taylor and colleagues compared the reasons for discontinuation of clozapine with a matched cohort of patients discontinuing risperidone injection, and found that *non-adherence* accounted for 48% of discontinuations (Taylor et al., 2009). Other rates of discontinuations due to *non-adherence* range from 31-55% (Ciapparelli et al., 2003; Atkinson et al., 2007; Krivoy et al., 2011; Davis et al., 2014; Mustafa et al., 2015). However, although *non-adherence* and *patient decision* have been identified as major reasons for discontinuation of clozapine, there has been no exploration of reasons behind this choice.

Adverse effects

Adverse drug reactions (*ADRs*) or intolerable side effects attributed to clozapine are a common cause of discontinuation. Previous studies indicate that 25-35% of discontinuations are due to *ADRs* (Atkinson et al., 2007; Taylor et al., 2009; Krivoy et al., 2011; Davis et al., 2014; Mustafa et al., 2015), although there have been reports of lower frequencies; 17% (Pai & Vella, 2012) and 9% (Ciapparelli et al., 2003). However, the role of ADRs may have been underestimated due to discontinuations secondary to *ADRs* being attributed *to non-adherence* or *patient decision*.

Haematological side effects, particularly neutropenia, are the most common *ADRs* cited as a reason to discontinue clozapine (Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). There is a wide range of other reasons reported, but common causes involve cardiovascular (hypotension, tachycardia), central nervous system (seizures, somnolence) and fever (Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). A recent review by

Nielsen and colleagues concluded that a number of these cited side effects do not necessarily warrant discontinuation (Nielsen et al., 2013). Although agranulocytosis should always lead to prompt discontinuation, previous research has shown that in many cases neutropenia is transient or not related to clozapine, and 70-80% can be reinstated on clozapine (Manu et al., 2012; Meyer et al., 2015). Furthermore, in some patients benign ethnic neutropenia (BEN) may be present, particularly in those of African and Middle Eastern descent (Rajagopal, 2005). Those with BEN have low baseline neutrophil levels and many can be successfully reinstated with adjusted monitoring thresholds (Nielsen et al., 2013). In regards to cardiovascular adverse effects, myocarditis and cardiomyopathy should cause immediate discontinuation (Manu et al., 2012). However, hypotension and tachycardia are common side effects of clozapine that could be managed by dose reduction, providing that myocarditis and cardiomyopathy are excluded (Nielsen et al., 2013). Furthermore, seizures can be managed by dose reduction or addition of antiepileptic medication (Nielsen et al., 2013).

Death

A study in 2007 by Atkinson and colleagues found that 12 out of 35 patients (35%) that discontinued clozapine died (Atkinson et al., 2007), which led to some concern over the causes of death in patients taking clozapine. Other cross-sectional studies of clozapine discontinuation have reported death to account for 10% (Mustafa et al., 2015) and 13% (Davis et al., 2014) of clozapine discontinuations. Taylor and colleagues, in a matched cohort of patients discontinuing risperidone injection, found that 13% of clozapine discontinuations were due to death, a rate that was significantly higher than those treated with risperidone (Taylor et al., 2009). However, patients taking clozapine are more likely to have a longstanding illness and greater antipsychotic exposure, thus limiting the

comparability of these groups. Furthermore, patients stabilised on clozapine tend to remain on the treatment for many years, a factor that is likely to confound results from a cohort of patients who discontinue treatment. Contrary to these findings, there is good evidence that clozapine decreases all-cause mortality (Hayes et al., 2015). Nonetheless, these studies emphasise the need for close monitoring of physical conditions when undergoing clozapine treatment (Taylor et al., 2009).

Inadequate response

The majority of previous studies have found that *inadequate response* to clozapine treatment is cited as a reason for only 2-3% of discontinuations (Taylor et al., 2009; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). In a comparison with risperidone, patients receiving clozapine were less likely to discontinue due to *inadequate response* (Taylor et al., 2009). However, two other studies reported larger estimates; Ciapparelli and colleagues reported *inadequate response* to account for 39% of discontinuations within two years and 28% within four years (Ciapparelli et al., 2000; Ciapparelli et al., 2003), and Krivoy and colleagues found that *insufficient response* accounted for 19% of discontinuations (Krivoy et al., 2011). This disparity may be due to the small sample size in these two studies, differences in the study populations and differences in the definition of *inadequate response*. Given that non-response to clozapine has been estimated between 40-70% (Kane et al., 1988; Lieberman et al., 1994b), this result is unlikely to reflect the true rates of inadequate response to clozapine but rather that it is seldom recorded as the primary reason to discontinue treatment.

Other reasons

There have been other cited reasons for clozapine discontinuation. Ciapparelli and colleagues found that 24% of discontinuations within four years were due to an inability to

travel to the treatment centre for monitoring (Ciapparelli et al., 2003). Davis and colleagues reported that 19% of discontinuations were due to administrative reasons (Davis et al., 2014) and a minority of females will discontinue due to pregnancy (Ciapparelli et al., 2003).

A limitation of studies assessing the reasons for clozapine discontinuation is the use of mutually exclusive categories. In many cases, the reasons for discontinuation will have been multifactorial. For example, a patient (or clinician) may be more likely to tolerate an ADR and be willing to persevere with clozapine if they are experiencing a good clinical response to clozapine, but might instead discontinue clozapine, citing adverse effects, in the absence of a clinical response. The decision to discontinue clozapine is likely to be a judgement as to the balance of the likely benefits versus harms of continuing versus stopping clozapine, taking into account the views of the patient and of his or her carers. Nonetheless, these studies have highlighted the key primary causes of discontinuation of clozapine.

1.6.2 Predictors of discontinuation

Several studies have attempted to identify patients that may be at an increased risk of clozapine discontinuation. An older age at clozapine initiation has been associated with greater rates of discontinuation (Munro et al., 1999; MacGillivray et al., 2003; Whiskey, 2003; Krivoy et al., 2011; Davis et al., 2014), an association that is not related to length of illness (Krivoy et al., 2011). MacGillivray and colleagues found that people who started clozapine at age 50+ were four times more likely to stop taking clozapine within three years than people ages 17-29 years (MacGillivray et al., 2003). There is evidence to suggest that older age at initiation is associated with non-patient-initiated but not patient-initiated discontinuations (Davis et al., 2014). Black African/American ethnicity in the USA and

African/Caribbean ethnicity in the UK has consistently been associated with a higher rate of clozapine discontinuation (Moeller et al., 1995; Munro et al., 1999; Rosenheck et al., 2000; Kelly et al., 2006; Davis et al., 2014). The increased rate of haematological side effects (specifically benign ethnic neutropenia) found in Black African/Caribbean patients (Moeller et al., 1995; Munro et al., 1999; Davis et al., 2014) has been suggested to account for this association. Krivoy and colleagues found an association between comorbid substance abuse and discontinuation of clozapine (Krivoy et al., 2011). However, this was not replicated by another study (Moeller et al., 1995), although the follow-up period was shorter. Davis and colleagues found a significant association for less symptomatic improvement and noted there were higher rates of discontinuation in females, although this was not statistically significant (Davis et al., 2014). One study found that patients with bipolar disorder were two times more likely to discontinue than patients with schizophrenia or schizoaffective disorder, despite the fact patients with bipolar disorder experienced a greater response to clozapine (Ciapparelli et al., 2000). Lastly, there is evidence to suggest that clinician experience can impact discontinuation rates. Whiskey and colleagues found that discontinuation rates were affected by the prescribing hospital and the lowest rates in their study were from a London hospital with a long history in prescribing clozapine (Whiskey, 2003). A lack of experience may explain why some patients are unnecessarily discontinued (Nielsen et al., 2010).

In conclusion, approximately 40% of patients will discontinue clozapine within 24 months of initiation, which carries a poor prognosis. The most common reasons for clozapine discontinuation identified in previous studies are *ADRS*, *patient decision* and *non-adherence*. An older age at clozapine initiation, Black African/Caribbean ethnicity and substance abuse have been found to increase risk of clozapine discontinuation. However, the majority of previous studies have not been conducted in patients receiving their first

trial of clozapine and thus the identified reasons for discontinuing may have been biased by previous clozapine trials. Furthermore, although *non-adherence* and *patient decision* have been identified as major reasons for discontinuation of clozapine, there has been no exploration of reasons behind this choice.

1.7. Clozapine response

Although the superior efficacy of clozapine has been consistently demonstrated, not all patients will respond to clozapine. This section provides an overview of studies relating to how response to clozapine is measured and defined, the proportion of patients that have been found to respond and predictors of clozapine response.

1.7.1 Clinical rating scales used in psychiatric research

There are several rating scales used in psychiatric research to assess response. One of the most widely used scales is the Brief Psychiatric Rating Scale (BPRS), which measures 18-24 different psychiatric symptoms, depending on the version, ranging from depression to anxiety and hallucinations (Overall & Gorham, 1962). The Positive and Negative Syndrome Scale (PANSS) measures 30 different symptoms specifically related to schizophrenia, and is widely used in the study of antipsychotic efficacy (Kay et al., 1987). Each symptom in the BPRS and PANSS is rated from 1-7. The Scale for the Assessment of Negative Symptoms (SANS) measures negative symptoms of schizophrenia, for example affective flattening, alogia, apathy and anhedonia (Andreasen, 1983), and the Scale for the Assessment of Positive Symptoms (SAPS) measures positive symptoms of schizophrenia, for example hallucinations, delusions, bizarre behaviour and formal thought disorder (Andreasen, 1984). Each symptom in the SAPS and SANS is rated from 0 to 5. The Clinical Global Impressions (CGI) scale is a global measure comprised of two scores (i) CGI-Severity (CGI-

S), and (ii) CGI-Improvement (CGI-I) (Guy, 1976). CGI-S is a seven-point scale measuring the severity of the illness in question and CGI-I is a seven-point scale measuring the change in psychopathology from baseline, frequently treatment initiation. Ratings take into account all available information (Busner & Targum, 2007). The CGI is often selected due to the ease and speed of application in comparison to the BPRS or PANSS, and has been demonstrated to have equivalent sensitivity (Leucht & Engel, 2006). General functioning scales such as the Global Assessment of Functioning (GAF), based on the Global Assessment Scale (Endicott et al., 1976), have been less frequently used.

Several studies have made direct comparisons between these measures (Leucht et al., 2006). A CGI-S score of 3 (mildly ill), 4 (moderately ill) and 5 (markedly ill) approximately corresponds to BPRS total scores of 31, 41 and 53 (Leucht et al., 2005a), and PANSS total scores of 58, 75 and 95 (Leucht et al., 2005b), respectively. A CGI-I score of 3 (minimally improved) corresponds to a 24-30% reduction in BPRS (Leucht et al., 2005a) and a 19-28% reduction in PANSS (Leucht et al., 2005b). A CGI-I score of 2 (much improved) approximately corresponds to a 40-53% reduction in BPRS score (Leucht et al., 2005a; Leucht & Engel, 2006) and 40-53% reduction in PANSS (Leucht et al., 2005b), although another study reported a 22-29% reduction in PANSS (Rabinowitz et al., 2010).

1.7.2 Defining clozapine response

The lack of consensus regarding how to define response to antipsychotic treatments has been a significant issue for some time (Suzuki et al., 2012). Consequently, there has been considerable variability in the definition of clozapine response used, resulting in limited comparability between studies. Several studies have used the absolute response criteria defined in Kane and colleagues' landmark clinical trial; a 20% decrease in BPRS total score, and either a post-treatment CGI-S score ≤ 3 or BPRS ≤ 35 (Kane et al., 1988). Other studies

have focused purely on symptom reduction, for example a ≥ 20% decrease in PANSS or BPRS total score (Meltzer et al., 1989a; Rosenheck et al., 1998; Umbricht et al., 2002; Ciapparelli et al., 2004; Usall et al., 2007; Kelly et al., 2010; Suzuki et al., 2012). Few studies have included GAF scores (Ciapparelli et al., 2003) or defined response specifically in terms of positive (SAPS) or negative (SANS) symptom reduction (Mauri et al., 2003; Meltzer et al., 2003b). More stringent thresholds (≥ 50% decrease in BPRS total score) have been used in longer-term studies (Ciapparelli et al., 2004). Because of these difficulties, other investigations have turned to more pragmatic outcomes such as time to rehospitalisation (Rosenheck et al., 2011; Nielsen et al., 2012) or time to discontinuation (Lieberman et al., 2005; Kroken et al., 2014).

A recent review recommended a response definition of ≥20% decrease in BPRS or PANSS total score or a CGI-Improvement score of 2 or 1 (Suzuki et al., 2012). However, defining response solely based on relative change ignores the importance of baseline levels (Mortimer, 2007) and will favour highly symptomatic patients because they have more room for improvement and thus the measurable effect of clozapine will be greater (Rosenheck et al., 1998). This is also important considering that in many studies where response is defined, patients with TRS were excluded. The use of a remission criteria, which is the clinical goal, has also been recommended (Mortimer, 2007). Due to results varying considerably by the response definition chosen, presenting a range of definitions has been recommended to evaluate whether effects remain consistent (Leucht et al., 2007b).

1.7.3 Proportion responding to clozapine

The wide variability in the proportion of patients reported to have responded to clozapine treatment appears to stem from, at least in part, the varying response definitions and

durations of treatment trial (Suzuki et al., 2012). Kane and colleagues found that 30% of patients with TRS responded to clozapine within six weeks (Kane et al., 1988). In a trial of 52 weeks, Lieberman and colleagues found that 50% of TRS and 76% of treatment-intolerant patients responded (Lieberman et al., 1994b). A long-term (48 months) naturalistic study found 65% of schizophrenia patients experienced a 50% reduction in BPRS (Ciapparelli et al., 2003). In general, other clinical trials and naturalistic studies fall within these reports, indicating that clozapine substantially reduces psychotic symptoms in 30-70% of TRS patients (Kane et al., 1988; Lieberman et al., 1994b; Wahlbeck et al., 2000; Semiz et al., 2007).

1.7.4 Timing of clozapine response

There has been substantial interest in the duration of clozapine treatment required to evaluate response to clozapine. If such a time could be identified, patients who have not shown significant improvement could be regarded as non-responders and their clozapine treatment discontinued. If this specified time is too short, patients who would have eventually experienced benefit may be discontinued, and if too long, nonresponsive patients may continue to unnecessarily receive treatment that will not benefit them. Previous studies have determined that response can be detected within the first 6-8 weeks of treatment (Rosenheck et al., 1999b; Suzuki et al., 2011a) and that early response is indicative of later response: response after one week of treatment predicted response at five weeks (Stern et al., 1994), and in another study response after four weeks predicted response at 16 weeks (Semiz et al., 2007). However, other studies indicate that trials up to six months may be required to detect non-response (Lieberman et al., 1994b) and some benefits of treatment may only become apparent after long-term follow-up, particularly for functional and quality of life measures (Rosenheck et al., 1999b). Although there have

been reports that a minority of patients may experience an improvement after six months of treatment (Wilson, 1996), there is limited evidence regarding a delayed response (Carpenter et al., 1995).

Studies investigating timing of response are often hindered by a lack of control for dose or clozapine plasma concentrations (Schulte, 2003). One study reported that all responders achieved response within 8 weeks of a dose escalation and on average 17 days after reaching the therapeutic dose at which clozapine response was achieved (Conley et al., 1997). Furthermore, in new responders at weeks 8, 12, and 24, in spite of a fixed clozapine daily dose, mean drug plasma levels progressively increased, up to the point where clinical response occurred (Fabrazzo et al., 2002). In addition, clozapine non-responders had a mean clozapine concentration below 260 ng/mL, which was significantly lower than the average in responders (above 400ng/mL) (Fabrazzo et al., 2002). There is evidence to suggest that some patients that do not respond under a normal dose may respond if plasma levels are brought above 350 ng/mL (Kronig et al., 1995).

1.7.5 Predictors of response

Predictors of clozapine response would be valuable in assisting clinicians in determining if clozapine treatment is likely to be beneficial. A number of studies have attempted to address this question (Chung & Remington, 2005; Suzuki et al., 2011c). This section describes the demographic and clinical, dosage and pharmacokinetic, and genetic predictors of response.

Demographic and clinical

Despite a large number of studies, there are few reliably replicated clinical or demographic predictors of clozapine response. There have been conflicting findings in regards to age at

initiation of clozapine (Meltzer et al., 1989a; Honer et al., 1995b; Rodriguez et al., 1998; Rosenheck et al., 1998; Sajatovic et al., 1998; Schall et al., 1999; Hofer et al., 2003; Mauri et al., 2003; Semiz et al., 2007; Kelly et al., 2010), age at onset of schizophrenia (Meltzer et al., 1989a; Lieberman et al., 1994a; Rodriguez et al., 1998; Mauri et al., 2003; Ciapparelli et al., 2004; Semiz et al., 2007; Kelly et al., 2010; Nielsen et al., 2012) and duration of illness (Lieberman et al., 1994b; Honer et al., 1995a; Schall et al., 1999; Umbricht et al., 2002; Hofer et al., 2003; Mauri et al., 2003). Female gender has been associated with a good response, independent of age of onset and chronicity (Mauri et al., 2003; Ciapparelli et al., 2004; Usall et al., 2007), but other studies have reported either a poor response (Lieberman et al., 1994a; Szymanski et al., 1996) or no effect (Hofer et al., 2003; Semiz et al., 2007). Furthermore, there have been mixed findings regarding a diagnosis of paranoid schizophrenia (Honigfeld & Patin, 1989; Meltzer et al., 1989a; Fenton & Lee, 1993; Lieberman et al., 1994b; Honer et al., 1995a; Rosenheck et al., 1998; Semiz et al., 2007) and the presence of EPS during typical antipsychotic treatment (Pickar et al., 1994; Umbricht et al., 2002). A naturalistic study reported that patients with bipolar disorder in comparison to schizoaffective disorder or schizophrenia had significantly better clinical response to clozapine (Ciapparelli et al., 2000; Ciapparelli et al., 2003; Ciapparelli et al., 2004), but this has not been replicated in an independent sample.

In contrast, findings have been consistent in regards to the level of functioning prior to clozapine treatment. An increasing number of hospitalisations, poor functioning and no history of independent living have been associated with a poor response to clozapine (Fenton & Lee, 1993; Honer et al., 1995a; Nielsen et al., 2012). A recent study investigating the impact of premorbid functioning on non-response to clozapine reported a trend for social withdrawal, poorer adaption to school and fewer peer relationships in childhood, suggesting that clozapine non-responders may represent a distinct subtype of patients

(Kelly et al., 2010). Findings have also been consistent in the lack of association with ethnicity (Lieberman et al., 1994a; Rosenheck et al., 1998; Kelly et al., 2010). One study has reported that smokers experienced greater therapeutic response compared to non-smokers, despite smoking less than when receiving conventional antipsychotics (McEvoy et al., 1999). Interestingly, increased weight gain has been associated with good clinical response, independent of age, initial weight, plasma clozapine levels and level of psychopathology (Leadbetter et al., 1992; Meltzer et al., 2003b).

The conflicting results of clinical and demographic predictors of clozapine response may be due to the considerable variability in response definitions and trial durations. Furthermore, most studies have been conducted in small sample sizes and thus are likely to be underpowered.

Baseline symptoms

The most consistently reported clinical baseline predictor of clozapine response is higher or more severe baseline symptoms as measured by the CGI, total BPRS or PANSS scores (Hasegawa et al., 1993; Lieberman et al., 1994b; Rosenheck et al., 1998; Ciapparelli et al., 2000; Umbricht et al., 2002; Semiz et al., 2007; Kelly et al., 2010). Specifically, high positive symptoms and low negative symptoms have been associated with good response (Rodriguez et al., 1998; Schall et al., 1999; Umbricht et al., 2002). However, the association with negative symptoms is unclear; a large study investigating this question found no differences in response between patients with high or low levels of negative symptoms or in patients with or without the deficit syndrome (Rosenheck et al., 1999a). The presence of suicidal ideation at baseline has been associated with good response (Ciapparelli et al., 2000). However, rather than implying that there is a superior effect of clozapine in patients with high positive or total symptom scores, the association may be simply

because highly symptomatic patients have more room for improvement (Rosenheck et al., 1998).

Dosage and pharmacokinetic factors

Analyses of clinical trials have indicated that dosages over 400 mg/day and up to 600 mg/day are required for optimal clozapine treatment (Davis & Chen, 2004; Kinon et al., 2004). One study demonstrated a significant dose effect (100 > 300 > 600 mg/day) with clozapine response after 16 weeks of treatment (Simpson et al., 1999) and a large naturalistic study reported that lower dose predicted a shorter time to hospital admission (Nielsen et al., 2012). However, a study comparing clinical trials across the USA and Europe found that although dose was higher in the USA (444 mg versus 284 mg), estimates of response were similar (Fleischhacker et al., 1994). However, European studies had fewer adverse effects, suggesting lower doses could be more tolerable and just as effective (Fleischhacker et al., 1994).

Although clozapine dose and plasma concentrations are correlated, there is a wide inter-individual variability. Thus, the relationship between clozapine response and plasma levels has been more commonly assessed than dose response studies (Mauri et al., 2007). Lower clozapine levels have been associated with increased rates of relapse (Xiang et al., 2006) and a number of studies indicate that a plasma concentration of at least 350-420 ng/mL is required for optimal clozapine response (Perry et al., 1991; Hasegawa et al., 1993; Kronig et al., 1995; VanderZwaag et al., 1996; Spina et al., 2000; Llorca et al., 2002; Mauri et al., 2007). Monitoring of plasma levels is widely utilised in the clinical management of clozapine treatment and thus constitutes the most utilised predictor of response. There are many factors that influence plasma clozapine levels including age, gender, smoking and metabolic factors (Haring et al., 1989; Lane et al., 1999; Kim, 2015).

Genetic

A twin study indicates that there may be a genetic susceptibility to clozapine response (Vojvoda et al., 1996). Pharmacogenetic studies have focused largely on candidate genes that are either directly involved in the drug mechanism of action, such as neurotransmitter receptors, or affect clozapine metabolism and thus influence plasma concentrations (Malhotra et al., 2004).

Neurotransmitter receptors

Initial studies of clozapine response focused on the dopamine D_4 receptor gene (*DRD4*) because of clozapine's high affinity for D_4 in comparison to D_2 receptors (Van Tol et al., 1991). Studies investigating the role of *DRD4* in clozapine response have focused on a 48-base-pair repeat polymorphism, but there have been conflicting findings (Shaikh et al., 1993; Rao et al., 1994; Kohn et al., 1997; Cohen et al., 1999; Hwang et al., 2012). Ozdemir and colleagues (Özdemir et al., 1999) implicated a repeat polymorphism within the first intron of *DRD4*. However, despite the functional nature of these polymorphisms, there is limited evidence to support their involvement in treatment response of clozapine (Malhotra et al., 2004).

The dopamine D_2 receptor gene (*DRD2*) has been examined given its action is critical for antipsychotic response. Previous studies have focused on the functional polymorphism -141C Ins/Del in the promoter region of *DRD2* (Malhotra et al., 1999). Although there have been a number of negative reports (Arranz et al., 1998c; Hwang et al., 2005), a recent meta-analysis of *DRD2* gene variation in antipsychotic response, including but not limited to clozapine, added support for the role of this variant (Zhang et al., 2010). Furthermore, an exploratory analysis of 12 SNPs in *DRD2* found an association between the *Taq1*A, *Taq1*B and rs1125394 markers and clozapine response, but only for African-American

patients (Hwang et al., 2005). More comprehensive examination of the role of *DRD2* may be required, but it appears this gene may be more important for other antipsychotics that have higher affinity for D_2 receptors (Zhang et al., 2010).

Genetic association studies of the dopamine D_3 receptor gene (*DRD3*) have focused on a variant that causes a serine-to-glycine substitution at amino acid position 9 (Ser9Gly, rs6280) (Hwang et al., 2010). A meta-analysis by Jonsson and colleagues concluded that responders to traditional antipsychotics were observed to have higher Ser allele, and homozygote genotype (Ser/Ser or Gly/Gly) frequencies, whereas the opposite was true for clozapine responders (Jonsson et al., 2003). However, a recent meta-analysis focusing solely on clozapine studies failed to replicate this finding, although there was a trend in the same direction (Hwang et al., 2010).

There are fewer studies examining the possible role of D_1 and D_5 dopamine receptors in clozapine response. Two studies have implicated variants within *DRD1* (Potkin et al., 2003; Hwang et al., 2007) and although a two-marker haplotype in *DRD5* was recently reported to be associated with negative symptom response to clozapine, there appears to be very little evidence for the role of *DRD5* (Hwang et al., 2012). Individually, dopamine receptor genes appear to have a minor effect on response to clozapine.

Due to clozapine's high affinity for the serotonin 5-HT_{2A} receptor, the role of serotonin genes has been investigated in numerous candidate studies. In 1995, Arranz and colleagues identified an association between the 102-T/C polymorphism in *5-HT_{2A}* and clozapine response (Arranz et al., 1995). Although there were a number of subsequent negative findings regarding this variant (Malhotra et al., 1996a; Masellis et al., 1998), a meta-analysis, including these negative studies, revealed a significant excess of 102C allele carriers in clozapine non-responders (Arranz et al., 1998b). Additional support came from

a study implicating 1438-G/A, a functional variant in the promoter region of *5-HT$_{2A}$*, and in strong linkage disequilibrium with 102-T/C (Arranz et al., 1998a). Furthermore, a less common polymorphism in *5-HT$_{2A}$*, 452-His/Tyr has been associated with response (Arranz et al., 1996; Arranz et al., 1998a; Arranz et al., 1998b; Masellis et al., 1998) although again there are conflicting findings (Malhotra et al., 1996a). Other serotonin-related genes have also been examined in pharmacogenetic studies of clozapine response. An association was initially reported between a cysteine to serine substitution at amino acid 23 (Cys23Ser) in *5-HT$_{2C}$* and clozapine response (Sodhi et al., 1995) but this has not been replicated in other studies (Malhotra et al., 1996b; Rietschel et al., 1997; Masellis et al., 1998). Although not previously thought to be associated (Gutiérrez et al., 2002), recent studies have implicated rs1062613 in *5-HT$_{3A}$* in clozapine response (Souza et al., 2010). Additional variants in serotonin genes include 267-T/C in *5-HT$_{6}$* (Yu et al., 1999; Masellis et al., 2001), *5-HT$_{7}$* (Masellis et al., 2001), as well as the serotonin transporter gene *SLC6A4* (Arranz et al., 2000a; Tsai et al., 2000; Kohlrausch et al., 2010).

A study by Arranz and colleagues identified a combination of six polymorphisms in genes coding for neurotransmitter receptors that predicted 76.9% of clozapine response, with a sensitivity of 95% (Arranz et al., 2000b). However, this was not been replicated in an independent sample (Schumacher et al., 2000). Further studies are required to understand the genetic contribution of neurotransmitter receptor genes to clozapine drug response.

Metabolism

The cytochrome P450 isoform CYP1A2 is primarily responsible for clozapine metabolism. Thus, polymorphisms within *CYP1A2* may affect clozapine response by impacting on clozapine plasma levels. The *CYP1A2*1F* (-163C>A, rs762551) polymorphism has been associated with a decreased response to clozapine (Ozdemir et al., 2001; Eap et al., 2004;

Balibey et al., 2011; de Brito et al., 2015). In a sample of 75 individuals, this polymorphism was not found to directly impact clozapine plasma levels (Jaquenoud Sirot et al., 2009). *ABCB1* encodes for the transmembrane transporter P-glycoprotein and has been demonstrated to affect plasma clozapine levels (Consoli et al., 2009; Jaquenoud Sirot et al., 2009). A recent study reported an association between polymorphisms in *ABCB1* and clozapine response (Lee et al., 2012b).

Despite a significant number of studies, there is a lack of consistent pharmacogenetic findings of clozapine response. This may due to small sample sizes making studies underpowered and the significant differences between studies in regards to sample characteristics and definition of clozapine response. All the studies to date take a candidate gene approach and thus a well-powered GWAS or comprehensive examination of these genes is required to further examine the role genetics in clozapine response.

1.8. Summary and limitations of existing literature

The clinical course of schizophrenia is highly heterogeneous and 20-30% of patients will remain symptomatic and significantly impaired despite antipsychotic treatment. Even though the superior efficacy of clozapine has been consistently demonstrated in the management of TRS, clozapine remains widely under-prescribed, due, at least in part, to the risk of haematological side effects of agranulocytosis and neutropenia. The aetiology of clozapine-induced agranulocytosis and neutropenia is currently unknown. The first, and only, genome-wide association study (GWAS) conducted by the Clozapine-Induced Agranulocytosis Consortium (CIAC) has provided substantial support for the role of genetics, specifically for *HLA-DQB1* and *HLA-B* (Goldstein et al., 2014). However, this sample is not independent of earlier studies. Although there have been many candidate

gene studies in this field, there has been significant overlap of samples (Zhang & Malhotra, 2013). The rare incidence of agranulocytosis has limited the availability of suitable patient samples and thus studies have suffered from small sample sizes. Lastly, over half of the individuals who develop clozapine-induced agranulocytosis or neutropenia do not have any of the identified risk variants. A comprehensive genetic analysis in a well-powered independent sample is required to further investigate the role of genetics in clozapine-associated agranulocytosis and neutropenia. This is the subject of Chapter 2.

A substantial proportion of patients will not tolerate clozapine, or find it sufficiently efficacious, leading to approximately 40% discontinuing within 24 months of initiation, which carries a poor prognosis. The most common reasons for clozapine discontinuation identified in previous studies are *ADRs*, *patient decision* and *non-adherence*. An older age at clozapine initiation, Black African/Caribbean ethnicity and substance abuse have been found to increase risk of clozapine discontinuation. However, the majority of previous studies have not been conducted in patients receiving their first trial of clozapine and thus the identified reasons for discontinuing may have been biased by previous clozapine trials. A cohort of patients initiating clozapine would be the most clinically informative study design to examine the reasons and timing of clozapine discontinuation. Furthermore, although non-adherence and patient decision have been identified as major reasons for discontinuation of clozapine, there has been no exploration of reasons behind this choice. Lastly, only one study has been conducted in the UK (Taylor et al., 2009), and given the differences in clozapine utilisation across health care systems, there may be limited generalisability of other studies to UK patients. I seek to address these limitations in Chapter 4.

Despite a significant number of studies, there is a lack of consistent findings regarding clinical response to clozapine. The somewhat conflicting results of clinical and demographic predictors may be due to the considerable variability in response definitions as well as trial durations, resulting in limited comparability between studies. Study durations tend to be short and consequently may not consider a group of patients that take longer to meet the response criteria, but are nonetheless responders (Meltzer et al., 1989a). Most studies are conducted in relatively small sample sizes and clinical trial data may not generalise to patients receiving clozapine in standard health care settings. Chapter 5 aims to address these limitations.

1.9. Aims and objectives

The aim of this thesis is to investigate and gain novel insights of treatment response and the adverse effects of clozapine. To achieve this aim I used a combination of genetic and epidemiological approaches. My three primary objectives were:

1. **Identify genetic risk variants associated with clozapine-associated neutropenia and agranulocytosis (Chapter 2).** To examine genetic associations with clozapine-associated neutropenia, I conducted analyses incorporating GWAS, HLA allele imputation and exome array variation in an independent, homogenous, UK sample of 66 clozapine-associated neutropenia cases and 5583 clozapine-treated controls. I then combined associated variants from these analyses in a joint meta-analysis with data from the CIAC study (Goldstein et al., 2014), giving the largest combined study sample of its kind to date.
2. **Examine the risk factors, reasons and timing of clozapine discontinuation (Chapter 4).** I aimed to assess the reasons for discontinuation of clozapine,

investigate the timing of these reasons, determine which ADRs lead to discontinuation, and characterise the patients who are at increased risk of discontinuing clozapine. For each of these aims, I also explored the differences between discontinuations resulting from a clinician-led decision and those from a patient decision. To achieve these aims I identified a two-year retrospective cohort of all patients starting their first clozapine trial over a five-year period (2007-2011, inclusive) in South London & Maudsley (SLaM) NHS Foundation Trust.

3. **Identify demographic and clinical predictors of clozapine response (Chapter 5).** I aimed to identify demographic and baseline clinical predictors of clozapine response, assess whether early improvement is indicative of long-term response, and determine the duration of treatment required to detect a response. To achieve these aims I retrospectively administered the Clinical Global Impressions (CGI) scale to case notes at the start of clozapine treatment and after 1, 2, 3, 6, 9, 12, 18 and 24 months of treatment to the two-year cohort study of patients with TRS receiving their first course of clozapine also used for Objective 2 (Chapter 4).

Chapter 2

Clozapine-associated Neutropenia

2.1. Summary

The antipsychotic clozapine is uniquely effective in the management of treatment-resistant schizophrenia, but its use is limited by its potential to induce agranulocytosis. The causes of this, and of its precursor neutropenia, are largely unknown although genetic factors are implicated. I sought risk alleles for clozapine-associated neutropenia in a sample of 66 cases and 5583 clozapine-treated controls who have never developed neutropenia, through a genome-wide association study (GWAS), imputed HLA alleles and exome array analysis. I then combined associated variants in a meta-analysis with data from the Clozapine-Induced Agranulocytosis Consortium (up to 163 cases and 7970 controls). The GWAS meta-analysis identified a novel genome-wide significant association with clozapine-associated neutropenia and rs149104283 (OR = 4.32, P = 1.79×10^{-8}), intronic to transcripts of *SLCO1B3* and *SLCO1B7*, members of a family of hepatic transporter genes involved in drug uptake. This group of genes has been previously implicated in adverse drug reactions, most prominently simvastatin-induced myopathy and also docetaxel-induced neutropenia. Exome array analysis provided evidence for gene-wide association between non-synonymous variants within *UBAP2* and *STARD9*. In addition, a previously reported association between neutropenia and a variant at *HLA-DQB1* was replicated in a subset of 61 clozapine-associated neutropenia cases and 305 clozapine-treated controls (OR = 15.6, P = 0.015, positive predictive value = 35.1%). These findings implicate biological pathways through which clozapine may act to cause this serious adverse effect.

2.2. Introduction

Clozapine is the only licensed medication for treatment-resistant schizophrenia (TRS), defined as a failure to respond to at least two antipsychotic trials of sufficient dose and duration. Although it is the only treatment with proven efficacy in this severely impaired group of patients, (Kane et al., 1988; Leucht et al., 2009) it is substantially under-prescribed (Royal College of Psychiatrists, 2012) due, at least in part, to the risk of haematological side effects of agranulocytosis and neutropenia (i.e., reductions of neutrophils to levels below 500/mm^3 or 1500/mm^3 respectively). The cumulative risk of agranulocytosis in those taking clozapine is 0.8% and for neutropenia is 2.9% (Atkin et al., 1996). The peak incidence for clozapine-induced blood disorders is in the first 6-18 weeks of treatment (Atkin et al., 1996; Munro et al., 1999). If undetected, compromised immune function secondary to agranulocytosis can be fatal, as happened in a series of patients when the drug was introduced in the 1970s, leading to its widespread withdrawal. Evidence of its marked effectiveness over other antipsychotics led to Federal Drug Agency (USA) approval in 1989 with stipulations about the need for regular blood monitoring to aid early detection of blood abnormalities. The monitoring system has been successful in reducing the prevalence of agranulocytosis and in the UK the associated fatality rates are now very low, estimated between 0.016% and 0.03% of patients treated with clozapine (Atkin et al., 1996; Munro et al., 1999). However, the requirement for blood monitoring limits the acceptability of the drug to patients, and poses an obstacle to its use in clinical practice (Patel, 2012).

The aetiology of clozapine-induced blood disorders is currently unknown. One of the best-supported hypotheses relates to the bioactivation of clozapine or a stable metabolite of clozapine to a chemically reactive nitrenium ion (Liu & Uetrecht, 1995; Maggs et al., 1995;

Pirmohamed & Park, 1997), which has been shown to cause dose-dependent apoptosis to neutrophils at therapeutic levels of clozapine (Williams et al., 2000; Pessina et al., 2006) as well as toxicity to stromal cells, the precursors of neutrophils in bone marrow (Pereira & Dean, 2006). The reason why only 0.8% of individuals treated with clozapine are affected by agranulocytosis has not been fully elucidated, though genetic causes contribute.

Until recently, genetic studies of clozapine-induced agranulocytosis and neutropenia have largely focused on candidate genes from the Human Leukocyte Antigen (HLA) region involved in immune response (Opgen-Rhein & Dettling, 2008). Although there have been many candidate studies, the rare incidence of clozapine-induced agranulocytosis has limited the availability of suitable patients and thus studies have been underpowered (Zhang & Malhotra, 2013). Nonetheless, there have been significant recent advances. In 2011, Athanasiou and colleagues reported a replicated association between *HLA-DQB1* 6672G>C and clozapine-induced agranulocytosis (Athanasiou et al., 2011). The first genome-wide association study (GWAS) study conducted by the Clozapine-Induced Agranulocytosis Consortium (CIAC) has provided substantial evidence for the role of *HLA-DQB1* and *HLA-B* (Goldstein et al., 2014). However, the sample used in CIAC is not independent of earlier studies. A small exome sequencing study did not identify any rare, functional variants that were significantly associated with clozapine-induced neutropenia (Tiwari et al., 2014).

2.2.1 Aims of the study

The aim of the study was to examine the genetic susceptibility to clozapine-associated agranulocytosis and neutropenia. The first objective was to conduct analyses incorporating GWAS, imputed classical *HLA* alleles and amino acid polymorphisms, and exome array in an independent UK sample. The second objective was to combine associated variants from

these analyses in a joint meta-analysis with the CIAC study (Goldstein et al., 2014), giving the largest combined study sample of its kind to date.

2.3. Method

2.3.1 Sample description

Study individuals were from CLOZUK (N=5493) and CardiffCOGS (Cognition in Schizophrenia, N=156) samples. All had a clinical or research diagnosis of schizophrenia. CLOZUK is comprised of individuals who were prescribed clozapine in the UK and have a clinical diagnosis of TRS (Hamshere et al., 2013; Rees et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The CLOZUK study individuals were acquired cross-sectionally over a period of three months in collaboration with Novartis Pharmaceuticals, one of the drug companies that provide clozapine (Clozaril®), in accordance with relevant ethics permissions and the UK Human Tissue Act. All individuals were anonymised and only basic demographic details of age, gender, ethnicity and diagnosis were provided. Twelve months after sample acquisition, the research team were informed of those that had developed neutropenia whilst taking clozapine and where available, the recorded lowest neutrophil counts of these individuals were supplied.

CardiffCOGS is a schizophrenia sample recruited from secondary mental health services in South Wales, UK (Carroll et al., 2011; Rees et al., 2014). As part of a comprehensive clinical interview, individuals were asked about lifetime clozapine use and occurrence of neutropenia. Clinical case notes were used to confirm neutropenia status and lowest recorded neutrophil levels were collected. Further detailed information of both samples and their ascertainment are described in an open access publication (Rees et al., 2014).

Clozapine-associated neutropenia cases (N=66, 58 from CLOZUK, 8 from CardiffCOGS) developed an absolute neutrophil count (ANC) ≤ 1500/mm^3 during treatment with clozapine. Following the approach of recent studies, (Goldstein et al., 2014; Tiwari et al., 2014) we assessed cases with agranulocytosis and neutropenia because the success of the monitoring system and pre-emptive drug withdrawal in the UK has made agranulocytosis extremely rare. This neutrophil count threshold is used in the UK as a trigger to discontinue clozapine. Controls (N=5583, 5435 CLOZUK, 148 CardiffCOGS) had received clozapine for a minimum of a year without developing an ANC ≤ 2000/mm^3. Those who had a test result (1500/mm^3 < ANC ≤ 2000/mm^3) were excluded from all analyses (n=20). No differences in age or sex were observed between clozapine-associated neutropenia cases and controls (**Table 2.1**). A chi-squared test demonstrated no evidence of difference in gender between controls and clozapine-associated neutropenia cases (X^2 = 1.59, P = 0.208). A Mann-Whitney-Wilcoxon test (as age was not normally distributed) showed no differences in age between controls and clozapine-associated neutropenia cases (P = 0.408). All individuals were of European ancestry, as determined by self-report and principal component analysis of GWAS data.

	Male gender N (%)	**Mean age (range)**
Controls (N=5583)	3992 (71.5)	41.9 (16-90)
Clozapine-associated neutropenia cases (N=66)	40 (60.6)	40.3 (20-64)

Table 2.1. *Sample characteristics. Gender and age of clozapine-associated neutropenia cases and clozapine-treated controls.*

2.3.2 Genotyping

Genotyping was performed at the Broad Institute, Cambridge, MA, USA. CardiffCOGS and part of the CLOZUK sample (40 cases and 3573 controls) were genotyped on Illumina

HumanOmniExpressExome-8v1 and the remainder of the CLOZUK sample (26 cases and 2098 controls) were genotyped on both Illumina HumanOmniExpress-12v1 and Illumina HumanExome BeadChip.

2.3.3 Genome-wide association study

Genotyping arrays provide an informative backbone of tag single nucleotide polymorphisms (SNPs) thus making large-scale whole-genome genotyping affordable. Quality control procedures and imputation were conducted at the Broad Institute using the Psychiatric Genomics Consortium (PGC) pipeline (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) and detailed in Appendix 1. Imputation was performed using IMPUTE2 (Howie et al., 2009) and a reference panel from the full 1000 Genomes Project dataset (freeze date August 2012). Imputation was conducted on a combined dataset of the two arrays.

I conducted principal component estimation using EIGENSTRAT (Price et al., 2006) to exclude outliers and assess population stratification (**Figure 2.1** and **Figure 2.2**). I included genotyping array as well as the first three principal components as covariates to account for population structure. Sensitivity analyses increasing the number of principal components included to either 5 or 10 did not alter the results or improve statistical inflation values (lambda or QQ plot inspection). SNPs with allele frequencies that differed between genotyping arrays at $P < 1 \times 10^{-5}$ were excluded (**Figure 2.3**). These 124 SNPs were imputed but confirmed to be non-ambiguous. Common SNPs with high imputation quality were selected for analysis (INFO > 0.8, MAF > 0.01 in cases and controls). Association analysis was performed using logistic regression in PLINK (Purcell et al., 2007) and SNPs functionally annotated using the Scripps genome advisor (Erikson et al., 2014). PLINK was used to identify index SNPs in relative linkage equilibrium (--clump-p1 0.0001 --

clump-p2 0.0001 --clump-r2 0.1 --clump-kb 3000) and the SNP with the highest association was selected as the index SNP. A genome-wide significance level (GWS) of $P < 5 \times 10^{-8}$ was used to determine significance, which corresponds to a Bonferroni multiple testing correction for 1 million SNPs in linkage equilibrium.

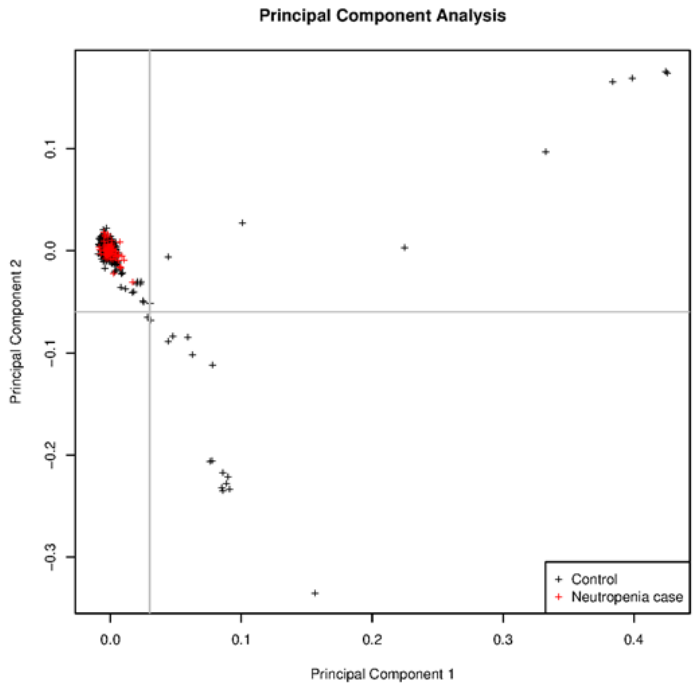

Figure 2.1. *Principal component analysis. Figure displays principal component 1 and 2. Points represent individual samples; black points represent clozapine-treated controls and red points represent neutropenia cases. Grey lines represent exclusions (top left corner included).*

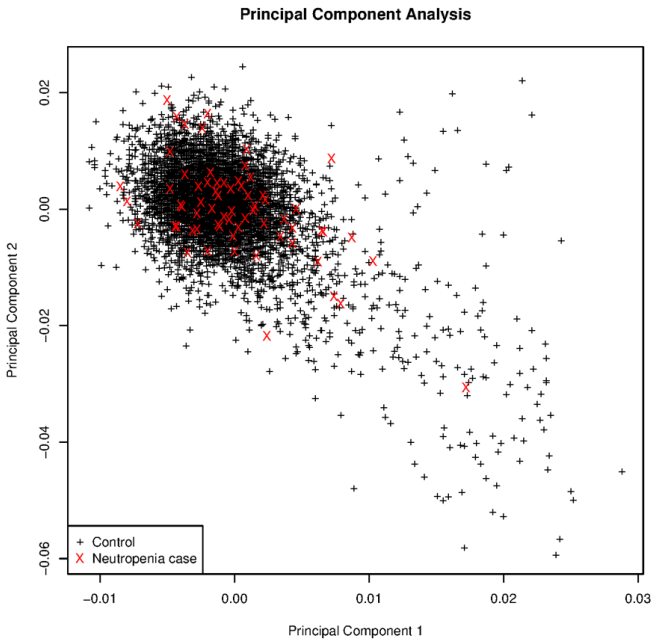

Figure 2.2. *Principal component analysis of included samples. Figure displays principal component 1 and 2. Points represent individual samples included in GWAS analysis; black points represent clozapine-treated controls and red points represent neutropenia cases.*

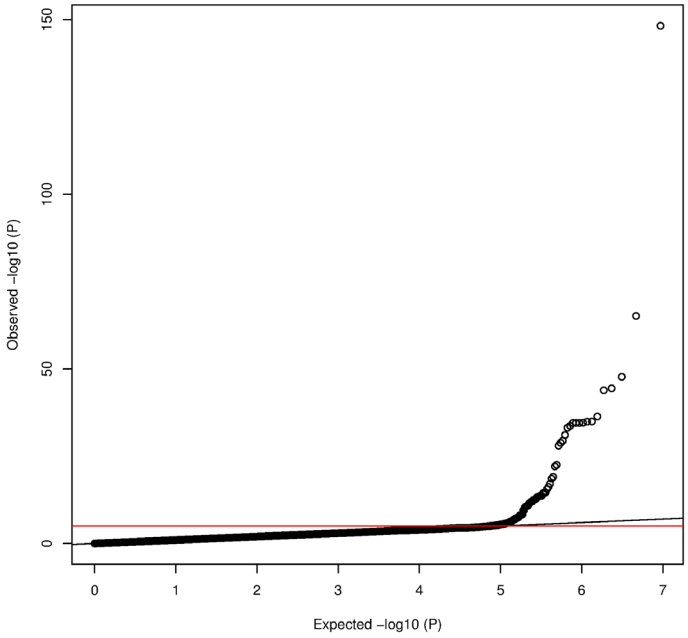

Figure 2.3. *SNP differences by genotyping array. QQ plot of GWAS comparing SNPs genotyped on HumanOmniExpressExome-8v1 and Illumina HumanOmniExpress-12v1. Logistic regression was conducted in PLINK to detect SNPS that performed differently between genotyping arrays. 124 SNPs with $P < 1 \times 10^{-5}$, represented by the red line, were excluded from GWAS analyses.*

2.3.4 HLA analysis

Classical *HLA* alleles and amino acid polymorphisms were imputed using SNP2HLA version 1.02 (Jia et al., 2013) using BEAGLE version 3.0.4 (Browning & Browning, 2007) from genotyped common variants using a reference dataset of 5225 individuals from the Type 1 Diabetes Genetics Consortium (T1DGC). I used the same procedures for SNP selection, analysis and covariate selection (three PCAs derived from GWAS, plus genotyping array) as described for the GWAS analysis above. Due to complex and extended linkage disequilibrium in the MHC, I did not identify index SNPs in relative linkage equilibrium.

We additionally genotyped a candidate SNP, *HLA-DQB1* 6672G>C (Athanasiou et al., 2011) (rs113332494), in 60 cases and 305 age and sex matched controls. This SNP was genotyped separately as it was not imputed with sufficient quality to be reported in the GWAS or HLA analyses, and was a strong candidate variant (Athanasiou et al., 2011). Genotyping was conducted at deCODE genetics using the Centaurus (Nanogen) platform (Kutyavin et al., 2006). Association with clozapine-associated neutropenia was tested using Fisher's exact test given low minor allele counts but to ensure there were no effect of population stratification, I also conducted a logistic regression including three PCAs derived from GWAS with 5×10^8 permutations to generate empirical p-values.

2.3.5 Exome array

The Illumina exome array is designed to genotype uncommon-to-rare coding variants previously observed in whole exome sequencing studies. Exome array data were available for 57 cases and 4958 controls. Complex quality control procedures were conducted as part of a schizophrenia case control analysis and are detailed in Appendix 2. I conducted principal component analysis using EIGENSTRAT (Price et al., 2006) with 14,743 common exome array variants in relative linkage equilibrium (MAF ≥ 0.05, $r^2 < 0.2$) to assess

population structure and identify outliers (**Figure 2.4**). Due to the relatively small case sample size, I did not apply a frequency filter to variants in this analysis.

Single variant association was conducted using logistic regression in PLINK with the first 10 principal components included as covariates. Adaptive permutations (between 10 and 1 x 10^9) were used to generate empirical p-values in logistic regression analyses due to inflated logistic regression statistics at low allele frequencies in comparison to a Fisher's Exact test or permuted p-values. PLINK was used to identify index SNPs in relative linkage equilibrium (--clump-p1 0.01 --clump-p2 0.01 --clump-r2 0.1 --clump-kb 3000). The SNP with the lowest permuted p-value (strongest association) was selected as the index SNP. Due to reduced reliability of genotype calling for rare variants (Goldstein et al., 2012), all exome array variants noted in this paper were subject to visual inspection of cluster plots.

To test for the effects of multiple functional variants in genes, I used SKAT-O (Lee et al., 2012a) with 2 x 10^6 permutations, including the first 10 principal components, for genes with at least two uncommon (MAF < 0.05), non-synonymous (missense, stop or splice) variants. The SKATBinary function within SKAT-O was utilised. Variants were allocated to genes according the RefSeq database. SKAT-O computes the association of a SNP set (for example a gene) with a phenotype of interest, and maximises power by adaptively using the data to optimally combine the burden test and the nonburden sequence kernel association test (SKAT) (Lee et al., 2012a). The burden tests assume that all variants are causal and affect the phenotype in the same way whereas the nonburden tests allows for protective, neutral and risk variant effects.

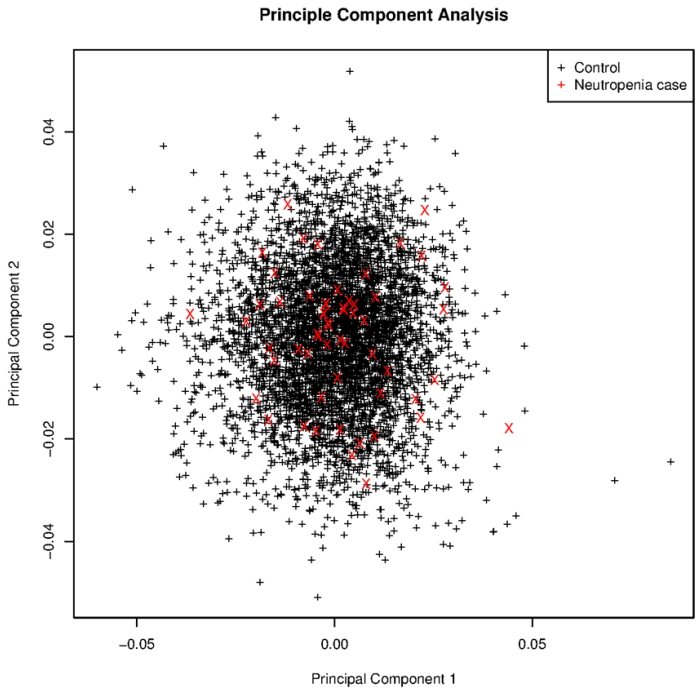

Figure 2.4. *Principal component analysis of exome array samples. Figure displays principal component 1 and 2 for each individual included in exome array analysis; black points represent clozapine-treated controls and red points represent clozapine-associated neutropenia cases.*

2.3.1 Secondary analysis of neutropenia below ≤ 1000/mm^3

Given that within the field there are valid concerns that a more stringent threshold may produce more reliable results, we additionally conducted secondary analyses on a subset of the more severely affected cases with ANC ≤ 1000/mm^3. We assessed the association of single variants with clozapine-associated neutropenia below ≤ 1000/mm^3 in GWAS (N=18), HLA imputation (N=18), and exome array (N=16) analyses. All analyses conducted were consistent with methods used for clozapine-associated neutropenia as described above.

2.3.1 Replication sample and analysis

We obtained summary statistics for associated SNPs from a recently published study by the CIAC (Goldstein et al., 2014), a comprehensive genetic association study in 163 clozapine-induced neutropenia cases (98 with ANC < 500 mm^3, 61 with 500 ≤ ANC ≤ 1000

mm^3 and 4 with ANC ≤ 1500/mm^3). The CIAC study included: GWAS (161 cases with clozapine-induced neutropenia, 249 clozapine exposed controls without neutropenia and 947 unexposed controls), *HLA* allele imputation (162 cases and 4319 unexposed controls), and exome array analysis (148 cases and up to 7970 unexposed controls). I combined the replication sample with both (i) clozapine-associated neutropenia and (ii) neutropenia ≤ 1000/mm^3 analyses. SNPs that were associated with clozapine-associated neutropenia at $P < 1 \times 10^{-4}$ from the GWAS, or $P < 0.05$ from the *HLA* variant analysis, were combined with the replication data in fixed-effects meta-analyses using PLINK to estimate a combined odds ratio weighted by the study's inverse standard error. If an index SNP was not present in replication data for GWAS, a proxy SNP in strong LD ($r^2 \geq 0.8$) was substituted and the s.e. weighted (s.e.w) to account for the lack of information: s.e.w=s.e./sqrt(r^2) (Green et al., 2013). The variants that were associated with clozapine-associated neutropenia from exome array analyses with $P < 0.01$ were combined with the replication data in a p-value based method in METAL (Willer et al., 2010), weighted by the square root of the total sample size. Due to differing analytical methods used by CIAC, it was not possible to combine our gene-based results in a joint analysis. CIAC used a Fisher's exact test to assess the number of carriers of a functional variant whereas SKAT-O assesses the frequency of variants. We used different p-value replication thresholds for the *HLA* and exome array to arrive at approximately the same number of variants.

2.4. Results

Figure 2.5 provides a summary of the study design and key results from the primary analysis assessing genetic susceptibility to clozapine-associated neutropenia.

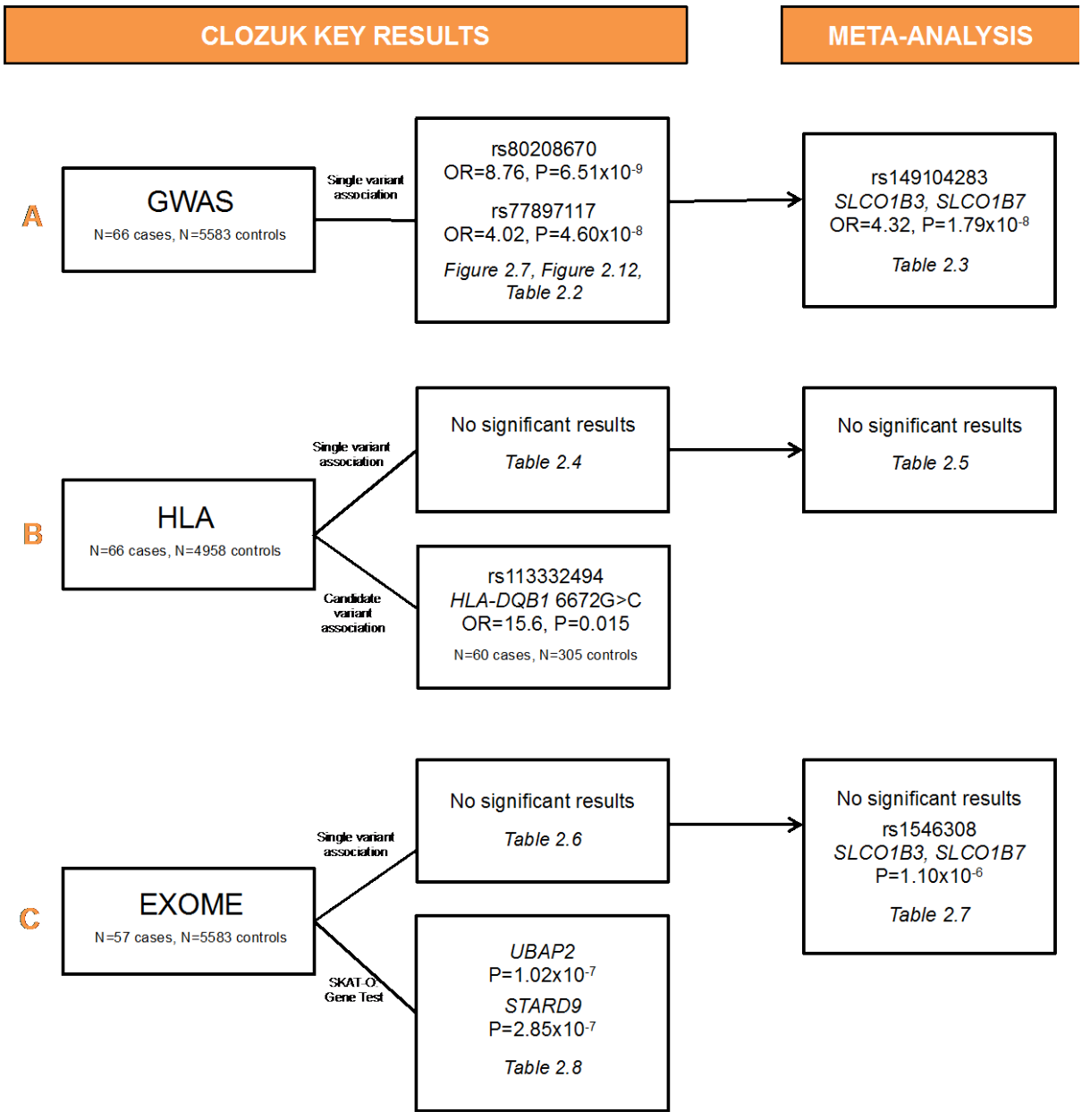

Figure 2.5. *Study design and key results. To investigate the association of genetic variants with clozapine-associated neutropenia, we conducted (**A**) genome-wide association study, (**B**) Human Leukocyte Antigen (HLA) allele imputation and genotyped a candidate variant of interest, HLA-DQB1 6672G>C/ rs113332494, and (**C**) exome array single variant and gene-based analysis. We then took forward the associated variants from GWAS, HLA and exome array analyses to a combined meta-analysis with the Clozapine-Induced Agranulocytosis Consortium (CIAC) study.*

2.4.1 GWAS

Discovery analysis

We performed a genome-wide association study of 7,559,010 genotyped and imputed common SNPs (QQ plot in **Figure 2.6**, λ_{GC} = 0.95). Two SNPs were associated with clozapine-associated neutropenia at the genome wide significance (GWS) level of P < 5 x 10^{-8} (Manhattan Plot in **Figure 2.7**). rs80208670 on chromosome 13 (OR = 8.76, 95% CI: 4.21-18.25, P = 6.51 x 10^{-9}) is 13kb downstream of *SLITRK1* and was present in 7.35% of cases and 1.24% of controls. rs77897117 on chromosome 1 (OR = 4.02, 95% CI: 2.46-6.57, P = 4.60 x 10^{-8}) is an intergenic variant and was present in 16.39% of cases and 5.24% of controls. **Table 2.2** lists the 10 most strongly associated SNPs in relative linkage equilibrium from the CLOZUK analysis. Our sample size had 80% power to detect an odds ratio (OR) ≥ 4 for alleles with MAF ≥ 0.10 at GWS (**Figure 2.8**).

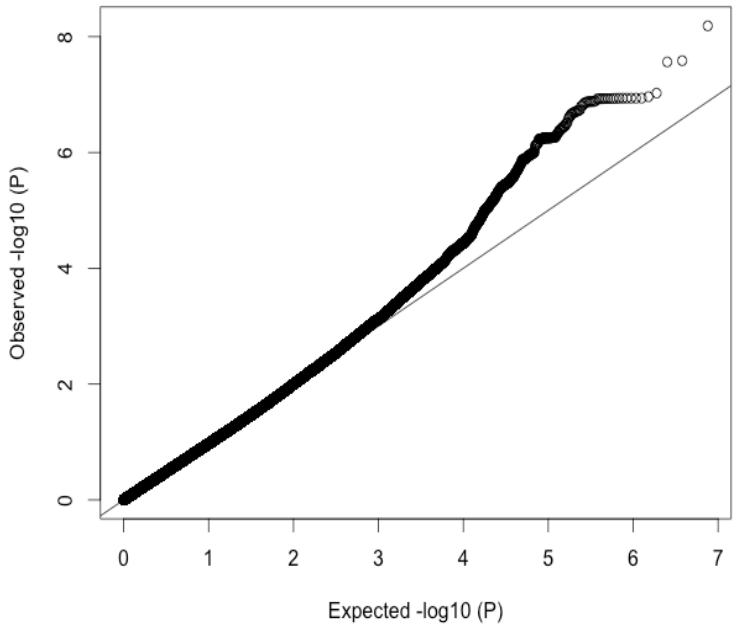

Figure 2.6. *QQ plot for clozapine-associated neutropenia GWAS. The –log10 observed logistic regression p-values (y-axis) are plotted against expected p-values (x-axis).* λ_{GC} *= 0.95.*

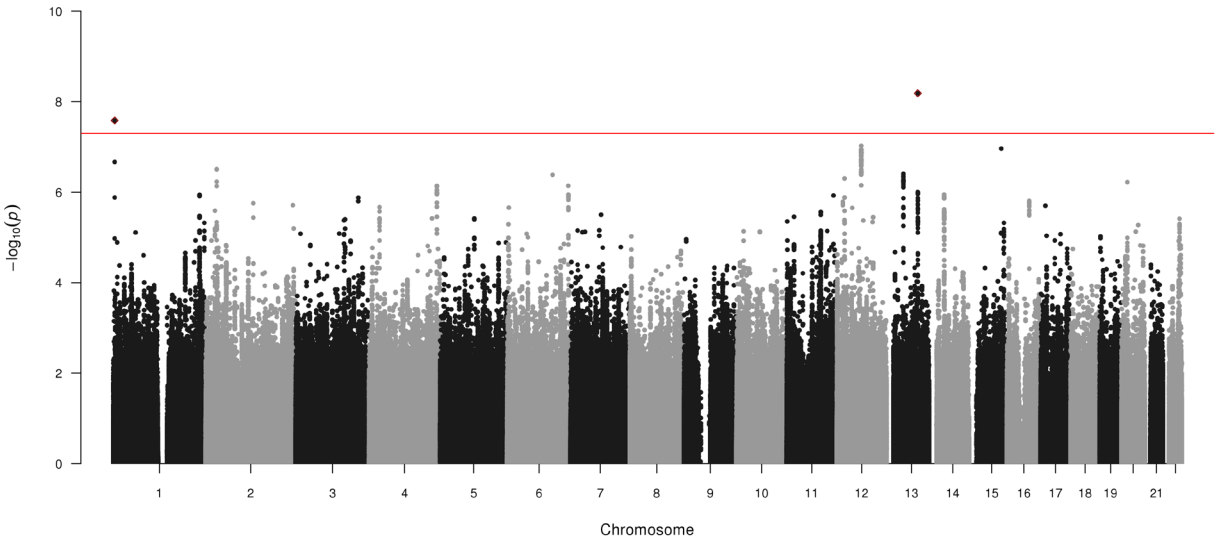

Figure 2.7. *Manhattan plot of clozapine-associated neutropenia GWAS. –log10 P-values (y-axis) for each SNP is presented on the basis of chromosomal position (x axis). The red line represents the genome wide significance level ($P < 5 \times 10^{-8}$).*

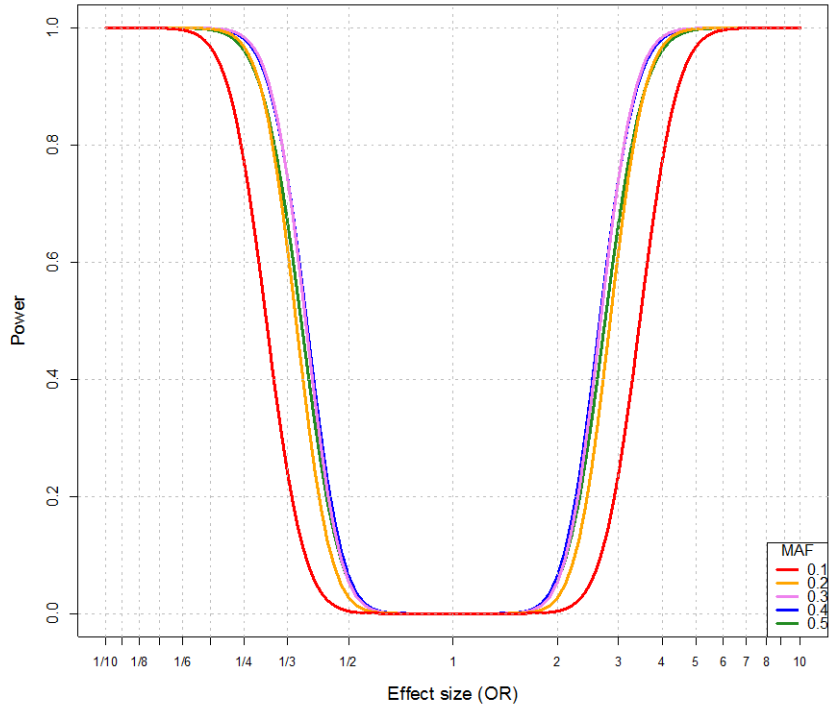

Figure 2.8. *Power calculation for clozapine-associated neutropenia GWAS. Power calculations derived from Quonto software (http://biostats.usc.edu/Quanto.html) using an additive disease model, 3% disease prevalence for 66 cases and 5583 controls (1:85 ratio) to detect GWS variant ($P = 5 \times 10^{-8}$) at minor allele frequencies of 0.1 (red), 0.2 (orange), 0.3 (pink), 0.4 (blue) and 0.5 (green) for effect sizes (OR) 1-10.*

Meta-analysis

In total, there were 266 relatively independent ($r^2 < 0.1$) SNPs associated with clozapine-associated neutropenia at $P < 1 \times 10^{-4}$ and I sought replication of these SNPs in the CIAC sample. Data was available for 256 SNPs and a proxy identified for a further SNP. rs116552069 was used as a proxy for rs16216021 ($r^2 = 0.96$, D'= 1, Appendix 3). **Table 2.2** details the association in CIAC for the 10 most strongly associated SNPs in the CLOZUK analysis. The variants that were GWS in the CLOZUK analysis, rs80208670 and rs77897117, were not significantly associated in CIAC (OR = 1.69, P = 0.27 and OR = 0.67, P = 0.28, respectively).

Table 2.3 lists the 10 most strongly associated SNPs from the meta-analysis. One SNP on chromosome 12 surpassed the GWS threshold for association with clozapine-associated neutropenia (OR = 4.32, $P = 1.79 \times 10^{-8}$). rs149104283 is intronic to transcripts of *SLCO1B3* and *SLCO1B7* (solute carrier organic anion transporter family, member 1B3 and member 1B7) and was present in 7.37% of cases vs. 1.52% of controls in our sample and 4.20% of cases vs. 1.67% of controls in the CIAC sample.

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Gene**	**Location**
13	rs80208670	84438088	C	6.51×10^{-9}	8.76	7.38	1.24	0.2728	1.69	2.32	1.37	1.56×10^{-7}	4.69	*SLITRK1*	13kb downstream
1	rs77897177	4362747	C	2.60×10^{-8}	4.02	16.39	5.24	0.2796	0.67	3.86	4.83	5.74×10^{-5}	2.31		Intergenic
12	rs114615084	65752916	T	9.44×10^{-8}	6.73	6.82	1.00	0.5116	0.65	0.91	1.24	1.36×10^{-5}	3.90	*MSRB3*	Intronic
15	rs77051678	86642215	T	1.09×10^{-7}	8.16	6.37	1.11	0.4718	0.64	1.10	1.51	3.86×10^{-5}	3.97		Intergenic
2	rs4464232	30007327	C	3.08×10^{-7}	3.89	12.88	3.60	0.4377	0.74	2.90	3.32	1.69×10^{-4}	2.27	*ALK*	Intronic
13	rs73181572	45617497	A	3.89×10^{-7}	5.02	8.81	1.91	0.1272	0.45	1.56	2.60	4.26×10^{-4}	2.60	*KIAA1704*	9kb downstream
6	rs117655439	122314323	C	4.15×10^{-7}	5.90	8.32	1.91	0.8544	1.09	1.79	1.67	2.49×10^{-5}	3.32		Intergenic
12	rs149104283	21083862	T	4.98×10^{-7}	6.20	7.37	1.52	3.61×10^{-3}	2.95	4.20	1.67	1.79×10^{-8}	4.32	*SLCO1B3, SLCO1B7*	Intronic, intronic
20	rs6135490	15721572	G	5.98×10^{-7}	3.39	17.14	6.11	0.7832	1.07	8.81	7.13	2.40×10^{-4}	1.87	*MACROD2*	Intronic
6	rs117329068	164581056	T	7.22×10^{-7}	7.32	5.95	1.10	0.8599	0.87	0.80	1.04	1.61×10^{-5}	4.65		Intergenic

Table 2.2. *Top 10 independent SNPs from discovery (CLOZUK) GWAS analysis. Results are ordered by CLOZUK analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency in controls (MAF U) for CLOZUK and CIAC sample, p-value and odds ratio (OR) for meta-analysis, gene and location to/in gene.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Nearest Gene**	**Location**
12	rs149104283	21083862	T	4.98×10^{-7}	6.20	7.37	1.52	3.61×10^{-3}	2.95	4.20	1.67	1.79×10^{-8}	4.32	*SLCO1B3, SLCO1B7*	Intronic, intronic
13	rs80208670	84438088	C	6.51×10^{-9}	8.76	7.38	1.24	0.2728	1.69	2.32	1.37	1.56×10^{-7}	4.69	*SLITRK1*	13kb downstream
1	rs184597564	82236406	A	2.48×10^{-5}	4.03	8.99	2.82	4.99×10^{-3}	2.40	5.18	2.52	8.01×10^{-7}	3.06	*ADGRL2*	Intronic
7	rs78900159	76968378	A	6.94×10^{-6}	4.01	9.12	2.44	0.0247	1.97	5.30	2.67	2.02×10^{-6}	2.79	*GSAP*	Intronic
10	rs16916041	63146547	T	7.40×10^{-6}	2.57	21.97	9.73	0.0205	1.58	13.29	9.23	2.05×10^{-6}	1.98	*TMEM26*	20kb downstream
16	rs11649311	25226020	T	9.70×10^{-5}	2.07	49.68	34.13	2.62×10^{-3}	1.51	42.33	34.33	2.26×10^{-6}	1.68	*AQP8*	2kb upstream
17	rs117202297	53769035	T	8.48×10^{-6}	6.25	5.60	1.22	0.1503	2.66	1.51	0.65	5.27×10^{-6}	4.97	*TMEM100*	28kb downstream
17	rs80282661	13252073	T	1.99×10^{-6}	6.15	6.29	1.21	0.2632	1.85	1.45	1.00	5.49×10^{-6}	4.17	*HS3ST3A1*	147kb downstream
1	rs185053659	60704250	A	7.76×10^{-6}	5.80	6.33	1.42	0.1012	2.13	2.26	1.33	8.05×10^{-6}	3.80	*C1orf87*	165kb upstream
14	rs78074145	40404458	C	1.13×10^{-6}	4.05	11.43	3.29	0.2716	1.44	3.88	3.34	1.08×10^{-5}	2.60	*FXB033*	503kb upstream

Table 2.3. *Top 10 SNPs from GWAS meta-analysis. Results are ordered by meta-analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency in controls (MAF U) for CLOZUK and CIAC sample, p-value and odds ratio (OR) for meta-analysis, name of nearest gene and location to/in gene.*

2.4.2 HLA analysis

Discovery analysis

We performed an association analysis of 7,751 imputed classical *HLA* alleles and amino acid polymorphisms (QQ plot in **Figure 2.9**, λ_{GC} = 0.94). No polymorphism was associated with clozapine-associated neutropenia at GWS ($P < 5 \times 10^{-8}$). **Table 2.4** lists the 10 most strongly associated SNPs from the discovery analysis. The most significant variant was intronic to *HLA-F-AS1* (OR = 0.57, P = 0.0033) and present in 31.06% of cases and 44.13% of controls.

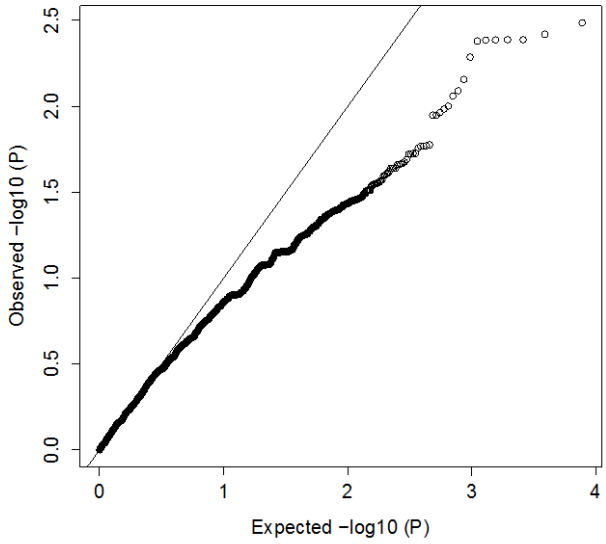

Figure 2.9. *QQ plot for clozapine-associated neutropenia imputed HLA analysis.* λ_{GC} *= 0.94.*

Meta-analysis

Data was available within CIAC for 102 of the 139 *HLA* variants associated with clozapine-associated neutropenia at P < 0.05. No imputed classical *HLA* allele or amino acid polymorphism was associated with clozapine-associated neutropenia at GWS in the combined meta-analysis. **Table 2.5** lists the 10 most strongly associated SNPs from the meta-analysis.

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Gene**	**Location**
6	rs2743951	29709234	A	0.003265	0.57	31.06	44.13	0.4443	1.09	45.99	44.01	0.3870	0.92	*HLA-F-AS1*	Intronic
6	rs1737069	29730730	A	0.003814	1.67	38.64	27.14	0.1476	0.83	24.07	27.60	0.5720	1.06		Intergenic
6	rs2394160	29703262	G	0.004095	0.58	31.06	43.78	0.5795	1.07	45.07	43.66	0.3121	0.91	*HLA-F, HLA-F-AS1*	Intronic
6	rs2523393	29705659	C	0.004095	0.58	31.06	43.78	0.5841	1.06	45.06	43.67	0.3101	0.91	*HLA-F, HLA-F-AS1*	Intronic
6	rs2523388	29707704	T	0.004111	0.58	31.06	43.78	0.5771	1.07	45.07	43.65	0.3144	0.91	*HLA-F-AS1*	Intronic
6	rs2523395	29702510	T	0.004122	0.58	31.06	43.78	0.5767	1.07	45.06	43.64	0.3145	0.91	*HLA-F, HLA-F-AS1*	Intronic
6	rs2735052	29701564	T	0.004176	0.58	31.06	43.76	0.5795	1.07	45.06	43.66	0.3146	0.91	*HLA-F, HLA-F-AS1*	Intronic
6	rs9258266	29725452	T	0.005186	2.50	7.58	3.10	-	-	-	-	-	-	*IFITM4P, HLA-F-AS1*	7kb/9kb upstream
6	rs2070600	32151443	A	0.006986	2.02	12.88	6.85	-	-	-	-	-	-	*AGER*	Missense
6	rs9258260	29723161	T	0.008124	1.86	16.67	9.65	0.1109	0.69	6.48	9.44	0.4705	1.13	*IFITM4P, HLA-F-AS1*	4kb/6kb upstream

Table 2.4. *Top 10 imputed HLA variants from CLOZUK analysis. Results are ordered by CLOZUK analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency in controls (MAF U) for CLOZUK and CIAC sample, p-value and odds ratio (OR) for meta-analysis, gene and location to gene.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Nearest Gene**	**Location**
6	rs1150753	32059867	C	0.0255	1.70	15.91	10.08	0.0448	1.41	12.98	10.1	0.0033	1.50	*TNXB*	Intronic
6	rs1269852	32080191	G	0.0252	1.70	15.91	10.08	0.0453	1.41	12.93	10.04	0.0033	1.51	*TNXB, ATF6B*	Intronic
6	rs3130288	32096001	A	0.0248	1.70	15.91	10.06	0.0480	1.41	12.93	10.06	0.0035	1.50	*ATF6B*	1kb ds
6	rs3131618	31434621	C	0.0425	1.60	16.67	11.16	0.0761	1.35	12.93	10.51	0.0085	1.44	*HCP5, PMSP, HCG26*	1kb ds, 1kb ds, 4kb ups
6	rs3131619	31434331	A	0.0471	1.59	16.67	11.25	0.1084	1.31	12.94	10.69	0.0135	1.41	*HCP5, PMSP, HCG26*	1kb ds, 1kb ds, 4kb ups
6	rs3094605	31430694	G	0.0468	1.59	16.67	11.24	0.1095	1.31	12.96	10.72	0.0136	1.40	*HCP5, PMSP, HCG26*	1kb ds, 1kb ds, 8kb ups
6	rs2734583	31505480	C	0.0469	1.58	16.67	11.2	0.1110	1.31	13.08	10.92	0.0137	1.40	*DDX39B*	Intronic
6	rs3094013	31434366	T	0.0471	1.59	16.67	11.25	0.1096	1.32	12.93	10.68	0.0137	1.41	*HCP5, PMSP, HCG26*	1kb ds, 1kb ds, 5kb ups
6	rs1150754	32050758	A	0.0276	1.60	21.21	14.5	0.2014	1.22	16.05	14.05	0.0194	1.35	*TNXB*	Intronic
6	rs1150755	32038550	A	0.0283	1.60	21.21	14.53	0.2024	1.22	16.07	14.1	0.0199	1.34	*TNXB*	Intronic

Table 2.5. *Top 10 variants from imputed HLA meta-analysis. Results are ordered by meta-analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency in controls (MAF U) for CLOZUK and CIAC sample, p-value and odds ratio (OR) for meta-analysis, name of nearest gene and location to gene.*

Genotyping of HLA-DQB1 6672G>C (rs113332494)

We additionally genotyped a candidate SNP, *HLA-DQB1* 6672G>C (rs113332494), in 60 cases and 305 age and sex matched controls as it was not imputed with sufficient quality. We found independent support for the association of rs113332494 (OR = 15.6, 95% CI: 1.6 - 151.4, P = 0.015), replicating previous reports of association with clozapine-induced agranulocytosis (Athanasiou et al., 2011). The association strengthened when considering only those with ANC below ≤ 1000/mm^3 (OR = 38.1, 95% CI: 3.4 – 430.9, P = 0.008). For the associated 'G' allele, there were three heterozygote carriers among 60 cases, and a single heterozygote carrier among 305 controls. Lowest neutrophil counts were available for two of the three 'G' case carriers, both of whom had a neutrophil level < 1000/mm^3 (700 and 900). The association was tested using a Fisher's exact test given the low minor allele counts but to ensure there were no effect of population stratification, I also conducted a logistic regression including three PCAs derived from GWAS, with 5 x 10^8 permutations to generate empirical p-values. After adjusting for GWAS PCAs, rs113332494 remained associated with clozapine-associated neutropenia (OR = 12.4, 95% CI: 1.12 - 137.4, P = 0.015) and neutropenia ≤ 1000/mm^3 (OR = 22.5, 95% CI: 1.01 - 502.4, P = 0.039).

Given the candidate design, the analyses of rs13332494 were limited to samples of European ancestry. However, if further population-based exclusions were applied, one of the heterozygote carriers would be classed as an outlier. When this individual is excluded from the analyses, the association attenuates to OR=10.5 (95% CI 0.94-116.7, P=0.070) for clozapine-associated neutropenia and OR=19.7 (95% CI 1.2-321.5, P=0.097) for neutropenia ≤ 1000/mm^3. A colleague, Antonio Pardinas, conducted admixture analyses to address the appropriateness of excluding this sample and wider population-based analyses relevant to this genetic variant and region. There was no evidence to suggest that past

admixture had caused a strong differentiation of the MHC region in this individual in comparison to other individuals with the same HLA genotype or to the wider population to which this individual belongs.

2.4.1 Exome array

Discovery analysis

I performed an association analysis of 115,029 variants genotyped on the exome array. No single variant exceeded a significance threshold of $P < 4.3 \times 10^{-7}$, corresponding to a Bonferroni correction for 115,000 variants tested (QQ plot displayed in **Figure 2.10**, λ_{GC} = 1.11). **Table 2.6** lists the 10 most strongly associated variants from the discovery analysis. The most significant variant was rs140003855, a missense variant in *NSUN2* ($P = 1.23 \times 10^{-5}$), present in 1.75% of cases and 0.01% of controls.

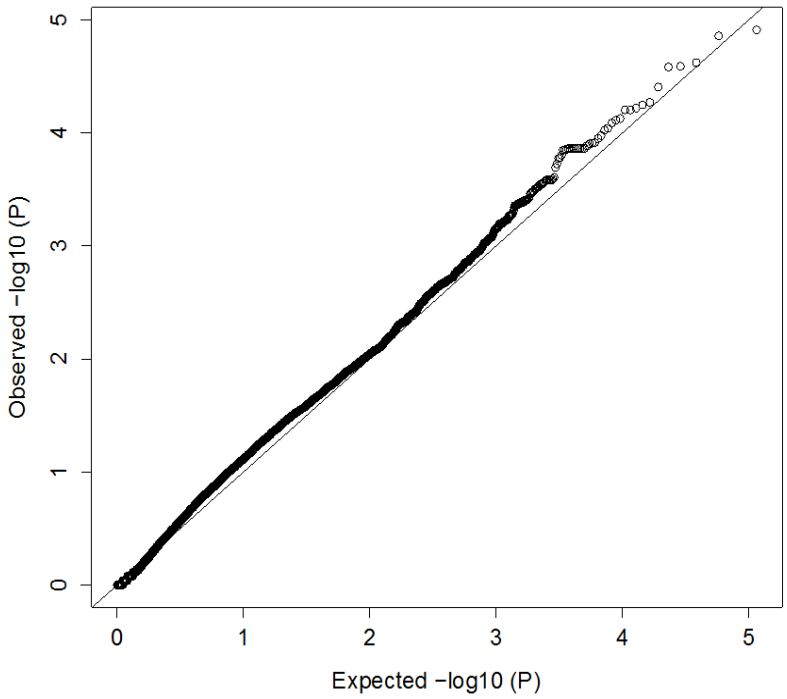

Figure 2.10. *QQ plot of exome array discovery analysis. The –log10 observed permuted logistic regression p-values (y-axis) are plotted against expected p-values (x-axis).* λ_{GC} *= 1.11.*

Meta-analysis

A total of 1138 independent variants ($r^2 < 0.1$) were associated with clozapine-associated neutropenia at $P < 0.01$ in the discovery analysis. Data was available for 1023 of these variants in CIAC. However, due to rarity of the variants in this analysis, only 279 variants had a computable odds ratio (OR) in both samples; it was common for the minor allele to be absent from either cases or controls. Although we used a p-value based method for the meta-analysis, the OR is required to determine the direction of effect. Thus, only these 279 variants could be included.

Table 2.7 lists the 10 most strongly associated variants from the exome array meta-analysis. No variant exceeded a significance threshold of $P < 4.3 \times 10^{-7}$. However, of interest is rs1546308 ($P = 1.10 \times 10^{-6}$), a missense variant in *SLCO1B7* and intronic to *SLCO1B3,* that is 92kb from the SNP that emerged from GWAS meta-analysis. rs1546308 is predicted to be benign using the SIFT algorithm (Kumar et al., 2009) and was present in 15.8% of cases vs. 5.8% of controls in our sample and 9.3% of cases vs. 5.6% of controls in the CIAC sample.

				CLOZUK				CIAC				Meta-analysis			
CHR	**Variant**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**Gene**	**Location**	**Function**
5	rs140003855	6620271	T	1.23×10^{-5}	287.7	1.75	0.01	1	0	0.00	0.00	-	*NSUN2*	Exonic	Missense
2	rs75073607	55408785	T	1.39×10^{-5}	16.53	4.39	0.31						*CLHC1*	Exonic	Missense
14	rs200759581	74062288	A	2.39×10^{-5}	92.6	1.75	0.03	1	0	0.00	0.06	-	*ACOT4*	Exonic	Stop gained
1	rs147055033	145562532	G	2.58×10^{-5}	7.235	7.02	1.14						*ANKRD35, NBPF10*	Exonic, intronic	Missense
9	rs189396476	123156869	C	2.61×10^{-5}	89.69	1.75	0.04	1	0	0.00	0.11	-	*CDK5RAP2*	Exonic	Missense
12	rs138912646	42711606	T	3.94×10^{-5}	9.279	5.26	0.54	0.1613	2.93	1.23	0.43	1.79×10^{-4}	*ZCRB1*	Exonic	Missense
6	rs141802559	118635315	A	5.37×10^{-5}	194.8	0.88	0.01	1	0	0.00	0.09	-	*SLC35F1*	Exonic	Missense
12	rs79149293	8975873	G	5.66×10^{-5}	11.33	4.39	0.46	0.0967	4.11	1.23	0.30	1.01×10^{-4}	*A2ML1*	Exonic	Missense
14	rs140098306	74973443	T	6.05×10^{-5}	194.1	0.88	0.01						*LTBP2*	Exonic	Missense
2	rs12233132	25328703	T	6.27×10^{-5}	2.143	41.23	25.53						*EFR3B*	Intronic	Intronic

Table 2.6. *Top 10 variants from exome array discovery (CLOZUK) analysis. Results are ordered by CLOZUK analysis P-value. Columns are: chromosome (CHR), variant ID (Variant), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency in controls (MAF U) for CLOZUK and CIAC sample, meta-analysis p-value, gene reference, location and function.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**Variant**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**Gene**	**Location**	**Function**
4	rs201099591	7436363	A	2.48×10^{-3}	67.45	0.88	0.02	1.31×10^{-4}	62.13	1.85	0.03	1.10×10^{-6}	*PSAPL1, SORCS2*	Exonic, Intronic	Missense[1]
7	rs17139320	63726370	G	3.96×10^{-3}	2.92	7.89	2.96	2.87×10^{-4}	3.214	7.41	2.67	3.70×10^{-6}	*ZNF679*	Exonic	Missense[1]
8	rs201071539	145003862	A	2.19×10^{-3}	-	0.88	0.00	1.69×10^{-3}	82.34	1.23	0.02	1.19×10^{-5}	*PLEC*	Exonic	Missense[1]
19	rs2591594	9076728	A	4.66×10^{-3}	0.51	19.30	31.91	3.26×10^{-3}	0.56	19.75	30.89	4.52×10^{-5}	*MUC16*	Exonic	Missense[2]
12	rs1546308	21176135	C	1.25×10^{-4}	3.00	15.79	5.83	0.06786	1.67	9.26	5.63	9.13×10^{-5}	*SLCO1B7, SLCO1B3*	Exonic, Intronic	Missense[1]
1	rs12073549	17720545	T	1.60×10^{-3}	2.02	23.68	13.68	0.01631	1.61	20.37	13.86	9.31×10^{-5}	*PADI6*	Exonic	Synonymous
15	rs117116488	89390513	T	2.76×10^{-4}	6.09	5.26	1.04	0.04477	3.18	2.47	0.79	9.73×10^{-5}	*ACAN*	Exonic	Missense[2]
12	rs79149293	8975873	G	5.66×10^{-5}	11.33	4.39	0.46	0.09674	4.11	1.23	0.30	1.01×10^{-4}	*A2ML1*	Exonic	Missense[3]
12	rs138912646	42711606	A	3.94×10^{-5}	9.28	5.26	0.54	0.1613	2.93	1.23	0.43	1.79×10^{-4}	*ZCRB1, PPHLN1*	Exonic, Intronic	Missense[1]
12	rs143584336	130921539	A	1.22×10^{-4}	24.33	2.68	0.13	0.1144	10.22	0.62	0.06	2.12×10^{-4}	*RIMBP2*	Exonic	Missense[1]

Table 2.7. *Top 10 variants from exome array meta-analysis. Results are ordered by meta-analysis P-value. Columns are: chromosome (CHR), variant ID (Variant), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency in controls (MAF U) for CLOZUK and CIAC sample, and meta-analysis p-value, gene reference, location and function. Predicted function of non-synonymous variants (Kumar et al., 2009): [1]benign, [2]possibly damaging, [3]probably damaging.*

Gene-based analysis

To assess the cumulative effects of rare functional variants in clozapine-associated neutropenia, I tested the association of 3,343 genes that had two or more uncommon (MAF ≤ 0.05), non-synonymous variants from the exome array using SKAT-O (QQ plot in **Figure 2.11**, λ_{GC} = 3.39). **Table 2.8** displays the 10 most significantly associated genes. There was evidence of association for two genes that exceeded a threshold of $P < 2.5 \times 10^{-6}$, which corresponds to a Bonferroni correction of 20,000 genes tested (MacArthur et al., 2014); *UBAP2* on chromosome 9 ($P = 1.02 \times 10^{-7}$) and *STARD9* on chromosome 15 ($P = 2.85 \times 10^{-7}$).

Gene	N variants	MAC controls (n=5415)	MAC cases (n=57)	Method	P-value
UBAP2	4	37	7	QA	1.02×10^{-7}
STARD9	10	776	30	UA	2.85×10^{-7}
ITFG3	3	10	4	ER	6.57×10^{-6}
CLHC1	3	597	11	UA	9.53×10^{-6}
GRIK3	3	5	3	ER	1.92×10^{-5}
AKAP9	8	167	10	QA	2.27×10^{-5}
PAK6	5	187	10	QA	4.38×10^{-5}
SMARCAD1	4	169	8	QA	8.76×10^{-5}
LYST	8	263	13	QA	1.39×10^{-4}
ASTN2	5	382	12	QA	1.46×10^{-4}

Table 2.8. *Top 10 genes associated with clozapine-associated neutropenia. Analysis conducted using SKAT-O to test association of cumulative effects of rare functional variants. Columns represent: Gene name (Gene), number of variants in analysis (N variants), total count of rare functional alleles within gene in controls (MAC controls), total count of rare functional alleles within gene in cases (MAC cases), Analysis method (Method; Efficient resampling (ER), Quantile adjusted moment matching (QA, No adjustment (UA)), and permuted p-value for gene (P-value).*

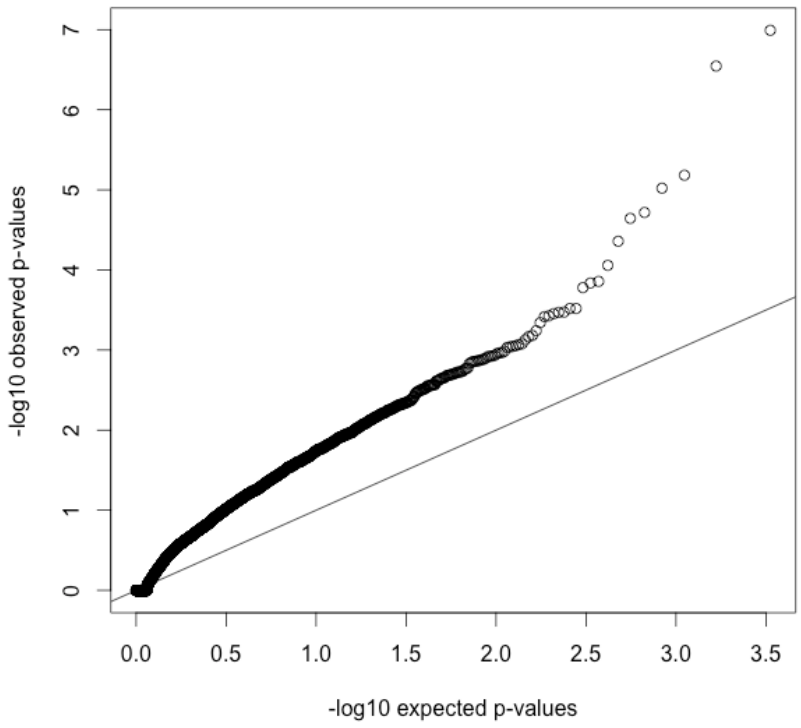

Figure 2.11. *QQ plot of gene-based SKAT-O analysis. The –log10 observed permuted SKAT-O p-values (y-axis) are plotted against expected p-values (x-axis).* $\lambda_{GC} = 3.39$.

2.4.2 Independence of rs1546308 and rs149104283

I investigated the independence of rs1546308 (missense variant in *SLCO1B7* and intronic in *SLCO1B3* from exome chip analysis) and rs149104283 (intronic variant in *SLCO1B3* and *SLCO1B7* from GWAS analysis) in samples with data available for both variants (55 cases and 4834 controls). The linkage disequilibrium between the two variants in our sample was $r^2 = 0.15$, D' = 0.84. In a conditional logistic regression, the strength of the association of rs1546308 with clozapine-associated neutropenia was attenuated from OR = 3.00 (95% CI: 1.735-5.189, P = 8.40×10^{-5}) to OR = 2.16 (95% CI: 1.093-4.251, P = 0.027) after adjusting for rs149104283. Haplotype analysis did not strengthen the association signal. Thus, these two findings are not independent, and the associated region spans *SLCO1B1*, *SLCO1B3* and *SLCO1B7*. **Figure 2.12** displays associated region 12p12.2 from the discovery analysis and **Figure 2.13** from the SNPs in meta-analysis.

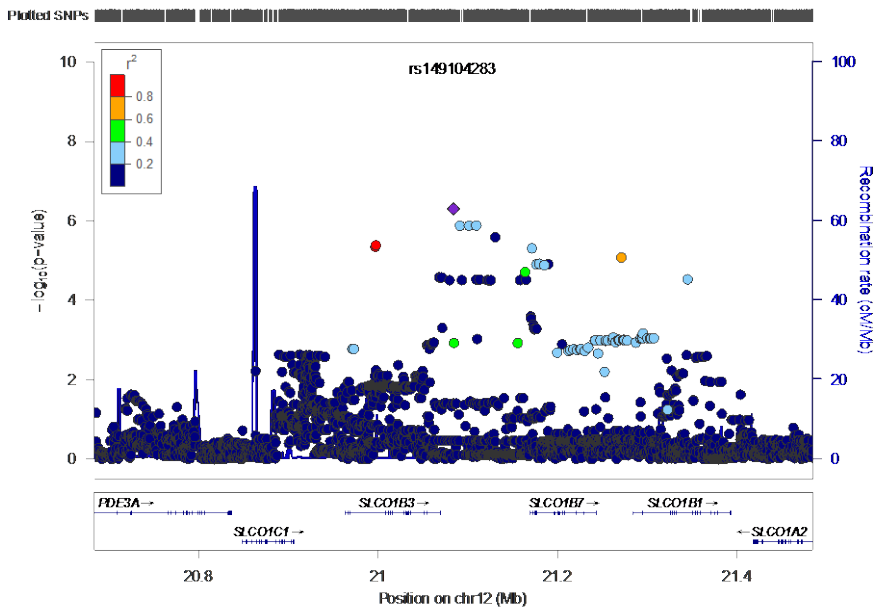

Figure 2.12. *LocusZoom plot of association of 12p12.2 in CLOZUK analysis. Genes within the region are shown in the lower panel, and the unbroken blue line indicates the recombination rate. Points represent the P-value for each SNP, with the top SNP rs149104283 shown in purple and the SNPs in the region coloured depending on their degree of correlation (r^2) with rs149104283 (as estimated on the basis of CEU HapMap haplotypes).*

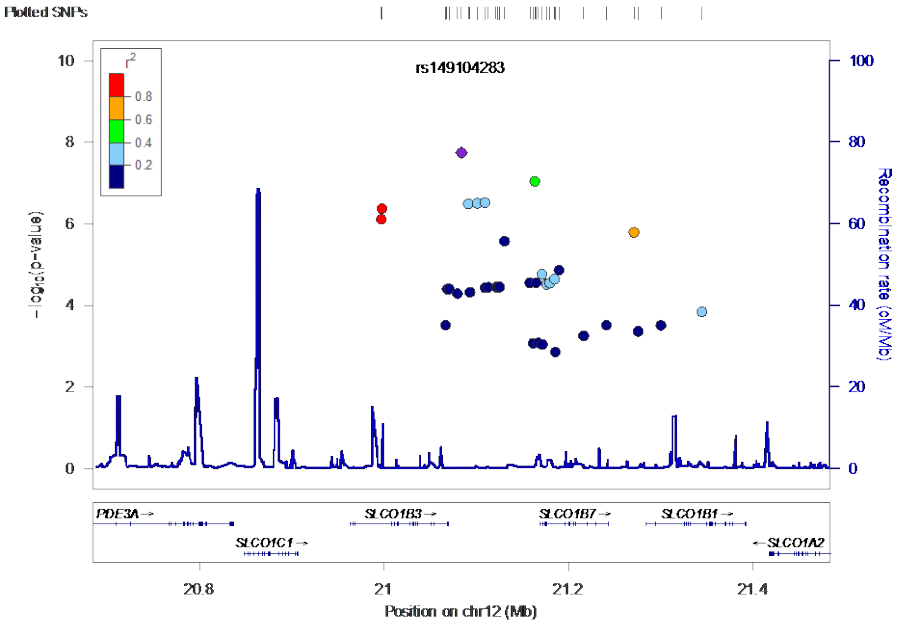

Figure 2.13. *LocusZoom plot of meta-analysis SNPs in 12p12.2. Genes within the region are shown in the lower panel, and the unbroken blue line indicates the recombination rate. Points represent the P-value for each SNP, with the top SNP rs149104283 shown in purple and the SNPs in the region coloured depending on their degree of correlation (r^2) with rs149104283 (as estimated on the basis of CEU HapMap haplotypes).*

2.4.3 Predictive test analysis

To assess the predictive value of the three key variants identified in this study, I calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) based on a prevalence of 3.7% for either agranulocytosis or neutropenia (**Table 2.9**). This prevalence is based on the cumulative risk of agranulocytosis (0.8%) and neutropenia (2.9%) in those taking clozapine (Atkin et al., 1996). The PPV and NPV values were adjusted by estimating the number of risk allele carriers based on the sensitivity and specificity values in a population with a prevalence of 3.7% (Parikh et al., 2008). rs149104283 was the intronic variant in *SLCO1B3* and *SLCO1B7* identified from the GWAS meta-analysis. rs1546308 was the missense variant in *SLCO1B7,* and intronic in *SLCO1B3,* identified in the exome array meta-analysis. rs113332494 (HLA-DQB1 6672G>C) was the genotyped candidate variant. The sensitivity for a test including these three risk variants was 29.17%, the specificity 90.61%, the PPV 9.94%, and the NPV 97.30%. The analyses indicate that 29.17% of individuals with clozapine-associated neutropenia or agranulocytosis will carry at least one of the three identified risk alleles and that individuals that have any of these risk alleles have a 9.94% risk of neutropenia or agranulocytosis.

	rs149104283	**rs1546308**	**rs149104283 & rs1536308**	**rs113332494**	**rs149104283, rs1536308 & rs113332494**
N Cases	64	56	55	60	48
N Controls	5391	4924	4832	305	245
Sensitivity	10.94	28.57	30.91	5.00	29.17
Specificity	97.61	88.57	89.26	99.67	90.61
PPV	13.97	8.15	9.28	35.13	9.94
NPV	96.86	97.22	97.32	96.73	97.30

Table 2.9. *Predictive test analysis. Analysis of utility of identified variants as a predictive test, based on a prevalence of 3.7%. Rows represent: number of cases in analysis (N Cases), number of controls in analysis (N Control), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV).*

2.4.4 Secondary analyses of neutropenia below ≤ 1000/mm^3

In secondary analyses, we assessed the association of single variants with clozapine-associated neutropenia below ≤ 1000/mm^3 in GWAS, HLA imputation, and exome array analyses, consistent with methods used for clozapine-associated neutropenia.

GWAS

A total of 6,231,759 genotyped and imputed variants were included in secondary analyses of a subset of the more severely affected cases with ANC ≤ 1000/mm^3 (N=18) (QQ plot in **Figure 2.14**, λ_{GC} = 0.97). **Table 2.10** lists the 10 most strongly associated SNPs from the discovery analysis. Two intronic loci reached GWS (Manhattan plot, **Figure 2.15**); rs76415963 in *SLX4IP* on chromosome 20 (OR =18.05, 95% CI: 6.79-48.00, P = 6.63 x 10^{-9}) and rs138818969 in *FAM228A* on chromosome 2 (OR = 15.55, 95% CI: 5.99-40.32, P = 1.67 x 10^{-8}). Our sample size for clozapine-associated neutropenia ≤ 1000/mm^3 analyses was 80% powered to detect a GWS association (P < 5 x 10^{-8}) with an effect size ≥ 10 and MAF ≥ 0.20 (**Figure 2.16**).

In total, there were 373 independent (r^2 < 0.1) SNPs associated with clozapine-associated neutropenia below ≤ 1000/mm^3 at P < 1 x 10^{-4} and we sought replication of these SNPs in the CIAC sample. Data was available for 361 SNPs and proxies identified for a further three SNPs. Appendices 3 details the proxy SNPs used and their LD with the original SNP. **Table 2.10** details the association in CIAC for the 10 most strongly associated SNPs in the CLOZUK analysis. Associations of rs76415963 in *SLX4IP* and rs138818969 in *FAM228A* were not replicated in CIAC (OR = 0.57, P = 0.31 and OR = 0.94, P = 0.97, respectively). **Table 2.11** lists the 10 most strongly associated SNPs from the meta-analysis. No SNP reached GWS in the meta-analysis. The most significantly associated SNP from the meta-analysis was rs143888465 in *SCN8A* on chromosome 12 (OR = 4.94, P = 3.72 x 10^{-6}).

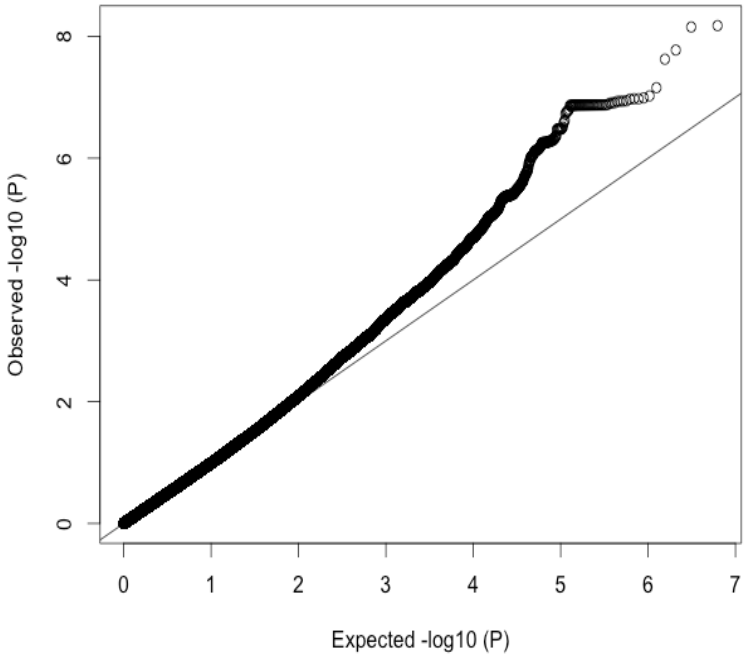

Figure 2.14. *QQ plot for clozapine-associated neutropenia ≤ 1000/mm^3 GWAS. The –log10 observed logistic regression p-values (y-axis) are plotted against expected p-values (x-axis). λ_{GC} = 0.97.*

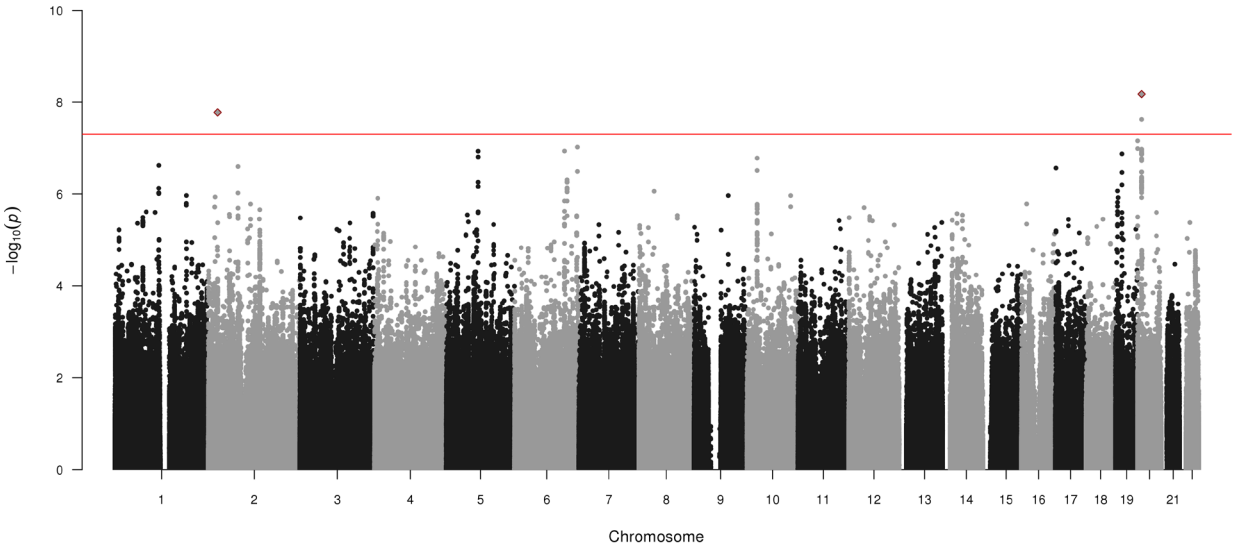

Figure 2.15. *Manhattan plot of clozapine-associated neutropenia ≤ 1000/mm^3 GWAS. –log10 P-values (y-axis) for each SNP is presented on the basis of chromosomal position (x axis). The red line represents the GWS level (P < 5 x 10^{-8}).*

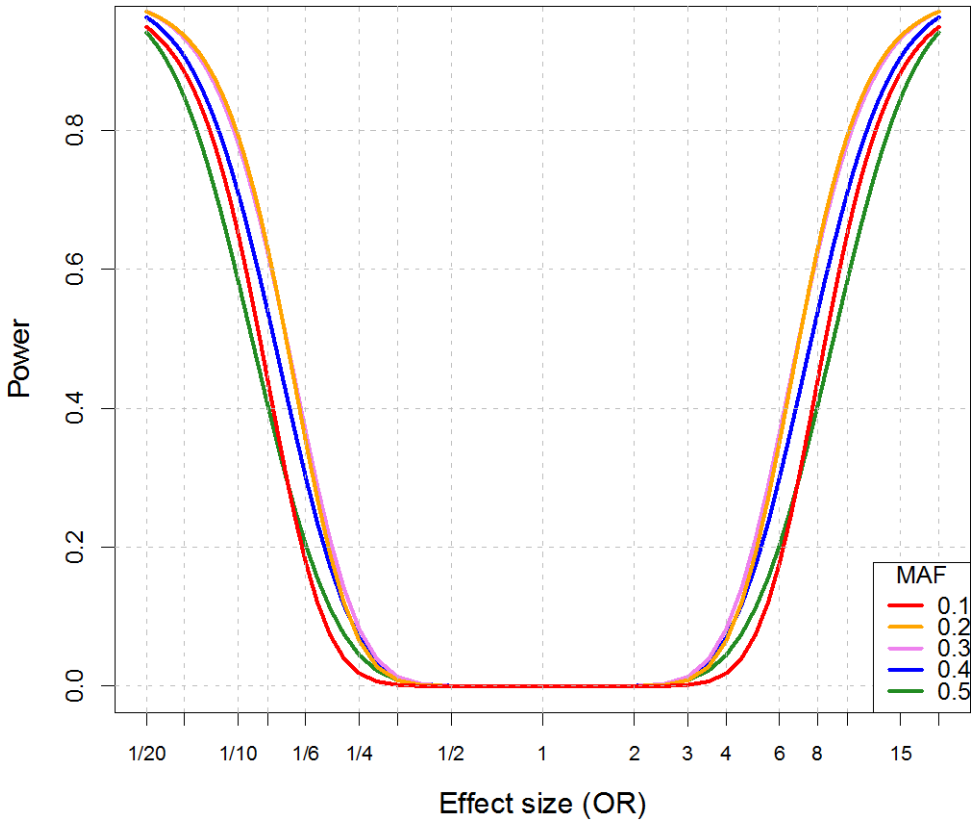

Figure 2.16. *Power calculation for clozapine-associated neutropenia ≤ 1000/mm^3 GWAS. Power calculations derived from Quonto software (http://biostats.usc.edu/Quanto.html) using an additive disease model, 1% disease prevalence for 18 cases and 5583 controls (1:312 ratio) to detect GWS variant (P = 5 x 10^{-8}) at minor allele frequencies of 0.1 (red), 0.2 (orange), 0.3 (pink), 0.4 (blue) and 0.5 (green) for effect sizes (OR) 1-10.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Gene**	**Location**
20*	rs76415963	10544516	T	6.63×10^{-9}	14.57	19.0	1.97	0.311	0.57	1.24	2.32	5.68×10^{-5}	4.66	*SLX4IP*	Intronic
2	rs138818969	24412084	A	1.67×10^{-8}	15.55	19.1	2.05	0.9384	0.97	2.28	2.26	1.48×10^{-4}	3.52	*FAM228A*	Intronic
20	rs2196239	80655	A	6.94×10^{-8}	6.73	41.7	9.8	0.7354	0.93	8.7	9.32	9.96×10^{-3}	1.62	*DEFB125*	3kb downstream
6	rs3817796	166875819	T	9.47×10^{-8}	6.91	41.7	10.1	0.7792	0.94	9.73	10.32	0.015	1.55	*RPS6KA2*	Intronic
6	rs74416570	132931444	A	1.15×10^{-7}	8.12	26.8	4.6	0.4751	0.77	2.89	4.31	2.04×10^{-3}	2.29	*TAAR3 / TAAR2*	1kb/7kb upstream
5	rs78442711	84190774	T	1.16×10^{-7}	9.25	24.0	3.84	0.8075	0.92	4.2	4.45	1.96×10^{-3}	2.24		Intergenic
19	rs12980608	17114605	T	1.33×10^{-7}	6.90	62.55	22.5	0.4255	1.12	25.14	23.33	6.74×10^{-3}	1.45	*CPAMD8*	Intronic
10	rs148473220	27242377	C	1.65×10^{-7}	10.45	19.44	2.39	0.9185	0.96	2.18	2.45	3.11×10^{-4}	3.11		Intergenic
1	rs149086962	117537116	C	2.39×10^{-7}	15.85	13.95	1.42	0.1011	0.24	0.59	1.49	3.83×10^{-4}	5.05	*PTGFRN / CD101*	4kb downstream / 7kb upstream
2	rs75471613	78795665	G	2.52×10^{-7}	10.07	19.41	2.44	0.8752	1.06	3.11	3.11	6.94×10^{-4}	2.62		Intergenic

Table 2.10. *Top 10 SNPs for clozapine-associated neutropenia ≤ 1000/mm^3 CLOZUK (discovery) GWAS. Results are ordered by CLOZUK analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency for controls (MAF U) in CLOZUK and CIAC sample, p-value and combined odds ratio (OR) for meta-analysis, gene and location to gene.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Gene**	**Location**
12	rs143888465	52010108	T	1.02×10^{-5}	11.07	11.20	1.17	0.018	2.87	2.61	1.23	3.72×10^{-6}	4.94	*SCN8A*	Intronic
8	rs548650	102585836	A	2.93×10^{-6}	10.91	13.17	1.46	0.074	2.31	2.71	1.37	7.48×10^{-6}	4.70	*GRHL2*	Intronic
2	rs78626772	111707226	A	1.65×10^{-6}	10.24	17.55	2.70	0.064	1.95	3.83	2.48	1.40×10^{-5}	3.52	*ACOXL*	Intronic
3	rs181373231	97994503	A	5.86×10^{-6}	15.08	10.89	1.08	0.237	2.34	0.05	0.98	1.62×10^{-5}	12.85	*OR5H2*	7kb upstream
1	rs141788696	83927911	T	2.44×10^{-6}	18.15	10.53	1.11	0.156	0.43	1.31	0.99	1.68×10^{-5}	0.16		Intergenic
8	chr8_131078867_I	131078867	I5	5.03×10^{-5}	9.86	11.11	1.41	0.016	2.56	3.51	1.25	1.80×10^{-5}	3.96	*ASAP1*	Insertion
1	rs17028665	112355413	A	2.63×10^{-5}	10.83	10.97	1.40	0.031	2.52	2.93	1.23	2.10×10^{-5}	4.29	*KCND3*	Intronic
5	rs114337922	2565908	G	8.42×10^{-5}	12.53	9.34	1.24	0.032	3.17	1.93	0.99	3.05×10^{-5}	0.18		Intergenic
6	rs9365903	166070843	C	8.19×10^{-5}	7.95	11.11	1.33	0.023	2.41	3.41	1.4	3.17×10^{-5}	0.27	*PDE10A*	Intronic
1	rs185053659	60704250	A	4.33×10^{-6}	14.31	12.31	1.42	0.101	2.13	2.26	1.33	3.45×10^{-5}	4.44		Intergenic

Table 2.11. *Top 10 SNPs for clozapine-associated neutropenia ≤ 1000/mm^3 GWAS meta-analysis. Results ordered by meta-analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency for controls (MAF U) in CLOZUK and CIAC sample, p-value and combined odds ratio (OR) for meta-analysis, gene and location to gene.*

Imputed HLA analysis

I performed an additional association analysis of clozapine-associated neutropenia ≤ 1000/mm^3 in 6,672 imputed classical *HLA* alleles and amino acid polymorphisms (QQ plot in **Figure 2.17**, λ_{GC} = 1.63). No polymorphism was associated at GWS ($P < 5 \times 10^{-8}$). **Table 2.12** lists the 10 most strongly associated SNPs from the discovery analysis. The most significant variant was rs3129963 (OR = 4.14, $P = 3.94 \times 10^{-5}$), located 5kb upstream of *BTNL2* and present in 44.44% of cases and 16.94% of controls.

Data was available within CIAC for 763 of the 839 *HLA* variants associated with clozapine-associated neutropenia ≤ 1000/mm^3 at $P < 0.05$. No imputed classical *HLA* allele or amino acid polymorphism was associated at GWS in the combined meta-analysis. **Table 2.13** lists the 10 most strongly associated SNPs from the meta-analysis. rs3129963, 5kb from *BTNL2*, was weakly associated in CIAC (OR = 1.32, P = 0.048) (**Table 2.12**). The most significant variant from the meta-analysis was rs3129891 (OR = 1.91, $P = 6.28 \times 10^{-8}$), located 2kb downstream of *HLA-DRA* and present in 44.44% of cases and 18.83% of controls in the CLOZUK sample and 29.93% of cases and 19.97% of controls in the CIAC sample. Among the top associated variants in the meta-analysis were amino acid changes in *HLA-B*, a gene been previously implicated in clozapine-induced agranulocytosis (Goldstein et al., 2014). Due to complex LD in this region, independent SNPs were not identified. Thus it is likely that the variants listed do not represent independent signals. **Figure 2.18** highlights the LD and association across *BTNL2, HLA-DRA* and other *HLA* genes in the CLOZUK sample. It was not possible to impute specific variants of interest in *HLA-DQB1* (Athanasiou et al., 2011; Goldstein et al., 2014) with sufficient accuracy and thus this region is considered separately (section 2.4.2).

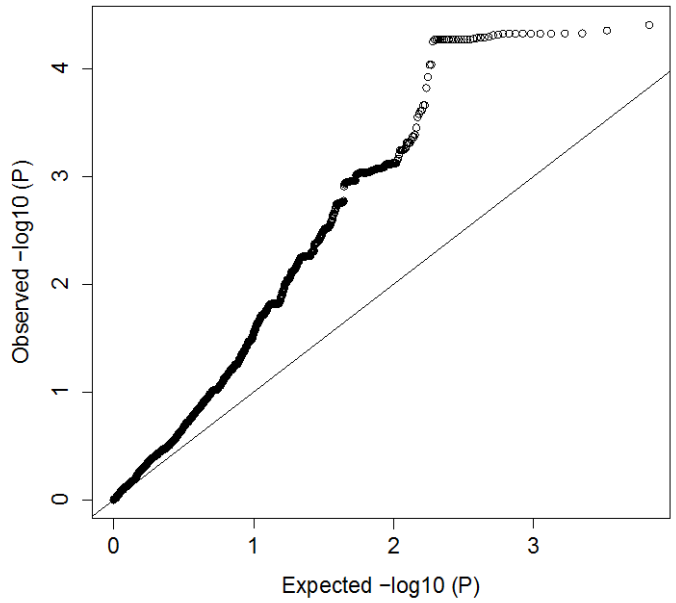

Figure 2.17. *QQ plot for clozapine-associated neutropenia ≤ 1000/mm^3 imputed HLA allele discovery analysis. The –log10 observed logistic regression p-values (y-axis) are plotted against expected p-values (x-axis). λ_{GC} = 1.63.*

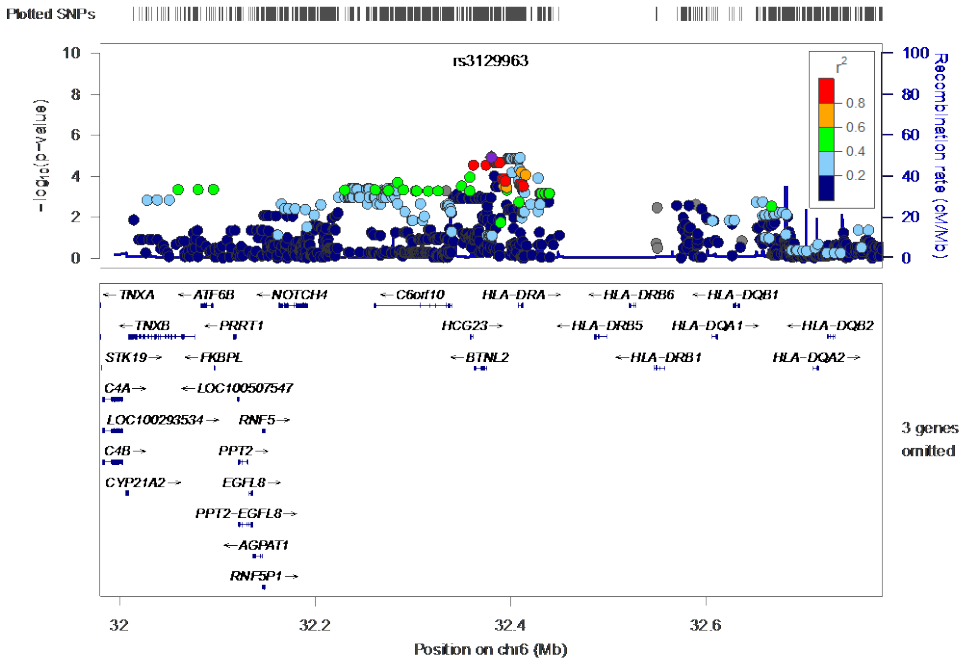

Figure 2.18. *LocusZoom plot of discovery HLA analysis for clozapine-associated neutropenia ≤ 1000/mm^3. Genes within the region are shown in the lower panel, and the unbroken blue line indicates the recombination rate. Points represent the P-value for each SNP, with the top SNP rs3129963 shown in purple and the SNPs in the region coloured depending on their degree of correlation (r^2) with rs3129963 (as estimated on the basis of CEU HapMap haplotypes).*

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Gene**	**Location**
6	rs3129963	32380208	G	3.94×10^{-5}	4.14	44.44	16.94	0.0476	1.32	20.99	17.14	6.98×10^{-4}	1.56	*BTNL2*	5kb upstream
6	rs3129881	32409484	T	4.44×10^{-5}	4.13	61.11	28.24	0.7967	0.97	25.77	26.47	0.2262	1.16	*HLA-DRA*	Intronic
6	rs3129872	32407153	T	4.72×10^{-5}	4.10	61.11	28.33	0.9087	1.01	27.78	27.62	0.1297	1.20	*HLA-DRA*	1kb upstream
6	rs2395177	32405076	C	4.75×10^{-5}	4.10	61.11	28.34	0.9794	1.00	27.52	27.56	0.1485	1.19	*HLA-DRA*	3kb upstream
6	rs2395172	32399842	C	4.75×10^{-5}	4.10	61.11	28.35	0.9732	1.00	28.15	28.30	0.1691	1.18	*HLA-DRA*	7kb upstream
6	rs3129848	32397049	A	4.75×10^{-5}	4.10	61.11	28.35	0.8605	1.02	27.78	27.45	0.1157	1.21		Intergenic
6	rs3129853	32398648	A	4.75×10^{-5}	4.10	61.11	28.35	0.9098	1.01	27.78	27.62	0.1301	1.20	*HLA-DRA*	9kb upstream
6	rs3129858	32400520	A	4.75×10^{-5}	4.10	61.11	28.35	0.9101	1.01	27.78	27.62	0.1302	1.20	*HLA-DRA*	7kb upstream
6	rs3135339	32399261	C	4.75×10^{-5}	4.10	61.11	28.35	0.9128	1.01	27.77	27.62	0.131	1.20	*HLA-DRA*	8kb upstream
6	rs3135342	32396615	A	4.75×10^{-5}	4.10	61.11	28.35	0.8603	1.02	27.78	27.45	0.1155	1.21		Intergenic

Table 2.12. *Top 10 imputed HLA alleles for clozapine-associated neutropenia ≤ 1000/mm^3 CLOZUK (discovery) analysis. Results ordered by CLOZUK P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency for controls (MAF U) in CLOZUK and CIAC sample, p-value and combined odds ratio (OR) for meta-analysis, gene and location of variant to gene.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**SNP**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**Gene**	**Location**
6	rs3129891	32415080	A	2.64×10^{-4}	3.44	44.44	18.83	1.05×10^{-5}	1.76	29.93	19.97	6.28×10^{-8}	1.91	*HLA-DRA*	2kb downstream
6	rs3129890	32414273	C	0.0012	2.96	47.22	23.15	6.18×10^{-4}	1.52	33.62	24.95	1.54×10^{-5}	1.64	*HLA-DRA*	2kb downstream
6	rs9268832	32427789	T	0.0071	2.51	61.11	38.44	4.89×10^{-4}	1.49	47.38	38.08	3.13×10^{-5}	1.57		Intergenic
6	rs7754768	32420179	C	0.0038	2.75	63.89	39.09	7.82×10^{-4}	1.47	48.13	39.03	4.21×10^{-5}	1.56	*HLA-DRA*	7kb downstream
6	SNP_C_31345234	31237255	G	0.0090	2.48	58.33	36.28	5.96×10^{-4}	1.49	42.89	34.90	4.40×10^{-5}	1.57	*HLA-C*	Intronic
6	SNP_B_31432910	31324931	A	0.0318	2.05	50.00	32.46	4.18×10^{-4}	1.51	40.56	31.62	5.36×10^{-5}	1.56	*HLA-B*	Exonic
6	AA_B_-23_31432910_L	31324931	P	0.0318	2.05	50.00	32.46	4.70×10^{-4}	1.51	40.53	31.66	6.04×10^{-5}	1.56	*HLA-B*	Exonic
6	AA_B_-21_31432904_M	31324925	P	0.0318	2.05	50.00	32.46	4.73×10^{-4}	1.51	40.53	31.66	6.08×10^{-5}	1.56	*HLA-B*	Exonic
6	SNP_B_31432808_G	31324829	P	0.0320	2.05	50.00	32.47	4.85×10^{-4}	1.51	40.51	31.66	6.28×10^{-5}	1.56	*HLA-B*	Exonic
6	SNP_B_31432809	31324830	G	0.0320	2.05	50.00	32.47	4.85×10^{-4}	1.51	40.51	31.66	6.28×10^{-5}	1.56	*HLA-B*	Exonic

Table 2.13. *Top 10 imputed HLA alleles from clozapine-associated neutropenia ≤ 1000/mm^3 meta-analysis. Results ordered by meta-analysis P-value. Columns are: chromosome (CHR), variant ID (SNP), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency for controls (MAF U) in CLOZUK and CIAC sample, p-value and odds ratio (OR) for meta-analysis, gene and location to gene. Nomenclature for (i) classical HLA alleles: HLA_[GENE]_[ALLELE], (ii) HLA Amino Acids: AA_[GENE]_[AMINO ACID POSITION]_[GENETIC POSITION]_[ALLELE], (iii) HLA intragenic SNPs: SNP_[GENE]_[POSITION]_[ALLELE], and (iv) Insertions / deletions: [VARIANT]_[GENE]_[POSITION]_[INSERTION/x=DELETION] i.e. one of above with x or insertion L=Leucine, M=Methionine, G=Glycine.*

Exome array analysis

I performed an additional analysis of 114,814 exome array variants in a subset of 16 cases with clozapine-associated neutropenia ≤ 1000/mm^3. No single variant exceeded a significance threshold of $P < 4.3 \times 10^{-7}$, corresponding to a Bonferroni correction for 115,000 variants tested (QQ plot displayed in **Figure 2.19**, $\lambda_{GC} = 0.94$). **Table 2.14** lists the 10 most strongly associated variants from the discovery analysis. The most significant variant was rs4445901 on chromosome 16 ($P = 3.08 \times 10^{-5}$), present in 78.12% of cases and 44.82% of controls.

A total of 1110 independent variants ($r^2 < 0.1$) were associated with clozapine-associated neutropenia ≤ 1000/mm^3 at $P < 0.01$ in the discovery analysis. Data was available for 990 of these variants in CIAC. However, due to rarity of the variants in this analysis, only 291 variants had a computable odds ratio (OR) in both samples; it was common for the minor allele to be absent from either cases or controls. Although we used a p-value based method for the meta-analysis, the OR is required to determine the direction of effect. Thus only these 291 variants could be included in our meta-analysis. **Table 2.15** lists the 10 most strongly associated variants from the exome array meta-analysis. No variant exceeded a significance threshold of $P < 4.3 \times 10^{-7}$. The most significant variant was rs17139320 ($P = 7.56 \times 10^{-6}$), a missense variant in *ZNF679* and present in 12.5% of cases vs. 2.96% of controls in the CLOZUK sample and 7.41% of cases vs. 2.67% of controls in the CIAC sample.

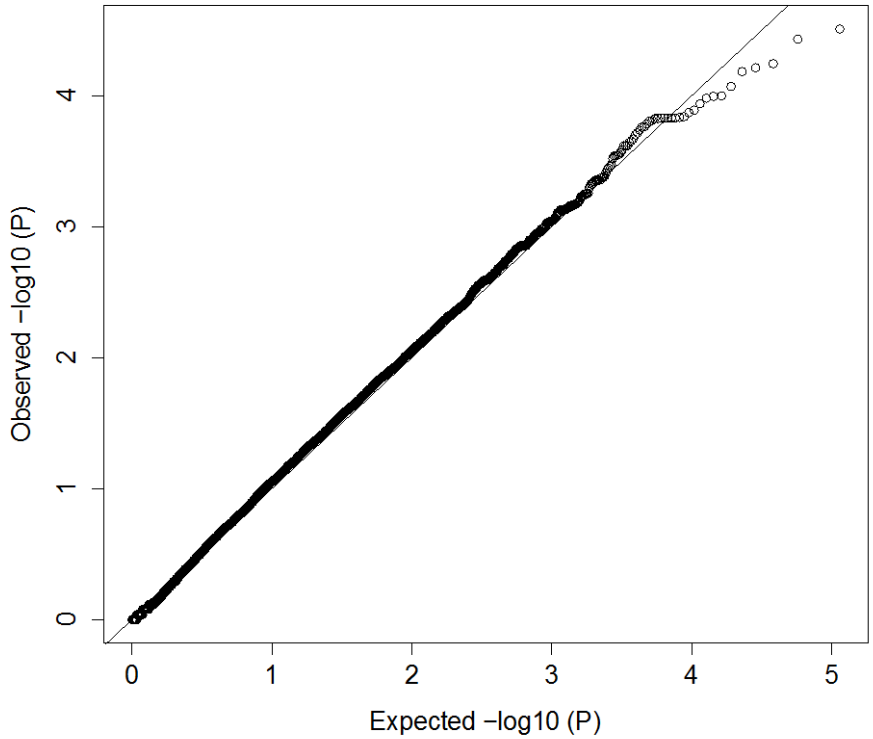

Figure 2.19. *QQ plot of clozapine-associated neutropenia below ≤ 1000/mm^3 exome array analysis. The –log10 observed permuted logistic regression p-values (y-axis) are plotted against expected p-values (x-axis).* $\lambda_{GC} = 0.94$.

				CLOZUK				CIAC				Meta-analysis			
CHR	**Variant**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**Gene**	**Location**	**Function**
16	rs4445901	51259725	T	3.08×10^{-5}	4.63	78.12	44.82	0.248	1.20	47.53	43.34	9.78×10^{-5}		Intergenic	
15	rs117116488	89390513	T	3.69×10^{-5}	16.49	12.50	1.04	0.045	3.18	2.47	0.79	2.51×10^{-5}	*ACAN*	Exonic	Missense
11	rs34809643	488541	A	5.68×10^{-5}	71.28	6.25	0.16	0.104	3.91	1.23	0.32	1.15×10^{-4}	*PTDSS2*	Exonic	Missense
10	rs7918793	27497191	A	6.11×10^{-5}	6.276	25.00	4.12	0.779	0.89	3.70	4.20	0.015	*ACBD5*	Exonic	Missense
15	rs147997234	54307871	A	6.53×10^{-5}	886.1	3.13	0.03	1	0	0.00	0.09	-	*UNC13C*	Exonic	Missense
1	rs184646465	236762890	C	8.48×10^{-5}	166.6	6.25	0.04	1	0	0.00	0.08	-	*HEATR1*	Exonic	Missense
20	rs1780680	35769647	T	1.00×10^{-4}	4.176	75.00	42.47	0.353	0.86	38.89	42.91	0.021	*MROH8*	Exonic	Missense
9	rs201613718	106864334	T	1.01×10^{-4}	3308	3.13	0.01	-	-	0.00	0.00	-	*SMC2*	Exonic	Missense
16	rs61732874	3293257	A	1.04×10^{-4}	66.82	6.25	0.17	0.429	1.85	0.62	0.33	0.0017	*MEFV*	Exonic	Missense
19	rs4808551	17111297	A	1.15×10^{-4}	4.18	43.75	16.99						*CPAMD8*	Exonic	Missense

Table 2.14. *Top 10 exome array variants from clozapine-associated neutropenia ≤ 1000/mm^3 CLOZUK (discovery) analysis. Results ordered by CLOZUK p-value. Columns are: chromosome (CHR), variant ID (Variant), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency for controls (MAF U) for CLOZUK and CIAC sample, meta-analysis p-value, gene reference, location and function.*

				CLOZUK				CIAC				Meta-analysis			
CHR	**Variant**	**Position**	**A1**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**OR**	**MAF A**	**MAF U**	**P-value**	**Gene**	**Location**	**Function**
7	rs17139320	63726370	G	8.00×10^{-3}	5.06	12.50	2.96	2.87×10^{-4}	3.21	7.41	2.67	7.56×10^{-6}	*ZNF679*	Exonic	Missense
20	rs150057397	3677326	A	3.24×10^{-3}	123.6	3.13	0.05	1.69×10^{-3}	82.34	1.23	0.02	1.71×10^{-5}	*SIGLEC1*	Exonic	Stop gained
15	rs117116488	89390513	T	3.69×10^{-5}	16.49	12.50	1.04	0.04477	3.18	2.47	0.79	2.51×10^{-5}	*ACAN*	Exonic	Missense
22	rs182012324	24237074	T	1.34×10^{-4}	62.38	6.25	0.17	0.02731	9.14	1.23	0.14	3.15×10^{-5}	*MIF*	Exonic	Missense
16	rs4445901	51259725	T	3.08×10^{-5}	4.63	78.13	44.82	0.2479	1.20	47.53	43.34	9.78×10^{-5}		Intergenic	
11	rs138991613	117693150	T	2.00×10^{-3}	81.16	3.13	0.07	0.01458	13.71	1.23	0.09	1.10×10^{-4}	*FXYD2,FXYD6-FXYD2*	Exonic	Missense
17	rs4792739	16322676	C	1.49×10^{-3}	5.87	15.63	3.23	0.01866	2.18	6.17	2.82	1.13×10^{-4}	*TRPV2*	Intronic	
11	rs34809643	488541	A	5.68×10^{-5}	71.28	6.25	0.16	0.1043	3.91	1.23	0.32	1.15×10^{-4}	*PTDSS2*	Exonic	Missense
1	rs12073549	17720545	T	3.15×10^{-3}	2.94	31.25	13.68	0.01631	1.61	20.37	13.86	1.64×10^{-4}	*PADI6*	Exonic	Synonymous
9	rs140343430	5922080	G	1.96×10^{-3}	53.04	3.13	0.11	0.0227	10.28	1.23	0.12	1.80×10^{-4}	*KIAA2026*	Exonic	Missense

Table 2.15. *Top 10 exome array variants from clozapine-associated neutropenia ≤ 1000/mm^3 meta-analysis. Resuts ordered by meta-analysis p-value. Columns are: chromosome (CHR), variant ID (Variant), chromosomal position (Position), minor reference allele (A1), p-value, odds ratio (OR), minor allele frequency in cases (MAF A), and minor allele frequency for controls (MAF U) for CLOZUK and CIAC sample, meta-analysis p-value, gene reference, location and function*

2.5. Discussion

I have conducted a multifaceted genetic analysis of clozapine-associated neutropenia in the largest combined sample studied to date. Using GWAS, I identified a novel association implicating a family of organic anion transporters involved in drug metabolism which have been previously associated with adverse drug reactions. I also found evidence for effects of uncommon non-synonymous variants within *UBAP2* and *STARD9* and provide independent replication of a previously identified variant in *HLA-DQB1*.

Novel association at SLCO1B3/SLCO1B7

The primary GWAS finding from the meta-analysis was a genome-wide significant association with clozapine-associated neutropenia for rs149104283, an intronic SNP for transcripts of both *SLCO1B3* and *SLCO1B7.* The associated region also contains a third member of this organic anion transporter family*, SLCO1B1. SLCO1B7* encodes a putative protein (OAT1B7) that is poorly characterised, based on coding sequence prediction, and its functionality is currently unknown. *SLCO1B3* and *SLCO1B1* share sequence homology and encode liver-specific organic anion-transporter polypeptides (OATP1B3 and OATP1B1) that are multipass transmembrane proteins expressed exclusively in the basolateral membrane of hepatocytes (Konig et al., 2000). They facilitate uptake of exogenous substances, including drugs, from the portal vein into hepatocytes, where the substance is subsequently modified either via metabolism with cytochrome (CYP) 450 enzymes or excreted (International Transporter Consortium et al., 2010).

Polymorphisms in *SLCO1B1* and *SLCO1B3* have been implicated in adverse reactions with other drugs. In 2008, a GWAS identified a missense variant rs4149056 in *SLCO1B1* that increased the risk of simvastatin-induced myopathy by increasing the area under the curve

(AUC) for simvastatin, particularly in those taking high doses (Search Collaborative Group et al., 2008). This prominent pharmacogenetic finding has been widely replicated and has led to recommendations for its use as a routine pre-emptive clinical test (Ramsey et al., 2014). Particularly relevant to the current study are reports of an association between rs11045585, an intronic variant in *SLCO1B3,* and severe leukopenia/ neutropenia induced by docetaxel, a chemotherapeutic agent, (Kiyotani et al., 2008), and that this may be secondary to alterations in the pharmacokinetics and bioavailability of the drug (Nambu et al., 2011; Yamakawa et al., 2011; Chew et al., 2012). These polymorphisms were not in high LD ($r^2 < 0.1$ for both) with the index SNP in this study although rs11045585 was weakly associated with neutropenia in our CLOZUK sample (OR = 1.62, P = 0.03).

Together, the findings suggest the hypothesis that genetic variants at *SLCO1B3* (and/or *SLCO1B1*) increase risk of clozapine-associated neutropenia through a pharmacokinetic mechanism. It is unclear whether clozapine plasma levels are associated with development of neutropenia (Hasegawa et al., 1994; Centorrino et al., 1995; Mauri et al., 1998). One of the best-supported hypotheses to explain clozapine's association with agranulocytosis relates to the bioactivation of clozapine, or a stable metabolite, to a chemically reactive nitrenium ion (Pirmohamed & Park, 1997). The propensity for nitrenium ions to cause apoptosis to neutrophils, or be toxic to neutrophil precursors, is dose dependent, lending support to the hypothesis that clozapine pharmacokinetics and bioavailability are related to its potential to cause neutropenia (Williams et al., 2000; Pereira & Dean, 2006).

Further studies are required to determine if *SLCO1B3* (and/or *SLCO1B1*) influence the uptake or metabolism of clozapine and sequencing of this chromosomal region is required to refine the association signal.

Rare variants

In analysis of exome array data I found evidence of association with neutropenia for uncommon non-synonymous variants in *STARD9* and *UBAP2*. *STARD9* is a mitotic kinesin and *STARD9*-depleted cancer cells have abnormal cellular morphology and undergo apoptosis (Torres et al., 2011). In addition, STARD9-depletion was found to synergise with the chemotherapeutic agent taxol, the use of which is dose-limited due to neutropenia (Torres et al., 2011). The function of *UBAP2* is undetermined though it has an ubiquitin-associated domain and is widely expressed across tissues including bone marrow. The ubiquitination pathway has been shown to modulate the granulocyte colony-stimulating factor receptor (Ai et al., 2008; Kindwall-Keller et al., 2008), a critical regulator of neutrophil production. A recent study reported the association of a missense variant in the ubiquitin gene *USP43* with clozapine-associated neutropenia (Tiwari et al., 2014).

No single variant from the exome array was significantly associated with clozapine-associated neutropenia or neutropenia ≤ 1000/mm^3, although a number of functional but nominally associated variants were identified. However, the exome array does not capture all exonic variation. Exome- wide sequencing would prove the role of functional exonic variants definitively.

A colleague, Elliott Rees, assessed the role of rare, exonic copy number variation (CNV) in clozapine-associated neutropenia. The identification and quality control of CNVs for the CLOZUK and CardiffCOGS samples has been previously described (Rees et al., 2014). CNVs were included if they had a frequency ≤ 0.01, contained ≥ 10 probes, and were ≥ 100kb in length. Samples that passed both CNV and GWAS quality control (63 cases and 5456 controls) were used to test genes for enrichment of exon disrupting CNVs using a 2-sided Fisher's exact test. Deletions and duplications were analysed separately and P-values were

adjusted for multiple testing by applying a Bonferroni correction of 20,000 genes. In this genome-wide analysis, no individual gene was significantly enriched for large, rare exonic CNVs. The CIAC study also failed to identify any CNV that was significantly associated with clozapine-associated neutropenia (Goldstein et al., 2014).

HLA genes

The final finding adds to the growing evidence implicating *HLA-DQB1* in clozapine-associated neutropenia, supporting the recently published CIAC study (Goldstein et al., 2014). There have been further reports implicating SNPs within *HLA-DQB1* (Yunis et al., 1995; Dettling et al., 2001; Athanasiou et al., 2011) although these samples and those in CIAC are overlapping; thus we provide the first fully independent replication implicating this locus in clozapine-associated neutropenia/agranulocytosis. *HLA-DQB1* belongs to the HLA class II beta chain paralogs and has been implicated in several autoimmune diseases including insulin-dependent diabetes and narcolepsy (Siebold et al., 2004). The *HLA-DQB1* variant alone has a positive predictive value of 35.1%. Whilst this is promising, the majority of those that develop neutropenia or agranulocytosis whilst taking clozapine are not carriers of this risk allele, or indeed the other alleles we have identified in this study.

Secondary analyses of clozapine-associated neutropenia ≤ 1000/mm^3 implicated variants in *BTNL2*, *HLA-DRA* and *HLA-B,* although none of these variants reached statistical significance. The CIAC study found evidence for association of missense variants in *BTNL2*, but this signal was not independent of that from *HLA-DQB1*. Variants in *HLA-B* have been previously implicated in clozapine-induced agranulocytosis (Yunis et al., 1992; Yunis et al., 1995; Valevski et al., 1998; Goldstein et al., 2014). Our findings suggest that *HLA* genes may have a greater impact in those with a more severe phenotype.

Strengths and limitations

Due to its rarity, genetic studies of clozapine-associated neutropenia are typically underpowered to detect associations that do not have a moderate to large effect. Thus, there may be causal variants of small effect that we were not able to detect in this study. However, a considerable strength of this study is the large control sample, all of which were treated with clozapine for at least a year without developing neutropenia. Nonetheless, the low statistical power in this study increases the likelihood that our findings may not represent true effects (Button et al., 2013). An important consideration is that our analyses included cases with neutropenia rather than agranulocytosis. It is now very rare to develop agranulocytosis because of the success of the monitoring system; in fact only four cases met this threshold in our sample. Lastly, the individuals examined in this study were limited to those of European ancestry and there may be differences in the clinical impact of associated variants between different populations.

Clinical Implications

Although the identified variants convey a substantially increased risk for clozapine-associated neutropenia, they are currently on their own unlikely to have clinical utility for pharmacogenetic testing due to low sensitivity and positive predictive value (Verbelen et al., 2015), particularly as there is currently no alternative treatment for those with TRS. Nonetheless our findings provide novel insights into putative biological processes underlying clozapine-associated neutropenia. We have indicated a potential link between the pharmacokinetics of clozapine and risk of neutropenia/agranulocytosis with potentially important clinical implications. The development of such understanding should help widen the availability of clozapine with beneficial impact on those with TRS.

Chapter 3

Methods (for studies presented in Chapter 4 and Chapter 5)

This chapter describes in detail the sample used for Chapters 4 and 5 of this thesis. Data for both chapters were derived from a two-year retrospective cohort study of all patients starting their first clozapine trial over a five-year period (2007-2011, inclusive) in South London & Maudsley (SLaM) NHS Foundation Trust. Details of the setting, ethical approval, sample selection, study design and exposure variables are presented within this chapter. Study-specific details of additional inclusion criteria, measures, and data analysis procedures are presented in the relevant chapters.

3.1. Setting

The study used data from the Case Register Interactive Search (CRIS) system; a large, anonymised case register derived from South London & Maudsley (SLaM) NHS Foundation Trust electronic case records (Stewart et al., 2009). SLaM is the largest secondary mental health care provider in Europe serving approximately 1.2 million people from four London boroughs; Lambeth, Croydon, Lewisham and Southwark. SLaM provides specialist general adult, child and adolescent, forensic, older adult, learning disabilities and addiction mental health services as well as a number of national services. Electronic case records were implemented in SLaM in 2006 to allow sharing of information across services. The electronic case records contain structured fields for fixed data (e.g. demographic details, medication, ICD-10 diagnosis and structured assessments) and unstructured fields for free text (e.g. correspondence, referrals, clinical assessments, care plans and ward rounds).

The introduction of electronic case records provided a unique opportunity to create a large case register that could be used for secondary research purposes. The CRIS system is a large, anonymous case register derived for research purposes from SLaM's electronic case records. There are in excess of 230,000 patients represented on the CRIS system (Stewart et al., 2009; Fernandes et al., 2013). CRIS was developed for use and is supported by the National Institute for Health Research (NIHR) Mental Health Biomedical Research Centre and Dementia Unit (BRC/U) and hosted by SLaM and King's College London. To protect the anonymity of the patients represented within CRIS, a unique BRC identifier is created from NHS numbers, which are not available for researchers, and any text within CRIS that matched the name, address or date of birth fields is replaced with 'ZZZZZ' (Fernandes et al., 2013).

A major advantage of CRIS is that in the UK the National Health Service (NHS) provides nearly 100% of the health care and thus the whole population is captured by these services. The system automatically updates itself and keeps a history of previous entries, so for example you can see current and all previous diagnoses made. The CRIS system allows the researcher to search any combination of structured and unstructured fields from electronic case records in order to select cases and specify the output fields that are required, which is exportable for further analysis. In addition, data from CRIS can be linked to other datasets including the Office of National Statistics (ONS), which records information relating to death, and Hospital Episode Statistics (HES) derived from structured information collected from general hospital episodes.

3.2. Ethical approval

Ethical approval for the use of CRIS as a research dataset was given by Oxfordshire Research Ethics Committee C (08/H0606/71) and permission for the studies in this PhD were granted by the CRIS oversight committee.

3.3. Sample selection

The cohort consisted of patients who had a primary ICD-10 diagnosis of a psychotic disorder (F20-F29, inclusive) and began a first trial of clozapine between 1 January 2007 and 31 December 2011. We selected this study period because electronic case records were fully implemented during 2006 and clozapine initiations on or before 31 December 2011 permitted a two-year duration to the time of data collection (January 2014). Patients were aged 18-65 years at the start of clozapine treatment and initiated clozapine under secondary mental health care services, either as an inpatient or outpatient. Patients who solely received tertiary care from SLaM national services (such as the National Psychosis Unit) and were not residing in SLaM were excluded because complete follow-up data were not always available and they were not representative of clozapine patients in general. Patients receiving care from national services are likely to have complex and co-morbid mental illness and were thus excluded in order to make results widely applicable.

The process of cohort identification is detailed in Figure 3.1. The sample was initially extracted using a General Architecture for Text Engineering (GATE) application, developed and validated against manual annotations (Cunningham et al., 2013; Hayes et al., 2015). GATE applications can take into account linguistic context when extracting data from free text, therefore distinguishing between a current prescription of clozapine and the mention of clozapine in other contexts (Hayes et al., 2015). The application used multiple data

sources to identify medication use including pharmacy dispensing events, structured medication field, clinical correspondence and free text entries, resulting in a high degree of sensitivity (Hayes et al., 2015). The application detected a total of 3242 patients, from approximately 230,000 represented in CRIS, whom had evidence of current or previous clozapine use. I then selected patients who had (i) first clozapine prescription between 1 November 2006 (extended due to the fact clinical discussions about clozapine can precede its initiation) and 31 December 2011, (ii) had entries that spanned more than a single day (iii) ICD-10 F20-F29 diagnosis, and (iv) aged 18 years or over on 31 December 2011 and 65 years or less on 1 January 2007. The data for the 799 patients who met these criteria were manually screened and study eligibility verified from their electronic clinical records. A total of 316 patients were included in the study (see **Figure 3.1**).

3.4. Design

The study comprised of a two-year retrospective cohort of all patients starting their first clozapine trial over a five-year period (2007-2011, inclusive) in SLaM. The study period began at clozapine initiation and ended with discontinuation, death or 24 months after treatment onset (whichever of these occurred first). In the event that an individual was lost to follow-up, their data was treated as censored. The date of clozapine initiation was derived from case notes, defined as the date the patient took their first dose of clozapine. The date of discontinuation was defined as the date the patient was last known to take clozapine (as stated in case notes), where this was followed by at least three consecutive months without clozapine treatment. Therefore, patients who stopped clozapine but were successfully re-titrated within three months were not classed as discontinued. The timing and the reasons for discontinuation were assessed in a case note review if the patient

discontinued clozapine treatment within 24 months of initiation (further details provided in section 5.3).

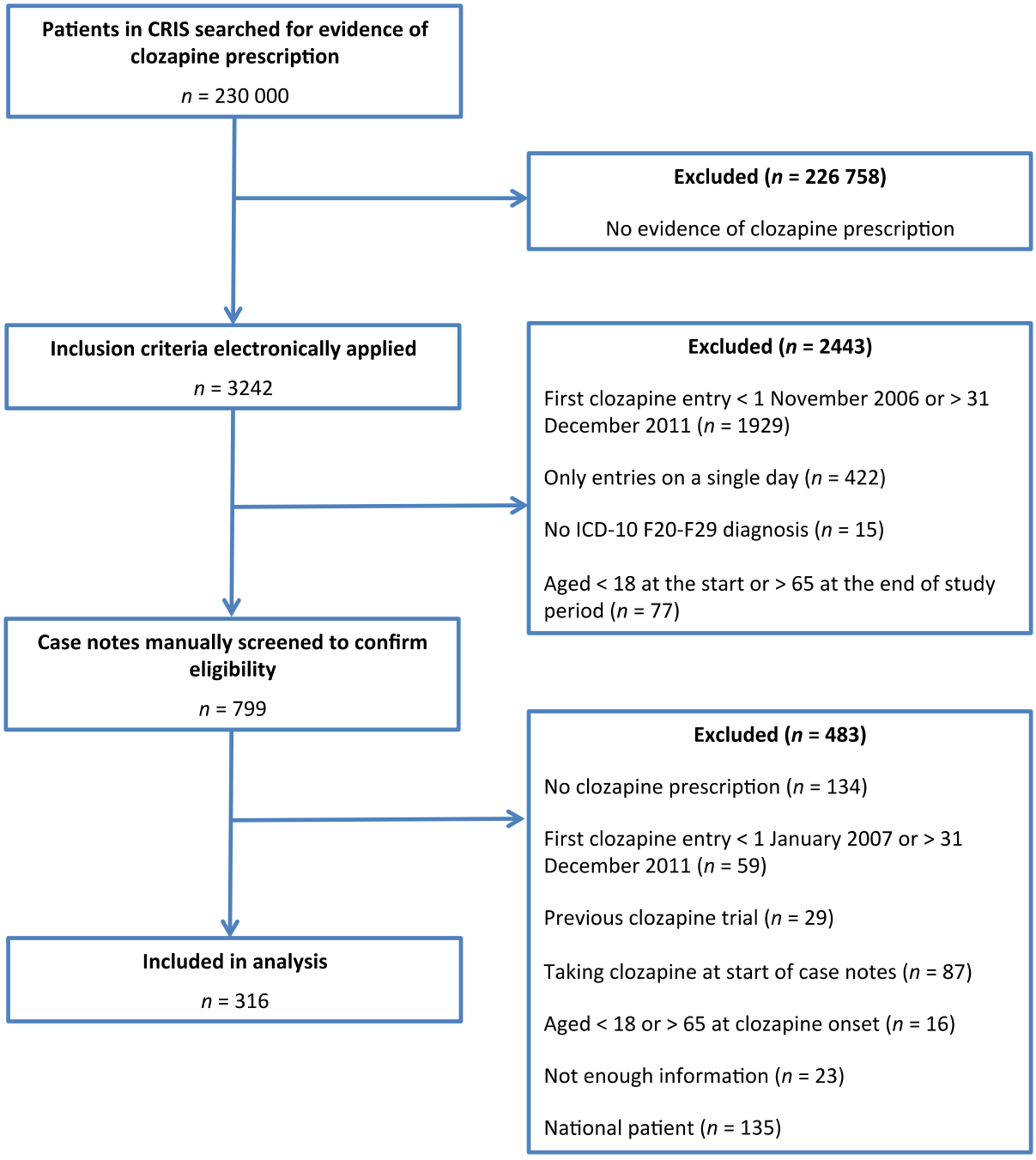

Figure 3.1. *Sample identification.*

3.5. Study clinical assessments

To assess response to clozapine treatment, I administered clinical research assessments retrospectively to CRIS case notes at the start of clozapine treatment (baseline) and 1, 2, 3, 6, 9, 12, 18 and 24 months after clozapine initiation. These time points were selected to account for the greater likelihood of change within the first few months of treatment. If the patient discontinued clozapine prior to the end of the study period, clinical assessments were administered until the date clozapine treatment was ceased.

We selected the Clinical Global Impressions (CGI) scale (Guy, 1976) as it is a widely accepted research tool that captures clinical improvement, originally designed for use in clinical trials, but has also been utilised in retrospective case note studies (Agid et al., 2011). We also selected this instrument for pragmatic reasons given that the majority of clinical notes were seen to contain the necessary information to make CGI ratings whereas there was often insufficient detail in order to rate more comprehensive rating scales retrospectively such as the Brief Psychiatric Rating Scale (BPRS) (Overall & Gorham, 1962) or the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987). This decision was informed by the experiences of a previous medical student who attempted to administer BPRS scales retrospectively from case notes and found that there was insufficient information to do so. The CGI (Guy, 1976) is a global measure that is comprised of two scores (i) CGI-Severity (CGI-S), and (ii) CGI-Improvement (CGI-I). CGI-S is a seven-point scale measuring the severity of the illness in question (detailed in **Table 3.1**) and CGI-I is a seven-point scale measuring the change in psychopathology from baseline, frequently treatment initiation (detailed in **Table 3.2**). Ratings rely on the rater's prior experience of the psychopathology in question and take into account all available information, such as symptoms, behaviour, psychosocial circumstances and the impact of the symptoms on the

patient's ability to function (Busner & Targum, 2007). The CGI-S and CGI-I scales were administered in this study in line with published guidance (Busner & Targum, 2007) and additional coding guidelines written by JW and SL. Information used for the CGI ratings came from the descriptive case notes within CRIS. Frequently used sources were ward round or multi-disciplinary team meeting summaries, medication reviews, nursing notes, admission details, discharge summaries and tribunal reports.

Baseline was defined as the week prior to clozapine initiation. Assessments at subsequent time points took a global impression of the time period from the last assessment. Several assessments, from different times and sources, were used to form a global impression of response to clozapine during that time period. Clinical information was used for a designated time point if the date of the assessment was closer than halfway between each assessment point (for example, if an assessment was one month and three weeks after the onset of clozapine, it would have been used for the month two assessment). Ratings were not made if the patient was non-adherent at that follow-up date, as it would not have reflected response to clozapine. Of 2106 possible time-point assessments, 2034 (97%) were made, ranging from 94% to 99% for each time point (**Table 3.3**).

CGI-Severity score		Description
1	*Normal, not at all ill*	*Not at all ill, symptoms of disorder not present past seven days*
2	*Borderline mentally ill*	*Subtle or suspected pathology*
3	*Mildly ill*	*Clearly established symptoms with minimal, if any, distress or difficulty in social and occupational function*
4	*Moderately ill*	*Overt symptoms causing noticeable, but modest, functional impairment or distress; symptom level may warrant medication*
5	*Markedly ill*	*Intrusive symptoms that distinctly impair social/occupational function or cause intrusive levels of distress*
6	*Severely ill*	*Disruptive pathology, behaviour and function are frequently influenced by symptoms, may require assistance from others*
7	*Extremely ill*	*Pathology drastically interferes in many life functions; may be hospitalised*

Table 3.1. *CGI-Severity guidelines, adapted from (Busner & Targum, 2007).*

CGI-Improvement score		Description
1	*Very much improved*	*Nearly all better; good level of functioning; minimal symptoms; represents a very substantial change*
2	*Much improved*	*Notably better with significant reduction of symptoms; increase in the level of functioning but some symptoms remain*
3	*Minimally improved*	*Slightly better with little or no clinically meaningful reduction of symptoms. Represents very little change in basic clinical status, level of care, or functional capacity*
4	*No change*	*Symptoms remain essentially unchanged*
5	*Minimally worse*	*Slightly worse but may not be clinically meaningful; may represent very little change in basic clinical status or functional capacity*
6	*Much worse*	*Clinically significant increase in symptoms and diminished functioning*
7	*Very much worse*	*Severe exacerbation of symptoms and loss of functioning*

Table 3.2. *CGI-Improvement guidelines, adapted from (Busner & Targum, 2007).*

Time point of assessment	Taking clozapine at time point (*n*)	Assessments completed (*n*)	Assessments completed (%)
Baseline	316	313	99.05
1 month	277	271	97.83
2 months	261	247	94.63
3 months	252	242	96.03
6 months	233	223	95.71
9 months	210	198	94.23
12 months	197	188	95.43
18 months	185	181	97.84
24 months	175	168	96.00
Total	2106	2034	96.58

Table 3.3. CGI assessments completed at each follow-up assessment.

Rater reliability

The CGI assessments were carried out by a single rater (SL), whom had extensive prior experience with clinical assessments used for research purposes (SCAN, CAPA), and in conducting retrospective case note reviews. JW (primary supervisor and consultant psychiatrist) provided initial training on administering the CGI and advice throughout the rating process. Formal consensus ratings were made between SL and JW for marginal assessments. To assess reliability, a colleague Johnny Downs (psychiatrist) rated 12 randomly selected patients (every 15th who had taken clozapine for at least 30 days). In total, 79 CGI-S and 66 CGI-I assessments were second rated. Intraclass correlations (ICC) were calculated to assess reliability, using a two-way mixed-effects model for absolute agreement. Higher values for ICC indicate greater inter-rater reliability: an ICC of 1 indicating perfect agreement and 0 random agreement. For CGI-S the ICC was 0.65 (95% CI: 0.51-0.76) and for CGI-I the ICC was 0.61 (95% CI: 0.43-0.74). These scores were both in the 'good' range (0.60-0.74) as outlined in established guidelines (Cicchetti, 1994). We did not assess test-retest reliability (repeatability) due to time constraints.

3.6. Exposure variables

Demographic and clinical details of age, gender, marital status, ethnicity, diagnosis, inpatient status and detention under the Mental Health Act were obtained from structured fields within CRIS. I was unable to collect data prior to clozapine initiation (such as age of onset, duration of untreated psychosis or theoretical delay to treatment) because data in CRIS was only available from 2006 onwards, when electronic records were fully introduced in SLaM. Age was defined as age at the date of clozapine initiation. Marital status was classified into currently married/cohabiting (married, cohabiting or civil partnership) and single (single, divorced, civil partnership dissolved, separated, widowed, surviving civil partnership or unknown). Self-reported ethnicity was coded as Black African/Caribbean (African, Caribbean and any other Black background) or other (White British, Irish, Indian, Bangladeshi, Pakistani, Chinese, and mixed or other ethnic group background). The decision to aggregate into these categorises was based on Black African/Caribbean ethnicity being the largest group within our sample, the relatively small cell counts of other ethnic groups, and approaches taken in the literature (Moeller et al., 1995; Davis et al., 2014). Diagnosis was classified into schizophrenia (ICD-10 code: F20) and non-schizophrenia F21-9 diagnosis (F21- F29, inclusive). If an individual had more than one psychotic disorder diagnosis, the diagnosis closest in date to the start of clozapine was selected. Inpatient status was determined by whether clozapine was initiated as an inpatient or outpatient. Legal status was determined by whether the patient was detained under the Mental Health Act or not at the time of clozapine initiation. Level of deprivation was calculated from the Index of Multiple Deprivation (IMD) for England (2007). The IMD is made up of seven individual measures of deprivation (income, employment, health deprivation and disability, education skills and training, barriers to housing and services,

crime, and living environment) and is an established score for investigating social deprivation. The IMD score is calculated for geographical areas, which are ranked from one (most deprived) to 32482 (least deprived). Deprivation ranks were divided to give three roughly equal groups: high (1-5500), intermediate (5501-10000) and low (10001-32482). The patient's home address closest to the start of the study period (1st January 2007) was used, with a separate category assigned to those who were homeless.

Chapter 4

Discontinuation of Clozapine

4.1. Summary

Clozapine is uniquely effective in the management of treatment-resistant schizophrenia (TRS). However, a substantial proportion of patients discontinue treatment and this carries a poor prognosis. I investigated the risk factors, reasons and timing of clozapine discontinuation in a two-year retrospective cohort study of 316 patients with TRS receiving their first course of clozapine. Reasons for discontinuation of clozapine and duration of treatment were obtained from case notes and Cox regression was employed to test the association of baseline clinical factors with clozapine discontinuation. A total of 142 (45%) patients discontinued clozapine within two years. By studying the reasons for discontinuations due to a *patient decision*, we found that adverse drug reactions (ADRs) accounted for over half of clozapine discontinuations. Sedation was the most common ADR cited as a reason for discontinuation and the risk of discontinuation due to ADRs was highest in the first few months of clozapine treatment. High levels of deprivation in the neighbourhood where the patient lived were associated with increased risk of clozapine discontinuation (HR=2.12, 95% CI 1.30-3.47). This study indicates that clinical management to reduce the burden of ADRs in the first few months of treatment may have a significant impact on clozapine discontinuation and help more patients experience the benefits of clozapine treatment.

4.2. Introduction

The superior efficacy of clozapine has been consistently demonstrated for those with TRS (Kane et al., 1988; McEvoy et al., 2006; Leucht et al., 2009), which is defined by the National Institute for Health and Care Excellence (NICE) as a failure to respond to two antipsychotic trials at a therapeutic dose for at least six weeks (National Institute for Health and Care Excellence, 2009). A third of patients with schizophrenia are estimated to be treatment-resistant (Meltzer, 1997) and thus potentially eligible for clozapine treatment.

Clinical trials indicate that clozapine substantially reduces psychotic symptoms in 30-60% of TRS patients (Kane et al., 1988; Lieberman et al., 1994b). In addition, treatment with clozapine in comparison to other antipsychotic medications has been associated with decreased rates of mortality (Hayes et al., 2015), suicide (Meltzer et al., 2003a; Tiihonen et al., 2009), aggression (Chengappa et al., 2002), and financial costs due to reduced hospitalisations (Honigfeld & Patin, 1990; Hayhurst et al., 2002). Despite this evidence, an estimated 40% of patients eligible for clozapine in the UK have not received a trial (Royal College of Psychiatrists, 2012) and in those that have, there is a four-year delay from eligibility to first treatment (Howes et al., 2012b). The widespread underutilisation of clozapine may be due in part to its significant side effect profile (Atkin et al., 1996; Henderson et al., 2000). Of particular importance is the risk of agranulocytosis, which necessitates regular blood monitoring (Atkin et al., 1996).

Discontinuation of clozapine can cause a rapid deterioration in psychotic symptoms (Seppala et al., 2005) and subsequent increased rates of compulsory treatment, hospitalisation, and poorer functioning (Atkinson et al., 2007; Wheeler et al., 2009b).

Unfortunately, approximately 40% of patients will discontinue clozapine treatment within 24 months of initiation (Ciapparelli et al., 2000; Whiskey, 2003; Davis et al., 2014).

Considering the favourable outcomes of clozapine treatment, and poor prognosis for those that discontinue, efforts have been made to identify patients that may be at increased risk and understand the causes of discontinuation. Older age at clozapine initiation, Black African/Caribbean ethnicity and substance abuse have been found to increase risk for clozapine discontinuation (Moeller et al., 1995; Krivoy et al., 2011; Davis et al., 2014). *Adverse effects*, *patient decision* and *non-adherence* have been identified as common reasons for clozapine discontinuation in previous studies (Atkinson et al., 2007; Taylor et al., 2009; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). Although *patient decision* and *non-adherence* have been identified as major reasons for discontinuation of clozapine, there has been no exploration of reasons behind this choice.

As far as we are aware, only one study has been in a systematically attained sample of patients receiving their first trial of clozapine (Davis et al., 2014), which allows timing to be studied in detail and comparisons made with patients that continue. In addition, reasons for discontinuation of subsequent trials may differ from the first. Only one study has been conducted in the UK (Taylor et al., 2009), and given the differences in clozapine utilisation across health care systems, there may be limited generalisability of other studies to UK patients.

4.2.1 Aims of the study

The aims of the study were to (i) assess the reasons for discontinuation of clozapine, (ii) investigate the timing of reasons for discontinuation, (iii) determine which ADRs lead to discontinuation, and (iv) characterise the patients who are at increased risk of discontinuing clozapine. For each of these aims, we explored the differences between

discontinuations resulting from a *clinician/clinical team decision* and those as a result of patient non-adherence, referred to as *patient decision*. To achieve these aims a two-year retrospective cohort study of all patients starting their first clozapine trial over a five-year period (2007-2011, inclusive) in South London & Maudsley (SLaM) NHS Foundation Trust was conducted.

4.3. Method

Full details of the setting, ethical approval and sample selection are presented in sections 3.1, 3.2 and 3.3, respectively.

4.3.1 Sample

The sample used for this analysis consisted of a cohort of patients who had an ICD-10 primary diagnosis of a psychotic disorder (F20-F29, inclusive) and began a first trial of clozapine between 1 January 2007 and 31 December 2011 in SLaM. Patients were aged 18-65 years at the start of clozapine treatment and initiated clozapine under standard secondary mental health care services, either as an inpatient or outpatient.

4.3.2 Outcome measure

I assessed the timing and the reasons for discontinuation in a case note review if the patient discontinued clozapine treatment within 24 months of initiation. The date of clozapine initiation was defined as the date the patient took their first dose of clozapine. The date of discontinuation was defined as the date the patient was last known to take clozapine, where this was followed by at least three consecutive months without clozapine treatment. Therefore, patients who stopped clozapine but were successfully re-titrated within three months were not classed as discontinued.

Reasons for discontinuation were obtained from case notes when explicitly stated by the patient's clinical team. These were categorised into mutually exclusive reasons, as outlined below, and were consistent with the previous literature (Atkinson et al., 2007; Taylor et al., 2009; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). If there were multiple reasons that spanned more than one category (of which there were only five instances), the most likely primary reason was inferred after discussion with a consultant psychiatrist (primary supervisor, JW).

Reasons for discontinuation were coded into categories consistent with the previous literature; (i) *adverse drug reaction* (ADR) defined as any unwanted or harmful reaction attributed to clozapine including intolerable side effects, (ii) *non-adherence not otherwise specified* defined as patient declining to take medication, not attending for blood monitoring, or missing doses without informing their clinical team and with no reason for doing so stated (iii) *inadequate response* defined as insufficient improvement in symptoms, (iv) *blood monitoring* defined as a stated dislike of either blood tests or inconvenience of frequent clinic visits, (v) *belief medication not required* defined as a patient belief that clozapine would not help them or that they did not need any medication, (vi) *delusional belief* held by the patient specifically regarding clozapine, (vii) *anticipated non-adherence* defined as pre-emptive discontinuation initiated by the clinical team as it was believed the patient would become non-adherent upon discharge from inpatient services, (vii) *death*, regardless of whether the cause was attributed to clozapine, and (ix) any *other* reason. If a patient discontinued due to non-adherence but cited a reason for doing so, they would be classified under the reason cited. To investigate differences in patients that were non-adherent, discontinuations were further classified as a *clinician-led decision* or *patient decision*. *Clinician-led decision* was defined as a discontinuation that was led by the clinical team, although in most cases this was a

consensual decision between the patient and clinical team. *Patient decision* was defined as discontinuation due to non-adherence by declining to take medication, not attending for blood monitoring, or missing doses without informing their clinical team.

The specific ADR was recorded if it was stated to be the reason for discontinuation. These were not classified into mutually exclusive causes because in the majority of cases a number of ADRs were cited per patient and since we wanted to reflect the broad adverse effect profile responsible for treatment discontinuation.

4.3.3 Exposure variables

Details of age, gender, marital status, ethnicity, diagnosis, inpatient status and detention under the Mental Health Act were obtained from structured fields within CRIS. Level of deprivation was calculated from the Index of Multiple Deprivation (IMD) for England (2007) for the patient's home address. Key definitions regarding these variables are described in section 3.6.

4.3.4 Analysis

A Kaplan-Meier survival curve was used to display the time to all-cause clozapine discontinuation and reasons for discontinuation. Having checked proportional hazard assumptions, a Cox regression was employed to model the association between all-cause clozapine discontinuation and gender, age, marital status, ethnicity, level of deprivation, diagnosis, inpatient status and detention under the Mental Health Act. Associations with all-cause discontinuation were assessed in a crude univariable analysis, and also in models that had been fully adjusted for all variables examined. Level of deprivation was entered into the model as a categorical dummy variable. A likelihood ratio test was used to test the appropriateness of entering age as a continuous variable. Interaction effects with age,

gender and ethnicity were investigated for variables significantly associated with all-cause discontinuation ($P < 0.05$). Sensitivity analyses were conducted whereby (i) death was classified as censored data rather than as a reason for all-cause discontinuation, and (ii) only patients with a diagnosis of schizophrenia (F20) were included. Competing-risks regression (Fine & Gray, 1999; Kim, 2007) was employed to model the impact of predictors on cause-specific discontinuation, whilst taking into account the other causes, firstly in a crude analysis and secondly, fully adjusted for all covariates examined. The specific causes of discontinuation investigated were; *ADRs*, *non-ADRs* (all reasons other than ADRs), *clinician-led decision* and *patient decision*. All statistical analyses were performed using STATA version 12 (StataCorp, 2011).

4.4. Results

4.4.1 Patient characteristics

A total of 316 patients were included in the study. Sample characteristics are presented in **Table 4.1**. The mean age at clozapine initiation was 36 years. The majority of the sample had a diagnosis of schizophrenia (n=285). Non-schizophrenia diagnoses included schizoaffective disorder (n=21), acute and transient psychotic disorder (n=3), unspecified nonorganic psychosis (n=3), persistent delusional disorder (n=2), schizotypal disorder (n=1), and other nonorganic psychotic disorder (n=1). In the *other* ethnicity category, 127 were White British, 17 Asian and 21 of other ethnicity. Of the 162 (51.3%) who were detained under the Mental Health Act at the time of clozapine initiation, the majority were under section 3 (detention for treatment, n=124) or under sections 37-49 (forensic, n=30).

Characteristic	**N**	**%**
Male gender	205	64.9
Mean age (years)	36.23	SD=10.9
Married or cohabiting	27	8.5
Black African/Caribbean ethnicity	151	47.8
Level of deprivation		
Low	84	26.6
Intermediate	112	35.4
High	100	31.7
Homeless	14	4.4
Schizophrenia diagnosis	285	90.2
Inpatient	262	82.9
Detained under Mental Health Act	162	51.3

Table 4.1. *Sample characteristics. SD = standard deviation. Percentages relate to total sample (n=316).*

4.4.2 Reasons for discontinuation

A total of 142 (45%) patients discontinued their first trial of clozapine within 24 months of initiation. **Table 4.2** details the reasons for clozapine discontinuation. In total, 65 discontinuations (45.8% of total discontinuations) were from a *clinician-led decision* and 74 (52.1%) were from a *patient decision*. Three patients died within the study period. Cause of death as determined by the case notes was available for two patients: one pulmonary embolism and the other 'natural causes'. The majority of discontinuations from a *clinician-led decision* were due to *ADRs* (n=54), and *other* reasons included periods spent in other countries (n=3), lack of response for tardive dyskinesia (n=1) and inability to obtain blood samples due to lack of venous access (n=1). It was possible to obtain reasons for 49 of the 74 discontinuations resulting from a *patient decision* and thus the remaining 25 patients were classified as *non-adherence NOS*. *ADRs* were the most common reason for discontinuation from a *patient decision* (n=26), followed by a *dislike of blood monitoring* (n=10), *inadequate response* (n=5), a *belief that the medication was not required* (n=4), and a *delusional belief* regarding clozapine (n=4). Combined, *ADRs* attributed to clozapine were responsible for over half of the total discontinuations (n=80). Discontinuations due to *blood monitoring* (n=11) and an *inadequate response* (n=8) were more frequent for *patient* than *clinician-led* discontinuations.

Reason for discontinuation	Clinician-led decision	Patient decision	Combined
	N (%)	N (%)	N (%)
Adverse drug reaction	54 (38.0)	26 (18.3)	80 (56.3)
Non-adherence NOS	-	25 (17.6)	25 (17.6)
Blood monitoring	1 (0.7)	10 (7.0)	11 (7.8)
Inadequate response	3 (2.1)	5 (3.5)	8 (5.6)
Belief medication not required	0 (0.0)	4 (2.8)	4 (2.8)
Delusional belief	0 (0.0)	4 (2.8)	4 (2.8)
Anticipated non-adherence	2 (1.4)	0 (0.0)	2 (1.4)
Other	5 (3.5)	0 (0.0)	5 (3.5)
Death	-	-	3 (2.1)
Total	65 (45.8)	74 (52.1)	142 (100.0)

Table 4.2. *Reasons for clozapine discontinuation. Columns represent discontinuations resulting from a clinician-led decision, patient decision, and combined total reasons. Percentages relate to total discontinued (n=142).*

4.4.3 Time to clozapine discontinuation

Figure 4.1 displays a Kaplan-Meier survival curve for the time to all-cause clozapine discontinuation and for overall reasons of *ADRs*, *non-adherence NOS*, *blood monitoring* and *inadequate response*. Due to the small number of observations, the timings of discontinuations due to reasons of a *belief medication is not required*, *delusional belief*, *anticipated non-adherence*, *death* and *other* were not displayed. Appendix 4 details the timings for all combined reasons.

A substantial proportion of those who initiated clozapine discontinued within the first few months: 12.3% within one month, 20% within three months and 38% within a year. The mean time to all-cause discontinuation was 5.9 months and the median 4.0 months (analysis restricted to those that discontinued within 24 months). The risk of discontinuations due to *ADRs* was highest in the first few months of clozapine treatment

(see **Figure 4.1**). By contrast, the risk of discontinuation due to *non-adherence NOS*, *blood monitoring* and *inadequate response* were evenly distributed across the study period.

In a comparison of all-cause clozapine discontinuation timings of *clinician-led decisions* and *patient decisions* (**Figure 4.2**), the risk in the first three months of treatment was higher for *clinician-led* than *patient* discontinuations. The frequencies of reasons of discontinuations in three monthly intervals due to *clinician-led* and *patient decision* are listed in appendix 5 and 6, respectively.

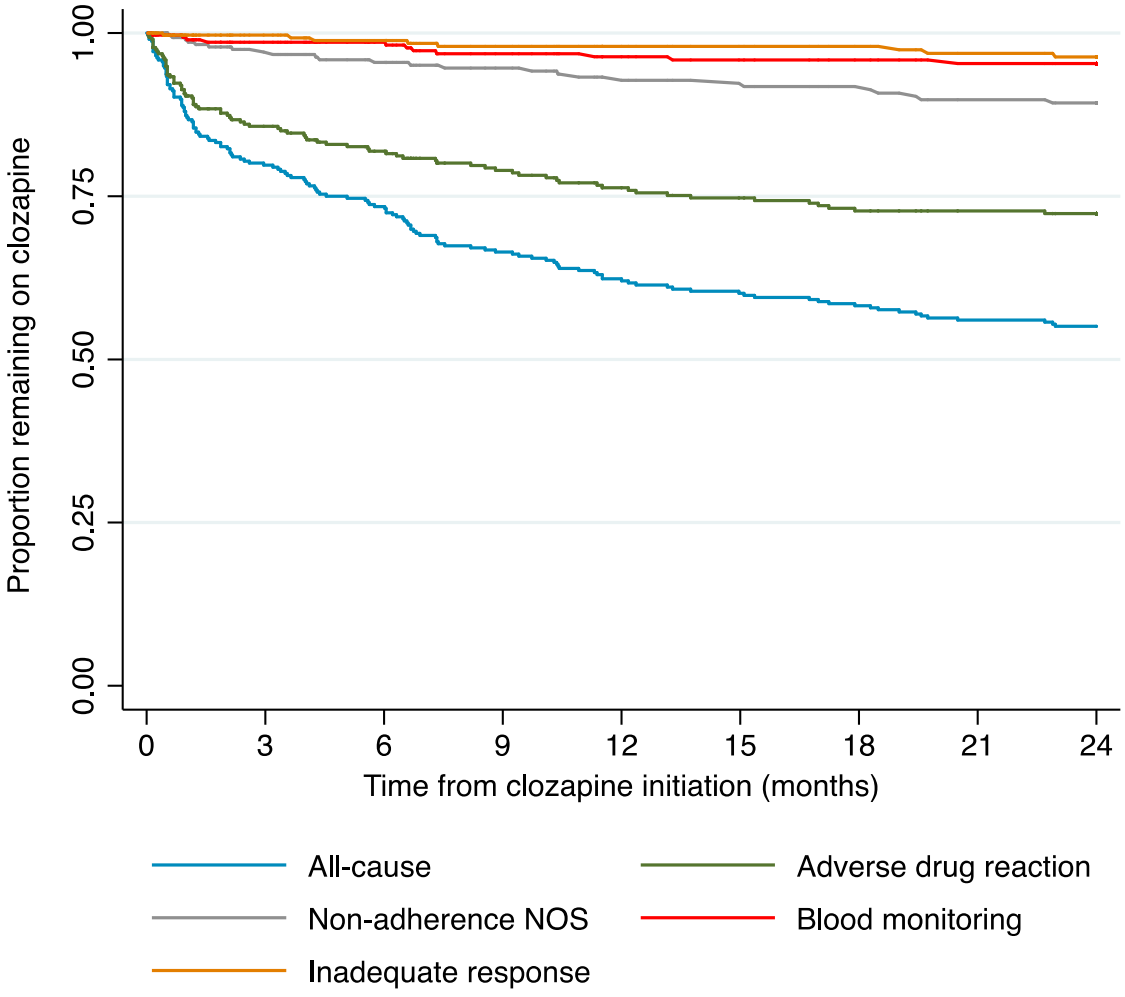

Figure 4.1. *Kaplan-Meier survival curve demonstrating proportion remaining on clozapine over initial 24 months of clozapine treatment. Blue line represents all-cause discontinuation. Other lines represent discontinuations due to adverse drug reactions (green), non-adherence not otherwise specified (grey), blood monitoring (red) and inadequate response (orange).*

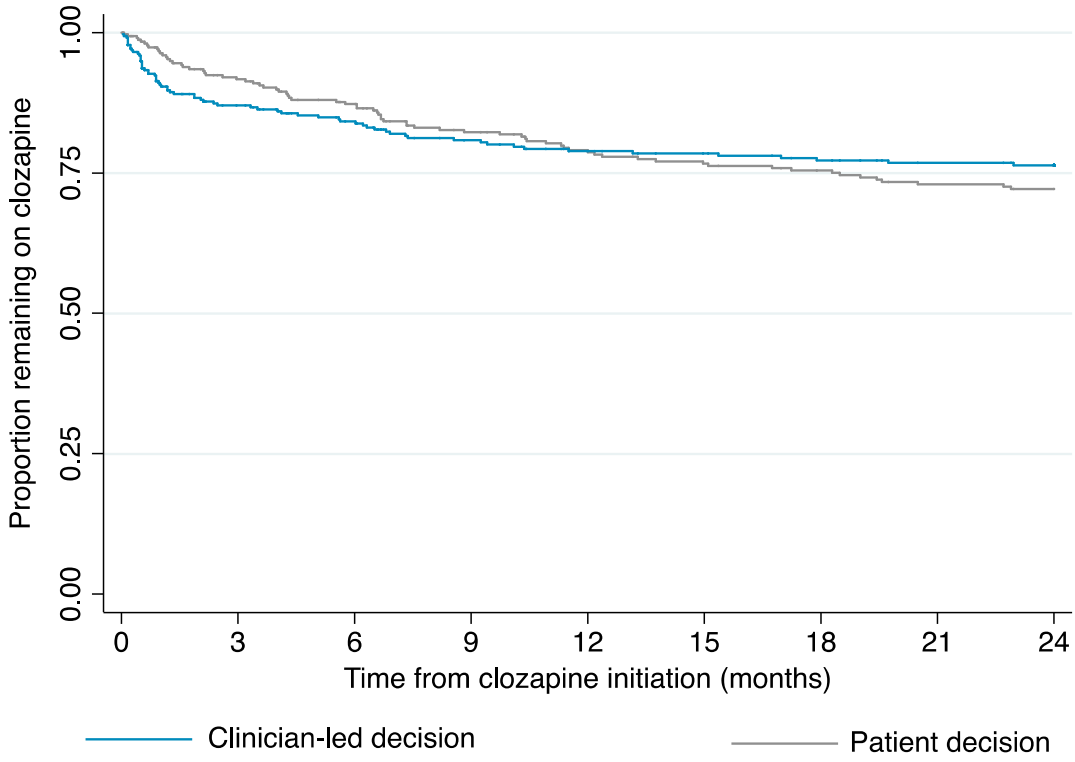

Figure 4.2. *Kaplan-Meier survival curve demonstrating cumulative timing of discontinuations due to a clinician-led decision or patient decision.*

4.4.4 Adverse drug reactions

The 80 patients who discontinued clozapine due to *ADRs* cited a total of 130 individual ADRs. **Figure 4.3** displays the proportion of discontinuations due to ADRs that were from a *clinician-led* or *patient decision* (frequencies listed in Appendix 7). Overall, sedation (n=28), neutropenia (n=15) and tachycardia (n=13) were the most common ADRs cited as a reason for discontinuation of clozapine. The most common ADRs cited for *clinician-led* discontinuations were neutropenia (n=15), sedation (n=13), tachycardia (n=12) and dizziness (n=8). The most common ADRs cited as a reason for discontinuation from a *patient decision* were sedation (n=15), followed by nausea (n=6), hypersalivation (n=4) and weight gain (n=4).

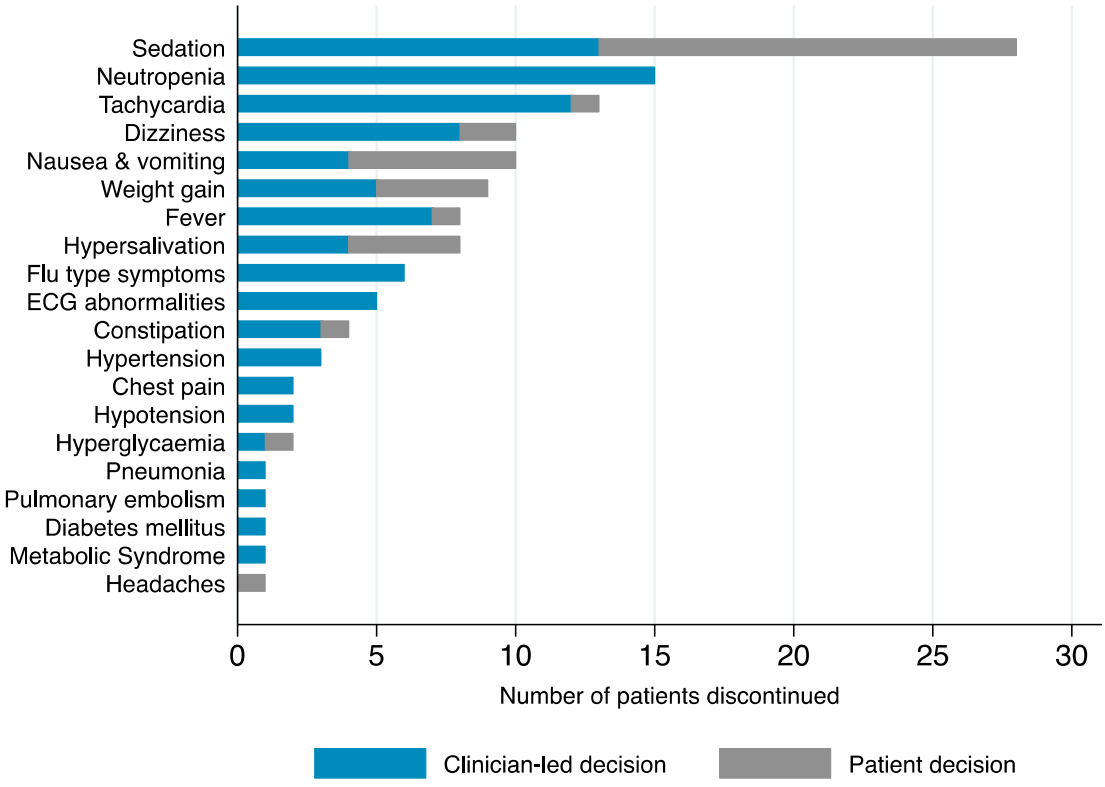

Figure 4.3. *Adverse drug reactions (ADRs) cited as a reason for discontinuation of clozapine for 80 patients (130 ADRs). ADRs are not exclusive and differentiated by whether the discontinuation was a clinician-led decision (blue) or patient decision (grey).*

4.4.5 Risk factors for discontinuation

Table 4.3 details the association of baseline clinical factors with all-cause clozapine discontinuation. In the fully adjusted model, intermediate (hazard ratio (HR) = 1.74, 95% CI 1.06-2.83) and high neighbourhood deprivation (HR = 2.12, 95% CI 1.30-3.47) were associated with increased risk for all-cause clozapine discontinuation. Black African/Caribbean ethnicity was associated with all-cause discontinuation in the crude analysis (HR = 1.55, 95% CI 1.11-2.16) but the association attenuated in the fully adjusted model (HR = 1.26, 95% CI 0.89-1.80). Gender, age, marital status, diagnosis, inpatient status and detention under the Mental Health Act were not associated with all-cause clozapine discontinuation in either the crude or fully adjusted models. No interaction effects were identified and there were no differences in sensitivity analyses where death was not classed as a cause of discontinuation (**Table 4.4**). In a second sensitivity analysis restricted to those with a schizophrenia diagnosis, initiating clozapine as an inpatient was associated with a reduced risk of discontinuation in the fully adjusted model (HR=0.53, 95% CI 0.31-0.90, **Table 4.5**).

Competing-risks regressions were used to investigate risk for cause-specific discontinuations. The association of predictors with discontinuations due to *ADRs* and *non-ADRs* are detailed in **Table 4.6**. There was a significant association between level of deprivation and *non-ADR* discontinuations, but this was no longer significant in the fully adjusted model. The association of predictors with discontinuation due to a *clinician-led decision* or *patient decision* were also investigated (**Table 4.7**). High deprivation was significantly associated with *patient decision* discontinuations, in both the crude and fully adjusted models (HR = 2.17, 95% CI 1.11-4.24).

Characteristic	Discontinued (n=142) N (%)	Continued (n=174) N (%)	Crude Hazard ratio (95% CI)	Crude P-value	Fully Adjusted[1] Hazard ratio (95% CI)	Fully Adjusted[1] P-value
Male gender	89 (62.68)	116 (66.67)	0.86 (0.61-1.21)	0.381	0.77 (0.53-1.10)	0.155
Age at clozapine onset (years, SD)	36.11 (11.29)	36.33 (10.66)	1.00 (0.98-1.01)	0.717	0.99 (0.98-1.01)	0.423
Currently married or cohabiting	14 (9.86)	13 (7.47)	1.26 (0.73-2.19)	0.407	1.40 (0.76-2.57)	0.284
Black African/Caribbean ethnicity	81 (57.04)	70 (40.23)	1.55 (1.11-2.16)	0.010	1.26 (0.89-1.80)	0.194
Level of deprivation						
Low	26 (18.31)	58 (33.33)	Ref		Ref	
Intermediate	53 (37.32)	59 (33.91)	1.72 (1.08-2.75)	0.024	1.74 (1.06-2.83)	0.027
High	55 (38.73)	45 (25.86)	2.21 (1.38-3.52)	9.10×10^{-4}	2.12 (1.30-3.47)	0.003
Homeless	6 (4.23)	8 (4.60)	1.44 (0.59-3.50)	0.419	1.51 (0.61-3.71)	0.373
Non-schizophrenia F20 diagnosis	14 (9.86)	17 (9.77)	1.01 (0.58-1.76)	0.966	0.82 (0.46-1.46)	0.497
Inpatient	115 (80.99)	147 (84.48)	0.84 (0.55-1.27)	0.401	0.66 (0.39-1.11)	0.119
Detained under Mental Health Act	81 (57.04)	81 (46.55)	1.32 (0.95-1.84)	0.104	1.34 (0.88-2.03)	0.168

Table 4.3. *Risk for clozapine discontinuation. Columns represent characteristics for those that discontinued or continued clozapine, hazard ratio and P-value from crude and fully adjusted Cox regression. [1]Fully adjusted includes all variables. Data for all 316 patients was available for each variable other than level of deprivation, which was available for 310 patients.*

Note: Follow-up period begins at start of clozapine treatment (from January 1, 2007 to December 31, 2011, inclusive) and ends with discontinuation, death or end of study period (24 months after treatment onset).

	Crude		Fully Adjusted[1]	
Characteristic	**Hazard ratio** (95% CI)	**P-value**	**Hazard ratio** (95% CI)	**P-value**
Male gender	0.88 (0.63-1.25)	0.487	0.79 (0.55-1.14)	0.210
Age at clozapine onset (years, SD)	0.99 (0.98-1.01)	0.620	0.99 (0.98-1.01)	0.422
Currently married or cohabiting	1.19 (0.67-2.11)	0.547	1.32 (0.70-2.47)	0.394
Black African/Caribbean ethnicity	1.53 (1.10-2.15)	0.013	1.27 (0.88-1.81)	0.197
Level of deprivation				
Low	Ref		Ref	
Intermediate	1.72 (1.08-2.75)	0.023	1.71 (1.05-2.79)	0.032
High	2.09 (1.30-3.34)	0.0023	1.98 (1.20-3.25)	0.007
Homeless	1.44 (0.59-3.51)	0.418	1.49 (0.61-3.68)	0.384
Non-schizophrenia F20 diagnosis	1.04 (0.60-1.80)	0.899	0.85 (0.48-1.52)	0.588
Inpatient	0.81 (0.54-1.24)	0.338	0.64 (0.38-1.08)	0.092
Detained under Mental Health Act	1.31 (0.93-1.83)	0.117	1.36 (0.89-2.08)	0.152

Table 4.4. *Sensitivity analysis of Cox regression including death as censored data rather than a reason for discontinuation. Columns represent hazard ratio and P-value from crude and fully adjusted Cox regression. [1]Fully adjusted includes all variables. Data for all 316 patients was available for each variable other than level of deprivation, which was available for 310 patients.*

	Crude		Fully Adjusted[1]	
Characteristic	**Hazard ratio** (95% CI)	**P-value**	**Hazard ratio** (95% CI)	**P-value**
Male gender	0.84 (0.58-1.20)	0.341	0.73 (0.50-1.08)	0.112
Age at clozapine onset (years, SD)	0.99 (0.98-1.01)	0.467	0.99 (0.97-1.01)	0.295
Currently married or cohabiting	1.22 (0.66-2.26)	0.536	1.46 (0.73-2.91)	0.279
Black African/Caribbean ethnicity	1.67 (1.17-2.38)	0.0046	1.34 (0.92-1.95)	0.128
Level of deprivation				
Low	Ref		Ref	
Intermediate	1.91 (1.16-3.14)	0.011	1.99 (1.18-3.33)	0.009
High	2.44 (1.49-4.01)	0.00043	2.32 (1.38-3.90)	0.001
Homeless	1.70 (0.69-4.19)	0.245	1.78 (0.71-4.46)	0.216
Inpatient	0.70 (0.45-1.07)	0.103	0.53 (0.31-0.90)	0.020
Detained under Mental Health Act	1.27 (0.90-1.80)	0.180	1.37 (0.88-2.14)	0.167

Table 4.5. *Sensitivity analysis for clozapine discontinuation only including patients with a schizophrenia diagnosis (ICD-10 F20) (n=285). Columns represent Cox regression hazard ratio and P-value from crude and fully adjusted Cox regression. [1]Fully adjusted includes all variables. Data for all 285 patients was available for each variable other than level of deprivation, which was available for 279 patients.*

Characteristic	Crude SHR (95% CI)	Crude P-value	Fully Adjusted[1] SHR (95% CI)	Fully Adjusted[1] P-value
i. Discontinuation due to ADRs				
Male gender	0.73 (0.47-1.14)	0.169	0.66 (0.41-1.07)	0.093
Age at clozapine onset (years, SD)	1.00 (0.98-1.02)	0.829	0.99 (0.97-1.02)	0.461
Currently married or cohabiting	1.74 (0.88-3.45)	0.112	1.79 (0.79-4.04)	0.164
Black African/Caribbean ethnicity	1.37 (0.88-2.13)	0.161	1.13 (0.68-1.88)	0.628
Level of deprivation				
Low	Ref		Ref	
Intermediate	1.70 (0.92-3.15)	0.089	1.79 (0.90-3.55)	0.097
High	1.66 (0.89-3.12)	0.112	1.68 (0.84-3.34)	0.141
Homeless	1.18 (0.35-3.91)	0.790	1.36 (0.39-4.72)	0.631
Non-schizophrenia F20 diagnosis	1.36 (0.71-2.63)	0.353	1.06 (0.50-2.23)	0.875
Inpatient	0.79 (0.46-1.37)	0.411	0.63 (0.31-1.27)	0.195
Detained under Mental Health Act	1.19 (0.76-1.84)	0.445	1.27 (0.73-2.23)	0.402
ii. Discontinuations not due to ADRs				
Male gender	1.15 (0.67-1.95)	0.618	1.07 (0.60-1.91)	0.815
Age at clozapine onset (years, SD)	1.00 (0.98-1.02)	0.933	1.00 (0.98-1.03)	0.905
Currently married or cohabiting	0.69 (0.23-1.83)	0.457	0.88 (0.32-2.44)	0.805
Black African/Caribbean ethnicity	1.65 (0.99-2.75)	0.054	1.42 (0.84-2.41)	0.191
Level of deprivation				
Low	Ref		Ref	
Intermediate	1.48 (0.72-3.04)	0.286	1.37 (0.67-2.80)	0.392
High	2.33 (1.16-4.69)	0.017	2.06 (0.98-4.31)	0.056
Homeless	1.71 (0.49-6.03)	0.401	1.54 (0.44-5.41)	0.504
Non-schizophrenia F20 diagnosis	0.61 (0.22-1.70)	0.345	0.64 (0.21-1.93)	0.424
Inpatient	0.96 (0.50-1.83)	0.900	0.78 (0.35-1.76)	0.553
Detained under Mental Health Act	1.43 (0.86-2.38)	0.169	1.39 (0.72-2.69)	0.327

Table 4.6. *Competing risks regression to model impact of predictors on (i) discontinuation due to ADRs, whilst taking into account the other causes of discontinuation and (ii) discontinuation due to reasons other than ADRs, whilst taking into account discontinuations due to ADRs. SHR = subhazard ratio.* [1]*Fully adjusted includes all variables. Data for all 316 patients was available for each variable other than level of deprivation, which was available for 310 patients.*

	Crude		Fully Adjusted[1]	
Characteristic	**SHR** (95% CI)	**P-value**	**SHR** (95% CI)	**P-value**
i. Clinician-led decision				
Male gender	0.87 (0.53-1.43)	0.589	0.87 (0.51-1.49)	0.603
Age at clozapine onset (years, SD)	1.00 (0.98-1.03)	0.765	1.00 (0.97-1.02)	0.879
Currently married or cohabiting	2.18 (1.10-4.33)	0.026	2.17 (0.95-4.95)	0.067
Black African/Caribbean ethnicity	1.45 (0.89-2.36)	0.131	1.26 (0.74-2.13)	0.399
Level of deprivation				
Low	Ref		Ref	
Intermediate	1.67 (0.84-3.35)	0.146	1.62 (0.76-3.48)	0.213
High	1.73 (0.86-3.50)	0.124	1.53 (0.71-3.31)	0.277
Homeless	1.43 (0.43-4.74)	0.561	1.68 (0.49-5.73)	0.405
Non-schizophrenia F20 diagnosis	1.75 (0.89-3.42)	0.102	1.43 (0.66-3.11)	0.360
Inpatient	0.68 (0.38-1.21)	0.188	0.48 (0.22-1.07)	0.072
Detained under Mental Health Act	1.26 (0.78-2.05)	0.341	1.56 (0.81-3.01)	0.183
ii. Patient decision				
Male gender	0.91 (0.57-1.44)	0.677	0.75 (0.46-1.25)	0.270
Age at clozapine onset (years, SD)	0.99 (0.97-1.02)	0.555	0.99 (0.97-1.02)	0.604
Currently married or cohabiting	0.54 (0.21-1.41)	0.210	0.66 (0.24-1.81)	0.422
Black African/Caribbean ethnicity	1.51 (0.96-2.39)	0.076	1.24 (0.75-2.07)	0.403
Level of deprivation				
Low	Ref		Ref	
Intermediate	1.55 (0.82-2.91)	0.176	1.59 (0.83-3.06)	0.165
High	2.15 (1.15-4.00)	0.016	2.17 (1.11-4.24)	0.024
Homeless	1.40 (0.39-4.96)	0.605	1.30 (0.34-4.89)	0.701
Non-schizophrenia F20 diagnosis	0.47 (0.18-1.25)	0.129	0.44 (0.15-1.25)	0.122
Inpatient	1.11 (0.60-2.05)	0.733	0.99 (0.48-2.02)	0.975
Detained under Mental Health Act	1.31 (0.83-2.07)	0.247	1.14 (0.64-2.01)	0.661

Table 4.7. *Competing risks regression to model impact of predictors on (i) discontinuations due to a clinician-led decision, taking into account discontinuations due to a patient decision and (ii) discontinuations due to a patient decision, taking into account discontinuations from a clinician-led decision. SHR = subhazard ratio.* [1]*Fully adjusted includes all variables. Data for all 316 patients was available for each variable other than level of deprivation, which was available for 310 patients.*

4.5. Discussion

In a retrospective cohort study, 45% of patients discontinued their first trial of clozapine within 24 months of initiation. *Adverse drug reactions* (*ADRs*) were responsible for over half of clozapine discontinuations and the risk of discontinuations due to *ADRs* was highest in the first few months of clozapine treatment. Neighbourhood deprivation was associated with an increased risk of all-cause clozapine discontinuation.

4.5.1 Reasons for discontinuation

This is the first study to examine in detail the reasons for discontinuations due to a *patient decision* and distinguish them from *clinician-led* or joint decisions to discontinue. Consistent with previous research, discontinuations due to a *patient decision* were more common than discontinuations involving the clinical team (Taylor et al., 2009; Davis et al., 2014). By studying, where possible, the reasons for discontinuation due to a *patient decision*, we found that *ADRs* accounted for over half of clozapine discontinuations. The results suggest that the role of ADRs has been underestimated as previous studies have used a restricted number of categories for discontinuation (i.e. *patient choice* and *non-adherence*), with no studies categorising the underlying reasons for non-adherence (Taylor et al., 2009; Krivoy et al., 2011; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). Furthermore, the results are consistent with studies that have shown a quarter to two thirds of non-adherence to other antipsychotics was attributable to ADRs (Fenton et al., 1997; Hudson et al., 2004).

The adverse effect most frequently cited was sedation, which accounted for 20% of all discontinuations. Interestingly, over half of discontinuations due to sedation were from discontinuations due to a *patient decision*. This is particularly worrying since sedation is

usually transient and can almost always be minimised by reducing the dose and/or titration rate of clozapine, adjusting the timing of the dose or partial substitution with less sedating drugs such as aripiprazole (Nair & MacCabe, 2014). Around 10% of patients who start clozapine are discontinuing for this reason; it is likely many could probably remain on clozapine if this adverse effect was more actively managed and monitored by the clinical team.

The second most common ADR was neutropenia, which occurred in 4.7% of the total sample. Previous research has shown that in many cases, neutropenia is not related to clozapine or is transient, and with the correct expertise, 80% can be reinstated on clozapine (Meyer et al., 2015). In this study, 10 of the 15 discontinuations due to neutropenia were in Black African/Caribbean patients and only a minority (3/10) had a haematological assessment for benign ethnic neutropenia. This raises the possibility that this condition remains under recognised (Whiskey et al., 2011).

A question that remains to be explored is whether the side effect profile of patients who discontinue clozapine differs from patients who continue with the treatment. It has been suggested that many discontinuations due to ADRs could be avoided (Nielsen et al., 2013), although the appropriateness of any given reason was not assessed in this study. Nonetheless, these findings suggest that prompt identification and appropriate management of ADRs has the potential to improve continuation of clozapine treatment.

Consistent with earlier reports (Taylor et al., 2009; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015), discontinuation due primarily to an *inadequate response* to clozapine was rare, occurring in only 2.5% of patients. Given that non-response to clozapine has been estimated between 40-70% (Kane et al., 1988; Lieberman et al., 1994b), this result is unlikely to reflect the true rates of non-response to clozapine but

rather that non-response is seldom recorded as the primary reason to discontinue treatment. A patient (or clinician) may be more likely to tolerate an ADR and be willing to persevere with clozapine if they are experiencing a good clinical response to clozapine, but might instead discontinue clozapine, citing adverse effects, in the absence of a clinical response. The decision to discontinue clozapine is likely to be a multifactorial one involving a judgement as to the balance of the likely benefits versus harms of continuing versus stopping clozapine, taking into account the views of the patient and of his or her carers. Nevertheless, the small percentage of patients who discontinue primarily due to inadequate response is striking. It could be driven partly by concern over risk of further relapse upon cessation (Seppala et al., 2005) and partly by a lack of any other evidence-based treatment options.

An interesting and novel insight was the observation that discontinuation of clozapine due to *dislike of blood monitoring* was reported in 3.5% of patients. This raises the question of whether rates would be higher in all those eligible for clozapine, an important issue given that low rates of clozapine prescription have been attributed to the burden of blood monitoring (Nielsen et al., 2010).

In this cohort of patients initiating clozapine, three patients died (2% of discontinuations) during the follow-up period. It would be prudent not to over interpret this finding, but it does contrast with cross-sectional studies of clozapine discontinuation, which reported death as accounting for 13% of clozapine discontinuations (Taylor et al., 2009; Davis et al., 2014; Mustafa et al., 2015). This inconsistency is likely due to differences in study design. Previous studies assessed all discontinuations over a study period, regardless of duration of clozapine treatment, whereas this study assessed the discontinuations within 24

months of initiation. Consequently, the mean duration of exposure to clozapine and the average age of patients are lower in this sample.

4.5.2 Risk factors for discontinuation

This is the first study to observe an association between level of social deprivation and risk of clozapine discontinuation. Furthermore, it was found this result was driven by discontinuations resulting from a *patient decision*. Previous studies have reported mixed results regarding the relationship between socio-economic status and non-adherence to medication (Kane et al., 2013), although it is not measured in many studies. It is likely social deprivation is a proxy marker for other factors that underlie discontinuation and non-adherence and further research is needed to determine whether particular characteristics of these patient groups increase risk for clozapine discontinuation or perhaps whether clinical teams supporting areas in high deprivation are under increased pressure or have more limited resources.

Consistent with previous studies, there were increased rates of all-cause clozapine discontinuation in Black African/Caribbean patients (Moeller et al., 1995; Davis et al., 2014); 54% of Black African/Caribbean patients discontinued compared with 40% of non-Black African/Caribbean patients. However, this association attenuated and was not statistically significant after adjusting for other factors. There was no evidence to support previous findings that higher age at clozapine initiation increased risk for discontinuation (MacGillivray et al., 2003; Krivoy et al., 2011; Davis et al., 2014).

4.5.3 Study limitations

A limitation of this study is its retrospective nature, specifically that the quality of data available was limited to information entered into the electronic case note system by the

patient's clinical team. However, benefits of this study design are that the results are reflective of routine clinical care and there was universal capture of patients commencing clozapine in a defined geographical area covering a population of 1.2 million people, with consequently little or no selection bias. The fact that informed consent was not required also eliminated the selection bias in favour of higher functioning patients that bedevils research on psychosis. Recall bias was minimised by the use of contemporaneous records and the minimal missing data allowed us to determine the reasons for discontinuation for all of the patients. Furthermore, CRIS incorporates routinely collected data from multiple sources, such as pharmacy dispensing information, to increase reliability.

The exclusion of patients who solely received tertiary care in SLaM national services (such as the National Psychosis Unit) may mean that our findings do not extend to a group of patients with particularly complex or co-morbid mental illness. However, patients who were residing in SLaM but received tertiary care were included in the study and so these findings should be widely applicable.

An additional limitation is that only the discontinuations within the first 24 months of treatment onset were assessed. However, previous studies show that a substantial proportion of those who discontinue do so within the first year of treatment (Pai et al., 2012; Davis et al., 2014). The proportion of patients that discontinued clozapine in this study is in the mid-range (20%-57%) of other studies whose durations ranged from 6 months to 15 years (Moeller et al., 1995; Laker et al., 1998; Ciapparelli et al., 2000; Hayhurst et al., 2002; MacGillivray et al., 2003; Krivoy et al., 2011; Pai & Vella, 2012; Davis et al., 2014). Additional confidence in the study design and its generalisability comes from the fact the frequencies of reasons for discontinuation, other than rates of death, are similar to studies with longer follow-ups suggesting discontinuations after two years are

not qualitatively different. Furthermore, the study design allowed an analysis of timing of first clozapine discontinuation and comparison with those that continued with treatment.

The sample is comparable to the population in London with a diagnosis of schizophrenia (Morgan et al., 2006) but has higher proportions of Black African/Caribbean ethnicity and lower proportions of Asian ethnicity in comparison to England as a whole (Stewart et al., 2009). However, the National Audit of Schizophrenia (2012) suggest that the service use in London is comparable to the rest of the UK (Royal College of Psychiatrists, 2012). The only other UK study investigating clozapine discontinuation was conducted in the same area as this study, but the samples are entirely independent (Taylor et al., 2009).

4.5.4 Clinical implications

Considering that clozapine is the most effective treatment for TRS (Kane et al., 1988; Lieberman et al., 1994b), it is important that avoidable discontinuation is minimised. By examining the reasons for discontinuations due to a patient decision we found that ADRs accounted for the majority of clozapine discontinuations. It is important that clinicians identify and treat ADRs attributed to clozapine, particularly in the first few months after treatment onset, before they lead to discontinuation. Patients who live in an area of high deprivation are at an increased risk of discontinuing clozapine and may need additional support to maintain engagement with treatment.

Chapter 5

Clozapine Response

5.1. Summary

Clinical trials indicate that 30-60% of patients with treatment-resistant schizophrenia (TRS) will respond to clozapine treatment. Predictors of response would be valuable in assisting clinicians in deciding if clozapine treatment is likely to be beneficial. The predictive factors and timing of long-term response to clozapine were investigated in a two-year retrospective cohort study of patients with TRS receiving their first course of clozapine. The Clinical Global Impressions (CGI) scale was administered from case notes at the start of clozapine treatment and after 1, 2, 3, 6, 9, 12, 18 and 24 months. Due to the substantial variability of clozapine response definitions used in previous studies, we considered several definitions of response. In total, 114 (41.2%) patients experienced an *absolute* response (CGI-I ≤ 2 and CGI-S ≤ 3), 48.4% of patients had a *relative* response (reduction ≥ 2 CGI-S scores), and 19.1% had an *exceptional* response (CGI-I score of 1) over the study period. In a fully adjusted Cox regression, female gender was significantly associated with a good response to clozapine (hazard ratio (HR) = 0.63, 95% CI 0.41-0.96), regardless of the response definition. In contrast there was an inconsistent association between baseline severity and response; higher baseline CGI severity was associated with poor *absolute* (HR = 0.43, 95% CI 0.31-0.60) and *exceptional* (HR = 0.60, 95% CI 0.38-0.95) response, but good *relative* response (HR = 3.24, 95% CI 2.34-4.50). Clinical improvement after one month of treatment was a strong predictor of response within 24 months. We found that 75% of *absolute* responders were at least much improved (CGI-I score of 2) at two months, which

increased to 92% at six months. Of the patients who were either minimally improved or not changed after two months of treatment, 23% and 38% went on to meet *absolute* and *relative* response criteria within 24 months, respectively. By six months this proportion reduced to 14%. The results suggest that responders are more likely to be female, tend to have a good initial response to clozapine. However, six months of treatment may be required to determine non-response.

5.2. Introduction

The superior efficacy of clozapine has been consistently demonstrated for those with treatment-resistant schizophrenia (TRS) (Kane et al., 1988; McEvoy et al., 2006; Leucht et al., 2009), defined by the National Institute for Health and Care Excellence (NICE) as a failure to respond to two antipsychotic trials at a therapeutic dose for at least six weeks (National Institute for Health and Care Excellence, 2009). A third of patients with schizophrenia are estimated to be treatment-resistant (Meltzer, 1997) and thus potentially eligible for clozapine treatment.

In addition to substantially reduced psychotic symptoms, treatment with clozapine in comparison to other antipsychotic medications has been associated with decreased rates of mortality (Hayes et al., 2015), suicide (Meltzer et al., 2003a; Tiihonen et al., 2009), aggression (Chengappa et al., 2002), and financial costs due to reduced hospitalisations (Honigfeld & Patin, 1990; Hayhurst et al., 2002). However, clinical trials indicate that only 30-60% of patients with TRS will respond to clozapine (Kane et al., 1988; Lieberman et al., 1994b) and previous studies indicate that this response can be detected within the first 6-8 weeks of clozapine treatment (Rosenheck et al., 1999b; Suzuki et al., 2011a).

An estimated 40% of patients eligible for clozapine in the UK have not received a trial (Royal College of Psychiatrists, 2012) and in those that have, there is a four-year delay from eligibility to first treatment (Howes et al., 2012b). The widespread underutilisation of clozapine may be due in part to its significant side effect profile (Atkin et al., 1996; Henderson et al., 2000). Of particular importance is the risk of agranulocytosis, which necessitates regular blood monitoring (Atkin et al., 1996). This burden of monitoring limits the acceptability of clozapine to patients, and poses an additional obstacle to clinician recommendation and use in clinical practice.

Predictors of clozapine response would be valuable in assisting clinicians in deciding if clozapine treatment is likely to be beneficial and may increase the number of patients who are likely to benefit being offered clozapine. A number of studies have attempted to address this question and there have been several reviews (Chung & Remington, 2005; Suzuki et al., 2011c). Severe baseline symptoms (Hasegawa et al., 1993; Lieberman et al., 1994b; Rosenheck et al., 1998; Ciapparelli et al., 2000; Umbricht et al., 2002; Semiz et al., 2007; Kelly et al., 2010) and an early response, even after one week of treatment (Stern et al., 1994; Semiz et al., 2007), have been associated with significantly better response to clozapine. Other clinical markers including premorbid functioning (Umbricht et al., 2002; Kelly et al., 2010), age at clozapine initiation (Hofer et al., 2003; Mauri et al., 2003; Semiz et al., 2007; Kelly et al., 2010), age at onset of schizophrenia (Ciapparelli et al., 2004; Semiz et al., 2007; Kelly et al., 2010; Nielsen et al., 2012), diagnosis (Fenton & Lee, 1993; Ciapparelli et al., 2004; Semiz et al., 2007), and previous extrapyramidal symptoms (Pickar et al., 1994; Umbricht et al., 2002) have yielded less consistent results. Reports regarding the role of gender have also been contradictory (Lieberman et al., 1994a; Szymanski et al., 1996; Hofer et al., 2003; Mauri et al., 2003; Ciapparelli et al., 2004; Semiz et al., 2007; Usall

et al., 2007) whilst results have been consistent in the lack of association between ethnicity and response (Lieberman et al., 1994a; Rosenheck et al., 1998; Kelly et al., 2010).

These somewhat conflicting reports may be due to the considerable variability in response definitions as well as trial durations, resulting in limited comparability between studies. Several studies have used the *absolute* response criteria defined by Kane and Colleagues; a 20% decrease in Brief Psychiatric Rating Scale (BPRS) total score, and either a post-treatment Clinical Global Impressions (CGI) severity score ≤ 3 or BPRS ≤ 35 (Kane et al., 1988). Other studies have focused purely on percentage change and adopted a *relative* response criteria, for example a ≥20% decrease in Positive and Negative Syndrome Scale (PANSS) or BPRS total scores (Usall et al., 2007; Suzuki et al., 2012). A recent review recommended a response definition of a CGI-Improvement score of 2 or better (Suzuki et al., 2012), which approximately corresponds to a 40-53% reduction in BPRS total score (Leucht et al., 2005a; Leucht & Engel, 2006).

The study duration of previous studies investigating clozapine response has tended to be short (Meltzer et al., 1989a) and consequently may not have considered a group of patients that take longer to meet the response criteria. This may be particularly important for patients starting clozapine, as they are likely to be highly symptomatic. In addition, many studies are conducted in relatively small sample sizes and clinical trial data may not be generalisable to patients receiving clozapine in standard health care settings.

5.2.1 Aims of the study

In a two-year retrospective cohort study of all patients starting their first clozapine trial over a five-year period (2007-2011, inclusive) in SLaM, the study aims were to: (i) select a primary response criterion by comparing the sensitivity and specificity of different response definitions against the groups outlined by group-based trajectory modeling, (ii)

identify demographic and baseline clinical predictors of clozapine response, (iii) assess whether early improvement is indicative of long-term response, and (iv) explore the timing of response to clozapine and the duration of treatment required to detect a response.

5.3. Method

Full details of the setting, ethical approval and sample selection are presented in sections 3.1, 3.2 and 3.3, respectively. The procedures used for clinical assessments are specified in section 3.5.

5.3.1 Sample

The sample used for this analysis consisted of a cohort of patients who had an ICD-10 primary diagnosis of a psychotic disorder (F20-F29, inclusive) and began a first trial of clozapine between 1 January 2007 and 31 December 2011 in SLaM. Patients were aged 18-65 years at the start of clozapine treatment and initiated clozapine under standard secondary mental health care services, either as an inpatient or outpatient. Patients who discontinued clozapine within the first month of treatment were excluded from this analysis, as they did not have an adequate clozapine trial and thus the level of response could not be reliably measured.

5.3.2 Clinical assessments

To assess response to clozapine, the Clinical Global Impressions (CGI) scale was administered at the start of clozapine treatment (baseline) and at 1, 2, 3, 6, 9, 12, 18 and 24 months after clozapine initiation. The CGI is a global measure that comprises two scores (i) severity (CGI-S, detailed in **Table 3.1**), and (ii) improvement (CGI-I, detailed in **Table 3.2**). Information used for the CGI ratings came from the descriptive case notes within CRIS.

Baseline was defined as the week prior to clozapine initiation. Assessments at subsequent time points took a global impression of the time period from the last assessment. Further details of procedures used are given in section 3.5.

5.3.3 Clozapine response

Due to variability of clozapine response definitions used in previous studies (Suzuki et al., 2011b; Suzuki et al., 2012), three definitions of response were considered: *absolute*, *relative* and *exceptional*. If the patient met the relevant criteria at any assessment over the study period they were classed as a responder. If the patient did not meet these criteria at any assessment they were classed as a poor responder.

***Absolute response*:** CGI-S score of 3 (mildly ill) or less and a CGI-I score of 2 (much improved) or less. This definition is focused on patients who achieved a good level of functioning and is reflective of frequently used criteria originally defined by Kane and colleagues (Kane et al., 1988), which included a post-treatment severity threshold in addition to improvement.

***Relative response*:** Reduction of 2 or more CGI-S scores. For example, if the patient's CGI-S score was 6 at baseline and reached a 4 within the study period they would be classified as a relative responder. This second definition is purely focused on the change from baseline.

***Exceptional response*:** CGI-I score of 1 (very much improved). This definition is focused on the patients that experienced the greatest benefit from clozapine treatment.

The study period began at clozapine initiation and ended with discontinuation, death, or after 24 months of treatment. The date of clozapine initiation was defined as the date the patient took their first dose of clozapine. The date of discontinuation was defined as the date the patient was last known to take clozapine, where this was followed by at least

three consecutive months without clozapine treatment. Time to clozapine response was defined as the duration from baseline to the assessment that the patient first met the relevant response criteria.

5.3.4 Exposure variables

Details of age, gender, marital status, ethnicity, diagnosis, inpatient status and detention under the Mental Health Act were obtained from structured fields within CRIS. Level of deprivation was calculated from the Index of Multiple Deprivation (IMD) for England (2007) for the patient's home address. Key definitions regarding these variables are described in section 3.6.

5.3.5 Analysis

To select a primary response definition, group membership was compared for each patient between that identified by group-based trajectory modeling (GBTM) and *absolute* and *relative* response definitions. GBTM is an application of finite mixture modeling and is designed to identify clusters of individuals with similar developmental trajectories (Nagin & Odgers, 2010). GBTM identifies homogenous groups based on growth parameters describing each patient's initial rating level and rate of change of CGI-S scores with each patient being assigned a probability of belonging to each group (Nagin, 2005; Jones & Nagin, 2013). In comparison to growth mixture modeling, it identifies groups to approximate a continuous distribution of trajectories of unknown shape, rather than assuming there are distinct subpopulations (Nagin & Odgers, 2010). I specified for GBTM to identify two groups: responders and poor responders. The definition of response with the optimal sensitivity and specificity was selected for the main analyses.

A Kaplan-Meier survival curve was used to display the time to clozapine response. Having checked proportional hazard assumptions, a Cox regression was employed to model the association between clozapine response and gender, age, marital status, ethnicity, level of deprivation, diagnosis, inpatient status, detention under the Mental Health Act and baseline CGI-S score. Associations with clozapine response were assessed in a crude analysis, and also fully adjusted for all covariates examined. Level of deprivation was entered into the model as a categorical dummy variable. A likelihood ratio test was used to test the appropriateness of entering age and baseline CGI-S as a continuous variable. Interaction effects with age, gender and ethnicity for variables associated with clozapine response at $P < 0.05$ were investigated. A sensitivity analysis was conducted excluding patients without ICD-10 schizophrenia diagnosis. Secondary analyses using the alternative definition of clozapine response (i.e. the definition not selected from the GBTM) were conducted to assess consistency of results. In addition, a Cox regression was used to investigate the association of the above factors with *exceptional* response. The association of baseline clinical and demographic factors with gender were assessed in a crude and fully adjusted logistic regression.

To assess the impact of early improvement on response to clozapine, Cox regression was employed to test the association between CGI-I score after one month of treatment and each clozapine response definition. Additional sensitivity analyses were conducted (i) adjusting for other significant predictors identified, (ii) fully adjusted for all covariates, and (iii) excluding patients that met response criteria within one month.

To investigate how long to wait for a response to clozapine the likelihood of a delayed response was assessed by calculating the proportion of patients with a CGI-I score of 3 or 4 (minimally improved or no change) at particular time points that subsequently met

response criteria. These groups were combined due to low numbers in each individual group and because a CGI-I score of 3 (minimally improved) represents a slight improvement with little or no clinically meaningful reduction of symptoms (Busner & Targum, 2007).

All statistical analyses were performed using STATA version 12 (StataCorp, 2011).

5.4. Results

5.4.1 Patient Characteristics

A total of 316 patients were initiated on clozapine during the study period, 39 of which discontinued treatment within 30 days. Thus, 277 patients were included in this analysis. **Table 5.1** provides sample characteristics. The mean age at clozapine initiation was 36 years. The majority of the sample had a diagnosis of schizophrenia (n=250) and the most common non-schizophrenia F21-9 diagnosis was schizoaffective disorder (n=18). Of the 143 (51.6%) that were detained under the Mental Health Act at the time of clozapine initiation, the majority were detained under a section 3 (detention for treatment, n=109) or under sections 37-49 (forensic, n=27). The mean baseline CGI-S score for the total sample at the time of clozapine initiation was 5.15 (SD 0.67, markedly ill).

Characteristic	**N**	**%**
Male gender	184	66.4
Mean age (years)	36.34	SD: 10.9
Married or cohabiting	21	7.6
Ethnicity		
White British / White other	111	40.1
Black African / Caribbean	133	48.0
Asian	15	5.4
Other	18	6.5
Level of deprivation		
Low	75	27.1
Intermediate	99	35.7
High	84	30.3
Homeless	13	4.70
Schizophrenia diagnosis	250	90.2
Inpatient	231	83.4
Detained under Mental Health Act	143	51.6
Mean baseline CGI-Severity	5.15	SD: 0.67

Table 5.1. *Sample characteristics. SD = standard deviation. Percentages relate to total sample (n=277). Data for all 277 patients was available for each variable other than level of deprivation, which was available for 271 patients.*

5.4.2 Defining clozapine response

A substantial proportion of the sample had much improved (CGI-I score of 2, n=162) or very much improved (CGI-I score of 1, n=53) over the study period in comparison to their condition at clozapine initiation. The remaining sample was either minimally improved (CGI-I score of 3, n=50) or experienced no change (CGI-I score of 4, n=12). **Figure 5.1** displays the mean CGI-S scores over the study period based on the patient's lowest (most improved) CGI-I score. The mean reduction over the study period of CGI-S score was 2.34 (from 5.04 to 2.70) for very much improved patients, 1.55 (from 5.19 to 3.64) for much improved patients, 0.76 (from 5.18 to 4.42) for minimally improved and 0.08 (from 4.92 to 4.83) for those who experienced no change. The raw data for **Figure 5.1** is presented in Appendix 8.

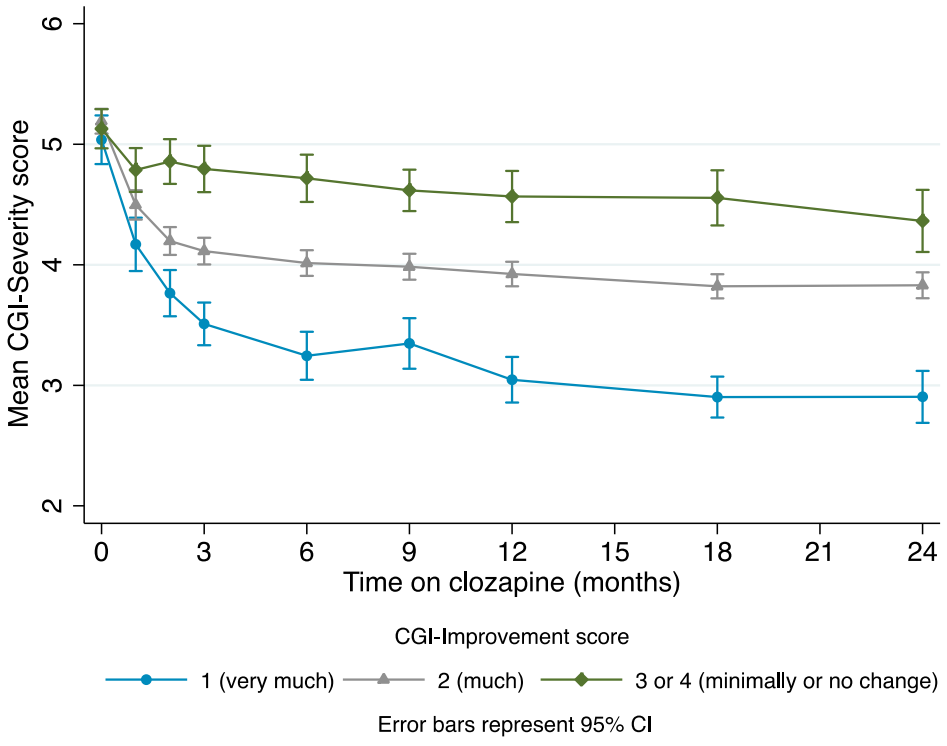

Figure 5.1. *Mean CGI-Severity scores at time assessment points for patients who had an improvement score of 1 (very much improved, blue), 2 (much improved, grey), and either 3 or 4 (minimally improved or no change, green). Error bars represent 95% confidence intervals.*

A total of 114 (41.2%) patients showed an *absolute* response (CGI-I ≤ 2 and CGI-S ≤ 3) to clozapine over the study period. Alternatively, 134 (48.4%) patients experienced a *relative* response (reduction ≥ 2 CGI-S score) and 58 (19.1%) demonstrated an *exceptional* response (CGI-I score of 1) to clozapine.

Group-based trajectory modeling (GBTM) classified 40.7% of the sample into one response trajectory and 59.3% of the sample into the second response trajectory (see **Figure 5.2**). The former group had more substantial reductions in severity and thus we refer to this group as responders. Using these groups as the gold standard, the sensitivity and specificity of the *absolute* response definition was 0.88 and 0.91, respectively. The *relative* response definition had a sensitivity of 0.68 and specificity of 0.65. Therefore, *absolute* response was selected as the primary response definition. Although the 163 (58.8%) other patients may have improved somewhat, for the purposes of these analyses they were classified as poor *absolute* responders.

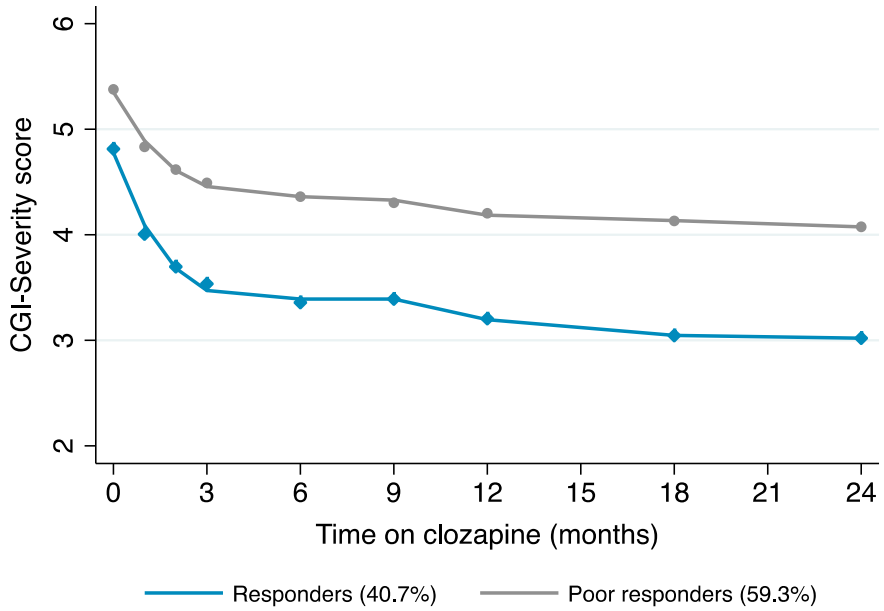

Figure 5.2. *Clozapine response groups as classified by group-based trajectory modeling (GBTM) from CGI-Severity scores. Percentages relate to proportion of total sample in each group.*

5.4.3 Predictors of response

Table 5.2 details the association of baseline clinical factors with *absolute* response to clozapine. In a fully adjusted Cox regression model, female gender (hazard ratio (HR) = 0.63, 95% confidence intervals (CI) 0.41-0.96) and lower CGI-S (less unwell) at baseline (HR = 0.43, 95% CI 0.31-0.60) were associated with good *absolute* response. Age, marital status, ethnicity, level of deprivation, diagnosis, inpatient status and detention under MHA were not associated with *absolute* response. No interaction effects were identified and there were no differences in a sensitivity analysis restricted to those with a schizophrenia diagnosis (**Table 5.6**).

I also conducted a secondary analysis considering the alternative definition of *relative* response (**Table 5.3**). In a fully adjusted Cox regression model, female gender (HR = 0.49, 95% CI 0.33-0.72) and a higher CGI-S at baseline was strongly associated with good *relative* response (HR = 3.24, 95% CI 2.34-4.50). In a further analysis regarding *exceptional* response, female gender (HR = 0.41, 95% CI 0.23-0.73) and lower CGI-S at baseline (HR = 0.60, 95% CI 0.38-0.95) were associated with an *exceptional* response to clozapine (**Table 5.4**).

Due to the observed relationship between gender and clozapine response, the association of other baseline clinical and demographic factors with gender was investigated (**Table 5.5**). In a fully adjusted logistic regression, male patients were significantly younger than females (HR = 0.95, 95% CI 0.93-0.98); the mean age at clozapine initiation was 34.6 years for males and 39.8 years for females. Males were also less likely to be of Black African/Caribbean ethnicity (HR = 0.52, 95% CI 0.11-0.64) and were more likely to live in an area of intermediate (HR = 2.27, 95% CI 1.13-4.56) or high (HR = 2.38, 95% CI 1.14-4.95) deprivation. In addition, males were also less likely to have non-schizophrenia F20

diagnosis (HR = 0.27, 95% CI 0.11-0.64). However, controlling for these factors did not impact on the strength of the association between gender and clozapine response.

Characteristic	Absolute responders (N=114)	Poor responders (N=163)	Crude		Fully Adjusted	
			Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Demographic factors						
Male gender	68 (59.65)	116 (71.17)	0.67 (0.46-0.98)	0.038	0.63 (0.41-0.96)	0.034
Age (mean years)	36.91 (SD: 10.32)	35.94 (SD: 11.35)	1.00 (0.99-1.02)	0.587	1.00 (0.98-1.02)	0.873
Married or cohabiting	12 (10.53)	9 (5.52)	1.59 (0.88-2.90)	0.127	1.12 (0.58-2.19)	0.736
Black African / Caribbean ethnicity	51 (44.74)	82 (50.31)	0.93 (0.64-1.35)	0.699	0.98 (0.65-1.47)	0.915
Level of deprivation						
Low	40 (35.09)	35 (21.47)	Ref		Ref	
Intermediate	37 (32.46)	62 (38.04)	0.79 (0.50-1.23)	0.296	0.93 (0.57-1.52)	0.775
High	31 (27.19)	53 (32.52)	0.82 (0.51-1.31)	0.412	1.01 (0.61-1.68)	0.976
Homeless	3 (2.63)	10 (6.13)	0.42 (0.13-1.35)	0.146	0.66 (0.20-2.19)	0.497
Clinical factors						
Non-schizophrenia F20 diagnosis	8 (7.02)	19 (11.66)	0.74 (0.36-1.51)	0.407	0.60 (0.28-1.26)	0.176
Inpatient	93 (81.58)	138 (84.66)	0.76 (0.48-1.23)	0.266	1.42 (0.79-2.55)	0.235
Detained under Mental Health Act	52 (45.61)	91 (55.83)	0.76 (0.53-1.10)	0.144	0.92 (0.59-1.43)	0.712
Baseline CGI-Severity (mean)	4.94 (SD: 0.71)	5.29 (SD: 0.61)	0.47 (0.35-0.63)	3.14×10^{-7}	0.43 (0.31-0.60)	5.05×10^{-7}

Table 5.2. *Predictors of absolute clozapine response, defined as CGI-I ≤ 2 and CGI-S ≤ 3. Columns represent characteristics for responders and poor responders, hazard ratio and P-value from crude and fully adjusted Cox regression. The fully adjusted model included all variables. Data for all 277 patients was available for each variable other than level of deprivation, which was available for 271 patients. Note: Follow-up period began at start of clozapine treatment and ended with response, discontinuation, death or end of study period (24 months after treatment onset).*

Characteristic	Relative responders (N=134)	Poor responders (N=143)	Crude		Fully Adjusted	
			Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Demographic factors						
Male gender	78 (58.21)	106 (74.13)	0.56 (0.40-0.79)	9.60×10^{-4}	0.49 (0.33-0.72)	2.50×10^{-4}
Age (mean years)	37.03 (SD: 10.81)	35.68 (SD: 11.03)	1.01 (0.99-1.02)	0.303	0.99 (0.98-1.01)	0.396
Married or cohabiting	12 (8.96)	9 (6.29)	1.21 (0.67-2.19)	0.525	1.29 (0.68-2.43)	0.432
Black African / Caribbean ethnicity	61 (45.5)	72 (50.35)	0.99 (0.70-1.38)	0.932	0.91 (0.62-1.34)	0.645
Level of deprivation						
Low	45 (33.58)	30 (20.98)	Ref		Ref	
Intermediate	43 (32.09)	56 (39.16)	0.76 (0.50-1.15)	0.198	0.81 (0.52-1.25)	0.342
High	37 (27.61)	47 (32.87)	0.83 (0.54-1.28)	0.405	0.85 (0.53-1.36)	0.505
Homeless	5 (3.73)	8 (5.59)	0.69 (0.27-1.73)	0.423	0.51 (0.20-1.32)	0.165
Clinical factors						
Non-schizophrenia F20 diagnosis	8 (5.97)	19 (13.29)	0.51 (0.25-1.05)	0.067	0.41 (0.20-0.87)	0.021
Inpatient	124 (92.54)	107 (74.83)	3.01 (1.58-5.74)	8.10×10^{-4}	1.87 (0.90-3.87)	0.092
Detained under Mental Health Act	77 (57.46)	66 (46.15)	1.50 (1.07-2.12)	0.020	1.00 (0.67-1.49)	0.981
Baseline CGI-Severity (mean)	5.44 (SD: 0.62)	4.87 (SD: 0.60)	3.06 (2.28-4.09)	5.88×10^{-14}	3.24 (2.34-4.50)	1.96×10^{-12}

Table 5.3. *Predictors of relative clozapine response, defined as a reduction of 2 or more CGI-S scales. Columns represent characteristics for responders and poor responders, hazard ratio and P-value from crude and fully adjusted Cox regression. Fully adjusted model included all variables. Data for all 277 patients was available for each variable other than level of deprivation, which was available for 271 patients. Note: Follow-up period began at start of clozapine treatment and ended with discontinuation, death or end of study period (24 months after treatment onset).*

Characteristic	Exceptional responders (N=53)	Poor responders (N=224)	Crude model		Fully Adjusted model	
			Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Demographic factors						
Male gender	26 (49.06)	158 (70.54)	0.43 (0.25-0.74)	0.002	0.41 (0.23-0.73)	0.003
Age (mean years)	37.02 (SD: 10.43)	36.17 (SD: 11.06)	1.01 (0.98-1.03)	0.625	1.00 (0.97-1.03)	0.936
Married or cohabiting	7 (13.21)	14 (6.25)	2.02 (0.91-4.48)	0.082	1.71 (0.71-4.12)	0.235
Black African / Caribbean ethnicity	25 (47.17)	108 (48.21)	1.07 (0.63-1.84)	0.794	1.30 (0.71-2.40)	0.394
Level of deprivation						
Low	19 (35.85)	56 (25.00)	Ref		Ref	
Intermediate	19 (35.85)	80 (35.71)	0.87 (0.46-1.65)	0.680	1.25 (0.62-2.50)	0.535
High	12 (22.64)	72 (32.14)	0.71 (0.34-1.46)	0.354	0.99 (0.45-2.16)	0.974
Homeless	2 (3.77)	11 (4.91)	0.66 (0.15-2.82)	0.572	0.98 (0.22-4.47)	0.984
Clinical factors						
Non-schizophrenia F20 diagnosis	3 (5.66)	24 (10.71)	0.56 (0.17-1.78)	0.322	0.42 (0.13-1.40)	0.159
Inpatient	47 (88.68)	184 (82.14)	1.57 (0.67-3.68)	0.297	2.57 (0.98-6.77)	0.056
Detained under Mental Health Act	24 (45.28)	119 (53.13)	0.82 (0.48-1.40)	0.463	0.69 (0.37-1.27)	0.231
Baseline CGI-Severity (mean)	5.04 (SD: 0.73)	5.17 (SD: 0.66)	0.73 (0.49-1.10)	0.135	0.60 (0.38-0.95)	0.031

Table 5.4. *Predictors of exceptional clozapine response, defined as CGI-I score of 1 (very much improved). Columns represent characteristics for responders and poor responders, hazard ratio and P-value from crude and fully adjusted Cox regression. Fully adjusted model included all variables. Data for all 277 patients was available for each variable other than level of deprivation, which was available for 271 patients. Note: Follow-up period began at start of clozapine treatment and ended with discontinuation, death or end of study period (24 months after treatment onset).*

Characteristic	Male (N=184)	Female (N=93)	Crude model		Fully Adjusted model	
			OR (95% CI)	P	OR (95% CI)	P
Demographic factors						
Age (mean years)	34.58 (SD: 9.90)	39.80 (SD: 12.05)	0.96 (0.93-0.98)	0.002	0.95 (0.93-0.98)	3.10×10^{-4}
Married or cohabiting	9 (4.89)	12 (12.90)	0.35 (0.14-0.86)	0.022	0.62 (0.22-1.74)	0.364
Black African / Caribbean ethnicity	87 (47.28)	46 (49.46)	0.92 (0.56-2.92)	0.732	0.52 (0.29-0.94)	0.031
Level of deprivation						
Low	42 (23.46)	33 (35.87)	Ref			
Intermediate	69 (38.55)	30 (32.61)	1.81 (0.97-3.38)	0.064	2.27 (1.13-4.56)	0.021
High	58 (32.40)	26 (28.26)	1.69 (0.92-3.36)	0.090	2.38 (1.14-4.95)	0.020
Homeless	10 (5.59)	3 (3.26)	2.62 (0.67-10.29)	0.168	3.11 (0.72-13.46)	0.129
Clinical factors						
Non-schizophrenia F20 diagnosis	12 (6.52)	15 (16.13)	0.36 (0.16-0.81)	0.014	0.27 (0.11-0.64)	0.003
Inpatient	150 (81.52)	81 (87.10)	0.65 (0.32-1.33)	0.241	0.59 (0.24-1.46)	0.249
Detained under Mental Health Act	94 (51.09)	49 (52.69)	0.94 (0.57-1.55)	0.801	1.23 (0.65-2.31)	0.528
Baseline CGI-Severity (mean)	5.13 (SD: 0.67)	5.19 (SD: 0.68)	0.86 (0.59-1.25)	0.423	0.92 (0.59-1.44)	0.722

Table 5.5. *Association of baseline demographic and clinical factors with gender. Percentages relate to total sample (n=277). Columns represent characteristics for male and female patients, odds ratio (OR) and P-value from crude and fully adjusted logistic regression. Fully adjusted model included all variables. Data for all 277 patients was available for each variable other than level of deprivation, which was available for 271 patients.*

	Crude		Fully Adjusted[1]	
Characteristic	**Hazard ratio** (95% CI)	**P-value**	**Hazard ratio** (95% CI)	**P-value**
Male gender	0.60 (0.41-0.89)	0.010	0.64 (0.41-0.98)	0.042
Age at clozapine onset (years, SD)	1.01 (0.99-1.02)	0.543	1.00 (0.98-1.02)	1.000
Currently married or cohabiting	1.53 (0.80-2.94)	0.199	1.32 (0.66-2.66)	0.434
Black African/Caribbean ethnicity	0.95 (0.65-1.39)	0.781	1.05 (0.69-1.60)	0.812
Level of deprivation				
Low	Ref		Ref	
Intermediate	0.80 (0.50-1.26)	0.336	0.93 (0.57-1.52)	0.784
High	0.79 (0.48-1.28)	0.334	0.89 (0.53-1.50)	0.660
Homeless	0.46 (0.14-1.48)	0.190	0.53 (1.16-1.73)	0.290
Inpatient	0.86 (0.51-1.44)	0.569	1.04 (0.57-1.87)	0.574
Detained under Mental Health Act	0.79 (0.54-1.16)	0.233	0.82 (0.52-1.27)	0.524

Table 5.6. *Sensitivity analysis for absolute clozapine response only including patients with a schizophrenia diagnosis (ICD-10 F20) (n=250). Columns represent Cox regression hazard ratio and P-value from crude and fully adjusted Cox regression. [1]Fully adjusted includes all variables. Data for all 250 patients was available for each variable other than level of deprivation, which was available for 244 patients.*

5.4.4 Early improvement predicts good response

After one month of treatment, those who met *absolute* response criteria within 24 months had a mean CGI-I score of 2.70 (SD 0.79) compared to 3.11 (SD 0.80) for poor responders. Cox regression models (**Table 5.7**) indicated a strong association between a lower (more improved) CGI-I score at one month and good *absolute* response by 24 months (HR = 0.56, 95% CI 0.44-0.71), which remained significant and not substantially reduced in strength after adjusting for gender and baseline severity, or after adjusting for all covariates used in the prediction analyses. In addition, CGI-I score at one month was also strongly associated with *relative* response (HR = 0.53, 95% CI 0.43-0.66), and *exceptional* response (HR = 0.42, 95% CI 0.27-0.63). Removing the 22 individuals that met *absolute* response criteria within the first month reduced the strength of the association (HR = 0.72, 95% CI 0.55-0.93).

	Hazard Ratio (95% CI)	**P**
Absolute response		
Crude	0.56 (0.44-0.72)	3.82×10^{-6}
Adjusting for gender and baseline severity	0.50 (0.38-0.64)	9.97×10^{-8}
Fully Adjusted	0.48 (0.36-0.63)	2.27×10^{-7}
Removing 22 that met response criteria within 1 month	0.72 (0.55-0.93)	0.013
Relative response		
Crude	0.53 (0.42-0.66)	2.51×10^{-8}
Adjusting for gender and baseline severity	0.53 (0.42-0.67)	1.93×10^{-7}
Fully Adjusted	0.49 (0.40-0.64)	9.53×10^{-8}
Exceptional response		
Crude	0.42 (0.28-0.62)	4.07×10^{-5}
Adjusting for gender and baseline severity	0.43 (0.29-0.64)	2.92×10^{-5}
Fully Adjusted	0.43 (0.28-0.65)	5.66×10^{-5}

Table 5.7: *Association between early improvement and clinical response.*

5.4.5 Timing of clozapine response

Figure 5.3 displays the Kaplan-Meier survival curve for time to *absolute* response. Of all patients starting their first trial of clozapine, 7% achieved *absolute* response after 1 month of treatment, 18% by 3 months, 24% by 6 months, 34% by 12 months and 41% by 24 months. Although patients continued to meet *absolute* response across the study period, change was more pronounced in the initial three months. Time to *relative* response shows a similar pattern (**Figure 5.5**). Rather than indicating delayed response, this pattern of results may reflect continued gradual improvement; 45% of the *absolute* responders were much improved (CGI-I score of 2) at 1 month, 75% by 2 months and 92% by 6 months.

Figure 5.4 displays the mean CGI-S and CGI-I scores over the study period for *absolute* responders and poor responders (*relative* response displayed in **Figure 5.6**). The CGI-S score for the total sample decreased by an average of 1.51 scores in 24 months, from 5.15 to 3.64. The mean CGI-S change for *absolute* responders was 2.09 (4.94 to 2.85) and for poor responders 1.08 (5.29 to 4.21).

Likelihood of delayed response

A third of patients (33.5%) who had either a CGI-I score of 3 (minimally improved) or 4 (no change in psychopathology) after one month of treatment went on to meet *absolute* response criteria within 24 months. This proportion reduced to 22.9% at 2 months, 20.7% at 3 months, 14.1% at 6 months, 13.5% at 9 months, 7.3% at 12 months, and 0% at 18 months. The results were comparable for *relative* response (38.1%, 37.6%, 27.6%, 14.1%, 13.5%, 12.2% and 10.3% at 1, 2, 3, 6, 9, 12 and 18 months respectively). The raw data for these figures are provided in Appendix 9.

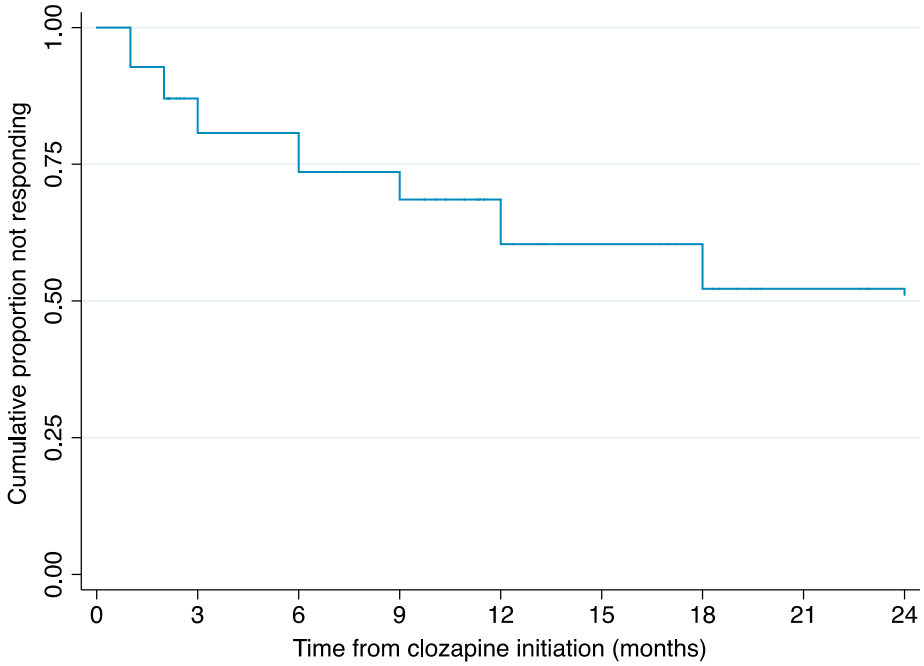

Figure 5.3. *Kaplan-Meier survival curve demonstrating proportion achieving absolute response to clozapine over initial 24 months of clozapine treatment.*

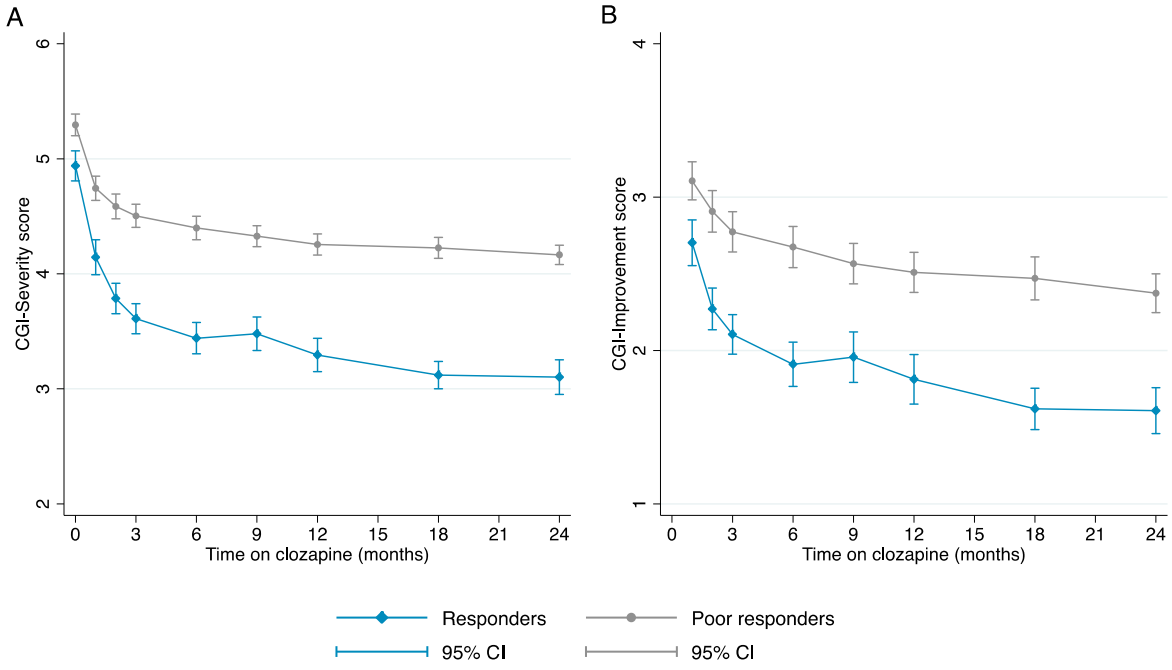

Figure 5.4*. Mean clinical assessment scores for responders (blue) and poor responders (grey) over initial 24 months of clozapine treatment. Graph (A) displays mean CGI-Severity score and (B) displays mean CGI-Improvement scores. Error bars represent 95% confidence intervals.*

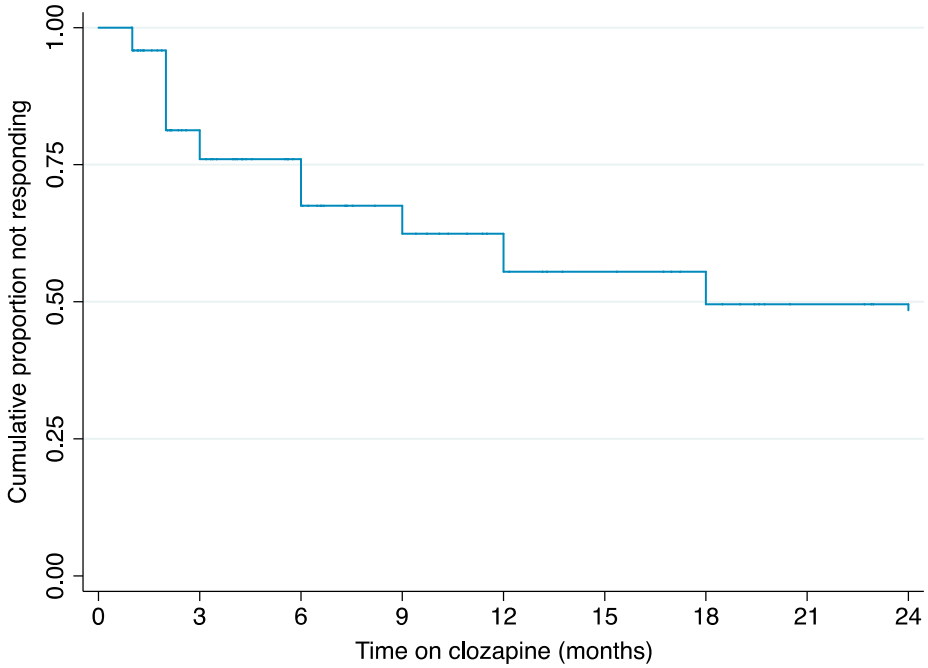

Figure 5.5. *Kaplan-Meier survival curve demonstrating proportion achieving relative response to clozapine over initial 24 months of clozapine treatment.*

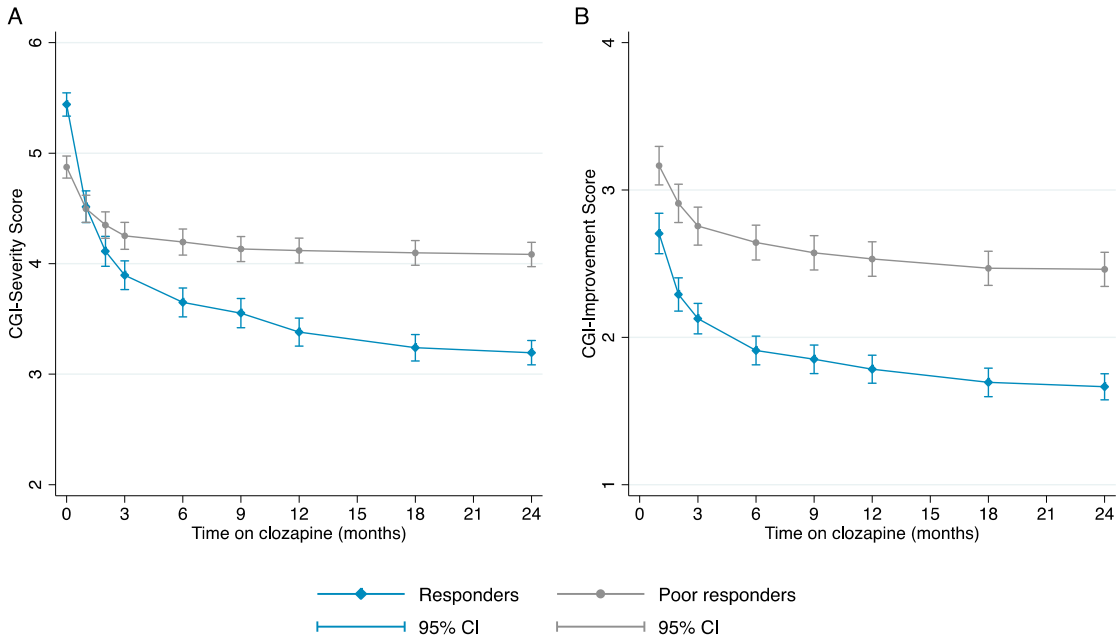

Figure 5.6. *Mean clinical assessment scores for relative responders (blue) and poor responders (grey) over initial 24 months of clozapine treatment. Graph A displays mean CGI-Severity score and graph B displays mean CGI-Improvement scores. Error bars represent 95% confidence intervals.*

5.5. Discussion

In a long-term retrospective cohort study of 277 patients with TRS, 41.2% of patients experienced an *absolute* response to their first trial of clozapine within 24 months. Considering alternative definitions, 48.4% of patients had a *relative* response and 19.1% had an *exceptional* response over the study period. We found that female gender was significantly associated with a good response to clozapine, regardless of the response definition. In contrast, the association of baseline severity with response was inconsistent; higher baseline severity was associated with poor *absolute* and *exceptional* response, but good *relative* response. Clinical improvement after one month of treatment was a strong predictor of response within 24 months. However, up to six months of treatment may be required to determine non-response to clozapine.

5.5.1 Gender and clozapine response

Female gender was significantly associated with a good response to clozapine and the strength of this association remained unchanged for different response definitions and after adjusting for potential confounders. Previous studies have reported conflicting findings regarding the role of gender (Lieberman et al., 1994a; Szymanski et al., 1996; Hofer et al., 2003; Mauri et al., 2003; Ciapparelli et al., 2004; Semiz et al., 2007; Usall et al., 2007). Small sample sizes, short study durations and differing response definitions are likely to have contributed to the lack of consistent findings. However, the findings in this study are supported by the SOHO study, one of the largest studies reported to date, which found that females had significantly better response to typical antipsychotics and clozapine (Usall et al., 2007). The SOHO study was similar to our study in regards to sample size (n=274), long follow-up period (six months), and definition of response.

There were important sociodemographic and clinical differences between males and females in our sample. Male patients were an average of five years younger than female patients at the start of clozapine treatment. Because it was not possible to adjust for the age of onset of schizophrenia since this data was not readily available in CRIS, this does not necessarily indicate a delay to clozapine treatment for females. However, a recent study demonstrated that female patients had a significantly longer delay to clozapine treatment (Ucok et al., 2015) and a UK study reported a mean delay to clozapine treatment of 39.5 months for males compared to 64.8 months for females, although this difference was not statistically significant (Howes et al., 2012b). The male patients in this study were also more likely to live in areas of higher deprivation, less likely to be of African/Caribbean ethnicity, and more likely to have a schizophrenia diagnosis. However, controlling for these factors did not impact on the strength of the association between gender and clozapine response.

Further investigations should aim to understand the underlying causes of the association between female gender and good clozapine response. For example, there may be an overrepresentation of other risk factors for poor response in males such as substance abuse, non-adherence, prominent negative symptoms and poor social functioning (Thorup et al., 2014). Previous studies have reported higher clozapine and norclozapine levels, after controlling for dose, in females compared to males and particularly male smokers (Haring et al., 1989; Kim, 2015), suggesting that females have a lower clearance of clozapine. Further studies controlling for clozapine levels, given they are strongly predictive of response (Mauri et al., 2007), may help to interpret the gender differences found in this study.

5.5.2 Baseline severity and clozapine response

The relationship between baseline severity and clozapine response was dependant on the definition of response used. Higher baseline CGI-S was strongly associated with good *relative* response, a definition solely indicating change. If an additional post-treatment severity threshold was included, indicating *absolute* response, a higher severity score predicted poor response. Previous studies have consistently reported an association between severe baseline symptoms and significantly better response to clozapine (Hasegawa et al., 1993; Lieberman et al., 1994b; Rosenheck et al., 1998; Ciapparelli et al., 2000; Umbricht et al., 2002; Semiz et al., 2007; Kelly et al., 2010) although many of these studies defined clozapine response purely in terms of symptom reduction (Meltzer et al., 1989a; Rosenheck et al., 1998; Umbricht et al., 2002; Ciapparelli et al., 2004; Kelly et al., 2010), closely fitting our *relative* response definition. Studies that have included a post-treatment severity threshold in their clozapine response definition have reported only trend level associations with baseline severity (Rodriguez et al., 1998; Semiz et al., 2007). Defining response solely based on symptom reduction will favour highly symptomatic patients because they have more scope for improvement and thus the measurable effect of clozapine on response will be greater (Rosenheck et al., 1998). Conversely, a definition that includes a severity threshold may favour patients with a lower severity because they are more likely to meet the criteria within the limited study period. Nonetheless, this study indicates that patients that are highly symptomatic at the start of clozapine treatment are more likely to experience greater improvement compared to baseline but are less likely to achieve an absolute response within 24 months. This study does not indicate that clozapine is more or less efficacious in highly symptomatic patients.

5.5.3 Association of other factors with clozapine response

There have been inconsistent reports regarding the role of age (Hofer et al., 2003; Mauri et al., 2003; Semiz et al., 2007; Kelly et al., 2010) and we found no evidence to suggest that the age at onset of clozapine treatment had any impact on clozapine response. Consistent with previous literature, we also found no association between ethnicity and clozapine response (Lieberman et al., 1994a; Rosenheck et al., 1998; Kelly et al., 2010). This strongly argues for clozapine being made available across the adult age range (18-65 in this study) and ethnic groups.

5.5.4 Timing of clozapine response

Improvement at one month was a strong predictor of good clinical response by 24 months and the strength of this association remained unchanged for different response definitions and after adjusting for potential confounders. This is consistent with previous studies which report early response to clozapine is indicative of later response; response after one week of treatment predicted response at 5 weeks (Stern et al., 1994), and response after 4 weeks predicted response at 16 weeks (Semiz et al., 2007). Our study expands on these previous findings in a naturalistic setting with a long-term follow-up.

There has been substantial interest in the duration of clozapine treatment required to detect a response to clozapine, with most studies concluding that response can be detected within the first 6-8 weeks of treatment (Rosenheck et al., 1999b; Suzuki et al., 2011a). However, we found that of the patients that were either minimally improved or not changed after two months of treatment, 23% would subsequently experience an *absolute* response and 38% a *relative* response within 24 months. After minimal or no improvement after six months of treatment, 14% went on to meet either *absolute* or *relative* response criteria in 24 months, indicating a small proportion of patients

experience a delayed response to clozapine. Conversely, we found that 75% of absolute responders were at least much improved (CGI-I score of 2) at two months, which increased to 92% at six months. These results indicate that although the majority of responders experience significant improvement within the first two months, this is not long enough to determine non-response. Further investigations using more advanced modeling techniques may provide valuable insights into this clinically important question. Nonetheless, our findings support current clinical practice to treat patients with clozapine for up to six months before determining the level of clinical response.

5.5.5 Definition of response

The somewhat conflicting results from previous studies investigating predictors of clozapine response may be due in part to the considerable variability in response definitions. Thus various response definitions were considered in this analysis and as far as we are aware, this is the first study to evaluate the sensitivity of response definitions against homogenous response groups identified by GBTM. We chose not to use the groups identified by GBTM as the primary outcome in this study to allow comparisons with the previous literature. An *absolute* response definition had higher sensitivity (0.88) and specificity (0.91) than *relative* response when compared to groups identified by GBTM. The definition of *absolute* response is focused on patients who achieved a good level of functioning and reflective of previously used criteria (Kane et al., 1988). A *relative* response criterion was considered because recent studies have tended to focus purely on the degree of change (Usall et al., 2007; Suzuki et al., 2012). The proportion of the sample that was classified as *absolute* and *relative* responders was comparable to previous studies (Kane et al., 1988; Lieberman et al., 1994b). Although a recommended definition of a CGI-I score of 2 or better was considered (Suzuki et al., 2012), which would correspond to a 40-

53% reduction in BPRS score (Leucht et al., 2005a; Leucht & Engel, 2006), a large proportion of our sample (78%) met this criteria within 24 months, possibly due to the longer follow-up period.

5.5.6 Study limitations

A limitation of this study is its retrospective nature, specifically that the quality of data available was limited to information entered into the electronic case note system by the patient's clinical team. However, strengths of this study design are that the results are reflective of routine clinical care, there was no recruitment bias, and recall bias was eliminated by the use of contemporaneous records. In addition, there was no attrition during the study period and minimal missing data. A further advantage of the study is that in the UK the National Health Service (NHS) provides nearly 100% of the health care and so the whole population is captured. The sample is comparable to the population in London with a diagnosis of schizophrenia (Morgan et al., 2006) but has higher proportions of Black African/Caribbean ethnicity and lower proportions of Asian ethnicity in comparison to England as a whole (Stewart et al., 2009). However, the National Audit of Schizophrenia (2012) suggests that the service use in London is comparable to the rest of the UK (Royal College of Psychiatrists, 2012).

It was not possible to control for concurrent medications, plasma levels or time until plateau of dose titration, which may have impacted on the timing of clozapine response (Fabrazzo et al., 2002; Schulte, 2003). A further limitation is that I was unable to collect data prior to clozapine initiation (such as age of onset, duration of untreated psychosis or theoretical delay to treatment) because data in CRIS was only available from 2006 onwards.

Although the CGI scale has been demonstrated to have equal validity to lengthier scales such as the BPRS (Leucht & Engel, 2006) and PANSS (Rabinowitz et al., 2010), the specific effects of clozapine on positive, negative or cognitive symptoms could not be analysed. The validity of the CGI has received some criticism (Beneke & Rasmus, 1992), and has been reported to be influenced by unrelated information such as adverse events (Busner et al., 2009). In addition, it is unclear whether change in CGI-S or CGI-I scales should be used to define treatment response (Jiang & Ahmed, 2009).

5.5.7 Clinical implications and conclusions

It is important to consider the clinical response definition when interpreting study results of clozapine response. This study indicates that clozapine responders are more likely to be female and further investigations should aim to understand the underlying causes of the association. We found that clozapine responders tend to have a good initial response to clozapine. However, up to six months of treatment may be required to determine non-response.

Chapter 6

Discussion

6.1. Summary of results

In this thesis I investigated three outcomes of clozapine treatment: neutropenia, discontinuation and clinical response. To examine the genetic susceptibility to clozapine-associated neutropenia, I conducted a multifaceted genetic analysis in the largest combined sample studied to date (Chapter 2). Using GWAS, I identified a novel genome-wide significant (GWS) association with rs149104283, a SNP intronic to transcripts of *SLCO1B3* and *SLCO1B7*, members of a family of hepatic transporter genes involved in drug uptake. In an analysis of exome array data I found evidence of association with clozapine-associated neutropenia for uncommon non-synonymous variants in *STARD9* and *UBAP2*. Furthermore, I replicated a previously reported association between neutropenia and a variant in *HLA-DQB1*. Thus Chapter 2 indicates variants that may increase susceptibility to clozapine-associated neutropenia and implicates biological pathways through which clozapine may act to cause this serious adverse effect.

A retrospective cohort of 316 patients with treatment-resistant schizophrenia (TRS) who received their first course of clozapine treatment over a five-year period was used to investigate clozapine discontinuation and clinical response. I found that 45% of patients discontinued clozapine within two years of starting treatment (Chapter 4). By studying the reasons for discontinuations due to a *patient decision* I found that *adverse drug reactions (ADRs)* accounted for over half of clozapine discontinuations. Sedation was the most common *ADR* cited as a reason for discontinuation, accounting for 20% of all

discontinuations, and the risk of discontinuation due to *ADRs* was highest in the first few months of clozapine treatment. Lastly, I found patients who live in an area of high deprivation are at an increased risk of discontinuing clozapine and may need additional support to maintain engagement with treatment. These findings highlight the importance for clinicians to identify and where possible treat ADRs attributed to clozapine before they lead to discontinuation.

In Chapter 5 I assessed the clinical predictors of clozapine response using several definitions: *absolute* response (CGI-I ≤ 2 and CGI-S ≤ 3), *relative* response (reduction ≥ 2 CGI-S scores), and *exceptional* response (CGI-I score of 1). I found that male gender was significantly associated with a poor response to clozapine and the strength of this association remained unchanged for different response definitions and after adjusting for potential confounders. There was an inconsistent association between baseline CGI severity and response; higher baseline severity was associated with a good *relative* response, but a poor *absolute* and *exceptional* response. In investigations of the timing of response, I found that clinical improvement after one month of treatment was a strong predictor of response within 24 months. Of the patients who were either minimally improved or not changed after two months of treatment, 23% and 38% went on to meet *absolute* and *relative* response criteria within 24 months, respectively. By six months this proportion reduced to 14%. This chapter indicates that clozapine responders are more likely to be female and tend to have a good initial response. However, more than two months of treatment may be required to determine non-response.

6.2. Key contributions to knowledge and clinical implications

6.2.1 Novel genetic associations with clozapine-associated neutropenia

The primary finding from the GWAS meta-analysis was a genome-wide significant association with clozapine-associated neutropenia for rs149104283, an intronic SNP for transcripts of both *SLCO1B3* and *SLCO1B7,* members of a family of hepatic transporter genes involved in drug uptake. The associated region in this study also contained a third member of this organic anion transporter family*, SLCO1B1.* Although the functionality of *SLCO1B7* is unknown, *SLCO1B3* and *SLCO1B1* encode liver-specific organic anion-transporter polypeptides (OATP1B3 and OATP1B1). These transmembrane proteins facilitate uptake of exogenous substances, including drugs, from the portal vein into hepatocytes, where the substance is subsequently modified either via metabolism with cytochrome (CYP) 450 enzymes or excreted (International Transporter Consortium et al., 2010). These novel findings suggest the hypothesis that genetic variants at *SLCO1B3* (and/or *SLCO1B1*) may increase risk of clozapine-associated neutropenia through a pharmacokinetic mechanism.

Support for this hypothesis comes from evidence implicating polymorphisms within *SLCO1B1* and *SLCO1B3* in adverse reactions of other drugs. A widely replicated missense variant in *SLCO1B1* has been demonstrated to increase the risk of simvastatin-induced myopathy by impacting the pharmacokinetics of simvastatin, leading to an increase in plasma concentrations, and is now recommended for use as a routine pre-emptive clinical test (Search Collaborative Group et al., 2008; Ramsey et al., 2014). Furthermore, an intronic variant in *SLCO1B3* has been associated with docetaxel-induced severe leukopenia/ neutropenia (Kiyotani et al., 2008), although there is inconclusive evidence regarding whether this is secondary to alterations in the pharmacokinetics and/or

bioavailability of the drug (Nambu et al., 2011; Yamakawa et al., 2011; Chew et al., 2012). If genetic variants at *SLCO1B3* and *SLCO1B1* increase the risk of clozapine-associated neutropenia through a pharmacokinetic mechanism, we may expect to observe a correlation with plasma concentrations of clozapine. There is currently limited evidence to suggest an association with clozapine plasma levels and agranulocytosis or neutropenia (Hasegawa et al., 1994; Centorrino et al., 1995; Combs et al., 1997; Mauri et al., 1998; Oyewumi et al., 2002); however, previous studies have either failed to include patients with abnormally low neutrophil levels (Centorrino et al., 1995; Combs et al., 1997; Mauri et al., 1998; Oyewumi et al., 2002) or control for treatment duration or dosage (Hasegawa et al., 1994; Centorrino et al., 1995). Furthermore, considering the marked inter-individual variation in clozapine plasma levels (Olesen et al., 1995), previous studies have been conducted in relatively small samples (Hasegawa et al., 1994).

The precise aetiology of clozapine-induced agranulocytosis and neutropenia is currently unknown. The best-supported hypotheses to date include a direct toxicity of clozapine metabolites, an immune-related mechanism, or a combination of the two. There is evidence indicating that agranulocytosis and neutropenia are caused by the activation of clozapine, or a stable metabolite of clozapine, to a chemically reactive nitrenium ion (Liu & Uetrecht, 1995; Maggs et al., 1995; Pirmohamed & Park, 1997). The propensity for nitrenium ions to cause apoptosis to neutrophils, or be toxic to neutrophil precursors, is dose dependent, lending support to the hypothesis that clozapine pharmacokinetics and bioavailability are related to its potential to cause neutropenia (Williams et al., 2000; Pereira & Dean, 2006).

I found evidence of association with neutropenia for uncommon non-synonymous variants in *STARD9* and *UBAP2*. *STARD9* is a mitotic kinesin and *STARD9*-depleted cancer cells have

abnormal cellular morphology and undergo apoptosis (Torres et al., 2011). In addition, STARD9-depletion was found to synergise with the chemotherapeutic agent taxol, the use of which is dose-limited due to neutropenia (Torres et al., 2011). The function of *UBAP2* is undetermined though it has an ubiquitin-associated domain and is widely expressed across tissues. A recent study reported the association of a missense variant in the ubiquitin gene *USP43* with clozapine-associated neutropenia (Tiwari et al., 2014). Our findings regarding *STARD9* and *UBAP2* have not been replicated and thus can only be interpreted cautiously.

6.2.2 *HLA-DQB1* 6672G>C and clozapine-associated neutropenia

I found independent support for the association of *HLA-DQB1* 6672G>C (rs113332494), an association that strengthened when considering only cases with ANC below ≤ 1000/mm^3. This finding adds to the growing evidence implicating *HLA-DQB1* in clozapine-associated neutropenia and agranulocytosis. HLA genes have been long hypothesised to be involved given their role in immune response and thus the majority of previous genetic association studies have primarily focused on candidate genes from the HLA region. Early candidate studies reported significant associations of variants in *HLA-DQB1* and clozapine-induced agranulocytosis (Yunis et al., 1995; Dettling et al., 2001) and in 2011, Athanasiou and colleagues reported a significant association with *HLA-DQB1* 6672G>C (OR = 16.9) (Athanasiou et al., 2011). Furthermore, the CIAC study reported a significant association of an amino acid polymorphism (126Q) in *HLA-DQB1* (Goldstein et al., 2014). Although we could not impute the amino acid polymorphism implicated in CIAC, it is in high linkage disequilibrium with *HLA-DQB1* 6672G>C (Goldstein et al., 2014). However, it is important to note that the samples used in the previous studies overlap. Due to the rarity of this side effect, there is a limited amount of independent samples available (Zhang & Malhotra,

2013). Thus, in this thesis I present the first fully independent replication implicating this locus in clozapine-associated neutropenia. Furthermore, the effect size in our study (OR = 15.6) was comparable to previous reports (Athanasiou et al., 2011). The association of *HLA-DQB1* adds support for an immune-related mechanism in clozapine-associated neutropenia, although the functional roles of the implicated polymorphisms are currently unclear.

6.2.3 Pharmacogenetic tests of clozapine-associated neutropenia

The goal of pharmacogenetic studies, as in Chapter 2, is to identify genetic variants that could be used to personalise medicine and tailor treatment choices for individual or groups of patients. A sensitive and reliable predictor of clozapine-associated neutropenia could conceivably be used as a test to identify patients at high risk (Zhang & Malhotra, 2013). For those at low risk, there could be a reduced need for regular monitoring, which limits the acceptability of clozapine to patients, and poses an obstacle to its use in clinical practice (Patel, 2012). Although the *HLA-DQB1* variant alone has a positive predictive value (PPV) of 35.1%, the majority of those that develop neutropenia whilst taking clozapine are not carriers of this risk allele, or indeed the other alleles we have identified in this study. The sensitivity for a test including all three risk variants (GWS intronic variant in *SLCO1B3* and *SLCO1B7*, missense variant in *SLCO1B7*, and *HLA-DQB1* 6672G>C) was 29.17%, the specificity 90.61%, the PPV 9.94%, and the NPV 97.30%. This indicates that 29.2% of individuals with clozapine-associated neutropenia will carry at least one of the three identified risk alleles and that individuals that carry any of these risk alleles have a 9.9% risk of clozapine-associated neutropenia. *HLA-DQB1* 6672G>C was marketed as a genetic predictive test on the basis of the study by Athanasiou and colleagues (Athanasiou et al., 2011), but the low sensitivity limited its clinical utility and it has now been withdrawn from

the market due to low uptake (Chowdhury et al., 2011). Although the identified variants in this thesis convey a substantially increased risk for clozapine-associated neutropenia, they are currently on their own unlikely to have clinical utility for pharmacogenetic testing due to low sensitivity and positive predictive value (Verbelen et al., 2015), particularly as there is currently no alternative treatment for those with TRS. Nonetheless our findings provide novel insights into putative biological processes underlying clozapine-associated neutropenia, particularly the potential link with the pharmacokinetics of clozapine. The development of such understanding should help widen the availability of clozapine with beneficial impact on those with TRS.

6.2.4 ADRs are the most common reason for discontinuation

In Chapter 4, I present the first study to examine the reasons for clozapine discontinuation due to a *patient decision*. By studying these reasons, I found that *ADRs* accounted for over half of clozapine discontinuations. Previous studies have consistently reported *patient decision* (or *non-adherence*) to be the most common cause of clozapine discontinuation, without further specifying reasons, and that 25-35% of discontinuations were due to *ADRs* (Atkinson et al., 2007; Taylor et al., 2009; Krivoy et al., 2011; Davis et al., 2014; Mustafa et al., 2015). My results suggest that the role of *ADRs* has been underestimated as previous studies have used a restricted number of categories for discontinuation (i.e. *patient choice* and *non-adherence*), with no studies categorising the underlying reasons for *non-adherence*.

I found that the adverse effect most frequently cited was sedation, which accounted for 20% of all discontinuations. Interestingly, over half of discontinuations due to sedation were from discontinuations due to a *patient decision*. This is a clinically important observation since sedation is usually transient and can almost always be minimised by

reducing the dose and/or titration rate of clozapine, adjusting the timing of the dose or partial substitution with less sedating drugs such as aripiprazole (Nair & MacCabe, 2014). If this adverse effect was more actively managed and monitored it is likely many could remain on clozapine. My results highlight the importance for clinicians to identify and treat ADRs attributed to clozapine, particularly in the first few months after treatment onset, before they lead to discontinuation.

6.2.5 Neighbourhood deprivation increases the risk of discontinuation

In Chapter 4, I present the first study to observe an association between level of social deprivation and risk of all-cause clozapine discontinuation. Furthermore, I found that this association was driven by *patient* rather than *clinician-led* discontinuations. Of note neighbourhood deprivation had no impact on clinical response (Chapter 5). No previous study has assessed the relationship between social deprivation and risk of clozapine discontinuation, but there have been studies investigating the relationship between socio-economic status and non-adherence to antipsychotic medication in general (Kane et al., 2013), although the results have been inconsistent. Previous studies have consistently reported increased rates of all-cause clozapine discontinuation in patients of Black African/Caribbean ethnicity (Moeller et al., 1995; Davis et al., 2014). However, I found that after controlling for neighbourhood deprivation, the association with ethnicity attenuated, indicating that this association could be related to factors relevant to deprivation rather than ethnicity. It is likely social deprivation is a proxy marker for other factors that underlie discontinuation and non-adherence and further research is needed to determine whether particular characteristics of these patient groups increase risk for clozapine discontinuation or perhaps whether clinical teams supporting areas in high deprivation are under increased pressure or have more limited resources. Nonetheless, this study

indicates that patients who live in an area of high deprivation are at an increased risk of discontinuing clozapine and may need additional support to maintain engagement with treatment.

6.2.6 Defining clinical response to clozapine is complex

Despite a significant amount of studies, there has been a lack of consistent findings regarding clinical and demographic predictors of clinical response to clozapine. The somewhat conflicting results may be due, in part, to the considerable variability in response definitions, resulting in limited comparability across studies. Thus, I considered various response definitions in the analysis of clozapine response (Chapter 5): *absolute* response (CGI-I ≤ 2 and CGI-S ≤ 3), *relative* response (reduction ≥ 2 CGI-S scores), and *exceptional* response (CGI-I score of 1). This was the first study to evaluate the sensitivity of response definitions against homogenous response groups identified by group-based trajectory modeling (GBTM). I found that *absolute* response had higher sensitivity and specificity than *relative* response in comparison to groups identified by GBTM, and was thus used as the primary outcome. The impact of different response definitions is highlighted in our findings regarding baseline severity. I found that higher baseline CGI-S was strongly associated with good *relative* response but poor *absolute* response. Previous studies reporting an association between severe baseline symptoms and significantly better response to clozapine have defined clozapine response purely in terms of symptom reduction, similar to our *relative* response definition (Hasegawa et al., 1993; Lieberman et al., 1994b; Rosenheck et al., 1998; Ciapparelli et al., 2000; Umbricht et al., 2002; Semiz et al., 2007; Kelly et al., 2010). Defining response solely based on symptom reduction will favour highly symptomatic patients because they have more scope for improvement and thus the measurable effect of clozapine on response will be greater (Rosenheck et al.,

1998). Conversely, a definition that includes a severity threshold may favour patients with a lower severity because they are more likely to meet the criteria within the limited study period. There was considerable variability in the level of response that individuals in our study experienced and the majority had at least minimal improvement. Determining a binary definition is an imperfect measure of response and my findings highlight the importance of considering the response definition used when interpreting study results and benefits of presenting a range of definitions to evaluate whether study effects remain consistent (Leucht et al., 2007b).

6.2.7 Male gender is associated with poor clozapine response

I found that male gender was significantly associated with a poor clozapine response (Chapter 5). The strength of this association remained unchanged for different response definitions and after adjusting for potential confounders. Previous studies have reported conflicting findings regarding the role of gender in clozapine response (Lieberman et al., 1994a; Szymanski et al., 1996; Hofer et al., 2003; Mauri et al., 2003; Ciapparelli et al., 2004; Semiz et al., 2007). Small sample sizes, short study durations and differing response definitions are likely to contribute to the lack of consistent findings. My findings are supported by the SOHO study, which found that females had significantly better response to typical antipsychotics and clozapine (Usall et al., 2007). The SOHO study was similar to our study in regards to a large sample size, a longer follow-up period, and the definition of clozapine response.

Further investigations should aim to understand the underlying causes of this association. For example, there may be an overrepresentation in males of other possible risk factors that we were not able to measure such as substance abuse, non-adherence, prominent negative symptoms and poor social functioning (Thorup et al., 2014). We found that the

males in our study were significantly younger than females at the onset of clozapine treatment, less likely to be of Black African/Caribbean ethnicity, and were more likely to live in an area of high deprivation and have a diagnosis of schizophrenia. However, controlling for these factors did not impact on the strength of the association between gender and clozapine response. Of note is that gender had no impact on rates of discontinuation (Chapter 4). Previous studies have reported higher clozapine and norclozapine levels, after controlling for dose, in females compared to males and particularly male smokers (Haring et al., 1989; Kim, 2015), suggesting that females have a lower clearance of clozapine. Further studies controlling for clozapine levels, given they are predictive of response (Mauri et al., 2007), may help to interpret the gender differences found in this study. We intend to address this clinically important question in a recently acquired sample.

6.2.8 Timing of clozapine response

I found that early improvement after one month of treatment was a strong predictor of good clinical response by 24 months (Chapter 5). The strength of this association remained unchanged for different response definitions and after adjusting for potential confounders. This is consistent with previous studies which report that early response to clozapine is indicative of later response: response after one week of treatment predicted response at five weeks (Stern et al., 1994), and in another study response after four weeks predicted response at 16 weeks (Semiz et al., 2007). Our study expands on these previous findings in a naturalistic setting with a longer-term follow-up.

There has been substantial interest in the duration of clozapine treatment required to detect a response to clozapine. If such a time point could be identified, patients who have not shown sufficient improvement could be regarded as non-responders and their

clozapine treatment augmented or discontinued. However, if this specified time is too short, patients who would have eventually experienced benefit may be discontinued, and if too long, nonresponsive patients may continue to unnecessarily receive treatment that will not benefit them. Previous studies have concluded that clozapine response can be detected within the first six to eight weeks of treatment (Rosenheck et al., 1999b; Suzuki et al., 2011a). However, other studies have suggested that clozapine trials should last far at least six months (Meltzer, 1992). Although we found that early response was predictive of later response, our analyses suggested that eight weeks would not be long enough to determine non-response. I found that of the patients that were either minimally improved or not changed (CGI-I score of 3 or 4) after two months of treatment, 23% would experience an *absolute* response and 38% a *relative* response within 24 months. After minimal or no improvement by six months of treatment, 14% went on to meet either *absolute* or *relative* response criteria within 24 months, indicating a small proportion of patients experienced a delayed response to clozapine. We found that only 75% of absolute responders were at least much improved (CGI-I score of 2) at two months, which increased to 92% at six months. It is important to note that minimally improved was defined in our study as 'slightly better with little or no clinically meaningful reduction of symptoms; represents very little change in basic clinical status, level of care, or functional capacity' (Busner & Targum, 2007). These results indicate that although the majority of those who respond to clozapine show signs of improvement within two months, at least six months of treatment may be required to determine non-response. Further investigations using more advanced modeling techniques may provide valuable insights into this clinically important question.

6.2.9 Outcome of clozapine treatment

There are currently no reliable predictors of clozapine treatment outcome, and thus clinicians are unable to determine a priori whether clozapine is likely to be beneficial. Previous studies have focused on specific outcomes such as clinical response and ADRs (particularly agranulocytosis, weight-gain and diabetes). A clinical challenge will be combining and weighing up the genetic and clinical predictors for each different outcome. However, discontinuation of clozapine encapsulates all kinds of drug failure and reflects real-world practice. It represents a synthesis of clinician and patient judgements that an assigned treatment was either insufficiently efficacious or tolerable (McEvoy et al., 2006). In analyses conducted recently and thus not presented in this thesis, I compared the validity of using discontinuation as a proxy of clinical response using the data presented in Chapters 4 and 5. Using Receiver Operating Characteristic (ROC) curve analyses, I found that the area under the curve (AUC) for clozapine discontinuation was 0.71 indicating a fair test for clinical response (defined here as a CGI-I score of 1 or 2). We determined that the best combination between sensitivity and specificity was at 24 months of treatment; with a sensitivity of 71%, specificity 66%, PPV 88% and NPV 40%. This illustrates that those who continued clozapine treatment beyond 24 months were very likely to have responded to clozapine (PPV = 88%). However, those that discontinued within 24 months are a combination of non-responders and responders that likely found clozapine intolerable (NPV = 40%). These findings support the concept that the use of a discontinuation phenotype encapsulates drug failure.

I have recently conducted a preliminary GWAS using clozapine discontinuation as a measure of clozapine failure. I considered 131 individuals who discontinued clozapine within 24 months as cases (those in whom adverse effects or lack of efficacy outweighed

benefits) and 6667 individuals who remained on clozapine for at least 24 months as controls (for whom benefits outweighed adverse effects) from a recently acquired clozapine sample from Leyden Delta. The most significant variant associated (which was GWS) with clozapine discontinuation was rs113821156 on chromosome 13, intronic to 5-Hydroxytryptamine Receptor 2A (HRT2A, also known as 5-HT_{2A}) and this was present in 4% of cases and 0.4% of controls. Clozapine has a high affinity for the serotonin 5-HT_{2A} receptor (Meltzer, 1999) and variants in 5-HT_{2A} have been previously implicated in candidate studies of clozapine response (Arranz et al., 1998a; Arranz et al., 1998b). Furthermore, serotonin impacts on appetite and variants within 5-HT_{2C} have been associated with the clozapine-induced weight gain (Muller & Kennedy, 2006; Lett et al., 2012). However, this analysis is still in the preliminary stages and replication will be required given the small sample size.

Monitoring of plasma concentrations of clozapine to confirm that therapeutic levels of clozapine have been achieved is widely applied in the clinical management of clozapine treatment. There are many factors that influence plasma clozapine levels including age, gender, smoking and metabolic factors (Haring et al., 1989; Lane et al., 1999; Kim, 2015). This thesis indicates the importance for careful management of plasma concentrations and dose of clozapine. I found that sedation was the most common ADR used as a reason for discontinuation, a side effect that can be minimised by reducing the dose and/or titration rate of clozapine (Chapter 4). Female gender was associated with greater clinical response to clozapine (Chapter 5) and previous studies indicate that females have a lower clearance of clozapine (Haring et al., 1989; Kim, 2015). Lastly, I implicated a pharmacokinetic mechanism for clozapine-associated neutropenia (Chapter 2). Together, these findings indicate that careful dose titration and on going monitoring of clozapine plasma levels should support more people to stay on clozapine.

6.3. Strengths and limitations

6.3.1 Clozapine-associated neutropenia

Due to its rarity, genetic studies of clozapine-associated neutropenia are underpowered to detect associations that do not have a moderate to large effect size. Thus, there may be causal variants of small effect that we were not able to detect in this study. However, a considerable strength of this study is the large control sample, all of which were treated with clozapine for at least a year without developing neutropenia. Although the CIAC study also had a large number of controls, the majority were not treated with clozapine (Goldstein et al., 2014). This disparity may have contributed to the lack of replication of some of our variants in the CIAC sample.

An important consideration is that our analyses included cases with both neutropenia and agranulocytosis. Large epidemiological studies have reported different demographic risk factors for agranulocytosis and neutropenia, suggesting that they may have distinct aetiologies (Flanagan & Dunk, 2008). However, it is now very rare to develop agranulocytosis because of the success of the monitoring system; in fact only four cases met this threshold in our sample. Given that within the field there are valid concerns that a more stringent threshold may produce more reliable results, I additionally conducted secondary analyses on a subset of the more severely affected cases with ANC ≤ 1000/mm^3. However, these samples were also included in the primary analyses thus limiting comparisons. A further limitation of this study is that there was very little information regarding the phenotype: Novartis only informed us of those that had developed neutropenia whilst taking clozapine and where available, the recorded lowest neutrophil counts of these individuals were supplied. However, individuals were excluded if another reason for the blood dyscrasia was suggested on the monitoring system and thus we can

be confident that the occurrence of neutropenia or agranulocytosis was related to clozapine.

Although GWAS allows a hypothesis free and affordable approach to identifying genetic susceptibility variants for a given disease or phenotype, a limitation is that many of the SNPs detected from GWAS are noncoding variants with an unknown impact (Ward & Kellis, 2012). Thus, subsequent investigations are required to determine the causal SNP(s) and any biological impact. Improved annotation of these signals together with recent advances in whole-genome sequencing should provide definitive answers in this regard.

A strength of this study is the analysis of both common and rare variants. Rare variants may have a larger effect in comparison to common variants, and thus may be more informative in the prediction of clozapine-associated neutropenia. However, a limitation of the exome array is that it does not capture all exonic variation. Exome- wide sequencing in a larger sample is required to prove the role of functional exonic variants definitively. Nonetheless, the approaches and subsequent findings in Chapter 2 contribute to the current understanding of clozapine-associated neutropenia and agranulocytosis.

6.3.2 Retrospective cohort used for Chapters 4 and 5

Chapters 4 and 5 were conducted in a retrospective cohort of 316 patients starting their first clozapine trial over a five-year period (2007-2011, inclusive) in South London & Maudsley (SLaM) NHS Foundation Trust. A limitation of this study design is its retrospective nature, specifically that the quality of data available was limited to information entered into the electronic case note system by the patient's clinical team. However, benefits of this study design are that the results are reflective of routine clinical care.

Only one other study has investigated clozapine discontinuation in a systematically attained sample of patients receiving their first trial of clozapine (Davis et al., 2014), which allows timing to be studied in detail and comparisons made with patients that continue. The previous studies of clozapine discontinuation that were not conducted in patients receiving their first trial of clozapine may have been biased by previous clozapine trials (Taylor et al., 2009; Pai & Vella, 2012). An alternative, prospective study of clozapine discontinuation could allow in-depth interviews with clinicians and patients to fully investigate reasons for discontinuation. However, this is a timely and costly approach. Furthermore, patients starting clozapine treatment are often severely impaired and consequently may be difficult to engage in research. Patients who are non-adherent may also be likely to disengage from research, leading to biased attrition.

The data I used was extracted from the Case Register Interactive Search (CRIS) system; a large, anonymised case register derived from SLaM electronic case records (Stewart et al., 2009). This allowed a universal capture of patients commencing clozapine in a defined geographical area covering a population of 1.2 million people, with consequently little or no selection bias. The fact that informed consent was not required also eliminated the selection bias in favour of higher functioning patients. Recall bias was minimised by the use of contemporaneous records and the minimal missing data allowed us to determine the reasons for discontinuation for all of the patients and administer a very high proportion of CGI assessments (96.6%). Furthermore, CRIS incorporates routinely collected data from multiple sources, such as pharmacy dispensing information, which increases reliability. A limitation of using CRIS is that data is only available from 2006 onwards, as this was when electronic records were fully introduced in SLaM. Although there is some data prior to this date, it is not consistently available. As a consequence, I was unable to

collect data prior to clozapine initiation such as age of onset, duration of untreated psychosis or theoretical delay to clozapine treatment.

The CRIS sample is comparable to the population in London with a diagnosis of schizophrenia (Morgan et al., 2006) but has higher proportions of Black African/Caribbean ethnicity and lower proportions of Asian ethnicity in comparison to England as a whole (Stewart et al., 2009). However, the National Audit of Schizophrenia (2012) suggest that the service use in London is comparable to the rest of the UK (Royal College of Psychiatrists, 2012).

6.3.3 Discontinuation of clozapine

The primary reasons for discontinuation in Chapter 4 were classified into mutually exclusive categories. This approach simplifies the data, making interpretation easier, and has been the method used in all previous studies (Taylor et al., 2009; Krivoy et al., 2011; Pai & Vella, 2012; Davis et al., 2014), thus allowing us to make direct comparisons. However, a limitation of this approach is that for many cases the reasons for discontinuation were multifactorial. For example, a patient (or clinician) may be more likely to tolerate an ADR if they are experiencing a good clinical response to clozapine, but might instead discontinue clozapine, citing adverse effects, in the absence of a clinical response. Chapter 4 addressed this limitation to some degree by integrating the reasons for non-adherence of clozapine. However, the reasons for non-adherence in our study were limited to what had been disclosed by the patient to the clinical team. The optimum approach to this question would be to interview patients and clinicians at the time of discontinuation or shortly after.

It is likely that our study has not captured the role that clinical response plays in discontinuation of clozapine. Chapter 4 indicates that discontinuation due primarily to an

inadequate response to clozapine is rare, occurring in only 2.5% of patients. This finding is consistent with earlier reports (Taylor et al., 2009; Pai & Vella, 2012; Davis et al., 2014; Mustafa et al., 2015). Chapter 5 indicates that, consistent with previous studies, approximately between 40-70% of patients will respond to clozapine (Kane et al., 1988; Lieberman et al., 1994b). The reason for why clozapine response is seldom recorded as the primary cause of clozapine discontinuation may be driven partly by concern over risk of further relapse upon cessation (Seppala et al., 2005) and partly by a lack of any other evidence-based treatment options.

6.3.4 Clozapine response

A key strength of the clozapine response analysis is the consideration of various response definitions and a long-term study period. The somewhat conflicting results from previous studies may be due, in part, to the considerable variability in response definitions and trial durations. Previous study durations have tended to be short and consequently may not have considered a group of patients that take longer to meet the response criteria (Meltzer et al., 1989a). An additional strength is the large sample size and the naturalistic setting. Many previous studies have derived data from clinical trials and thus may not be generalisable to patients receiving clozapine in standard health care settings.

We selected the CGI scale (Guy, 1976) to measure clozapine response as it is a widely accepted research tool that captures clinical improvement, originally designed for use in clinical trials, but has also been utilised in retrospective case note studies (Agid et al., 2011). We also selected this instrument for pragmatic reasons given that the majority of clinical notes contained the necessary information to make CGI ratings whereas there was often insufficient detail in order to rate more comprehensive rating scales retrospectively such as the BPRS (Overall & Gorham, 1962) or the PANSS (Kay et al., 1987). This decision

was informed by the experiences of a previous medical student who attempted to retrospectively rate BPRS scales from case notes and found that there was insufficient information to do so. The CGI scale has been demonstrated to have equal validity to the BPRS (Leucht & Engel, 2006) and PANSS (Rabinowitz et al., 2010). However, there are several limitations of the CGI. The validity of the CGI has received some criticism (Beneke & Rasmus, 1992), and ratings have been reported to be influenced by unrelated information such as adverse events (Busner et al., 2009). Furthermore, because it is a global scale, the specific effects of clozapine on positive, negative or cognitive symptoms could not be analysed. We were also unable to assess whether improvements made in general psychopathology translated to improvements in quality of life or functional measures. For example, a previous study found that although female gender was associated with a good response based on the CGI, there was no difference between males and females based on a quality of life scale (Usall et al., 2007).

6.4. Suggestions for future work

There are a number of possible, closely related analyses to those presented in this thesis that have the potential to further our understanding of treatment response and adverse effects of clozapine. Firstly, further studies are required to determine if *SLCO1B3, SLCO1B7* or *SLCO1B1* mediate clozapine uptake and to investigate what affect the identified polymorphisms in this thesis may have. Sequencing of this chromosomal region would refine the association signal and potentially identify the causal variant. Although we found that no single variant from the exome array was significantly associated with clozapine-associated neutropenia, coverage of the exome array is incomplete and exome-wide sequencing in a larger sample is needed to prove the role of functional exonic variants definitively. In addition, further studies are required to replicate the association of

uncommon non-synonymous variants in *STARD9* and *UBAP2*. We have plans to conduct these analyses in a recently acquired sample, described below.

In a larger sample, further studies could dissect the neutropenia and agranulocytosis phenotype. For example, to investigate whether neutropenia or agranulocytosis that occurs early on in clozapine treatment has a distinct aetiology from occurrences after many years of treatment and whether agranulocytosis and neutropenia have distinct causes. Furthermore, studies to date have been unable to differentiate between a transient neutropenia, a fall and then return to normal neutrophil counts, and neutropenia that progresses to agranulocytosis (Flanagan & Dunk, 2008). Lastly, future studies could specifically examine the group of individuals that develop a subsequent neutropenia or agranulocytosis upon rechallenge with clozapine.

We have recently acquired an additional sample of approximately 7000 individuals that were prescribed clozapine in the UK by the pharmaceutical company Leyden Delta, 200 of whom have had an occurrence of neutropenia or agranulocytosis. This sample was acquired as part of CRESTAR, a project funded by a grant from the European Union via the Seventh Framework Programme for Research and Technological Development. A significant advantage of this sample is the substantial phenotypic information in comparison to CLOZUK and the suggestions outlined above can be readily answered in this new sample. Another area of research that warrants attention is the genetic aetiology of agranulocytosis and neutropenia in individuals of non-European ancestry. An unpublished GWAS analysis conducted by Professor Dan Rujescu, from the University of Halle in Germany and as part of CRESTAR, includes individuals from many populations. Furthermore, we have plans to address this question in the sample from Leyden Delta. Sample size has proven to be a limiting factor in genetic studies of clozapine-associated

agranulocytosis and neutropenia. Thus, we are involved in initial discussions to contribute to a large meta-analysis involving CLOZUK, CIAC, CRESTAR and the new sample from Leyden Delta. These combined samples would amount to at least 600 cases with neutropenia and the identification of sensitive genetic markers should make significant advances in current understanding of the aetiology and could comprise a clinically useful pharmacogenetic test.

The findings in this thesis indicate that the role of a pharmacokinetic mechanism in the occurrence of agranulocytosis and neutropenia warrants further investigation. The first step could be to re-assess the relationship between plasma clozapine levels and neutropenia. Furthermore, a GWAS of plasma clozapine levels may help to understand the aetiology underlying the inter-individual variability in plasma levels and potentially have widespread implications given their role in several ADRs and clozapine response.

The approach used for the clozapine-associated neutropenia analysis provides a proof of principal for an approach that could be used for a genetic investigation of other clozapine outcomes such as clinical response. To date, no GWAS of clozapine response or discontinuation has been published.

A question that remains to be explored is whether the side effect profile of patients who discontinue clozapine differs from patients who continue with the treatment. It would be worthwhile for future studies to investigate the reasons for patient-led discontinuations and non-adherence, ideally by interviewing patients directly at the time of discontinuation or shortly after. A further area of future research indicated in this thesis is the contribution of neighbourhood and social deprivation to clozapine discontinuation. It is likely social deprivation is a proxy marker for other factors that underlie discontinuation and non-adherence and further research is needed to determine whether particular characteristics

of these patient groups increase risk for clozapine discontinuation or perhaps whether clinical teams supporting areas in high deprivation are under increased pressure or have more limited resources.

Further investigations should aim to understand the underlying causes of the association between gender and response to clozapine. To assess the duration of clozapine treatment required to detect a response, additional studies are required using more advanced modeling techniques. Finally, defining the outcome phenotypes of treatment response, discontinuation, and clozapine-associated neutropenia presented a considerable challenge. This has lead to an appreciation of the importance and challenge in defining treatment outcomes, even for superficially simple phenotypes.

6.5. Conclusions

The work presented in this thesis contributes to the understanding and prediction of clozapine treatment outcomes. This research indicates that genetic variants at *SLCO1B3* (and/or *SLCO1B1*) increase the risk of clozapine-associated neutropenia, implicating a pharmacokinetic mechanism and providing novel insights into the aetiology of clozapine-associated neutropenia. Furthermore, this thesis adds to the growing evidence implicating *HLA-DQB1* in clozapine-associated neutropenia and agranulocytosis. Although the identified variants convey a substantially increased risk for clozapine-associated neutropenia, they are currently on their own unlikely to have clinical utility for pharmacogenetic testing. By examining the reasons for discontinuations due to a patient decision, it was found that ADRs accounted for the majority of clozapine discontinuations. These findings indicate the importance for clinicians to identify and treat ADRs attributed to clozapine, particularly in the first few months after treatment onset, before they lead to

discontinuation. Patients who live in an area of high deprivation are at an increased risk of discontinuing clozapine and may need additional support to maintain engagement with treatment. This thesis highlights the importance of considering clinical response definitions in interpreting study results. Lastly, clozapine responders are more likely to be female and tend to have a good initial response. However, up to six months of treatment may be required to determine non-response. This thesis contributes to the understanding of the causes of different clozapine treatment outcomes and aids identification of reliable predictors of clozapine outcome, which should ultimately aid clinicians in determining whether clozapine is likely to be beneficial.

Appendix

Appendix 1: GWAS quality control and imputation procedures

Quality control procedures and imputation was conducted at the Broad Institute as part of the PGC2 pipeline (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The quality control parameters for retaining SNPs and subjects were: SNP missingness < 0.05 (before sample removal), subject missingness < 0.02, autosomal heterozygosity deviation (Fhet < 0.2), SNP missingness < 0.02 (after sample removal), and SNP Hardy-Weinberg equilibrium P > 5x10-6. Imputation was performed using IMPUTE2 (Howie et al., 2009) (chunk size of 3 Mb and default parameters) with a reference dataset consisting of 2,186 phased haplotypes from the full 1000 Genomes Project dataset (August 2012, 30,069,288 variants, release "v3.macGT1").

After imputation, SNPs with high imputation quality (INFO > 0.8) and low missingness (< 0.01) were identified for further quality control. After linkage disequilibrium pruning (r^2 > 0.02) and frequency filtering (MAF > 0.05), there were 19,551 autosomal SNPs across all 49 datasets of European ancestry in PGC2. This SNP set was used for robust relatedness testing and population structure analysis. Relatedness testing was done with PLINK (Purcell et al., 2007) and pairs of subjects with π > 0.2 were identified and one member of each pair removed at random after preferentially retaining cases over controls and trio members over case-control members.

Appendix 2: Exome array quality control procedures

First pass QC was performed using PLINK (Purcell et al., 2007) on genotypes called using GenCall. Initial QC for probe base exclusions included Hardy-Weinberg equilibrium (HWE)

$P < 1x10^{-8}$, call rate < 98%, and non-autosomal location. Initial QC for subject exclusions were based on call rate < 98%, as well as relatedness based on identity by descent (IBD) analysis (PI-HAT > 0.1), heterozygosity, and Principal Component Analysis PCA. PCA was run using EIGENSTRAT (Price et al., 2006) based on 3022 SNPs with MAF > 1%. The sample and healthy controls were merged with 1100 samples from 11 populations using the HapMap 3 dataset (Ripke et al., 2013), and outliers that did not cluster near to the HapMap European individuals were removed to minimize ancestral heterogeneity. In total we excluded 1204 individuals and 16841 markers prior to the zCall post-processing procedure.

zCall is a post-processing step designed to improve the calling of SNVs (Goldstein et al., 2012). We applied zCall to batches using batch-specific intensity data. Markers were subsequently excluded if they were monomorphic, had call rates < 99%, had HWE $P<10^{-6}$ in any batch, or had a difference in call rate between batches > 1%. We also excluded probes where the allele frequencies differed between the two groups of healthy controls (blood donors versus 1958 Birth cohort) at P < 0.001, or between the cases assayed on the two types of chip at P<0.0005. These p-value thresholds were derived from QQ-plots of the within healthy control and within case analyses. Lastly, we excluded variants that did not show a sufficient difference in mean intensity between different genotype clusters (GenTrain score < 0.4, cluster separation metric < 0.08).

We applied a further round of QC to the individuals based on the Z-call genotypes, excluding samples on the basis of call rate (>99% required for inclusion), heterozygosity (separately for variants above and below 1% MAF) and concordance between database and genetically determined sex.

Appendix 3: Proxy SNPs used in clozapine-associated neutropenia and neutropenia ≤1000/mm^3 meta-analyses

SNP	CHR	BP	Proxy SNP	Distance (bp)	r^2	D'
Clozapine-associated neutropenia						
rs116216021	6	3417882	rs116552069	11430	0.96	1
Clozapine-associated neutropenia ≤ 1000/mm^3						
rs111698467	8	25624468	rs113539283	84249	0.807	0.992
rs111242461	10	37404727	chr10_37367098_I	37629	0.840	0.932
rs76415963	20	10544516	rs74762384	7575	0.814	1

Proxy SNPs used for clozapine-associated neutropenia and clozapine-associated neutropenia ≤ 1000/mm^3 meta-analyses. Columns are: SNP = name of original variant identified in CLOZUK analysis; CHR = chromosome; BP = base pair position (hg19); Proxy SNP = proxy SNP identified within CIAC; Distance (bp) = distance between original and proxy SNP; r^2 = LD between original and proxy SNP; D' = LD between original and proxy SNP.

Appendix 4: Number of patients who discontinued clozapine by three monthly intervals for each overall reason for discontinuation

Month	N taking clozapine	ADR	Non-adherence NOS	Inadequate response	Blood monitoring	Belief medication not required	Delusional belief	Anticipated non-adherence	Death	Other
x ≤ 3	316	44	8	1	4	2	2	0	1	2
3 > x ≤ 6	252	11	4	2	0	1	0	0	0	2
6 > x ≤ 9	232	8	2	2	4	1	0	2	2	1
9 > x ≤ 12	210	7	4	0	1	0	2	0	0	0
12 > x ≤ 15	197	4	1	0	1	0	0	0	0	0
15 > x ≤ 18	190	5	1	0	0	0	0	0	0	0
18 > x ≤ 21	184	0	4	2	1	0	0	0	0	0
21 > x ≤ 24	177	1	1	1	0	0	0	0	0	0
Total		80	25	8	11	4	4	2	3	5

Number of patients who discontinued clozapine by three monthly intervals for each overall reason for discontinuation. N taking clozapine is the number of patients still taking clozapine at the beginning of that time period

Appendix 5: Timing of clinician-led discontinuations

Month	N taking clozapine	ADR	Inadequate response	Blood monitoring	Anticipated non-adherence	Other
x ≤ 3	316	36	0	1	0	2
3 > x ≤ 6	252	6	0	0	0	2
6 > x ≤ 9	232	3	1	0	2	1
9 > x ≤ 12	210	5	0	0	0	0
12 > x ≤ 15	197	1	0	0	0	0
15 > x ≤ 18	190	3	0	0	0	0
18 > x ≤ 21	184	0	1	0	0	0
21 > x ≤ 24	177	0	1	0	0	0
Total		54	3	1	2	5

Timing of clinician-led discontinuations. Number of patients who discontinued clozapine from a clinician-led decision for each reason by three monthly intervals from initiation. N taking clozapine is the number of patients still taking clozapine at the beginning of that time period.

Appendix 6: Timing of discontinuations due to a patient decision

Month	N taking clozapine	ADR	Inadequate response	Blood monitoring	Belief medication not required	Delusional belief	Non-adherence NOS
x ≤ 3	316	8	1	3	2	2	8
3 > x ≤ 6	252	5	2	0	1	0	4
6 > x ≤ 9	232	5	1	4	1	0	2
9 > x ≤ 12	210	2	0	1	0	2	4
12 > x ≤ 15	197	3	0	1	0	0	1
15 > x ≤ 18	190	2	0	0	0	0	1
18 > x ≤ 21	184	0	1	1	0	0	4
21 > x ≤ 24	177	1	0	0	0	0	1
Total		26	5	10	4	4	25

Timing of discontinuations due to a patient decision. Number of patients who discontinued clozapine from a patient decision by three monthly intervals from initiation. N taking clozapine is the number of patients still taking clozapine at the beginning of that time period.

Appendix 7: Specific ADRs cited as a reason for discontinuation

ADR	Clinician-led decision N	Patient decision N	Overall N
Sedation	13	15	28
Neutropenia	15	0	15
Tachycardia	12	1	13
Dizziness	8	2	10
Nausea & vomiting	4	6	10
Weight gain	5	4	9
Fever	7	1	8
Hypersalivation	4	4	8
Flu type symptoms	6	0	6
ECG abnormalities	5	0	5
Constipation	3	1	4
Hypertension	3	0	3
Chest pain	2	0	2
Hypotension	2	0	2
Hyperglycaemia	1	1	2
Pneumonia	1	0	1
Pulmonary embolism	1	0	1
Diabetes mellitus	1	0	1
Metabolic Syndrome	1	0	1
Headaches	0	1	1

Adverse drug reactions (ADRs) cited as a reason for discontinuation of clozapine for 80 patients (130 ADRs). ADRs are not exclusive and differentiated by whether the discontinuation was a clinician-led decision or patient decision.

Appendix 8: Data for Figure 5.1.

	CGI-I of 1 (very much improved)					CGI-I score of 2 (much improved)					CGI-I score of 3 or 4 (minimally improved or no change)				
Time	**N**	**Mean**	**SE**	**L95% CI**	**U95% CI**	**N**	**Mean**	**SE**	**L95% CI**	**U95% CI**	**N**	**Mean**	**SE**	**L95% CI**	**U95% CI**
0	53	5.04	0.10	4.84	5.24	162	5.19	0.05	5.09	5.29	62	5.13	0.08	4.97	5.29
1	53	4.17	0.11	3.95	4.39	157	4.50	0.06	4.37	4.62	61	4.79	0.09	4.61	4.97
2	51	3.76	0.10	3.57	3.96	147	4.20	0.06	4.08	4.31	49	4.86	0.09	4.67	5.04
3	49	3.51	0.09	3.33	3.69	149	4.11	0.06	4.00	4.22	44	4.80	0.10	4.60	4.99
6	49	3.24	0.10	3.05	3.44	135	4.01	0.05	3.91	4.12	39	4.72	0.10	4.52	4.91
9	46	3.35	0.10	3.14	3.56	125	3.98	0.05	3.88	4.09	34	4.62	0.08	4.45	4.79
12	43	3.05	0.09	2.86	3.24	118	3.92	0.05	3.82	4.03	30	4.57	0.10	4.35	4.78
18	41	2.90	0.08	2.73	3.07	118	3.82	0.05	3.72	3.92	27	4.56	0.11	4.33	4.78
24	42	2.90	0.11	2.69	3.12	106	3.83	0.05	3.72	3.94	22	4.36	0.12	4.11	4.62

Data for Figure 5.1: Mean CGI-Severity scores at time assessment points for patients who had an improvement score of 1 (very much improved, blue), 2 (much improved, grey), and either 3 (minimally improved, green) or 4 (no change, orange). N = number of patients still taking clozapine at the beginning of that time period. SE = standard error of mean. L95% CI = lower 95% confidence interval. U95% CI = upper 95% confidence interval.

Appendix 9: Proportion experiencing delayed response

Time	Total N	*Absolute* response (N)	*Absolute* response (%)	*Relative* response (N)	*Relative* response (%)
1	176	59	33.52	67	38.07
2	109	25	22.94	41	37.61
3	87	18	20.69	24	27.59
6	64	9	14.06	9	14.06
9	52	7	13.46	7	13.46
12	41	3	7.32	5	12.20
18	29	0	0.00	3	10.34

Proportion experiencing delayed response. Columns represent time of assessment, total number of patients with a CGI-I score of 3 or 4 (minimally improved or no change) at that time who are still taking clozapine, number and percentage that subsequent to this time meet absolute response criteria, number and percentage that subsequent to this time meet relative response criteria.

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