STUDIES ON THE ASSOCIATION BETWEEN GENETIC POLYMORPHISMS AND CLOZAPINE DRUG RESPONSE IN TREATMENT RESISTANT SCHIZOPHRENIA

THESIS

Submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai,

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DOCTOR OF PHILOSOPHY

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CERTIFICATE

I approve and certify that the work embodied in the thesis entitled, **"Studies on the association between genetic polymorphisms and clozapine drug response in treatment resistant schizophrenia"**, submitted by **Dr. R. Anto Praveen Rajkumar** for the award of the Degree of Doctor of Philosophy in Psychiatry is a bonafide record of research done by him during the period of study under my supervision and guidance. He carried out this work in the Department of Psychiatry, Christian Medical College, Vellore. This thesis has not been submitted to any university previously in part or full for the award of any Degree / Diploma / Associateship/ Fellowship or of any other similar title.

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DECLARATION

I hereby declare that this thesis entitled, **"Studies on the association between genetic polymorphisms and clozapine drug response in treatment resistant schizophrenia"**, submitted for the award of the Degree of Doctor of Philosophy in Psychiatry is an original research work completed by me. I confirm that this thesis has not been submitted to any university previously in part or full for the award of any Degree / Diploma / Associateship/ Fellowship or of any other similar title.

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R. Anto Praveen Rajkumar

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CHAPTER 1

INTRODUCTION

1.1. Treatment-resistant Schizophrenia:

Schizophrenia is a severe disabling neuropsychiatric disorder (1). It is almost equally prevalent in all societies around the world. The lifetime prevalence of schizophrenia is estimated around 1%. It affects men and women equally. It often starts in adolescence or in early adulthood and follows a chronic or episodic course. Both genetic and environmental factors contribute towards the complex etiopathogenesis of schizophrenia. Despite the absence of any laboratory tests, reliable clinical diagnoses of schizophrenia can be made by the standard international diagnostic criteria (2). Patients with schizophrenia suffer positive psychotic symptoms, such as delusions and hallucinations, negative symptoms, such as motivation and social deficits, as well as cognitive difficulties (3). They are usually treated with antipsychotic medications and with comprehensive psychosocial interventions. One third of patients with schizophrenia recover well with their treatment. Another one third continue to suffer moderate symptoms, while other third of patients remain resistant to their treatment (4). Prevalence of treatment-resistance among patients with schizophrenia varies between 20% and 60%, depending on the definition for Treatment-resistant schizophrenia. Various definitions for Treatment-resistant schizophrenia differ on the duration of illness, number of failed antipsychotic drug trials, definition for an adequate antipsychotic drug trial and on the severity of persisting psychopathology (5). Clozapine is the antipsychotic medication of choice to treat the patients with Treatment-resistant schizophrenia (6). It is a serotonin dopamine antagonist (SDA), which also acts on histaminergic, adrenergic and cholinergic receptors (7).

1.2. Clinical need to predict response to Clozapine:

Clozapine is associated with unparalleled clinical benefits and with life threatening adverse effects. Superior clinical efficacy of clozapine to treat patients with Treatment-resistant schizophrenia has been established beyond any doubt (8). The advantages of clozapine include its ability to reduce negative symptoms, cognitive symptoms, risk for violence and risk for suicide (9). Clozapine has low propensity to produce movement disorders (6). However, it can lead to serious adverse effects, such as seizures, agranulocytosis, myocarditis, pulmonary embolism and metabolic syndrome (10). Its disadvantages include sub-optimal response in 40-70% patients with Treatment-resistant schizophrenia (11), the need for periodic leukocyte monitoring and high cost. This combination of high benefits and high risks makes the therapeutic decision-making process, before starting clozapine, difficult for many patients, their families and psychiatrists. When psychiatrists offer clozapine to patients with Treatment-resistant schizophrenia, their discussions often involve the difficulties in predicting clinical responses and adverse effects of clozapine. Hence, there is a clinical need to reduce these uncertainties (12). Ability to identify patients who are likely to improve with clozapine, and to prevent unnecessary use of clozapine in patients, who are unlikely to improve with it, is desired worldwide. Studies investigating the predictors of response to clozapine attempt to serve this clinical need.

1.3. Predictors of response to clozapine:

Research on the factors associated with response to clozapine among patients with Treatment-resistant schizophrenia has focused on clinical (13-18), pharmacogenetic (19-22) as well as on other biological predictors, such as electroencephalography changes and cerebrospinal fluid markers (23, 24). Clinical variables, such as baseline psychopathology and premorbid functioning, have been reported as reliable predictors of response to clozapine

(12). Female gender (16), earlier age of onset (16, 25), non-paranoid subtype (26), longer duration of illness (23), poor baseline quality of life (27) and serum clozapine levels below 350 ng/ml (23) have been reported as the potential clinical predictors of non-response to clozapine. Oral clozapine dose (28-34), female gender (29-31, 33, 35), age (29, 35) and smoking (28, 29) have been proposed as the potential clinical predictors for serum clozapine levels. Pharmacogenetic associations between *HTR2A* gene polymorphisms and response to clozapine (36-40) as well as associations between *HTR2C* gene polymorphisms and clozapine induced weight gain have been established (41, 42). However, findings of many pharmacogenetic and clinical studies, evaluating the predictors of response to clozapine, have been inconsistent or contradictory so far.

1.4. Importance of *CYP1A2* and *HTR3A* gene polymorphisms:

Genes, which regulate the pharmacokinetics and pharmacodynamics of clozapine, are often investigated as the potential candidate genes to predict response to clozapine. Cytochrome P-450 1A2 (CYP1A2), a member of the cytochrome P-450 mixed function oxidase system, is the principal determinant of clozapine metabolism (43). CYP1A2 enzyme activity influences the serum clozapine levels (44-46) and inadequate serum clozapine levels lead to suboptimal clinical responses to clozapine in many patients (47-49). Fixed oral doses of clozapine can produce up to 45 fold inter-individual variability among its serum levels (47). The *CYP1A2* gene (Gene ID: 1544), located in 15q24.1, codes for the CYP1A2 enzyme. A single nucleotide polymorphism (SNP) of this gene, *CYP1A2*1C* (rs2069514) causes decreased enzyme activity in vivo. Another SNP *CYP1A2*1F* (rs762551) leads to higher inducibility of the enzyme by smoking (45) and caffeine consumption (46, 50). Ultra-rapid CYP1A2 activity, due to *CYP1A2*1F*, has been reported to yield low serum clozapine levels and treatment-resistance to clozapine (45).

Antagonism of type 3 serotonin (5HT3) receptor contributes to the superior clinical efficacy of clozapine (7, 51). The 5HT3 antagonist, Ondansetron, can augment clinical response to clozapine (52). *HTR3A* gene (Gene ID: 3359), located in 11q23.1, codes for 5HT3A subunits, which are essential for the formation of functional 5HT3 receptors (53). The pharmacogenetic associations between *5HT3A* gene SNP (rs1062613 and rs2276302) and clinical response to clozapine have been reported (22). Minor allele of rs1062613 increases HTR3A expression (54). rs1062613 modulates neuronal activation in human amygdala (54). It is associated with bipolar disorder, harm avoidance, aggression, anxiety and social desirability (55). T/T genotype of rs1062613 is associated with the need for higher daily antipsychotic dosage (56) and with the time to therapeutic response to antipsychotic medications among patients with schizophrenia (57). Hence, this research had the impetus to evaluate the pharmacogenetic associations between *HTR3A* and *CYP1A2* gene polymorphisms and clinical response to clozapine.

1.5. Need for this research:

There is a clinical need to identify the clinical and pharmacogenetic factors associated with response to clozapine in Treatment-resistant Schizophrenia (12). Such predictors will render therapeutic decision making process easier for the patients, who are likely to improve with clozapine, and will prevent unnecessary use of clozapine in patients, who are unlikely to improve with it. Pharmacogenetics of schizophrenia has promised a lot of clinical utility, but it has achieved only limited clinical success so far (58-60). The poor replication of the results of pharmacogenetic studies is often explained by clinical heterogeneity and by varying definitions for treatment responses (61, 62). However, pharmacogenetic studies of clozapine seldom investigate rich clinical data or employ multiple outcome definitions (22, 63). Most studies evaluating the clinical and pharmacogenetic predictors of response to clozapine (13-

18) were not specific to patients with Treatment-resistant schizophrenia (44, 64-66). They have recruited patients without treatment-resistance and patients with schizoaffective disorders. They have rarely employed standard instruments to assess premorbid adjustment, traumatic life events, cognition and disability. Many studies have not estimated serum clozapine levels, which influence the clinical responses of their participants. Hence, this research aimed to investigate the clinical and pharmacogenetic predictors of response to clozapine, exclusively in patients with Treatment-resistant Schizophrenia, with structured assessment of various clinical variables, estimation of serum clozapine levels and employing multiple outcome definitions.

Paucity of pertinent research on the predictors of response to clozapine among Indian patients and the ethnic differences in the plasma levels of clozapine among Asian patients highlight the need for such research in India (67). Moreover, current consensus guidelines for therapeutic drug monitoring in psychiatry strongly recommends monitoring of serum clozapine levels in all clinical settings to establish clinical efficacy and to ensure the safety of patients, treated with clozapine (68). As therapeutic drug monitoring of clozapine is not feasible in many clinical settings in India, this research included an objective to identify clinical proxy measures of serum clozapine levels in such settings.

CHAPTER 2

AIMS AND OBJECTIVES

2.1. Objectives:

The objectives of this research are,

(i) To investigate the associations between various clinical variables and response to clozapine in patients with Treatment-resistant Schizophrenia.

(ii) To evaluate the associations between four single nucleotide polymorphisms (SNP) in the *CYP1A2* gene (*CYP1A2*1C, CYP1A2*1D, CYP1A2*1E, CYP1A2*1F*) and response to clozapine in patients with Treatment-resistant Schizophrenia.

(iii) To evaluate the associations between these *CYP1A2* gene SNP and adverse effects related to clozapine in patients with Treatment-resistant Schizophrenia.

(iv) To evaluate the associations between these *CYP1A2* gene SNP and serum clozapine levels in patients with Treatment-resistant Schizophrenia.

(v) To evaluate the associations between these *CYP1A2* gene SNP and disability as well as cognition in patients with Treatment-resistant Schizophrenia.

(vi) To study the associations between two SNP in the *HTR3A* gene (rs1062613 and rs2276302) and response to clozapine in patients with Treatment-resistant Schizophrenia.

(vii) To study the associations between these *HTR3A* gene SNP and adverse effects related to clozapine in patients with Treatment-resistant Schizophrenia.

(viii) To study the associations between these *HTR3A* gene SNP and disability as well as cognition in patients with Treatment-resistant Schizophrenia.

(ix) To investigate the associations between various clinical variables and serum clozapine levels in patients Treatment-resistant Schizophrenia.

(x) To evaluate the influence of various clinical predictors and differing outcome definitions on the observed pharmacogenetic associations.

2.2. Research hypotheses:

This research is based on the following hypotheses,

(i) Clinical variables, including male gender, earlier age of onset, longer duration of untreated psychosis and poor premorbid functioning, are likely to be associated with non-response to clozapine in patients with Treatment-resistant Schizophrenia.

(ii) *CYP1A2*1C* is likely to be more frequent among the responders than the non-responders to treatment with clozapine.

(iii) *CYP1A2*1F* is likely to be more frequent among the non-responders than the responders to treatment with clozapine.

(iv) *CYP1A2*1C* is likely to increase the serum clozapine levels. *CYP1A2*1F* is likely to decrease the serum clozapine levels.

(v) *CYP1A2*1F* is likely to be associated with higher levels of cognitive deficits and disability among patients with Treatment-resistant Schizophrenia.

(vi) Minor alleles of *HTR3A* gene SNP (rs1062613 and rs2276302) are likely to be more frequent among the responders than the non-responders to treatment with clozapine.

(vii) These two *HTR3A* SNP are likely to be associated with less cognitive deficits and disability among patients with Treatment-resistant Schizophrenia.

(viii) These two *HTR3A* SNP are likely to be associated with less adverse effects related to clozapine in patients with Treatment-resistant Schizophrenia.

(ix) Clinical variables, including oral dose, age as well as female gender, are likely to increase the serum clozapine levels and smoking is likely to decrease the serum clozapine levels in patients with Treatment-resistant Schizophrenia.

(x) Differing outcome definitions are likely to influence the pharmacogenetic associations of clozapine among patients with Treatment-resistant Schizophrenia.

CHAPTER 3

REVIEW OF LITERATURE

3.1. Schizophrenia:

Schizophrenia is a devastating clinical syndrome, which usually starts its course in adolescence or in early adulthood (69). It is one among the top ten causes of disease related disability in the world (70). A recent systematic review has found 104 papers, evaluating the extensive psychosocial disability caused by schizophrenia (1). While comparing with the general population, the patients with schizophrenia have a standardized mortality ratio of 2.6 for all-cause mortality (71). Life time risk of suicide among patients with schizophrenia is around 5% (72). Patients with schizophrenia are also prone for premature mortality, secondary to cardio vascular complications (71). Schizophrenia affects men and women almost equally. However, the onset is usually earlier and the illness is often more severe in men (69). Despite extensive research for more than a century, our understanding of schizophrenia remains poor and the currently available treatments for it are only modestly effective (70).

3.1.1. Clinical heterogeneity of schizophrenia:

Schizophrenia is an umbrella term (73), which was coined by Eugene Bleuler in 1911. The advent of this term replaced an earlier term and Emil Kraepelin's concept of *Dementia Praecox*. Dementia Praecox was a relatively more homogenous clinical syndrome, characterised by early onset, chronic deteriorating course and the clinical symptoms of delusions as well as hallucinations (74). Bleuler's concept of schizophrenia was broad and has been criticized to be over inclusive (75). It included the patients with late onset illness, patients with shorter duration of illness, patients who suffered less functional deterioration and patients who did not have any positive psychotic symptoms, under the diagnosis of schizophrenia. The contemporary

diagnostic classification systems in psychiatry, International Classification of Diseases – 10th edition (ICD-10) and Diagnostic and Statistical Manual of mental disorders – 5th edition (DSM-5) (2), continue to use the term schizophrenia, but they do not follow the Bleuler's concept of schizophrenia, characterized by the splitting of psychic functions (75).

The ICD -10 and the DSM-5 do not agree on the duration, symptoms and the subtypes of schizophrenia (76). The Cohen's kappa coefficient evaluating the diagnostic congruence between the ICD -10 and the Diagnostic and Statistical Manual of mental disorders – IV edition text revision (DSM-IV TR) (77) definitions of schizophrenia was estimated as 0.61 (78). There are no pathognomonic clinical symptoms to diagnose schizophrenia. Presence of only one clear symptom or two less clear symptoms among the lists of symptoms in the ICD -10 diagnostic criteria indicates the diagnosis of schizophrenia (77). DSM-5 has recently increased the threshold to a minimum of two symptoms for diagnosing schizophrenia and has given up the subtyping of schizophrenia (2). Patients, who were diagnosed to have schizophrenia by using the same diagnostic criteria, may not share any similarity in their clinical presentations. These patients often vary widely in their clinical symptoms, course of illness, severity of illness and their prognosis (79). Hence, schizophrenia is not a single medical disease. It is a heterogeneous group of clinical disorders (80).

3.1.2. Multifactorial aetiology of schizophrenia:

The aetiology of schizophrenia remains unknown**.** As schizophrenia is a heterogeneous group of clinical disorders, underlying causes are expected to be multiple and diverse (81). Search for the aetiology of schizophrenia started with its psychosocial theories. Such theories include the psychoanalytic, psychodynamic, learning, cognitive (82), family and social theories for schizophrenia. The relative importance of nature and nurture towards the causation of

schizophrenia has long been a debate (83). The current understanding is that schizophrenia is a brain disorder, caused by both biological and environmental factors (81).

Neurodevelopmental models explain schizophrenia by abnormal brain development due to multiple genetic and environmental hits (84). Despite the absence of gliosis, neurodegenerative models suggest on-going degenerative processes with loss of neuronal function during the course of schizophrenia. Models combining these two theories explain that the primary pathology in schizophrenia is a neurodevelopmental abnormality and that later environmental factors precipitate the onset of illness as well as the subsequent progression of neurodegenerative processes (85). The precipitating environmental causes include cannabis use (86), other substance use, recent migration, urbanization, viral infections (87) and stressful life events (88). Early environmental insults, such as obstetric complications (89) and childhood trauma (90), predispose to future development of schizophrenia. Research on the biological causes of schizophrenia has focused mainly on the genetic factors and the biochemical factors. Dysregulation of dopamine, glutamate, serotonin, norepinephrine, Gamma Amino Butyric Acid (GABA), glutamate, other excitatory amino acids, and the neuropeptides contribute to schizophrenia (91). Because of this multifactorial aetiology, unidimensional causal models emphasizing only genetic or non-genetic factors often fail to explain the clinical heterogeneity of schizophrenia. Causal models for schizophrenia, which combine genetic and non-genetic factors together, have gained wide acceptance (92).

3.1.3. Outcome definitions for schizophrenia:

Outcome measures in schizophrenia are diverse (93). These measures include symptom outcomes, cognitive outcomes, neurobiological outcomes, hospitalisation, social outcomes and economic outcomes. They may be clinician rated or patient reported. They may categorise outcomes in schizophrenia or rate them on a clinical continuum. Remission rates in

schizophrenia vary widely between 17% and 88%, depending on the employed outcome definition for remission (94). Symptom rating scales are widely used to define outcome in schizophrenia, but their limitations are well recognised (95). Brief Psychiatric Rating Scale (BPRS) (96) is the most extensively used symptom rating scale, followed by Clinical Global Impression and Positive and Negative Syndrome Scale, to define outcome in schizophrenia (95). However, the threshold to categorise outcome in schizophrenia, by using BPRS cut-off scores, remains uncertain. Absence of uniform BPRS cut-off scores to define response in schizophrenia caused significant variability in the results of many clinical trials (97).

Remission Criteria for schizophrenia with cross-scale correspondence have been proposed in 2005 (98). They defined symptomatic remission by maintenance over six months of simultaneous ratings of mild or less on selected positive, disorganization and negative symptoms of schizophrenia. They support complementing BPRS with the Scale for Assessment of Negative Symptoms (3). However, the validity and clinical utility of these remission criteria for schizophrenia have not yet been established (99). Despite the uncertain validity of the psychiatric diagnostic categories, clinical psychiatrists and researchers can make reliable diagnosis of schizophrenia, by employing the diagnostic criteria of ICD-10 or DSM-IV TR. However, they lack any uniform criteria to define outcome in schizophrenia. Various research groups define the outcome in schizophrenia by their own arbitrary choice of outcome definitions (97). Their findings need to be interpreted within the context of varying outcome definitions for schizophrenia.

3.1.4. Treatment-resistant Schizophrenia:

Akin to schizophrenia, there is a lack of agreement regarding the criteria for treatment-resistant schizophrenia (TRS) and its outcome definition (5). Prevalence of treatment-resistance among patients with schizophrenia varies between 20% and 60%, depending on the employed

definitions (4). Kane *et al* initially defined the TRS by the following historical and psychopathologic severity criteria (100),

(i) During the preceding five years, there were at least three failed antipsychotic medication trials, from at least two different chemical classes, without significant symptomatic relief. Each antipsychotic medication trial should be at dosages equivalent to or greater than 1000 mg/day of chlorpromazine for a period of six weeks.

(ii) There was no period of good functioning within the preceding five years.

(iii) Illness severity is ensured by 18 items BPRS total scores of 45 or more as well as by two or more of the following four BPRS items, conceptual disorganization, suspiciousness, hallucinatory behaviours and unusual thought content, are rated at least with moderate severity.

(iv) Clinical Global Impressions Scale rating is moderately ill or worse.

Subsequent definitions for TRS revised the duration of illness, number of failed antipsychotic medication trials, antipsychotic dosage equivalence, duration of antipsychotic trial and the cross-sectional severity of psychopathology (5). There are arguments for and against to include cognitive impairments, subjective well-being, treatment intolerance, functional disability and social deficits in the definition of Treatment-resistant schizophrenia (101). Current recommendations do not include the minimum duration of illness in the definition of TRS (102). Suggested modification of Kane's criteria define the Treatment-resistant schizophrenia by the following,

(i) There were at least two failed antipsychotic medication trials from different chemical classes. Each antipsychotic medication trial should be at dosages equivalent to or greater than 600 mg/day of chlorpromazine for a period of six weeks.

(ii) Illness severity is ensured by minimum Clinical Global Impressions Scale rating of moderately ill and by Global Assessment of Functioning scale (103) total scores of 50 or below.

Outcome definitions of Treatment-resistant schizophrenia are also diverse. Prospective studies define the response in TRS by relative symptomatic improvement from the baseline. They often categorise the outcome in TRS by 20% reduction in the total scores of BPRS or of Positive and Negative Syndrome Scale. Cross-sectional studies define that by post-treatment BPRS total scores of 35 or less (102). Available literature reports wide variability of response rates in Treatment-resistant schizophrenia. Varying outcome definitions of TRS contribute towards such variability among the response rates (5, 102).

3.2. Clozapine:

Clozapine is a dibenzodiazepine ($C_{18}H_{19}N_4Cl$) with a molecular weight of 326.8. Clozapine was first synthesised in 1958. It was the first antipsychotic medication that dissociated antipsychotic efficacy and extra pyramidal adverse effects. Clozapine was first introduced in Europe in 1971. Because of the reports of clozapine induced agranulocytosis, the manufacturer voluntarily withdrew it in 1975. The seminal work of Kane *et al* highlighted the efficacy of clozapine among patients with Treatment-resistant schizophrenia in 1988 (100). In 1989, United States Food and Drug Administration (FDA) approved the use of clozapine for the patients with Treatment-resistant schizophrenia. FDA made the periodic monitoring of leucocyte counts mandatory for the patients on clozapine (104). It approved clozapine for reducing the risk of suicidal behaviours in patients with schizophrenia in 2002. Clozapine still remains as the drug of choice for the management of Treatment-resistant Schizophrenia (6).

3.2.1. Pharmacokinetics of clozapine:

After oral administration of clozapine, its absorption is almost complete. However, the average bioavailability is between 50 and 60%, because of variable first pass metabolism (104). Food does not affect the bioavailability of clozapine. Peak plasma levels are usually reached in about two hours after oral administration of clozapine. Steady state plasma levels are achieved within a week, if twice daily dosing schedule is used. At the steady state conditions, the elimination half-life is about 12 hours. Clozapine is extensively metabolized in the liver by the cytochrome P450 mixed-function oxidase system. The two major metabolites of clozapine are N-desmethyl clozapine (Norclozapine) and clozapine N-oxide. Norclozapine is also pharmacologically active (105). Cytochrome P-450 1A2 (CYP1A2) enzyme is the principal determinant of clozapine metabolism by N-demethylation (43). N-oxidation of clozapine is mainly determined by CYP3A4 enzyme and a flavin monooxygenase. Other Cytochrome P-450 enzymes, CYP2C19, CYP2D6, CYP2E1 and CYP3A3 also contribute to the metabolism of clozapine. Agents, such as cigarette smoking, which induce CYP1A2 enzyme increase the metabolism of clozapine and lead to lower serum clozapine levels. Agents, such as theophylline, ciprofloxacin and fluvoxamine, which inhibit CYP1A2 enzyme decrease the metabolism of clozapine and lead to higher serum clozapine levels (104). Clozapine and its metabolites are highly bound to the plasma proteins. The unbound fractions of clozapine, norclozapine and clozapine N-oxide in the serums of patients with schizophrenia are 5.5%, 9.7% and 24.6% respectively (105). After glomerular filtration, clozapine is largely reabsorbed by the renal tubules. However, the metabolites of clozapine undergo net tubular secretion. Renal clearance values of clozapine, norclozapine and clozapine N-oxide among patients with schizophrenia are 11%, 300% and 640% of the creatinine clearance, respectively (105). Eighty per cent of the orally administered clozapine is excreted in the urine or the faeces as its metabolites and their conjugated products.

3.2.2. Pharmacodynamics of clozapine:

Clozapine is a serotonin (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_{3B}, 5-HT_6 and 5-HT_7) dopamine (D_1-D_4) antagonist (SDA), which also acts on histaminergic, adrenergic and cholinergic receptors (7). Various mechanisms have been proposed to explain the superior clinical efficacy of clozapine in patients with Treatment-resistant schizophrenia. Clozapine has mild affinity towards D_2 receptors, which may explain its propensity to cause less extrapyramidal symptoms. It has higher affinity for the $5HT_2$ receptor. Low D_2 to $5HT_2$ blockade ratio has been proposed to explain its differential antipsychotic activity that is selective on the mesolimbic dopamine system (104). Faster dissociation of clozapine from the D_2 receptors reduces the risk for extrapyramidal adverse effects (106). Ten-fold higher affinity for the D_4 receptors than for the D_2 receptors, antagonism of $5HT_{3A}$ receptors (7, 51), stronger α_1 and α_2 antagonism, strong affinity for the muscarinic receptors and preferential blockade of D_1 receptors over D_2 receptors may explain the superior clinical efficacy of clozapine (104). Clozapine is a partial agonist at the $5HT_{1A}$ receptor, so it contributes to the improvement of depression, anxiety, and the negative symptoms of schizophrenia (107). It differs from other antipsychotic medications by its stronger affinity for H_1 receptors and by its lower affinity for sigma receptors (104). Antagonism of $5HT_7$ receptors in the suprachiasmatic nucleus may contribute to the improvements on circadian rhythmicity among the patients with schizophrenia, treated by clozapine (104).

3.2.3. Clinical benefits of clozapine:

The principal clinical indication for clozapine is Treatment-resistant schizophrenia. Kane *et al* showed that 30% of the severely ill patients with Treatment-resistant schizophrenia improved with clozapine, while only 4% of them improved with chlorpromazine (100). Several subsequent studies confirmed the superior efficacy of clozapine to treat patients with Treatment-

resistant schizophrenia (104). Two recent large effectiveness studies, Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) phase 2E (108) and the UK Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS) 2 trial (8) added evidence to the superior efficacy of clozapine over other typical and atypical antipsychotic medications. The CUtLASS 2 trial showed that clozapine produced greater reductions in psychopathology than olanzapine, quetiapine and risperidone. Patients, treated with clozapine, continue their medication longer than the patients on other atypical antipsychotic medications (109). Clozapine reduces the negative symptoms of schizophrenia as well as the risk for suicide (9). It also reduces the risk of violence and aggression among the patients with schizophrenia (110). Total mortality within 7-11 years interval of cumulative exposure was less for patients on clozapine than the patients on other antipsychotic medications (111). A review on the effects of clozapine on the cognitive functioning in schizophrenia suggests that clozapine improves memory, verbal learning, verbal fluency, and psychomotor speed, but it has less effect on executive functions and working memory (112). The superior clinical efficacy of clozapine to treat Treatment-resistant schizophrenia has also been observed in adolescent (113) and in older patients (114).

Clozapine benefits patients with severe movement disorders and patients who cannot tolerate other antipsychotic medications. There are few promising options other than the use of clozapine for the adult patients with Treatment-resistant bipolar affective disorder (115) or with Treatment-resistant psychotic depression (116). Evidence based reviews support the efficacy of clozapine for treating the psychotic symptoms in patients with Parkinson’s disease (117). Evidence to support the use of clozapine for other clinical indications is limited.

3.2.4. Adverse effects of clozapine:

FDA mandates clozapine to carry five black box warnings for agranulocytosis, seizures, myocarditis, other adverse cardiovascular and respiratory effects, and for increased mortality in older patients with dementia related psychosis (104). The incidence of agranulocytosis has been estimated as 0.38% among patients, treated with clozapine (118). The pathophysiology of clozapine induced agranulocytosis remains unknown. Immunologically mediated reactions, genetic risk factors and cytotoxicity of clozapine metabolites may contribute to clozapine induced agranulocytosis (119). The agranulocytosis can be life threatening and it demands periodic stringent monitoring of white blood cell counts. Estimates of the incidence of clozapine related myocarditis vary from 0.01% to 0.2% (120). IgE-mediated hypersensitivity reaction has been proposed as the mechanism underlying clozapine related myocarditis (119). Patients, treated with clozapine, are at five times greater risk of developing cardiomyopathy than the general population (120). Clozapine treatment has also been reported to be associated with venous thromboembolism and pulmonary embolism (121). Clozapine lowers seizure threshold and the relationship between clozapine and seizures is dose-dependent. Clozapine related seizures occur in 2.7% of patients, treated with doses between 300 and 600 mg/day, and in 4.4% of patients, treated with doses more than 600 mg/day (119).

Other cardiovascular adverse effects of clozapine include tachycardia, orthostatic hypotension, ECG changes, and hypertension. Gastrointestinal adverse effects of clozapine are nausea, vomiting, constipation, bloating, delayed gastric emptying and paralytic ileus. Antagonism of $5HT_3$ receptors and antimuscarinic properties contribute to these gastrointestinal adverse effects of clozapine (104). Excessive salivation is a common distressing adverse effect, which occurs in around 30% of patients on clozapine (122). Clozapine leads to the following central nervous system adverse effects, sedation, delirium, myoclonus, fatigue and obsessive

compulsive symptoms (123). Akathisia, tremors, extrapyramidal adverse effects and neuroleptic malignant syndrome are less frequent, but they can occur in patients, treated with clozapine (124). Long term treatment of clozapine is associated with weight gain, hyperglycaemia and dyslipidaemia (10). Estimates of the prevalence of metabolic syndrome among the patients, treated with clozapine, vary from 28% to 46% (125, 126). Around 6% of clozapine treated patients develop urogenital adverse effects, such as incontinence, increased frequency, increased urgency, urinary retention, enuresis and sexual dysfunction (104). Other clozapine related adverse effects are thermoregulatory abnormalities, heat stroke (127), eosinophilia, elevated liver enzymes, hepatotoxicity (128), sweating, dry mouth and blurring of vision (104, 119).

3.2.5. Therapeutic drug monitoring of clozapine:

Current consensus guidelines for therapeutic drug monitoring in psychiatry strongly recommends monitoring of serum levels of clozapine in all clinical settings to establish the therapeutic efficacy and to ensure the safety of patients, treated with clozapine (68). Fixed oral doses of clozapine are known to produce up to 45 fold inter-individual variability among its serum levels (47). Inadequate serum clozapine levels lead to suboptimal therapeutic responses to clozapine in many patients. Ensuring serum clozapine levels above a minimum threshold value can enhance the clinical responses to clozapine in such patients (47-49). Moreover, serum clozapine levels above a maximum threshold value increase the risk for its toxicity (35) and for clozapine related seizures (129). Presence of such a therapeutic window and the predisposition for high inter-individual variability support the routine therapeutic drug monitoring of clozapine in all clinical settings. Estimation of serum clozapine levels is more essential for the patients, who show poor response to treatment with clozapine, who are intolerant to usual therapeutic doses of clozapine, and for the patients who require clozapine dose titration above 600 mg/day (129, 130).

The minimum threshold value for serum clozapine level is suggested as 350 ng/ml (28, 48, 49) to ensure optimal therapeutic responses during acute treatment with clozapine. Above this cut-off value, the relationship between serum clozapine levels and clinical responses to clozapine remains uncertain (48, 129, 131). The minimum threshold value for serum clozapine level can be less for maintenance therapy of Treatment-resistant schizophrenia (132). This lower limit of the therapeutic window for serum clozapine levels is still being debated between 250 and 420 ng/ml (28, 35, 47-49, 129, 130). There are more uncertainties over the upper limit of the therapeutic window for serum clozapine levels, beyond which clinical benefits are less or outweighed by adverse effects. A recent review on this topic has not established any maximum threshold value for serum clozapine levels but has suggested the presence of a ceiling effect of therapeutic responses for serum clozapine levels above the range of 600-838 ng/ml (133). A safety related upper limit of the therapeutic window for serum clozapine levels has not been established based on the risks for seizures, myocarditis, paralytic ileus, or for other serious clozapine related adverse effects so far (133).

3.2.6. Clinical predictors of response to clozapine:

As described in section 1.2, there is a clinical need to identify the factors associated with response to clozapine among patients with Treatment-resistant schizophrenia (12). Previous studies on the factors associated with response to clozapine have focussed mainly on genetic (19-22) and other biological markers, such as quantitative electroencephalography (QEEG) changes and low ratio of cerebrospinal homovanillic acid/ 5-hydroxyindoleacetic acid levels (23, 24). However, many biological predictors are inconclusive or pending replication (21) and their clinical utility is currently doubtful (60). Clinical psychiatrists often consider many clinical variables, while discussing the option of clozapine with their patients with Treatment-resistant schizophrenia.

Clinical variables, such as baseline psychopathology and premorbid functioning have been reported as reliable predictors of response to clozapine. Their associations with response to clozapine have been replicated without any contradictory evidence so far (12). Other studies, which investigated the clinical predictors of response to clozapine (13-18), have identified male gender (16), later age of onset (16, 25), paranoid subtype (26), shorter duration of illness (23), baseline quality of life (27), past history of neuroleptic induced extra pyramidal symptoms (15, 23, 134), affective symptoms (27) and less negative symptoms at baseline (15, 25) as the potential clinical predictors of response to clozapine. The associations between these potential clinical predictors and response to clozapine are inconclusive due to contradicting research findings. There is a consistent lack of association between race and response to clozapine (16, 27). Cognitive domains, semantic fluency and orthographic fluency, have been reported as significant clinical predictors of response to clozapine (135). Available literature have identified oral clozapine dose (28-34), female gender (29-31, 33, 35), age (29, 35) and smoking status (28, 29) as the potential clinical predictors for serum clozapine levels. However, the associations between these potential clinical predictors and serum clozapine levels have not been confirmed by subsequent studies (30, 33, 34, 136)

3.3. Psychiatric genetics:

Psychiatric genetics studies the genetic mechanisms underlying the aetiology, pathophysiology and the management of psychiatric disorders. It is not a new subspecialty in psychiatry. It has a long and turbulent history (137). Hereditary aspects of psychiatric disorders have been recognised since the beginning of the history of psychiatry. Morel coined the term ‘Hereditary madness’ in 1860 (138). The first large scale family study of schizophrenia was published in 1916. The genealogic demographic department at the German research institute for psychiatry for investigating the genetics of psychiatric disorders was founded in Munich in

1917. Hans Luxenburger conducted the first twin study of schizophrenia in 1928. Erik Strömgren introduced his weighted age correction method for family studies of schizophrenia in 1935 (138). Inopportunely, Eugenics and the German racial hygiene movement made the history of psychiatric genetics turbulent (139).

After World War II, psychiatric genetics steadily regained its status. Fini Schulsinger, David Rosenthal and Seymour Kety conducted their adoption studies of schizophrenia in 1960s (140). Unravelling the genetic basis of Huntington's disease in 1983 boosted the interest in psychiatric genetics. However, the incompatibility between Mendelian disease genetics and psychiatric disorders slowed the progress of psychiatric genetics for a while (141). Tim Crow organized the first congress of psychiatric genetics in Cambridge in 1989. Then, the advances in the molecular biology methods and the computing facilities made psychiatric genetics as the fastest expanding field within psychiatry (137). The completion of human genome project broadened the horizons of psychiatric genetics in 2001. First meta-analysis of Genome Wide Association Studies of schizophrenia was published in 2009 (142). Today, psychiatrists, molecular biologists and bioinformaticians work together closely to understand the genetic basis of psychiatric disorders. The international society for psychiatric genetics conducts the world congress of psychiatric genetics to present the advancements in this field every year (143).

3.3.1. Research methods in psychiatric genetics:

Research methods in psychiatric genetics can be broadly classified in to genetic epidemiology, gene finding methods and molecular genetics (144). Genetic epidemiology includes twin, family and adoption studies. These methods quantify the degree of familial aggregation and the heritability of psychiatric disorders for a given population. They assess the aggregate aetiological importance of all genetic risk factors, irrespective of their locations within the human genome or their individual effect sizes. Gene finding methods are linkage

and association studies, which investigate the linkage disequilibrium of a particular phenotype with DNA markers or candidate genes, respectively. They examine the genomic location and identity of susceptibility genes for the psychiatric disorders (144). Genome scan of all human chromosomes with closely spaced polymorphic markers is employed for the linkage studies. There are parametric and non-parametric linkage analyses. Non-parametric linkage studies are preferred in psychiatry because of the difficulties in specifying the mode of genetic inheritance and the disease allele frequencies for the parametric linkage models (145). Candidate genes, identified on the basis of prior biochemical and pharmacological evidence, are often investigated during the association studies. Microarrays (146) and next generation sequencing methods (147) made the Genome Wide Association Studies (GWAS) of schizophrenia possible. Analyses of GWAS data pose major computing challenges to ensure appropriate corrections for multiple testing and to minimize the false discovery rates (145) .

Molecular genetic methods elucidate the critical DNA variants and study the biological pathways from DNA to the psychiatric disorders (144). Investigating functional implications of the DNA variants are essential to understand the mechanisms underlying psychiatric disorders and to find new therapeutic avenues. Transgenic mice models and other animal models help to understand the functions of specific genes, associated with schizophrenia (148). Gene expression studies investigate the differential RNA expression among patients with psychiatric disorders. Such studies often investigate post mortem brain tissues, lymphocytes, immortalized cell lines or neural cell cultures (149). Genome wide gene expression studies and Transcriptome analyses by next generation RNA sequencing aim to elucidate the involvement of genes in schizophrenia by identifying the differentially expressed genes between the patients and healthy controls (150). As the Encyclopedia of DNA Elements (ENCODE) project has recently mapped regions of transcription, transcription factor association, chromatin structure, histone modification, and biochemical functions for 80% of the

human genome (151), we can now interpret the functional implications of significant GWAS signals more efficiently than ever before.

Epigenetics deals with heritable phenotypes due to changes involving genetic material without alterations in the DNA sequence. The environment may influence the functioning of the genes by various epigenetic mechanisms (152). Chromatin immunoprecipitation sequencing (ChIP Seq) as well as Chromatin immunoprecipitation sequencing and microarray hybridization (ChIP-chip) help to understand the changes in the binding sites of DNA associated proteins and regulatory domains in schizophrenia (149). Methyl-CpG binding domain protein enriched genome sequencing (MBD-seq) has been introduced as a cost effective screening tool for methylome wide epigenetic association studies in schizophrenia (153). Proteome analyses, using mass spectroscopy, investigate the differentially expressed proteins in schizophrenia (154).

3.3.2. Genetics of schizophrenia:

Schizophrenia is a polygenic multifactorial disorder (155). It is governed by complex gene environment interactions (156). Heritability of schizophrenia has been estimated up to 81% (157). However, subsequent searches for genetic variants, which exert large effects on the pathogenesis of schizophrenia, have not been successful (158). Many common and rare genetic variants, carrying small effect sizes, contribute to the risk of developing schizophrenia (141, 142). The Schizophrenia Psychiatric Genome Wide Association Study Consortium (PGC) combined the data of 17 GWAS on schizophrenia, comprising a stage one discovery sample of 21,856 individuals and a stage two replication sample of 29,839 independent people, and identified seven loci for genome wide significant associations with schizophrenia (159). The association between these seven loci, 1p21.3, 2q32.3, 8p23.2, 8q21.3, 10q24.32, 6p21.32-p22.1 as well as 18q21.2-q24.33, and schizophrenia have been extensively replicated (160). However, the GWAS positive association signals explain only a

small proportion of the heritability of schizophrenia (161). Common variants, studied by the Schizophrenia Psychiatric GWAS Consortium, could capture only 23% of variation in the liability to schizophrenia (162). Copy number variations, epistasis, pleiotropy, presence of phenocopies, limitations of current sequencing methods, overestimation of heritability, gene environment interactions and epigenetic mechanisms have been proposed as possible reasons for this missing heritability of schizophrenia and other complex traits (163, 164). Genetic association studies, employing endophenotypes for schizophrenia, suggest multiple independent pathways mediating pathogenesis in the clinically heterogeneous group of schizophrenias (165).

Human genome dwells within the environment and its expression is shaped by the environment (166). The environment can regulate the expression of genome by epigenetic mechanisms, such as DNA methylation and histone modification. More than 100 genes, including *GAD1* and *RELN*, have been identified to display altered methylation status in schizophrenia (167, 168). Hypermethylation and decreased transcription of the Reelin promoter as well as hypomethylation of the MB-COMT promoter with consequent increased transcription have been reported in schizophrenia (168). The association between *BRD1* gene, regulating histone acetylation, and schizophrenia has been replicated by three studies (169-171). Non-coding RNA molecules, especially micro RNA dysregulation, contribute to the genetics of schizophrenia (172). One of the genome wide significant loci (1p21.3), identified by the Schizophrenia Psychiatric GWAS Consortium, contains the primary transcript for MIR137 (Micro RNA 137). MIR137 is involved in regulating adult neurogenesis and neuronal maturation (173). MIR137 variant genotype has been associated with earlier age of onset and reduced white matter integrity among patients with schizophrenia (174). Studies evaluating the interactions between the human genome, proteome and the envirome continue to enhance our understanding of schizophrenia (175).

3.3.3. Pharmacogenetics of schizophrenia:

Patients with schizophrenia differ widely in their ability to metabolize drugs and to respond to them. Genetic factors alter pharmacokinetics and pharmacodynamics of many antipsychotic medications. They contribute to the interindividual variations among the clinical response and toxicity of antipsychotic medications (176). Pharmacogenetics is defined as the study of sequence variations and functional implications of genes modifying drug response or toxicity (58). It aims to select the drugs with the greatest likelihood of benefit and the least likelihood of harm to individual patients, based on their genetic makeup. Because of the quest for the individualized therapy and the ability to predict clinical outcome, pharmacogenetics of schizophrenia has flourished over the last decade (176).

Several *DRD3*, *ABCB1* (G2677T), *HTR6*, *ADR1A*, *MC2R*, *SLC6A2* (G1287A) gene polymorphisms and long allele of *SLC6A4* have been associated with good clinical response to olanzapine among patients with schizophrenia. *COMT* (Val108/158Met) and *GRM3* gene polymorphisms have predicted significant improvement of negative symptoms of schizophrenia during treatment with olanzapine (177). AKT1 (rs380330 and rs2494732), *BDNF*, *CYP3A4*, *COMT*, *GRM3*, *DRD2*, *DRD3*, *HTR1A*, *HTR2A*, *HTR2C*, *HTR6*, *MDR1*, *RGS4* and *SLC6A4* (178) gene variations have been reported to be associated with clinical responses to risperidone among patients with schizophrenia. *DRD2* gene polymorphisms have been reported to be associated with clinical responses to haloperidol, chlorpromazine and to aripiprazole. The associations between CYP2D6 single nucleotide polymorphisms and response to haloperidol were reported to be significant (177). Several studies have evaluated the pharmacogenetic associations for tardive dyskinesia, antipsychotic induced weight gain and extrapyramidal symptoms (179). Pharmacogenetic associations between antipsychotic induced weight gain and *HTR2C* as well as *MC4R* genes were consistent (179). *ADRA1A*,

COMT, *CYP2D6*, *DRD4* and *HTR2A* gene polymorphisms were significantly associated with tardive dyskinesia but these associations have not yet been replicated (177).

Genome wide pharmacogenomics analysis of response to antipsychotic medications has identified a single nucleotide polymorphism rs17390445, in the intergenic region on 4p15, with genome wide significance. Associations between response to antipsychotic medications and polymorphisms in the *ANKS1B* as well as *CNTNAP5* genes were close to the threshold for genome wide significance (180). Despite these promising findings, only one pharmacogenetics test has been approved for clinical use in psychiatry by FDA so far (181). This test is not routinely recommended for the patients with schizophrenia (182). In spite of the diligent efforts to tailor personalized treatment with antipsychotic medications, pharmacogenetics of schizophrenia has achieved only limited clinical success so far (58-60). Clinical heterogeneity of schizophrenia and varying outcome definitions for treatment responses continue to challenge the translation of pharmacogenetics to clinical benefits (61, 62).

3.3.4. Pharmacogenetics of clozapine:

Pharmacogenetics of clozapine is more extensively evaluated than pharmacogenetics of other antipsychotic medications (183). It aims to identify the patients, who are most likely to benefit with clozapine, and to prevent the unnecessary exposure of many potential non-responders to serious adverse effects of clozapine. Pharmacogenetic associations between *HTR2A* gene polymorphisms, especially 102-T/C as well as His452Tyr alleles, and response to clozapine were consistent (36-40). Pharmacogenetic association between *HTR2C* gene polymorphisms and clozapine induced weight gain has also been established (41, 42). Results of several studies evaluating the pharmacogenetic associations between responses to clozapine and *DRD4* gene (11p15.5) polymorphisms have been inconclusive so far (184-188).

Pharmacogenetic studies of clozapine have suggested its association with *DRD2* gene polymorphisms (21). Associations between response to clozapine and *DRD1* gene (5q35.1) (189), *DRD3* gene (3q13.3) (190) as well as *SLC6A3* gene (5p15.3) polymorphisms (191) have been reported. *HTR6* gene polymorphisms have been reported to have a modest relationship with treatment response to clozapine (192). *COMT, DTNPB1* and *GFRA2* gene polymorphisms have been found to be associated with good clinical response to clozapine among patients with schizophrenia. *DRD4* 48bp five repeat allele, *SLC6A4* short allele and *HTR2A* gene polymorphisms have been reported to be associated with poor clinical response to clozapine (177). However, these pharmacogenetic associations of clozapine have not been replicated so far. A recent pharmacogenetic study, evaluating 127 polymorphisms of 27 candidate genes, did not replicate these associations, but reported a significant association between rs7787082 and rs10248420 of *ABCB1* gene and non-response to clozapine, after appropriate corrections for multiple testing (193). The CRESTAR collaboration is currently evaluating the pharmacogenomic biomarkers for response to clozapine among patients with Treatment-resistant schizophrenia in Europe (194).

Pharmacogenetic association studies involving *HTR1A*, *HTR7* (195), alpha adrenergic (196) and histaminergic receptor (197) gene polymorphisms did not show any relationship with treatment response to clozapine. Despite the limited clinical utility of the pharmacogenetic predictors for response to clozapine at present, pharmacogenetic models, combining the positive association signals, have been attempted to predict the likelihood of positive response to clozapine (198). Human leukocyte antigen (HLA) system DBQ1*0201 and DBQ1*0502 have been reported to increase the risk for clozapine induced agranulocytosis (177). *CYP2D6* and *MPO* gene polymorphisms were not significantly associated with clozapine induced agranulocytosis (199). Pharmacogenetics of clozapine has not identified any significant predictors for other clozapine related adverse effects (179).

3.4. Cytochrome P450 1A2:

Human cytochrome P450 superfamily (CYP) includes many diverse enzymes, which catalyse the metabolism of numerous clinically, physiologically, and toxicologically important organic compounds. The CYP1 family contains CYP1A1, CYP1A2, and CYP1B1 enzymes. CYP1A2 is almost exclusively expressed in liver. It is one of the major CYPs in human liver (13–15%). CYP1A1 is predominantly extra-hepatic and is expressed in intestines, lung, placenta, and in lymphocytes (200). As DNA and amino acid sequences of CYP1A1 and CYP1A2 are 74% identical, their substrate specificities often overlap (201). CYP1A2 metabolises caffeine, theophylline, more than 110 structurally diverse therapeutic drugs, herbal compounds, drugs of abuse, procarcinogens and many endogenous substances, including melatonin, bilirubin and estradiol (201).

3.4.1. CYP1A2 enzyme:

CYP1A2 enzyme is a phase I oxidative enzyme. Human CYP1A2 enzyme (uniProt: P05177) is made of 515 amino acids and has a molecular mass of 58,294 Da. It metabolises around 8–10% of clinical drugs, which are metabolised by all CYP enzymes. Crystal structure of CYP1A2 was described in 2007. It contains 12 α-helices, which are designated A–L and four β-sheets, which are named 1–4 (202). It has a planar active site cavity that is well adapted for the oxidation of large aromatic compounds. The substrates of CYP1A2 usually contain a planar ring that can readily fit the narrow planar active site of the CYP1A2 enzyme. The topology of the active site of CYP1A2 is identified by multiple residues on helix F and helix I, which form two parallel substrate binding platforms on either side of the cavity. CYP1A2 has six substrate recognition sequences (SRS) and many functionally important residues, which recognise its substrates. Both SRS and non-SRS regions of CYP1A2 play important roles in the substrate enzyme interactions. Site Directed Mutagenesis, homology modelling and X-ray

crystallographic studies help us to understand the mechanisms underlying substrate enzyme interactions of CYP1A2 (201).

Phenacetin, caffeine, and theophylline are often used as the model substrates for evaluating the activity of CYP1A2 in vivo by their marker reactions, phenacetin O-deethylation, caffeine N3-demethylation, and theophylline N-demethylation, respectively (203). Sixty fold interindividual variations in CYP1A2 activity, due to both environmental and genetic factors, have been reported (204). Drugs, including, fluvoxamine, fluoxetine (205), venlafaxine (206), isoniazid (207), fluoroquinolone antibiotics (208), oral contraceptives (209), and some herbal medicines (210) can reduce the activity of CYP1A2 by reversible or irreversible inhibition. Such inactivation or reversible inhibition of CYP1A2 by drugs leads to clinically important drug interactions. Reduced metabolic clearance of the substrate drugs elevates their serum levels and the risks for their toxicity. Drugs, such as quinine (211) as well as carbamazepine (212), smoking, coffee intake (213), and dietary polycyclic aromatic hydrocarbons induce CYP1A2 activity (201). They usually induce CYP1A2 by aromatic hydrocarbon receptor (AhR) mediated pathways. AhR is a ligand activated transcription factor and a basic helix–loop–helix (bHLH) protein that belongs to the Per-Arnt-Sim (PAS) family of transcription factors (214). Binding of an inducing ligand to AhR sets off sequential signalling events leading to increased transcription of *CYP1A2* gene. CYP1A2 induction increases the metabolic clearance of the substrate drugs and reduces their serum levels.

3.4.2. *CYP1A2* gene:

The human *CYP1A2* gene (gene ID: 1544) has been mapped to 15q24.1. Complementary DNA (cDNA) corresponding to the CYP1A2 gene was first isolated in 1987 (215). The *CYP1A2* gene spans 7.8 kilo bases, comprising seven exons, six introns and an enhancer region (201, 216). It shares a common 5’ flanking region with *CYP1A1* gene and

these genes are separated only by a 23 kb segment without any open reading frames (216). Nucleotide sequences of exons 2, 4, 5 and 6 in *CYP1A1* and in *CYP1A2* are similar. Phylogenetic analysis of CYP1A genes suggested that the *CYP1A2* gene resulted from the duplication of *CYP1A1* gene around 350 million years ago, during the evolution of mammals and birds (217). *CYP1A2* gene has one transcript (ENST00000343932), which is 2728 bp long and codes for the CYP1A2 enzyme. *CYP1A2* gene is pleomorphic and has at least 26 nonsynonymous single nucleotide polymorphisms, which modify the amino acid sequence (201).

*CYP1A2*1A* is the wild allele of the *CYP1A2* gene. *CYP1A2*1*C, *CYP1A2*1*D, *CYP1A2*1E* and *CYP1A2*1F* have been more extensively evaluated than other single nucleotide polymorphisms of *CYP1A2* gene (201). The *CYP1A2*1C* allele has G>A in the 5' flanking region of *CYP1A2*. It has been reported to cause reduced enzyme activity and probable reduced expression of the enzyme (218). *CYP1A2*1D* allele has a T nucleotide deletion in the 5' flanking region of *CYP1A2*. It was not found to affect CYP1A2 activity among Swedes and Koreans (219). *CYP1A2*1E* has T>G in the first intron of *CYP1A2*. It has been found to be more frequent among the Asians and Africans than the Caucasians (220). *CYP1A2*1F* has A>C in the first intron of *CYP1A2*. It is a common allele and is the most extensively evaluated *CYP1A2* allele (201). It increases CYP1A2 enzyme activity and leads to higher inducibility of the enzyme (50, 221).

There are prominent interethnic differences in the CYP1A2 activity. CYP1A2 poor metabolisers are more frequent among the Asian and African populations than the Caucasians (219, 222). Asian patients with schizophrenia have been found to have 30–50% higher serum clozapine levels than the Caucasians (223). Genetic factors alone may explain 35-75% of the high inter-individual variability among the CYP1A2 enzyme activity (201). Marked inter-

individual variations (15-40 fold) have been documented in the expression of CYP1A2 mRNA levels. Other genetic mechanisms, regulating CYP1A2 activity, include mRNA degradation, protein degradation, cis-acting and trans-acting transcriptional factors, alternative splicing, regulatory microRNAs, epistasis as well as epigenetic mechanisms such as DNA methylation and histone modifications (201, 224, 225). *CYP1A2* gene knockout mice were first reported in 1996 (226). They were viable but showed deficient metabolism of drugs. *CYP1A1/1A2/1B1* triple knockout mice have been reported in 2008. They showed greater risk of in-utero death, slower development, hydrocephalus, hermaphroditism, and cystic ovaries (227).

3.4.3. *CYP1A2* gene polymorphisms and clozapine:

CYP1A2 is the principal determinant of clozapine metabolism by N-demethylation and N-oxidation (43). CYP1A2 enzyme activity influences the serum clozapine levels (44-46). Inadequate serum clozapine levels are associated with suboptimal clinical response to clozapine in many patients (47-49). Ultra-rapid CYP1A2 activity, due to *CYP1A2*1F* polymorphism, has been associated with low serum clozapine levels and treatment-resistance to clozapine (44, 45). *CYP1A2*1F* has also been associated with higher induction of the enzyme by smoking (45) and heavy caffeine consumption (46). These studies suggested high clinical utility of *CYP1A2* genotyping for the patients on clozapine (45). CYP450 pharmacogenetic test chips have been introduced to the market (59, 228). However, there were at least three studies, which did not find any association between *CYP1A2* single nucleotide polymorphisms and serum clozapine levels as well as clozapine treatment response (64-66). A recent study, evaluating 27 candidate genes for pharmacogenetics of clozapine, was also negative for the associations between *CYP1A2* SNP and treatment responses to clozapine (193). *CYP1A2*1C* (229) and *CYP1A2*1F* (230) have been reported to be associated with increased severity of tardive dyskinesia among patients with

schizophrenia (177). However, the genetic associations between *CYP1A2* gene polymorphisms and schizophrenia as well as tardive dyskinesia were not significant, after appropriate corrections for multiple testing (231).

3.5. $5HT_3$ and clozapine:

Clozapine is a competitive antagonist of type 3 serotonin ($5HT_3$) receptor. Antagonism of $5HT_3$ receptor has been proposed as one of the mechanisms explaining the superior clinical efficacy of clozapine (7, 51). An in vivo microiontophoretic study has confirmed the modulation of $5HT_3$ response in the medial prefrontal cortex of the rats by clozapine (232). The $5HT_3$ antagonist, ondansetron, can augment the clinical response to clozapine (52) and to other antipsychotic medications among patients with schizophrenia (233). Typical antipsychotic medications, such as flupentixol, phenothiazines and haloperidol, have also been reported to exert non-competitive inhibition of $5HT_3$ receptors (234).

3.5.1. $5HT_3$ receptor:

Serotonin (5-hydroxytryptamine) neurotransmitter signals via numerous pharmacologically defined cell surface receptors. $5HT_3$ receptors are the only ligand gated ion channels among the myriad of serotonin receptors. They are fast activating non-selective cation channels (235), widely expressed in the central nervous system (236). Activation of $5HT_3$ receptors leads to membrane depolarization, increase in intracellular Ca^{2+} (237), release of neurotransmitters, and Ca^{2+} entry into lymphocytes (235). $5HT_3$ receptors mediate emetic response, inflammatory response, pain reception, anxiety, cognition, affect modulation and substance dependence (235).

Similar to other ligand gated ion channels, $5HT_3$ receptors are pentameric proteins, made of five subunits (238). The compulsory subunit of $5HT_3$ receptors is $5HT_{3A}$ (239). $5HT_{3B}$ subunit was cloned in 1999 (53). Three more subunits, $5HT_{3C}$, $5HT_{3D}$, and $5HT_{3E}$, were identified later (240). $5HT_{3A}$ subunits are essential for the formation of functional $5HT_3$ receptors (239). They form $5HT_3$ receptors either alone as homopentamers or in combination with other subunits as heteropentamers (53). Pre-synaptic $5HT_{3A}$ receptors determine the intracellular Ca^{2+} levels and modulate the release of several neurotransmitters. Post-synaptic $5HT_{3A}$ receptors mediate fast synaptic neurotransmission and modulate learning as well as memory processes (51).

3.5.2. *HTR3A* gene:

$5HT_{3A}$ subunit is encoded by *HTR3A* gene that has been mapped to chromosome 11q23.1. *HTR3A* gene spans 15,433 bases and has nine exons (241). It has six protein coding transcripts, HTR3A-001 (2432 bp), HTR3A-002 (2221 bp), HTR3A-003 (1994 bp), HTR3A-201 (2331 bp), HTR3A-202 (2235 bp), and HTR3A-203 (1385 bp), as well as, two other transcripts, HTR3A-004 (1385 bp) and HTR3A-005 (906 bp). 593 single nucleotide polymorphisms of *HTR3A* gene have been reported so far (242). rs1062613 is a missense mutation (Pro16Ser) within the upstream regulatory region of *HTR3A* gene (243). It is a functional important polymorphism, because its minor allele T increases the expression of *HTR3A* gene (54). Variations within the primary sequence of $5HT_3$ receptors have been proposed to be crucial for the antipsychotic potency and the safety profile of clozapine (51).

A functional neuroimaging study has reported that rs1062613 modulates neuronal activation in human amygdala (54). rs1062613 has been associated anxiety symptoms and with altered amygdalar reactivity during emotional face processing (244). Genetic association between rs1062613 and bipolar disorder was also significant (243). rs1062613 has been reported to be associated with harm avoidance, aggression, social desirability (55).

Significant interactions between rs1062613 and other *HTR3A* gene polymorphisms have been found to impact nicotine dependence (245). T/T genotype of rs1062613 has been associated with the need for higher daily antipsychotic dosage during maintenance therapy of schizophrenia (56). It has been reported to be associated with the time to therapeutic response to antipsychotic medications among patients with schizophrenia (57). However, a recent study, evaluating 29 single nucleotide polymorphisms of *HTR3A* gene among 943 patients with schizophrenia and 2,343 healthy controls, did not find any association between *HTR3A* gene polymorphisms and schizophrenia (246). Similarly, associations between *HTR3A* gene polymorphisms and suicidal behaviours among patients with schizophrenia were also not significant (247).

3.5.3. *HTR3A* gene polymorphisms and clozapine:

The first two pharmacogenetic studies, evaluating the associations between *HTR3A* gene polymorphisms and response to clozapine, did not find any significant associations (20, 63). However, a recent study has reported that the allelic and genotypic associations between rs1062613 as well as rs2276302 and treatment response to clozapine was significant (22). Only the pharmacogenetic association of rs1062613 was significant, after corrections for multiple testing with 100,000 permutations. We present further details of the studies, which evaluated the pharmacogenetic associations between *HTR3A* gene polymorphisms and response to clozapine among patients with schizophrenia, in Table 1.

Table 1: Studies evaluating the pharmacogenetic associations between *HTR3A* gene polymorphisms and response to clozapine

Study	Participants	*HTR3A* SNPs	Outcome definition	Clinical predictors	Results
Arranz et al. (2000) (20)	200 British patients with schizophrenia. TRS was not an explicit inclusion criterion.	rs1062613 1596-A/G	Retrospective assessment using GAS (103). GAS response threshold was not specified.	Not assessed	Allelic p for, rs1062613 = 0.79 1596-A/G = 0.85
Gutierrez et al. (2002) (63)	263 British patients with DSM-III-R schizophrenia. TRS was not an explicit inclusion criterion.	rs1062613 1596-A/G	Retrospective assessment for 20 points improvement in GAS scores after a minimum of 3 months treatment.	Not assessed	Allelic p for, rs1062613 = 0.36 1596-A/G = 0.78
Souza et al. (2010) (22)	140 (82% Caucasians) patients with DSM-III-R or IV schizophrenia. "*Almost all of them were treatment refractory or intolerant to typical antipsychotics*"	rs1062613 rs2276302 rs1176713 rs1150226	More than 20% reduction of BPRS total scores from the baseline.	Not assessed	Both rs2276302 and rs1062613 were nominally significant. only rs1062613 was significant after 100000 permutations (P=0.041)

TRS: Treatment-resistant Schizophrenia; GAS: Global Assessment Scale; DSM: Diagnostic and Statistical Manual of Mental Disorders; BPRS: Brief Psychiatric Rating Scale; SNP: Single Nucleotide Polymorphism

CHAPTER 4

SCOPE AND PLAN OF WORK

4.1. Scope of this research:

There is a clinical need to develop a valid model to predict the responses to clozapine and related adverse effects in Treatment-resistant schizophrenia (12). Individually tailored pharmacotherapy is desired worldwide. Schizophrenia is not a single disease, but a complex heterogeneous polygenic multi-factorial disorder (142). Hence, available models with limited clinical or pharmacogenetic predictors explain only a fraction of variability observed in the clinical responses to clozapine. Combining clinical and pharmacogenetic predictors may identify more patients, who are most likely to benefit with clozapine, and may prevent the unnecessary exposure of many potential non-responders to serious adverse effects. Studies investigating the complex relationship between candidate gene polymorphisms, clinical predictors, serum clozapine levels and clozapine treatment responses are sparse (22). As this research investigates these variables together, it has more potential to develop a combined clinical and pharmacogenetic model which will help the psychiatrists to predict the clinical responses and adverse effects of clozapine in Treatment-resistant Schizophrenia.

International standard criteria to define the treatment responses in schizophrenia are not available. Varying outcome definitions lead to poor replication of the results of pharmacogenetic studies in schizophrenia (61, 62). Pharmacogenetic studies of clozapine seldom employ multiple outcome definitions (22, 63). As this research systematically evaluates the influence of multiple outcome definitions over the pharmacogenetic associations, it has the potential to guide future research on this topic.

4.2. Plan of work:

This research planned to investigate its objectives by conducting two pharmacogenetic association studies and two cross-sectional observational studies. They are,

1. A cross-sectional observational study to evaluate the clinical predictors of response to clozapine in patients with Treatment-resistant Schizophrenia.

2. A cross-sectional observational study to evaluate the clinical predictors of serum clozapine levels in patients with Treatment-resistant Schizophrenia.

3. A pharmacogenetic association study to evaluate the association between *CYP1A2* gene single nucleotide polymorphisms and clinical responses to clozapine in patients with Treatment-resistant schizophrenia.

4. A pharmacogenetic association study to evaluate the association between *HTR3A* gene single nucleotide polymorphisms and clinical responses to clozapine in patients with Treatment-resistant schizophrenia.

We planned to combine clinical and pharmacogenetic information to develop a model to predict clinical response to clozapine.

CHAPTER 5

PATIENTS AND METHODS

5.1. Study design:

A cross-sectional observational study was employed to evaluate the clinical variables associated with responses to clozapine and with the serum clozapine levels in patients with Treatment-resistant schizophrenia. A case control design framework was used to identify the clinical predictors for non-response to clozapine. Pharmacogenetic association studies were employed to investigate the associations between *CYP1A2* gene as well as *HTR3A* gene Single Nucleotide Polymorphisms and clinical responses to clozapine among these patients.

5.2. Setting:

This research was conducted in the Department of Psychiatry, Christian Medical College (CMC), Vellore, a tertiary referral centre for the management of psychiatric disorders. It is situated in the southern Indian state of Tamil Nadu. Tamil Nadu is one of the most industrialized and one of the most populous states in India. It has better education, health and development indices than those for the rest of the India. The principal language of the state is Tamil. Vellore district is situated in the north central part of Tamil Nadu. It lies between 12° 15’ to 13° 15’ North latitudes and 78° 20’ to 79° 50’ East longitudes. It is spread over an area of 4314.29 km^2. Vellore city is the headquarters of the district of Vellore and houses the Christian Medical College. Christian Medical College Hospital is a multidisciplinary tertiary care hospital that has been providing services to the patients from Tamil Nadu and the rest of the India since 1900.

Department of Psychiatry, Christian Medical College, has two general adult psychiatry units, one psychiatric rehabilitation unit, one child and adolescent psychiatry unit and another unit for the intellectually disabled. This department has 122 inpatient beds. It provides short-term care for the patients with organic psychiatric disorders, substance use disorders, schizophrenia, other psychoses, mood disorders, and anxiety disorders. The emphasis is on a multi-disciplinary approach and on eclectic care using a wide variety of psychological as well as pharmacological therapies. The department has daily outpatient and regular follow-up clinics. The frequency of follow-up is dependent on the severity of the illness. Patients with schizophrenia are initially treated with either dopamine antagonists or serotonin dopamine antagonists. Clozapine is never used as the first line antipsychotic medication. It is reserved for the patients with Treatment-resistant schizophrenia, severe tardive dyskinesia and for those with treatment refractory bipolar affective disorders. Leukocyte counts and metabolic parameters of the patients receiving clozapine are periodically monitored (248). Detailed medical records of treatment are maintained for all patients. Most of our outpatients with schizophrenia live in the community with their families. Their medications are directly provided by their first degree relatives or spouses, who report any degree of non-adherence to the treating psychiatrists during periodically scheduled follow-up visits.

5.3. Recruitment of participants:

All consecutive patients, who attended the clinics of Department of Psychiatry, Christian Medical College, Vellore, and satisfied our eligibility criteria, were invited to participate in this research. The eligibility criteria were listed below,

(i) Diagnostic and Statistical Manual of Mental Disorders - IV edition Text Revision (DSM IV-TR) diagnosis of schizophrenia (77).

(ii) Treatment-resistant Schizophrenia has been established in the past after failure to respond at least two adequate antipsychotic trials, as documented by the treating psychiatrists in their medical records. An adequate antipsychotic trial was defined by 600 mg chlorpromazine equivalents for duration of at least six weeks with good drug compliance. The two adequate antipsychotic trials included at least one adequate trial with a Serotonin Dopamine Antagonist.

(iii) On stable dose regimen of clozapine for at least twelve weeks preceding recruitment, with good drug compliance during that period.

(iv) Origin of South Indian ethnicity.

(v) The patient as well as his/ her first degree relatives or spouse were willing to provide written informed consent to participate in this research.

The following patients were excluded,

(i) Patients who had severe neurological illnesses, precluding the assessment.

(ii) Patients who had intellectual disability.

(iii) Patients who had sensory impairment, precluding the assessment.

(iv) Patients who refused consent to participate in this research.

5.4. Clinical Assessment:

Seven standard instruments and a structured questionnaire were employed to assess the psychopathology, disability, cognition, traumatic life events, premorbid adjustment and socio-demographic, clinical as well as treatment data of the participants.

5.4.1. Brief Psychiatric rating scale (BPRS):

The Brief Psychiatric Rating Scale was developed by Overall and Gorham as a short scale for measuring the severity of psychiatric symptomatology (96). BPRS covers a broad range of areas including thought disturbance, emotional withdrawal, anxiety, depression, hostility and suspiciousness. It is a clinician-rated scale which can be administered within 30 minutes. BPRS ratings include patient interview and observation. Hence, it can be used to rate patients with very severe impairment. It has 18, 19, 20 and 24 item versions. The 19 items BPRS has an alpha coefficient of 0.83. This research employed the 18 items version of BPRS. These 18 items are rated on a seven point item specific Likert scale from 1 to 7 with a total score ranging from 18 to 126. BPRS has a good inter-rater reliability of 0.72 to 0.87. Validity of BPRS is measured by the correlations with other measures of symptom severity, especially those assessing schizophrenia symptomatology. BPRS has good psychometric properties (96) and is used widely in clinical settings. Psychopathology of all participants was assessed using BPRS.

5.4.2. Abnormal Involuntary Movements Scale (AIMS):

The AIMS is a clinical examination and rating scale developed to measure antipsychotic drug induced dyskinesia (249). It is a clinician rated scale which can be completed within 10 minutes. It has 12 items, each of which is rated on an item specific five point severity scale ranging from 0 to 4. Changes in global severity and over individual areas can be monitored over time. Ten items cover the movements and two items concern dental factors, which can complicate the diagnosis of dyskinesia. In the presence of extended antipsychotic exposure and the absence of other conditions causing dyskinesia, mild dyskinetic movements in two areas or moderate movements in one area suggest a diagnosis of tardive dyskinesia. AIMS is useful for monitoring patients for the development of tardive

dyskinesia and for tracking changes in the severity of tardive dyskinesia during follow up. This scale is routinely employed in many clinical settings. All participants were assessed using AIMS for the presence of tardive dyskinesia. If tardive dyskinesia was present, AIMS would measure its cross-sectional severity.

5.4.3. Addenbrooke's Cognitive Examination (ACE-R):

The ACE-R is a brief cognitive test battery, which incorporates the following sub-domains, orientation, attention, memory, verbal fluency, language and visuo-spatial. ACE-R has very good internal consistency, convergent validity and is sensitive to early cognitive dysfunction (250). Construct validity is best for the memory and verbal fluency sub-domains, which show good concordance with standard neuropsychological tests. ACE-R can be administered within 20 minutes and is widely used in many clinical settings. The total score of ACE-R ranges between 0 and 100, with higher scores indicating better cognitive function. The total score of 100 is made by 10 for orientation, eight for attention, 26 for memory, 14 for verbal fluency, 26 for language and 16 for visuo-spatial ability. Raw scores are used for all items except for verbal fluency, that utilizes a scaled scoring system for the letter and category fluency. ACE-R incorporates the questions on the Mini Mental Status Examination (MMSE) (251), but does not include many tests of executive functions. Unlike MMSE, age, level of education and gender do not strongly influence the predictive outcome of ACE-R (252). The cognitive functioning of all participants was assessed using ACE-R.

5.4.4. WHO Disability Assessment Scale II (WHODAS II):

The WHODAS II is a generic health status instrument validated to assess the disability status of physically and psychiatrically ill (253). It assesses the following six domains: communication, mobility, self-care, interpersonal, life activities, and participation (254). WHODAS II is available in 12 items as well as 36 items versions and in many

languages. The 12 items version was used in this research to assess the disability of our participants. Each item is rated on a five point Likert scale ranging from zero to four. The total score of WHODAS II 12 items version ranges between 0 and 48, with higher scores indicating more disability. Psychometric properties of WHODAS-II have been extensively evaluated and were found to be excellent, except for the presence of a ceiling effect (255).

5.4.5. Childhood Traumatic Events Scale (CTES):

The CTES briefly assesses six early traumatic experiences prior to the age of seventeen. These traumatic events include bereavement, parental discord, violence, sexual abuse, physical illness and other major life events (256). CTES also rates the degree to which the individuals confided these traumatic events. Each of the six items is rated on two seven point Likert scales ranging from one to seven. The first Likert scale rates the severity of the traumatic event and the next scale scores the degree to which individuals confided the traumatic event. CTES also records the age of the participant when he/ she experienced the particular traumatic event. CTES has been used to assess the impact of early life stress on the patients with schizophrenia (257).

5.4.6. Recent Traumatic Events Scale (RTES):

The RTES assesses seven traumatic experiences within the previous three years. These traumatic experiences include bereavement, divorce, violence, sexual abuse, physical illness, occupational change and other major life events (256). RTES also rates the degree to which the individuals confided these traumatic events. Each of the seven items is rated on two seven point Likert scales ranging from one to seven. The first Likert scale rates the severity of the traumatic event and the next scale records the degree to which individuals confided the traumatic event. Recent life stress of all participants was assessed using RTES.

5.4.7. Premorbid Assessment Scale (PAS):

The PAS is a widely used rating scale to reliably assess premorbid functioning retrospectively. It provides reliable and consistent data on premorbid functioning. The PAS contains 36 items assessing levels of functioning before the onset of psychosis. Each item is scored on a seven point Likert scale, ranging from zero to six. Lower scores indicate better premorbid adjustment. The PAS assesses the following domains: sociability and withdrawal, peer relationships, scholastic performance, adaptation as well as socio-sexual relationships. It evaluates the following four periods in life: childhood (up to 11 years), early adolescence (12-15 years), late adolescence (16-18 years) and adulthood (19 years and beyond). The PAS has already been employed to assess the premorbid functioning of patients with schizophrenia (258) and other non-affective psychoses (259). Available literature support the predictive and concurrent validity of the PAS and the validity of retrospective self-reported data on premorbid functioning among patients with schizophrenia (258). The weighted intra-class correlation for absolute agreement and consistency of PAS is 0.77 (260). Premorbid adjustment of all participants was assessed using the PAS. PAS ratings were based on interviews with the participants and with their first degree relatives.

5.4.8. Structured questionnaire:

A structured questionnaire (Appendix 10.3) was employed to collect socio-demographic and clinical data of the participants. This structured questionnaire included data on developmental delay, obstetric complications, urbanization, recent migration, caffeine as well as grape juice consumption, smoking and anthropometric measures. The current and past data on their pharmacological as well as psychological treatment were elicited by personal interviews and by verifying their medical records. Self-reported data on clozapine related adverse effects were collected using a detailed check list.

5.4.9. Translation of instruments:

The Childhood Traumatic Event Scale, Recent Traumatic Event Scale and Premorbid Adjustment Scale were translated into Tamil, the first language of the participants. Bilingual psychiatric and occupational therapy professionals translated these instruments. Then, bilingual professionals, who had not seen the original version of the instruments, back translated the translated versions to English. The final Tamil versions of the instruments were obtained by consensus among the translators while focusing on content, conceptual, semantic as well as technical equivalence.

5.5. Data collection:

After the participants were recruited in this research, a psychiatrist (Dr. S. Bhuvaneshwari) individually assessed the psychopathology of every participant by using the Brief Psychiatric Rating Scale. She examined the participants for the presence of tardive dyskinesia using Abnormal Involuntary Movements Scale. When tardive dyskinesia was present, the AIMS recorded its severity. An independent senior research fellow (Ms. Chitra Chittibabu), who was blind to the clozapine response status of the participants, employed other clinical assessment instruments and the structured questionnaire, described in section 5.4. She assessed various clinical variables by detailed personal interviews with the participants and their first degree relatives or spouses. The medical records of all participants were accessed with their consent. The author (R. Anto Praveen Rajkumar) was blind to the clozapine response status and to the clinical data of the participants during this research. He carried out the *CYP1A2* and *HTR3A* genotyping of all participants. Thus, three independent investigators collected data on treatment responses, genotypes and on clinical variables. They remained blind to each other's findings until the completion of this research.

5.5.1. Training of the personnel:

The senior research fellow (Ms. Chitra Chittibabu) underwent a structured training program, which included a one week orientation program to psychiatry as well as a four week training program on schizophrenia, practical issues in psychiatric assessment and project methodology. She observed interviewing and managing outpatients and inpatients with Treatment-resistant schizophrenia in the department of psychiatry, CMC, Vellore. A psychologist (Mrs. Archana Padmakar) trained this research fellow to use the Addenbrooke's Cognitive Examination- Revised. The author (R. Anto Praveen Rajkumar) trained her to employ other clinical assessment instruments. Before recruiting the participants, she assessed ten patients with schizophrenia by employing the clinical assessment instruments, under the supervision of the author.

5.5.2. Quality control procedures:

Two stage quality control procedures were employed to ensure the quality of our data entry. All data were entered twice and were checked for any discrepancies. Second checks of all data collection codebooks were performed. After the completion of this research, interviewing 10% of participants and their primary caregivers, selected by simple random sampling, were repeated to verify the accuracy of our data collection.

5.6. *CYP1A2* genotyping:

Nine ml of peripheral venous blood samples were collected from all participants by venepuncture, 12 hours after their last intake of oral clozapine dose. Six ml of collected blood was used to estimate serum clozapine levels. Three ml of collected blood remained in the Ethylene Diamine Tetraacetate (EDTA) tube for isolating genomic DNA. The author

performed genotyping of all samples in the molecular genetics laboratories of the department of haematology and of the department of biochemistry, CMC, Vellore.

5.6.1. Training of the author:

A professor of molecular genetics (Prof. B. Poonkuzhali) trained the author (R. Anto Praveen Rajkumar) on the practical laboratory skills and on the essential procedures of molecular genetics. The author was trained on genomic DNA extraction, polymerase chain reactions, gel electrophoresis, restriction enzyme digestion reactions and on direct DNA sequencing. *CYP1A2* genotyping of all samples were performed under the supervision of Prof. B. Poonkuzhali, department of haematology, CMC, Vellore.

5.6.2. Separating leucocytes from the whole blood:

The white blood cells (WBC) from the whole blood were separated by using the following procedures,

(i) Three ml blood sample were transferred into an appropriately labelled 15 ml tube and added nine ml of Red Blood Cell (RBC) lysis solution. The RBC lysis solution was made of,

a) Ammonium Bicarbonate = 0.072 g

b) Ammonium Chloride = 7.000 g

c) Water to make the volume to one litre

(ii) The mixture was frozen at minus 20 degrees for 10 minutes.

(iii) The mixture was centrifuged with 4000 Revolutions Per Minute (RPM) at four degrees for 10 minutes and then the supernatant was discarded.

(iv) The pellet was washed with the RBC lysis solution at least twice.

(v) 10 ml of the RBC lysis solution was added and the contents were mixed by vortex.

(vi) The contents were frozen at minus 20 degrees for 10 minutes.

(vii) The contents were centrifuged with 4000 RPM at four degrees for 10 minutes and then the supernatant was discarded.

(viii) One ml of the RBC lysis solution was added and the contents were transferred to an appropriately labelled 1.75 ml tube.

(ix) The tubes were centrifuged for two minutes and the supernatant was discarded with the help of a micropipette.

(x) The WBC pellet was stored in a minus 80 degree freezer until further processing.

5.6.3. Extracting genomic DNA from the WBC pellets:

Genomic DNA from the WBC pellet was extracted using QIAamp DNA mini kit (Qiagen GmBH, Germany). The following procedures were employed,

(i) One ml of WBC lysis solution to the WBC pellet was added at room temperature. The WBC lysis solution was made of,

a. Disodium EDTA (0.5 M; PH 8.0) = 25.0 ml

b. Sodium Chloride = 2.19 g

c. 1M Hydrochloric acid for adjusting the PH to 8.0

d. Water to make the volume to 500 ml.

(ii) The WBC pellet was broken and the contents were mixed by vortex.

(iii) The contents were transferred to an appropriately labelled 15 ml tube.

(iv) Two more ml of WBC lysis solution was added and the tubes were kept in 37 degrees water bath overnight.

(v) The contents were mixed next morning by vortex until the solution was less viscous.

(vi) One ml of protein precipitating solution (Qiagen GmBH, Germany) was added and the contents were mixed by vortex for twenty seconds.

(vii) The contents were centrifuged with 4000 RPM at four degrees for five minutes.

(viii) Four ml of 99% isopropanol was added. The contents were mixed by inverting the tube to precipitate DNA.

(ix) Two 1.75 ml tubes were taken and labelled. 750 µl of absolute alcohol and 250 µl of water were added in one of that tube.

(x) After mixing the contents of the tube, the precipitated DNA was transferred to that tube. The contents were inverted and mixed to wash the precipitated DNA.

(xi) The DNA was transferred to the second 1.75 ml tube and was dried at room temperature for 15 minutes.

(xii) 50 to 200 µl of DNA hydration solution (Qiagen GmBH, Germany) was added depending on the size of the DNA pellet and the contents were mixed by tapping.

(xiii) The tubes were kept in 55 degrees water bath for one hour and then the DNA were stored at four degrees until further processing.

The quantity and the quality of the isolated DNA were measured using Nanodrop spectrophotometer (Thermo scientific, Wilmington, USA). The concentration (in ng/µl) and the purity (260/280 nm) of the DNA were recorded. The 260/280 nm value was ensured to be above 1.80. When it was below 1.80, equal volume of absolute alcohol was added to precipitate the DNA again. Then, the DNA was washed with 70% alcohol and the procedures were repeated.

5.6.4. Polymerase chain reaction (PCR):

Polymerase chain reactions for this research used the appropriate primers (261) to amplify the *CYP1A2* gene regions flanking the *CYP1A2*1C, CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F* SNP. Those primer sequences were presented in Table 2.

Table 2: Primer sequences to amplify the *CYP1A2* gene regions flanking the four SNP

Primer name	Primer sequence (5'→ 3')
*CYP1A2*1C* Forward primer	GCTACACATGATCGAGCTATAC
*CYP1A2*1C* Reverse primer	CAGGTCTCTTCACTGTAAAGTTA
*CYP1A2*1D* Forward primer	TGAGCCATGATTGTGGCATA
*CYP1A2*1D* Reverse primer	AGGAGTCTTTAATATGGACCCAG
*CYP1A2*1E* Forward primer	CACTCACCTAGAGCCAGAAGCTC
*CYP1A2*1E* Reverse primer	AGAGCTGGGTAGCAAAGCCTGGA
*CYP1A2*1F* Forward primer	TGAGGCTCCTTTCCAGCTCTCA
*CYP1A2*1F* Reverse primer	AGAAGCTCTGTGGCCGAGAAGG

The DNA samples were diluted to 200 ng/µl concentration by adding 1 X TE solution. The TE solution was made of,

(i) TRIS chloride (1.0 M; PH 7.4) = 1.0 ml

(ii) EDTA (0.25 M; PH 8.0) = 0.4 ml

(iii) Water to make the volume to 100 ml.

Sterile TE buffer was added to the lyophilized primer powders to form the stock primer solutions at the concentration of 200 pmol/ µl. The stock primer solutions were stored at minus 20 degrees. Then, the stock primer solutions were diluted to form the working primer solutions at the concentration of 10 pmol/ µl by adding five µl of stock primer solution and 95µl of nuclease free water.

200 µl PCR tubes were carefully labelled and PCR was set up by mixing the following,

(i) Genei^{TM} Red Dye PCR master mix (Genei, Bangalore, India) = 12.5 µl

(ii) Double distilled Water = 9.5 µl

(iii) Forward primer (10 pmol/µl) = 1.0 µl

(iv) Reverse primer (10 pmol/µl) = 1.0 µl

(v) Diluted DNA (200 ng/µl) = 1.0 µl

The total reaction volume was 25 µl. One negative control tube was kept for every batch of PCR. The negative control tube did not have any DNA sample but had one more µl of double distilled water. PCR programs were presented in Table 3.

Table 3: PCR programs to amplify the *CYP1A2* gene regions flanking the four SNP

STAGE	*CYP1A2*1C* & *CYP1A2*1D*	*CYP1A2*1E* & *CYP1A2*1F*
Initial Denaturation	94 degrees for five minutes	94 degrees for five minutes
Denaturation Annealing Extension	94 degrees for 30 seconds 57 degrees for 30 seconds 72 degrees for 30 seconds (30 cycles)	94 degrees for 30 seconds 60 degrees for 30 seconds 72 degrees for 30 seconds (30 cycles)
Final Extension	72 degrees for seven minutes	72 degrees for seven minutes
Holding Temperature	Four degrees forever	Four degrees forever

5.6.5. Checking amplification of post PCR products:

The amplification of post PCR products was checked using agarose gel electrophoresis. 3% agarose gels were prepared using 0.5X TBE buffer and Ethidium bromide dye. 0.5X TBE was obtained buffer by diluting 10X TBE buffer. 10X TBE buffer was made of,

(i) TRIS base = 108 g

(ii) Boric acid = 55 g

(iii) EDTA (0.25 M; PH 8.0) = 80 ml

(iv) Water to make the volume to 1000 ml.

The gels were placed in a buffer tank, filled with 0.5X TBE buffer. Five µl of post PCR product or negative control was loaded in each well. 100 base pair DNA ladder was loaded in the adjacent well. Gel electrophoresis was performed at 120 V for 20 minutes. Then, the gels were placed under ultra violet (UV) light in a gel documentation system. The presence of correct molecular weight bands of the post PCR products and for the absence of any bands in the negative control were confirmed.

5.6.6. Restriction enzyme digestion reactions:

*CYP1A2*1C, CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F* were genotyped using previously published restriction fragment length polymorphisms (RFLP) method (261). Amplified post PCR products were subjected to restriction enzyme digestion reactions with appropriate restriction enzymes, *Bse*L I, *Nde* I, *Bsu*RI and *Bsp* 120I (Fermentas-Genetix biotech Asia, New Delhi, India) respectively for *CYP1A2*1C, CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F*.

5.6.6.1. Restriction enzyme digestion reaction for *CYP1A2*1C*:

600 µl tubes were labelled carefully and the restriction enzyme digestion reactions were set up by mixing the following,

(i)	Double distilled water	=	15.5 µl
(ii)	Post PCR product of *CYP1A2*1C*	=	2.0 µl
(iii)	Buffer tango (Fermentas-Genetix biotech Asia, New Delhi)	=	2.0 µl
(iv)	*BseL I* enzyme (Fermentas-Genetix biotech Asia, New Delhi)	=	0.5 µl

The total reaction volume was 20 µl. The tubes were kept in 55 degrees water bath overnight. Post PCR product of *CYP1A2*1C* contained 568 base pairs. *BseL I* restriction enzyme cut the wild allele in two places and made three fragments with lengths of 343, 132 and 93 base pairs. However, it cut the variant allele in only one place and made only two fragments with lengths of 475 and 93 base pairs.

5.6.6.2. Restriction enzyme digestion reaction for *CYP1A2*1D*:

600 µl tubes were labelled carefully and the restriction enzyme digestion reactions were set up by mixing the following,

(i) Double distilled water = 15.5 µl

(ii) Post PCR product of *CYP1A2*1D* = 2.0 µl

(iii) Buffer O (Fermentas-Genetix biotech Asia, New Delhi) = 2.0 µl

(iv) *Nde I* enzyme (Fermentas-Genetix biotech Asia, New Delhi) = 0.5 µl

The total reaction volume was 20 µl. The tubes were kept in 37 degrees water bath overnight. Post PCR product of *CYP1A2*1D* contained 167 base pairs. *Nde I* restriction enzyme did not cut the wild allele and the post digestion fragment remained with the length of 167 base pairs. However, it cut the variant allele in one place and made two fragments with lengths of 148 and 19 base pairs.

5.6.6.3. Restriction enzyme digestion reaction for *CYP1A2*1E*:

600 µl tubes were labelled carefully and the restriction enzyme digestion reactions were set up by mixing the following,

(i) Double distilled water = 15.0 µl

(ii) Post PCR product of *CYP1A2*1E* = 2.0 µl

(iii) Buffer R (Fermentas-Genetix biotech Asia, New Delhi) = 2.0 µl

(iv) *Bsu*RI enzyme (Fermentas-Genetix biotech Asia, New Delhi) = 1.0 µl

The total reaction volume was 20 µl. The tubes were kept in 37 degrees water bath overnight. Post PCR product of *CYP1A2*1E* contained 239 base pairs. *Bsu*RI restriction enzyme cut the wild allele in one place and made two fragments with lengths of 205 and 34 base pairs. However, it cut the variant allele in two places and made three fragments with lengths of 175, 34 and 30 base pairs.

5.6.6.4. Restriction enzyme digestion reaction for *CYP1A2*1F*:

600 µl tubes were labelled carefully and the restriction enzyme digestion reactions were set up by mixing the following,

(i) Double distilled water = 15.0 µl

(ii) Post PCR product of *CYP1A2*1F* = 2.0 µl

(iii) Buffer B (Fermentas-Genetix biotech Asia, New Delhi) = 2.0 µl

(iv) *Bsp 120I* enzyme (Fermentas-Genetix biotech Asia, New Delhi) = 1.0 µl

The total reaction volume was 20 µl. The tubes were kept in 37 degrees water bath overnight. Post PCR product of *CYP1A2*1F* contained 265 base pairs. *Bsp 120I* restriction enzyme did not cut the wild allele and the post digestion fragment remained with the length of 265 base pairs. However, it cut the variant allele in one place and made two fragments with lengths of 211 and 54 base pairs.

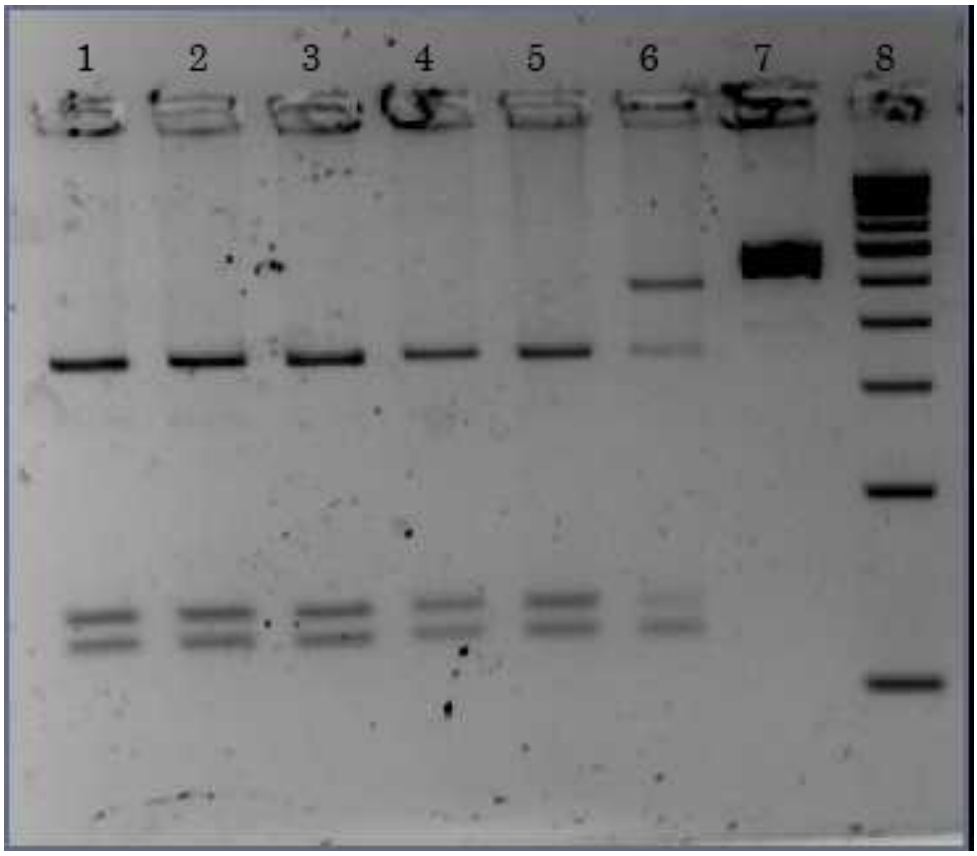
5.6.7. Calling *CYP1A2* genotypes:

The restriction enzyme digestion products were separated by another agarose gel electrophoresis. 3% agarose gels were used for genotyping *CYP1A2*1C, CYP1A2*1E* and *CYP1A2*1F*. 3.5% agarose gels were used for genotyping *CYP1A2*1D.* The gels were prepared using 0.5X TBE buffer and Ethidium bromide dye. The gels were placed in a buffer tank, filled with 0.5X TBE buffer. 15 µl of restriction enzyme digestion products, mixed with one µl of Bromo phenol blue dye (Bromo phenol blue 0.25 % and sucrose 40.0 % were dissolved in 100 ml of water) were loaded into each well. Five µl of undigested post PCR product was loaded in the adjacent well. Then, 100 base pair DNA ladder was loaded in the next well. All gel electrophoreses were performed at 90 V for 60 minutes. After the electrophoresis, the gels were placed under UV light in a gel documentation system. The number of post digestion bands and their sizes were checked. The *CYP1A2*1C, CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F* genotypes could be identified by their characteristic patterns on the gels.

5.6.7.1. Calling *CYP1A2*1C* genotypes:

Figure 1 provides an example for the genotyping of *CYP1A2*1C.* In Figure 1, 100 base pairs (bp) DNA ladder was loaded in lane 8. Undigested Post PCR product of *CYP1A2*1C* containing 568 base pairs was loaded in lane 7. The genotypes could be identified by the following characteristic patterns,

Figure 1: Genotyping *CYP1A2*1C*



(i) Wild genotype (GG) produced three bands at 343 bp, 132 bp and at 93 bp.

(ii) Heterozygous variant (GA) genotype produced four bands at 475 bp, 343 bp, 132 bp and at 93 bp.

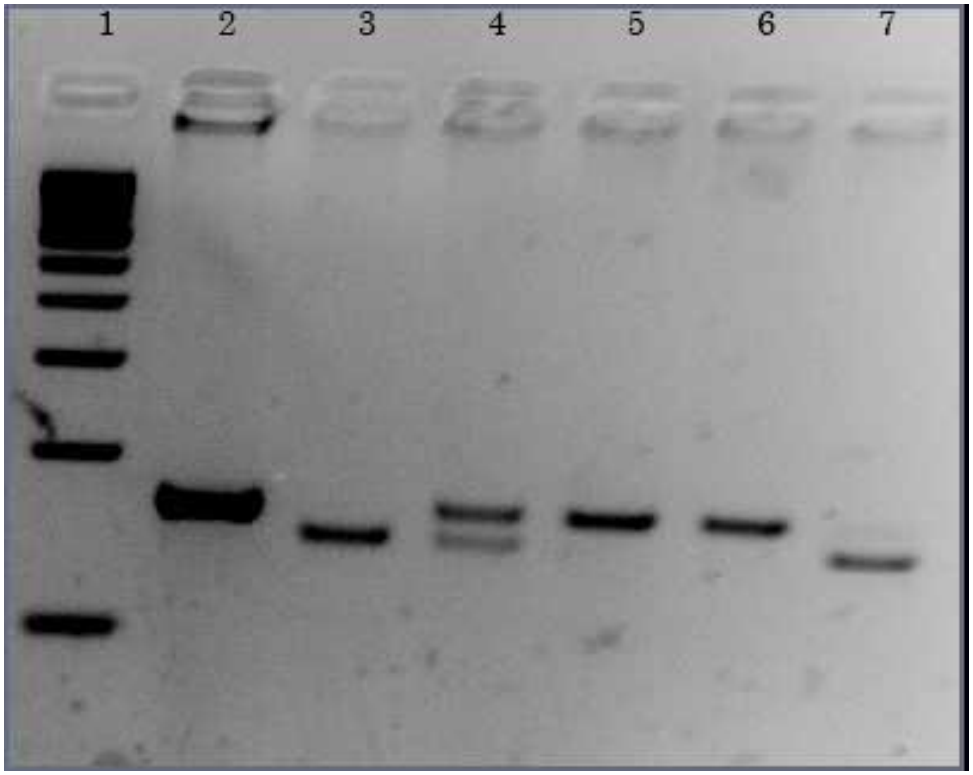
(iii) Homozygous variant (AA) genotype produced two bands at 475 bp and at 93 bp.

Hence, samples loaded in the lanes one to five were identified as the wild GG genotype and the sample loaded in the sixth lane was identified as the heterozygous variant GA genotype.

5.6.7.2. Calling *CYP1A2*1D* genotypes:

Figure 2 provides an example for the genotyping of *CYP1A2*1D.*

Figure 2: Genotyping *CYP1A2*1D*



In Figure 2, 100 base pairs (bp) DNA ladder was loaded in lane 1. Undigested Post PCR product of *CYP1A2*1D* containing 167 base pairs was loaded in lane 2. The genotypes could be identified by the following characteristic patterns,

(i) Wild genotype (TT) produced one band at 167 bp.

(ii) Heterozygous variant (T del-T) genotype produced two bands at 167 bp and at 148 bp.

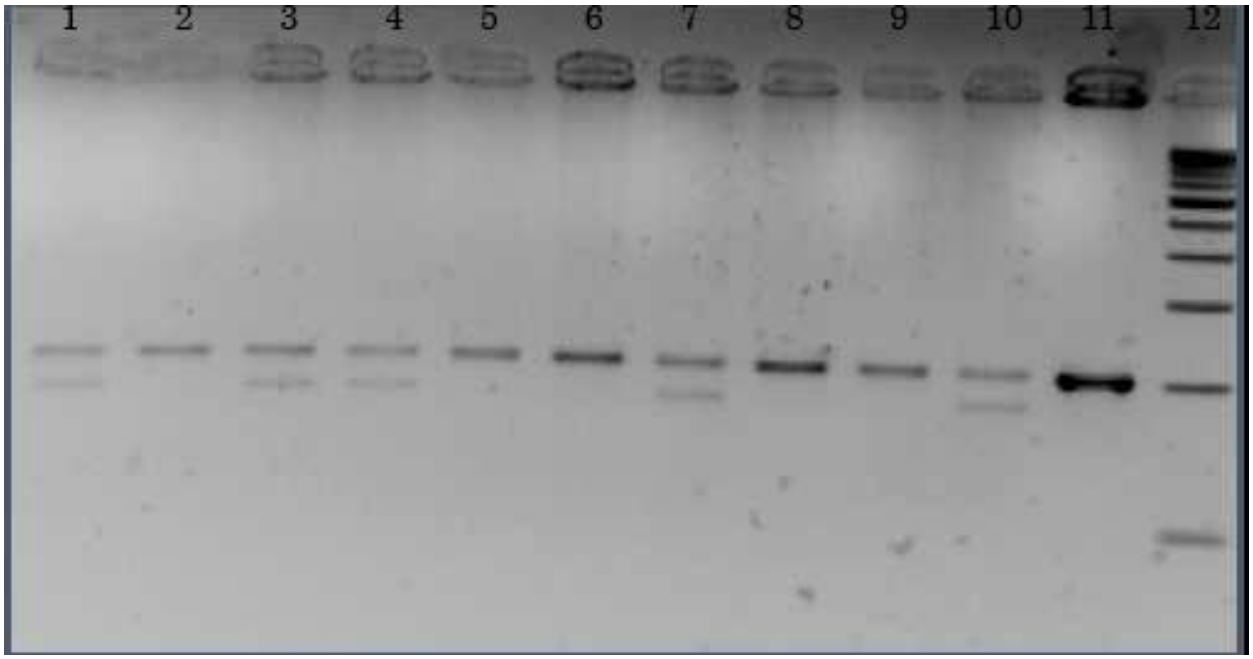
(iii) Homozygous variant (del-T del-T) genotype produced one band at 148 bp.

The 19 bp band was not visible after the gel electrophoresis. Hence, samples loaded in the fifth and sixth lanes were identified as the wild TT genotype. The sample loaded in the fourth lane was identified as the heterozygous variant genotype and the samples loaded in the third and seventh lanes were identified as the homozygous variant genotypes.

5.6.7.3. Calling *CYP1A2*1E* genotypes:

Figure 3 provides an example for the genotyping of *CYP1A2*1E.*

Figure 3: Genotyping *CYP1A2*1E*



In Figure 3, 100 base pairs (bp) DNA ladder was loaded in lane 12. Undigested Post PCR product of *CYP1A2*1E* containing 239 base pairs was loaded in lane 11. The genotypes could be identified by the following characteristic patterns,

(i) Wild genotype (TT) produced one band at 205 bp.

(ii) Heterozygous variant (TG) genotype produced two bands at 205 bp and at 175 bp.

(iii) Homozygous variant (GG) genotype produced one band at 175 bp.

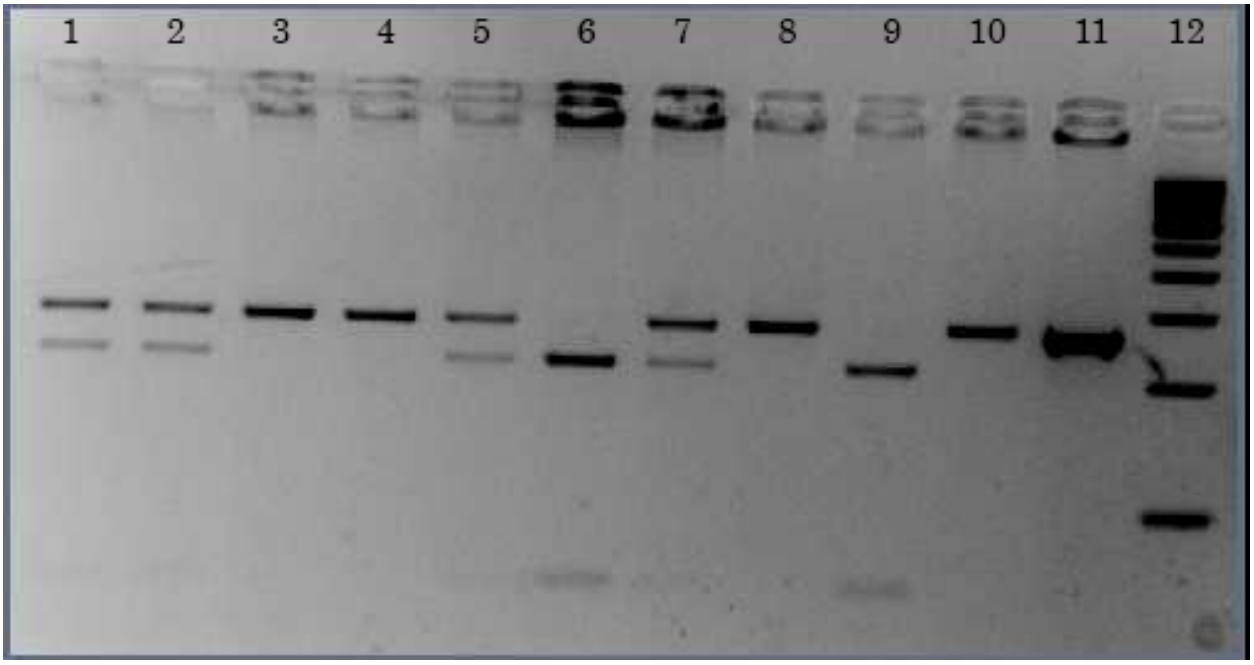
The 30 bp and 34 bp bands were not visible after the gel electrophoresis. Hence, the samples loaded in the lanes, numbered 2, 5, 6, 8 and 9 were identified as the wild TT

genotype. The samples loaded in the lanes, numbered 1, 3, 4, 7 and 10 were identified as the heterozygous variant TG genotypes.

5.6.7.4. Calling *CYP1A2*1F* genotypes:

Figure 4 provides an example for the genotyping of *CYP1A2*1F*.

Figure 4: Genotyping *CYP1A2*1F*



In Figure 4, 100 base pairs (bp) DNA ladder was loaded in lane 12. Undigested Post PCR product of *CYP1A2*1F* containing 265 base pairs was loaded in lane 11. The genotypes could be identified by the following characteristic patterns,

(i) Wild genotype (AA) produced one band at 265 bp.

(ii) Heterozygous variant (AC) genotype produced three bands at 265 bp, 211 bp and at 54 bp.

(iii) Homozygous variant (CC) genotype produced two bands at 211 bp and at 54 bp.

The 54 bp bands were often barely visible after the gel electrophoresis. Hence, the samples loaded in the lanes, numbered 3, 4, 8 and 10 were identified as the wild AA genotype. The samples loaded in the lanes, numbered 1, 2, 5 and 7 were identified as the heterozygous variant AC genotypes. The samples loaded in the sixth and the ninth lanes were identified as the homozygous variant CC genotypes.

5.7. *HTR3A* genotyping:

Another 200 nanograms of genomic DNA, isolated from the whole blood by the procedures described in the sections 5.6.2 and 5.6.3, were used for genotyping two *HTR3A* gene Single Nucleotide Polymorphisms (rs1062613 and rs2276302).

5.7.1. Polymerase chain reaction (PCR):

Polymerase chain reactions were employed to amplify the *HTR3A* gene regions flanking the two SNP rs1062613 and rs2276302. Primer sequences, used in those polymerase chain reactions, were presented in Table 4.

Table 4: Primer sequences to amplify the *HTR3A* gene regions flanking the two SNP

Primer name	Primer sequence (5'→ 3')
rs1062613 Forward primer	TACTCCTTGGGGAAACATGG
rs1062613 Reverse primer	GAGTGTGGGGAGGAGCAAG
rs2276302 Forward primer	TGCTGACCACCTACATCTGG
rs2276302 Reverse primer	GGTTTGGAGGGTTTCTCCTC

The DNA samples were diluted to 200 ng/µl concentration by adding 1X TE solution. Sterile TE buffer was added to the lyophilized primer powders to form the stock primer solutions at the concentration of 200 pmol/ µl. The stock primer solutions were stored at minus 20 degrees. The stock primer solutions were diluted to form the working primer solutions at the concentration of 10 pmol/ µl by adding five µl of stock primer solution and 95µl of nuclease free water.

200 µl PCR tubes were carefully labelled and the PCR was set up by mixing the following,

(i)	Genei^{TM} Red Dye PCR master mix (Genei, Bangalore, India)	=	12.5 µl
(ii)	Double distilled Water	=	9.5 µl
(iii)	Forward primer (10 pmol/µl)	=	1.0 µl
(iv)	Reverse primer (10 pmol/µl)	=	1.0 µl
(v)	Diluted DNA (200 ng/µl)	=	1.0 µl

The total reaction volume was 25 µl. One negative control tube was kept for every batch of PCR. The negative control tube did not have any DNA sample but had one more µl of double distilled water. PCR programs were presented in Table 5.

Table 5: PCR programs to amplify the *HTR3A* gene regions flanking the two SNP

STAGE	*HTR3A* rs1062613	*HTR3A* rs2276302
Initial Denaturation	94 degrees for five minutes	94 degrees for five minutes
Denaturation Annealing Extension	94 degrees for 30 seconds 55 degrees for 30 seconds 72 degrees for 30 seconds (30 cycles)	94 degrees for 30 seconds 53 degrees for 30 seconds 72 degrees for 30 seconds (30 cycles)
Final Extension	72 degrees for seven minutes	72 degrees for seven minutes
Holding Temperature	Four degrees forever	Four degrees forever

5.7.2. Checking amplification of post PCR products:

The amplification of post PCR products were checked using agarose gel electrophoresis. 2% agarose gels were prepared using 0.5X TBE buffer and Ethidium bromide dye. The gels were placed in a buffer tank, filled with 0.5X TBE buffer. Five µl of post PCR product or negative control was loaded in each well. 100 base pair DNA ladder was loaded in the adjacent well. Gel electrophoresis was performed at 100 V for 20 minutes. Then, the gels were placed under ultra violet (UV) light in a gel documentation system. The presence of correct molecular weight bands of the post PCR products and for the absence of any bands in the negative control were confirmed.

5.7.3. Direct DNA sequencing:

Direct DNA sequencing helps to determine the order of the nucleotides in a DNA strand. It is based on the chain termination method, introduced by Frederick Sanger (262). The chain termination method involves single stranded DNA template, forward or reverse primer, DNA polymerase, Deoxyribo Nucleotide Triphosphates (dNTPs), and Dideoxyribo Nucleotide Triphosphates (ddNTPs). Unlike dNTPs, Dideoxyribo Nucleotide Triphosphates lack a hydroxyl group on their 3' carbon. This hydroxyl group is essential to form a phosphodiester bond with the next nucleotide in the sequence. Hence, ddNTPs, when integrated into a DNA sequence, prevent the addition of further nucleotides and terminate elongation of the DNA strand. Direct DNA sequencing involves selective incorporation of chain-terminating ddNTPs during multiple cycles of in vitro DNA replication. When these ddNTPs are radioactively or fluorescently labelled, they can be read using automated sequencing machines. Our amplified post PCR products were subjected to direct DNA sequencing using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) in an Applied Biosystem 3130 Genetic Analyzer (Applied Biosystems, California, USA).

5.7.3.1. Sequencing reactions:

Akin to Polymerase Chain Reaction, sequencing reaction involves the three major steps of denaturation, annealing and extension, which are repeated for 30 or more cycles. However, there are three major differences between PCR and sequencing reactions. First, only one strand of DNA is copied in the sequencing reaction, because only one primer is used in it. Secondly, PCR leads to exponential amplification of DNA strands, but sequencing reactions lead to linear amplification of the number of copies of the DNA strand. Thirdly,

PCR usually starts with genomic DNA, but the sequencing reactions often start with amplified post PCR products.

Sequencing reactions were employed to sequence the *HTR3A* gene regions flanking the two SNP rs1062613 and rs2276302. The amplified post PCR products were diluted to 20 folds by adding nuclease free water. Our stock primer solutions were diluted to form the working primer solutions at the concentration of one pmol/ µl by adding one µl of stock primer solution and 199 µl of nuclease free water. 200 µl tubes were labelled carefully and the sequencing reactions were set up by mixing the following,

(i)	Sequencing buffer (Applied Biosystems, California, USA)	=	1.0 µl
(ii)	Forward primer (one pmol/µl)	=	1.6 µl
(iii)	RR mix (Applied Biosystems, California, USA)	=	0.5 µl
(iv)	Diluted post PCR product	=	1.0 µl
(v)	Double distilled Water	=	5.9 µl

The total reaction volume was 10 µl. The program for our sequencing reactions was presented in Table 6.

Table 6: Sequencing reaction program to sequence *HTR3A* regions flanking the two Single Nucleotide Polymorphisms

STAGE	***HTR3A* rs1062613 & rs2276302**
Denaturation	96 degrees for 15 seconds
Annealing	50 degrees for 20 seconds
Extension	60 degrees for four minutes
25 cycles	
Holding Temperature	15 degrees forever

Sequencing reactions were repeated using reverse primers. These sequencing reactions were set up by mixing the following,

(i) Sequencing buffer (Applied Biosystems, California, USA) = 1.0 µl

(ii) Reverse primer (one pmol/µl) = 1.6 µl

(iii) RR mix (Applied Biosystems, California, USA) = 0.5 µl

(iv) Diluted post PCR product = 1.0 µl

(v) Double distilled Water = 5.9 µl

The total reaction volume was 10 µl. The program, presented in table 6, was followed for these sequencing reactions.

5.7.3.2. Purifying the extension products:

Some fluorescently labelled Dideoxyribo Nucleotide Triphosphates in the RR mix of the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) may remain unincorporated to the DNA strands after the sequencing reaction. They can interfere with identifying nucleotides and can obscure the sequencing data, especially in the early part of the sequence. Hence, there was a need to purify the extension products before employing automated capillary electrophoresis to identify the sequence of the nucleotides. The extension products were cleaned by the following procedures,

(i) The sequencing products (10 µl) were mixed with 30 µl of sequencing injection buffer (Applied Biosystems, California, USA) within the wells of a post clean-up tray.

(ii) 20 Hg mm pressure was applied for five minutes and the fluid on the reverse of the tray was removed by wiping it with a filter paper.

(iii) 40 µl of sequencing injection buffer (Applied Biosystems, California, USA) was added into each well of the tray.

(iv) 20 Hg mm pressure was applied for five minutes and then the fluid on the reverse of the tray was removed by wiping it with a filter paper. This step was repeated once.

(v) The extension product was allowed to dry for two minutes.

(vi) The extension products were eluted with 30 µl of sequencing injection buffer (Applied Biosystems, California, USA).

5.7.4. Calling *HTR3A* genotypes:

The purified extension products were loaded in the Applied Biosystem 3130 Genetic Analyzer (Applied Biosystems, California, USA) to sequence the HTR3A gene regions flanking the two SNP rs1062613 and rs2276302. The purified extension products had mixture of DNA strands of differing lengths, which ended with fluorescently labelled ddNTPs. Applied Biosystem 3130 Genetic Analyzer separated these DNA strands using capillary electrophoresis. The auto-sampler of the genetic analyser sequentially brought each purified extension product into one end of a glass capillary, filled with a separation polymer. It separated the DNA strands on the separation polymer with a resolution of single base pair. There was a detector window at the end of the glass capillary. When the fluorescently DNA labelled strands reached that detector window, a laser beam excited the fluorescent molecule. Emitted fluorescence from the labelled ddNTPs was captured once per second by a charge coupled device camera at particular wave length bands. As the four types of ddNTPs had been labelled with distinctly coloured fluorescent dyes, their emitted fluorescence differed on their wave lengths accordingly. Each nucleotide had its own wavelength and colour, so the Applied Biosystem 3130 Genetic Analyzer could detect the sequence of the nucleotides in the *HTR3A* gene regions.

Sequencing results were derived in the form of chromatograms, which were graphs of coloured peaks corresponding to the location of nucleotides in the sequence. Each nucleotide was identified by its unique coloured peak. The colour code for the nucleotides were,

(i) Blue peak indicated C

(ii) Green peak indicated A

(iii) Black peak indicated G

(iv) Red peak indicated T

The sequences were aligned and identified rs1062613 and rs2276302 SNP using SeqScape Sequence alignment software (Applied Biosystems, California, USA). The *HTR3A* gene regions flanking these SNP were sequenced once with the forward primer and then with the reverse primer. Hence, the genotypes of rs1062613 as well as rs2276302 were confirmed in both directions. *HTR3A* genotyping of all samples were performed under the supervision of Prof. B. Poonkuzhali, department of haematology, CMC, Vellore.

5.8. Serum clozapine assay:

Peripheral venous blood samples were collected from all participants, 12 hours after their last intake of oral clozapine dose. Their serum clozapine levels were estimated by deproteinization with diethyl ether and subsequent high-performance liquid chromatography (HPLC) in the department of clinical pharmacology, Christian Medical College, Vellore. The specific reversed phase HPLC method, which had previously been developed and validated for the determination of serum clozapine levels, was followed (263). During this method, clozapine was extracted from the serum by liquid-liquid extraction with diethyl ether. One ml serum from each participant was pipetted into a tube and an internal standard (100 µl of 10 µg/ml diazepam) was mixed with that. One ml of 50 mM phosphate buffer (pH 7.0) was added to the tube and then extracted the mixture with three ml of diethyl ether. The contents were mixed by vortex for 10 minutes and then were centrifuged at 4000 RPM for 10 minutes. Two ml of supernatant was collected in another tube and was evaporated under reduced pressure at 35°C. The dried residue was reconstituted with 250 µl of a mobile phase. The mobile phase was made of,

(i) Acetonitrile = 40%

(ii) Methanol = 10%

(iii) 0.5% Triethylamine (pH 5.5) = 50%

50 µl of reconstituted solution was injected into the high-performance liquid chromatography column. HPLC flow rate was one ml/min. After the chromatographic separation, clozapine was detected at 250 nm wavelength by using an Ultra Violet- Visible (UV-VIS) detector. The serum clozapine levels were measured as ng/ml.

5.9. Outcome definitions:

Many researchers prefer to define the response to clozapine by greater than 20% reduction in the total score of the Brief Psychiatric Rating Scale (BPRS) (264). However, most clinical psychiatrists do not define non-response based on a change on the scores of any rating scales. They often prefer to define that by the presence of persistent positive or negative symptoms of schizophrenia (265). Hence, the response to clozapine was defined dichotomously by the widely employed cross-sectional threshold of having BPRS total score of 35 or less (264, 265). Differing BPRS cut-off scores to define the response to antipsychotics in schizophrenia are known to cause significant variability in the results of many clinical trials (97). Hence, this research decided to evaluate the clinical and the pharmacogenetic associations using five more BPRS based outcome definitions. Those outcome definitions for response to clozapine were presented below,

(i) BPRS total score below the 25th percentile of the BPRS total scores of all participants.

(ii) BPRS total score below the median value of the BPRS total scores of all participants.

(iii) BPRS total score below 75th percentile of the BPRS total scores of all participants.

(iv) Scores of mild or less severity in five individual items of BPRS rating suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization and unusual thought content. These five BPRS items have already been proposed to define the remission criteria for schizophrenia (98).

(v) Scores of moderate or above severity in not more than one of the five BPRS items rating suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization and unusual thought content.

5.10. Data analyses:

All study variables were initially analysed using descriptive statistics. The distributions of all continuous variables were assessed by Q-Q plots and by the one sample Kolmogorov–Smirnov tests.

5.10.1. Analysing the clinical variables:

Appropriate tests of statistical significance were performed to evaluate the observed differences among the clinical variables between those who responded to clozapine and those who did not respond. The odds ratios with 95% confidence intervals were calculated for various hypothesised clinical variables to predict non-response to clozapine. When a clinical variable was absent in either the responder or in the non-responder group, the Fisher's exact test was employed to assess its statistical significance, as the odds ratios could not be calculated. When some continuous clinical variables were needed to be converted to categorical measures, they were divided into two categories by their median values (266). Multiple logistic regression models were employed to calculate the adjusted odds ratios for the clinical variables to predict non-response to clozapine while accounting for the effects of age, gender and serum clozapine levels. Nagelkerke pseudo R^2 statistics were used to know

the coefficients of determinations of such multiple logistic regression models. Hosmer Lemeshow tests were used to assess goodness of fit of these models.

As the distribution of serum clozapine levels was non-parametric, the Spearman rank order correlation analyses were employed to assess the bivariate correlations between serum clozapine levels and various hypothesised clinical variables. Appropriate regression diagnostics were performed. They found that employing linear regression models to evaluate the correlates of serum clozapine levels would not be valid. The associations between serum clozapine levels and various hypothesized clinical explanatory variables were studied with non-parametric robust regression models, using the statistical software STATA 12.1 (StataCorp, College station, Texas, USA) *rreg* command. Robust regression models are valid, despite the presence of influential outliers and the non-normality of residuals. Ordinary least squares regression was initially performed to compute absolute residuals, which were scaled by their median residual value. After estimating Huber weights and Tukey biweights, iteratively reweighted least squares regression was performed to estimate the robust regression coefficients. Multiple robust regression statistics were performed for the clinical variables predicting serum clozapine levels while adjusting for the effects of age, sex and for oral clozapine doses. A non-parametric dosing equation was developed to predict the serum clozapine levels using clinical proxy measures.

The serum clozapine levels were dichotomously categorized into two categories of adequate levels and inadequate levels based on the minimum threshold value of 350 ng/ml (28, 48, 49). The odds ratios with 95% confidence intervals were calculated for the hypothesized clinical variables to predict inadequate serum clozapine levels. Multiple logistic regression models were used to calculate the adjusted Odds Ratios for the clinical variables to predict inadequate serum clozapine levels while accounting for the effects of age, sex and

oral clozapine doses. Nagelkerke pseudo R^2 statistics were used to determine the proportion of variability explained by these models and the Hosmer–Lemeshow tests were used to assess their goodness of fit. The clinical variables were analysed using the statistical software packages, SPSS 16.0 (IBM, New York, New York, USA) and STATA 12.1 (StataCorp, College station, Texas, USA).

5.10.2. Analysing the pharmacogenetic associations:

The *CYP1A2* and *HTR3A* allele frequencies were calculated among our participants. These allele frequencies were checked whether they were in Hardy-Weinberg Equilibrium (HWE). As our participants were dichotomously divided into clozapine responders and non-responders, the allelic odds ratios were calculated with 95% confidence intervals to predict non-response to clozapine. The Cochran Armitage Test for Trend (CATT) was employed to assess the statistical significance of the pharmacogenetic associations between *CYP1A2* as well as *HTR3A* genotypes and clinical responses to clozapine. Appropriate corrections for multiple testing using permutation based statistics were performed to confirm the observed allelic and genotypic associations. Multiple logistic regression models were employed to study these pharmacogenetic associations, using different outcome definitions, while adjusting for the effects of clinical predictors. Nagelkerke pseudo R^2 statistics were performed to assess the coefficients of determinations of such multiple logistic regression models. The medians of psychopathology, disability as well as cognition scores and the serum clozapine levels between *CYP1A2* genotypes, were compared using Kruskal Wallis tests. The prerequisite sample size and post hoc power to evaluate these pharmacogenetic associations were estimated by using Quanto 1.2.4 software (267). Other analyses were performed using the statistical software packages, STATA 12.1 (StataCorp, College station, Texas, USA) and PLINK v1.07 (268).

5.11. Sample size estimation:

An earlier study has documented the correlation coefficient between serum clozapine levels and oral clozapine doses as high as 0.7 (33). The required sample size was estimated with an anticipated correlation coefficient of 0.3, 80% power as well as 5% with alpha error as 79 for a two-sided test to evaluate the associations between the clinical variables and the serum clozapine levels.

A previous study using Brief Psychiatric Rating Scale (96) for the assessment of clinical outcome has reported that 44.3% patients with Treatment-resistant Schizophrenia were non-responders to clozapine (25). The minor allele (C) frequency of CYP1A2*1F (rs762551) in the Asian population is 0.386 (269). The prerequisite sample size was estimated as 34 cases of clozapine non-responders for an unmatched case control study two-sided test, with 5% alpha error, 80% power as well as with an odds ratio of 2.5 to evaluate the pharmacogenetic association between this *CYP1A2* SNP and the response to clozapine. The variant allele (del-T) frequency of CYP1A2*1D (rs35694136) is 0.414 (270). The prerequisite sample size was estimated to be 33 cases of clozapine non-responders for an unmatched case control study two-sided test with 5% alpha error, 80% power as well as with an odds ratio of 2.5 to evaluate the pharmacogenetic association between this *CYP1A2* SNP and the response to clozapine.

Minor allele frequency of *HTR3A* SNP rs1062613 is 15.1 % among Asian populations (271). While assuming that at least 40% patients respond to clozapine (22), The prerequisite sample size was estimated as 44 clozapine responders for an unmatched case control study two-sided test, with 5% alpha error, 80% power, as well as with an anticipated odds ratio of 4.0 to evaluate the pharmacogenetic association between this *HTR3A* SNP and the response to clozapine. Hence, this research decided to recruit at least 100 consecutive patients

including a minimum of 44 responders and a minimum of 34 non-responders to clozapine in this research.

5.12. Ethical considerations:

The protocol of this study was approved by the Institutional Review Board of Christian Medical College, Vellore. A fact sheet about the details of this study was provided to all participants and to their first degree relatives. The fact sheet explained the nature and purpose of this research, involved procedures, expected duration of involvement and the possible benefits of this research. The participants were assured of the confidentiality of their personal information and findings. They were informed that such information would be processed only for the research purposes in connection with our objectives. They were educated about their right to withdraw their consent at any point of time without any prior notice, which would not bear any consequences over their ongoing psychiatric care. These details were discussed and written informed consent (Appendix 10.2) was obtained from the participants and from their first-degree relatives or spouses. The medical records of all participants were accessed with their consent.

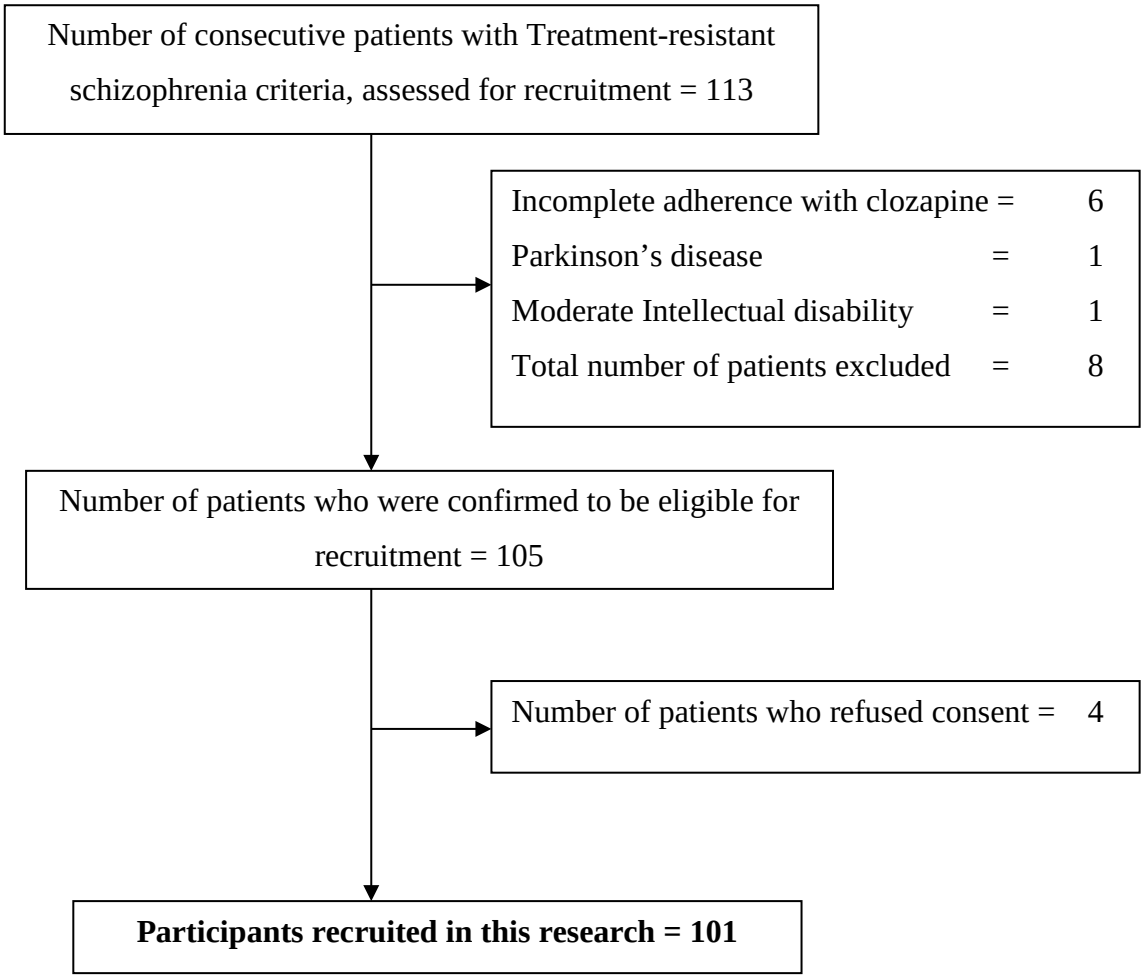
CHAPTER 6

RESULTS AND ANALYSIS

6.1. Recruitment of the participants:

The flowchart explaining recruitment of the patients was presented as Figure 5.

Figure 5: Flowchart for the recruitment of participants



113 consecutive south Indian patients with Treatment-resistant schizophrenia were assessed. Six patients, who were not completely compliant with clozapine within the past 12 weeks, were excluded. One patient with severe Parkinson's disease and another with moderate intellectual disability were also excluded. Among the 105 patients who were confirmed to be

eligible, 101 consented to participate, making the participation rate as 96.2%. Common reasons for refusing consent were lack of interest in our research objectives and reluctance to provide the blood sample. Participants (n=101) and those excluded (n=12) did not differ significantly on gender (χ^2= 0.04; df= 1; p= 0.84), age (t= -0.41; df= 111; p= 0.16) or on their duration of illness (t= -1.27; df= 111; p= 0.21).

6.2. Participant characteristics:

The sociodemographic profiles of all participants were presented in Table 7.

Table 7: Sociodemographic profiles of the participants (N=101)

Characteristic	N (%)	Mean (SD)
Male gender	73 (72.3)	
Age (years)		35.43 (9.43)
Number of years of education		11.86 (3.89)
Monthly family income (INR)		4733 (6062)
Marital status: single/ separated	66 (65.3)	
Currently unemployed	60 (59.4)	
Urban residence	58 (57.4)	
High caffeine intake [a]	24 (23.8)	
Current smoking	19 (18.8)	
Smoking ≥ one pack/day	17 (16.8)	

[a] Three or more cups of coffee or tea intake/day

The majority of the participants were men, unemployed and were living in urban areas. Their mean age was 35.43 years (SD 9.43). 89.1% of the participants (N= 90) had completed secondary school education. 55 participants (54.5%) owned their accommodation and others were living in rented accommodation in the community. None of them were living alone.

The clinical and treatment profiles of all participants were presented in Table 8. Most of the participants had paranoid subtype of schizophrenia. Most of them had the onset of their illness before the age of 30 (N=86; 85.1%). 61 participants (60.4%) had suffered schizophrenia for 10 or more years. Their average duration of antipsychotic drug treatment for schizophrenia was 113.64 months (SD 78.46). Their duration of clozapine treatment ranged from four to 174 months, with a median value of 28 months. More than half of the participants (53.5%) had received clozapine for more than two years. The oral doses of clozapine ranged from 100 to 650 mg/day, with a median value of 350 mg/day. Serum clozapine levels of the participants ranged from 104 to 2547 ng/ml, with a median value of 428 ng/ml. None of our participants were concurrently taking Carbamazepine or oral contraceptives pills.

The prevalence of clozapine related adverse events among our participants were presented in Table 9. The mean Body Mass Index (BMI) of our participants was 24.54 (SD 4.64) Kg/ m^2. As patients, who had developed neutropenia on clozapine, were not re-challenged with clozapine in the department of psychiatry, CMC, Vellore, none of our participants had past history of neutropenia or agranulocytosis.

Table 8: Clinical and treatment profiles of the participants (N=101)

Characteristic	N (%)	Mean (SD)
Family history of schizophrenia	17 (16.8)	
Age of onset of illness (years)		23.07 (7.22)
Duration of illness (years)		12.40 (6.77)
Duration of Untreated Psychosis (months)		11.21 (13.38)
Paranoid sub-type	85 (84.2)	
Past history of catatonia	5 (4.9)	
Presence of Abnormal Involuntary Movements	11 (10.9)	
Total duration of treatment (months)		113.64 (78.46)
Total duration of clozapine (months)		41.61 (39.58)
Oral dose of Clozapine (mg)		340.84 (119.04)
Serum clozapine level (ng/ml)		550.53 (378.46)
Concurrent intake of SSRI medication	8 (7.9)	
Concurrent intake of Valproate	9 (8.9)	
Brief Psychiatric Rating Scale total score		34.73 (12.45)
ACE-R total score		63.11 (20.78)
WHODAS-II total score		17.49 (12.98)
Childhood Traumatic Events Scale total score		8.32 (10.48)
Recent Traumatic Events Scale total score		5.94 (8.71)
Premorbid Adjustment Scale total score		54.83 (21.29)

SSRI- Selective Serotonin Reuptake Inhibitor; ACE-R: Addenbrooke's Cognitive Examination- Revised; WHODAS-II: World Health Organization Disability Assessment Scale- II

Table 9: Prevalence of adverse effects related to clozapine among the participants (n=101)

Adverse effects	n (%)
Excessive sedation on clozapine [a]	77 (76.2)
Sialorrhoea on clozapine	47 (46.5)
Nausea/ vomiting	21 (20.8)
Constipation	21 (20.8)
Clozapine related sexual dysfunction	13 (12.9)
Dyslipidemia	12 (11.9)
Clozapine related Seizures	9 (8.9)
Nocturnal enuresis	6 (5.9)
Neutropenia	0 (0)
Currently overweight (25 ≤ BMI < 30)	23 (22.8)
Obesity (BMI ≥ 30)	15 (14.9)

[a] Sleeping more than 9 hours /day; BMI- Body Mass Index in Kg/m^2.

6.3. Clinical variables predicting response to clozapine:

6.3.1. Clinical predictors of response to clozapine:

The first outcome definition defined the clozapine responders as those participants with the BPRS total scores of 35 or less. While employing this outcome definition, there were 65 (64.4%) clozapine responders and 36 (35.6%) non-responders. Table 10 compared the sociodemographic, clinical and treatment profiles of such clozapine responders as well as non-responders.

Table 10: Socio-demographic, clinical and treatment profiles of the clozapine responders (n=65) and non-responders (n=36)

Characteristic	Responders N(%) / Mean (SD)	Non-responders N(%) / Mean (SD)	χ^2/ t / U [a]	P value
Male gender	50 (76.9)	23 (63.9)	0.88	0.35
Age (years)	35.46 (9.07)	35.36 (10.18)	0.20	0.84
Number of years of education	11.91 (3.84)	11.78 (4.02)	1112	0.68
Monthly family income (INR)	5065 (6833)	4135 (4363)	986.50	0.19
Body mass index (Kg/m^2)	24.85 (4.50)	23.99 (4.90)	1.20	0.24
Family history of schizophrenia	11 (16.9)	6 (16.7)	0.00	0.97
Age of onset of illness (years)	22.32 (6.10)	24.42 (8.82)	1076	0.51
Duration of illness (years)	13.14 (7.31)	11.06 (5.51)	1.33	0.19
DUP (months)	11.37 (14.65)	10.92 (10.92)	1065	0.45
Presence of tardive dyskinesia	5 (7.7)	6 (16.7)	1.92	0.17
Paranoid sub-type	56 (86.2)	29 (80.6)	0.55	0.46
Past history of catatonia	**0 (0)**	**5 (13.9)**	**-**	**0.005[c]**
Duration of treatment (months)	117.62 (85.30)	106.47 (64.82)	0.40	0.69
Duration of clozapine (months)	45.83 (42.24)	34.00 (33.47)	991	0.20
Oral dose of Clozapine (mg)	**321.92 (101.98)**	**375.00 (140.03)**	**-2.42**	**0.02**
Serum clozapine level (ng/ml)	503.23 (260.37)	635.93 (523.07)	-1.85	0.07
High caffeine intake [b]	15 (23.1)	9 (25.0)	0.05	0.83
Smoking ≥ one pack/day	**7 (10.8)**	**10 (27.8)**	**4.79**	**0.03**
BPRS total score	**27.94 (3.79)**	**47.00 (13.27)**	**0.00**	**< 0.001**
ACE-R total score	**67.65 (18.64)**	**54.61 (22.77)**	**3.21**	**0.002**
WHODAS-II total score	16.49 (12.71)	19.28 (13.45)	-1.72	0.09
CTES total score	8.62 (10.56)	7.78 (10.46)	1101.50	0.62
RTES total score	5.55 (7.84)	6.64 (10.17)	1156.50	0.92
PAS total score	53.46 (17.31)	57.31 (27.15)	-0.97	0.34

[a] Chi square or independent samples T test or Mann Whitney U test between responders and non-responders; [b] Three or more cups of coffee or tea intake/day; [c] Fisher exact test P value (two tailed); DUP: Duration of Untreated Psychosis; INR: Indian Rupees; Clozapine responders: participants with BPRS total scores of 35 or less.

Table 11: Clinical variables associated with non-response to clozapine (n=101)

Clinical Variable	Responders (n=65) n (%)	Non-Responders (n=36) n (%)	Bivariate statistics	
			Odds ratio (95% CI)	p-value
Female gender	15 (23.1)	13 (36.1)	1.88 (0.77-4.60)	0.16
Never married/ divorced	39 (60.0)	27 (75.0)	2.00 (0.81-4.93)	0.13
Completed school education	57 (87.7)	33 (91.7)	1.54 (0.38-6.23)	0.54
Family history of schizophrenia	11 (16.9)	6 (16.7)	0.98 (0.33-2.92)	0.97
Paranoid subtype	56 (86.2)	29 (80.6)	0.67 (0.23-1.97)	0.46
Onset before 18 years of age	17 (26.2)	10 (27.8)	1.09 (0.44-2.71)	0.89
Past history of catatonia	**0 (0)**	**5 (13.9)**	**--**	**0.005[a]**
Any Axis II diagnosis	3 (4.6)	3 (8.3)	1.88 (0.36-9.83)	0.46
Duration of illness > 5 years	60 (92.3)	30 (83.3)	0.42 (0.12-1.48)	0.18
DUP more than one year	17 (26.2)	12 (33.3)	1.41 (0.58-3.43)	0.45
Prior poor drug compliance [b]	25 (38.5)	13 (36.1)	0.90 (0.39-2.10)	0.82
Past history of ECT	24 (36.9)	13 (36.1)	0.97 (0.41-2.25)	0.94
Clozapine level < 350 ng/ml	18 (27.7)	11 (30.6)	1.15 (0.47-2.81)	0.76
Clozapine duration > one year	52 (80.0)	25 (69.4)	0.57 (0.22-1.45)	0.24
High Caffeine intake [c]	15 (23.1)	9 (25.0)	1.11 (0.43-2.87)	0.83
Smoking ≥ one pack/day	**7 (10.8)**	**10 (27.8)**	**3.19 (1.09-9.30)**	**0.03**
Excessive sedation on clozapine [d]	**45 (69.2)**	**32 (88.9)**	**3.56 (1.11-11.40)**	**0.03**
Sialorrhoea on clozapine	28 (43.1)	19 (52.8)	1.48 (0.65-3.35)	0.35
Abnormal Involuntary Movements	5 (7.7)	6 (16.7)	2.40 (0.68-8.51)	0.18
Childhood trauma [e]	33 (56.8)	15 (41.7)	0.69 (0.30-1.58)	0.38
Recent Trauma [f]	26 (40.0)	12 (33.3)	0.75 (0.32-1.76)	0.51
Cognitive deficits [g]	**27 (41.5)**	**26 (72.2)**	**3.66 (1.52-8.83)**	**0.004**
Poor premorbid adjustment [h]	28 (43.1)	19 (52.8)	1.48 (0.65-3.35)	0.35

[a] Fisher's exact test, Odds ratios could not be calculated; [b] After initiating antipsychotic therapy, had been off medication for longer than six months duration; [c] Three or more cups of coffee or tea intake/day; [d] Sleeping more than 9 hours/day; [e] Childhood Traumatic Event Scale total score > 4; [f] Recent Traumatic Event Scale total score > 2; [g] Addenbrooke's Cognitive Examination- Revised total score < 68; [h] Premorbid Adjustment Scale total score > 54; DUP: Duration of Untreated Psychosis; ECT: Electro Convulsive Therapy; Clozapine responders: participants with BPRS total scores of 35 or less.

The bivariate analyses for the clinical variables predicting response to clozapine were presented in Table 11. Past history of catatonia, smoking more than one pack/day, excessive sedation and cognitive deficits were significantly associated with non-response to clozapine. Our multiple logistic regression models estimated the adjusted odds ratios for the clinical variables to predict non-response to clozapine, while adjusting for the effects of age, gender and serum clozapine levels. While employing the outcome definition of BPRS total scores of 35 or less to define response to clozapine, our multivariate analyses confirmed the findings of bivariate analyses, presented in Table 11. A multiple logistic regression model including these four multivariate significant clinical variables could explain 31.9% of variability observed in the response to clozapine (Nagelkerke R^2= 0.319). Hosmer Lemeshow test confirmed the goodness of fit of this model (χ^2= 0.25; df= 5; P= 0.99).

Multiple logistic regression statistics were repeated using more outcome definitions for non-response to clozapine. The results of those multivariate analyses were presented in Table 12. The clinical predictors varied, when different outcome definitions were employed. However, past history of catatonia, defined by one or more catatonic symptoms listed in Diagnostic and Statistical Manual of Mental Disorders - IV edition Text Revision (DSM IV-TR), remained as a consistent clinical predictor of non-response, while using all definitions. In order to dispel the concerns over the dichotomization of response to clozapine, we performed multiple linear regression analyses with BPRS total scores as the dependent variable, adjusted for the effects of age, gender and serum clozapine levels. Past history of catatonia (β= 22.75; SE= 5.16; t= 4.41; p<0.001), smoking more than one pack/day (β= 5.40; SE= 2.64; t= 2.05; p= 0.04) and cognitive deficits (β= 7.73; SE= 2.41; t= 3.20; p= 0.002) were significantly associated with worse responses to clozapine, as evidenced by higher BPRS total scores.

Table 12: Multivariate analyses [a] of the clinical predictors for non-response to clozapine, while employing multiple outcome definitions

Outcome definition for non-response	Non-responders n (%)	Past history of Catatonia	Smoking ≥ one pack / day	Excessive sedation [b]	Cognitive deficits [c]	Paranoid subtype	High caffeine intake [d]	Female gender
BPRS total score > 35	36 (35.6)	**p= 0.005[e]**	**5.03 (1.52-16.64) p= 0.008**	**4.25 (1.19-15.20) p= 0.03**	**3.64 (1.43-9.23) p= 0.007**	NS	NS	NS
BPRS total score ≥ 38 (75th percentile)	25 (24.8)	**p= 0.001[e]**	NS	NS	**3.71 (1.28-10.79) p= 0.02**	**0.27 (0.08-0.87) p= 0.03**	NS	NS
At least one of the five BPRS items[f] scored moderate or above	30 (29.7)	**18.16 (1.81-82.38) p= 0.01**	NS	NS	NS	NS	NS	NS
At least two of the five BPRS items[f] scored moderate or above	15 (14.9)	**46.34 (4.51-76.66) p= 0.001**	**15.61 (2.24-08.70) p= 0.006**	NS	NS	NS	**7.28 (1.77-9.98) p= 0.006**	**4.54[g] (1.30-15.84) p= 0.02**

NS: statistically not significant (p value ≥ 0.05); [a] Multiple logistic regression models to calculate Adjusted Odds Ratios (95% CI), accounting for the effects of age, gender and serum clozapine levels (ng/ml); [b] Sleeping more than 9 hours/day; [c] Addenbrooke's Cognitive Examination- Revised total score < 68; [d] Three or more cups of coffee or tea intake/day; [e] Fisher's exact test two tailed p value; [f] Suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization and unusual thought content; [g] Adjusted for the effects of age and serum clozapine levels (ng/ml)

6.3.2. Clinical predictors of adverse events related to clozapine:

The multiple logistic regression models for the clinical variables associated with clozapine induced excessive salivation (*Sialorrhoea*), while adjusting for the effects of serum clozapine levels (ng/ml), were presented in Table 13. The associations between the hypothesized clinical variables and clozapine induced sialorrhoea were not statistically significant. The multiple logistic regression models for the clinical variables associated with obesity (Body mass index above 30 Kg/m^2), while adjusting for the effects of serum clozapine levels (ng/ml), were presented in Table 14. Poor premorbid adjustment was significantly associated with obesity.

The multiple logistic regression analyses for the clinical variables associated with clozapine related sexual dysfunction, while adjusting for the effects of serum clozapine levels (ng/ml), were showed in Table 15. Men and those participants, who had better premorbid adjustment, were significantly more likely to report clozapine related sexual dysfunction. The multiple logistic regression analyses for the clinical variables associated with clozapine related seizures, while adjusting for the effects of serum clozapine levels (ng/ml), were presented in Table 16. Participants, aged above 42 years (75^{th} percentile), were significantly more likely to have the history of clozapine related seizures in the past. Moreover, serum clozapine levels above 750 ng/ml increased the risk of seizures by five times, after adjusting for the effects of age and oral clozapine dose (Adjusted OR 5.15; 95% CI 1.11-23.88; P= 0.03). The multiple logistic regression models for the clinical variables associated with clozapine related nocturnal enuresis, while adjusting for the effects of serum clozapine levels (ng/ml), were presented in Table 17. Participants with severe psychopathology (BPRS total scores above the 75^{th} percentile) were significantly more likely to experience clozapine related nocturnal enuresis.

Table 13: Association between the Clinical variables and clozapine induced sialorrhoea

Clinical variable	With Sialorrhoea (n=47) n (%)	Without Sialorrhoea (n=54) n (%)	Multivariate statistics [a]	
			Adjusted odds ratio (95% CI)	p-value
Female gender	12 (25.5)	16 (29.6)	0.80 (0.33-1.94)	0.62
Age ≥ 35 years	21 (44.7)	28 (51.9)	0.76 (0.35-1.68)	0.50
Age ≥ 42 years (75^{th} percentile)	10 (21.3)	16 (29.6)	0.66 (0.26-1.64)	0.37
Family history of schizophrenia	8 (17.0)	9 (16.7)	1.06 (0.37-3.02)	0.92
Paranoid subtype	39 (83.0)	46 (85.2)	0.82 (0.28-2.40)	0.72
Onset before 18 years of age	12 (25.5)	15 (27.8)	0.89 (0.36-2.16)	0.79
Past history of catatonia	3 (6.4)	2 (3.7)	2.03 (0.32-12.88)	0.45
Any Axis II diagnosis	4 (8.5)	2 (3.7)	2.41 (0.42-13.83)	0.33
Duration of illness > 5 years	39 (83.0)	51 (94.4)	0.28 (0.07-1.14)	0.08
DUP more than one year	9 (19.1)	20 (37.0)	0.40 (0.16-1.00)	0.05
Prior poor drug compliance [b]	18 (38.3)	20 (37.0)	1.04 (0.46-2.34)	0.92
Past history of ECT	16 (34.0)	21 (38.9)	0.79 (0.35-1.79)	0.57
SSRI co-medication	3 (6.4)	5 (9.3)	0.70 (0.16-3.14)	0.65
Valproate co-medication	3 (6.4)	6 (11.1)	0.42 (0.09-1.93)	0.27
High Caffeine intake [c]	11 (23.4)	13 (24.1)	1.04 (0.41-2.65)	0.93
Smoking ≥ one pack/day	6 (12.8)	11 (20.4)	0.55 (0.19-1.65)	0.29
BPRS score ≥ 38 (75^{th} percentile)	13 (27.7)	12 (22.2)	1.25 (0.50-3.14)	0.63
Presence of tardive dyskinesia	4 (8.5)	7 (13.0)	0.66 (0.18-2.45)	0.54
Recent Trauma [d]	17 (36.2)	21 (38.9)	0.93 (0.41-2.10)	0.86
Cognitive deficits [e]	28 (59.6)	25 (46.3)	1.67 (0.75-3.70)	0.21
Poor premorbid adjustment [f]	23 (48.9)	24 (44.4)	1.21 (0.55-2.65)	0.64

[a] Odds ratios adjusted for the effects of serum clozapine levels (ng/ml); [b] Had been off antipsychotics for six months or more in the past; [c] Three or more cups of coffee or tea intake/day; [d] Recent Traumatic Event Scale total score > 2; [e] Addenbrooke's Cognitive Examination- Revised total score < 68; [f] Premorbid Adjustment Scale total score > 54; DUP: Duration of Untreated Psychosis;; ECT: Electro Convulsive Therapy; SSRI: Selective Serotonin Reuptake Inhibitor; BPRS: Brief Psychiatric Rating Scale.

Table 14: Association between the clinical variables and obesity (N=101)

Clinical variable	With obesity (n=15) n (%)	Without obesity (n=86) n (%)	Multivariate statistics [a]	
			Adjusted Odds ratio (95% CI)	p-value
Female gender	7 (46.7)	21 (24.4)	2.76 (0.89-8.61)	0.08
Age ≥ 35 years	5 (33.3)	44 (51.2)	0.45 (0.14-1.45)	0.18
Age ≥ 42 years (75th percentile)	4 (26.7)	22 (25.6)	1.04 (0.30-3.62)	0.95
Family history of schizophrenia	3 (20.0)	14 (16.3)	1.25 (0.31-5.05)	0.75
Paranoid subtype	13 (86.7)	72 (83.7)	1.30 (0.26-6.43)	0.75
Onset before 18 years of age	6 (40.0)	21 (24.4)	2.13 (0.67-6.76)	0.20
Past history of catatonia	0 (0)	5 (5.8)	--	1.00[b]
Any Axis II diagnosis	2 (13.3)	4 (4.7)	3.33 (0.54-20.41)	0.19
Duration of illness > 5 years	13 (86.7)	77 (89.5)	0.75 (0.14-3.90)	0.73
DUP more than one year	4 (26.7)	25 (29.1)	0.90 (0.26-3.13)	0.87
Prior poor drug compliance [c]	4 (26.7)	34 (39.5)	0.57 (0.17-1.94)	0.37
SSRI co-medication	2 (13.3)	6 (7.0)	1.94 (0.35-10.72)	0.45
Valproate co-medication	1 (6.7)	8 (9.3)	0.92 (0.10-8.62)	0.94
High Caffeine intake [d]	2 (13.3)	22 (25.6)	0.40 (0.08-1.93)	0.25
Smoking ≥ one pack/day	1 (6.7)	16 (18.6)	0.32 (0.04-2.61)	0.29
BPRS score ≥ 38 (75th percentile)	5 (33.3)	20 (23.3)	1.80 (0.54-5.96)	0.34
Presence of Tardive Dyskinesia	2 (13.3)	9 (10.5)	1.21 (0.23-6.32)	0.82
Childhood trauma [e]	8 (53.3)	40 (46.5)	1.37 (0.45-4.16)	0.58
Recent Trauma [f]	6 (40.0)	32 (37.2)	1.08 (0.35-0.33)	0.90
Cognitive deficits [g]	9 (60.0)	44 (51.2)	1.50 (0.49-4.64)	0.48
Poor premorbid adjustment [h]	**11 (73.3)**	**36 (41.9)**	**3.80 (1.11-12.94)**	**0.03**

[a] Odds ratios adjusted for the effects of serum clozapine levels (ng/ml); [b] Fisher's exact test; [c] Had been off antipsychotics for six months or more in the past; [d] Three or more cups of coffee or tea intake/day; [e] Childhood Traumatic Event Scale total score > 4; [f] Recent Traumatic Event Scale total score > 2; [g] Addenbrooke's Cognitive Examination- Revised total score < 68; [h] Premorbid Adjustment Scale total score > 54; DUP: Duration of Untreated Psychosis; SSRI: Selective Serotonin Reuptake Inhibitor; BPRS: Brief Psychiatric Rating Scale.

Table 15: Association between the clinical variables and clozapine related sexual dysfunction (N=101)

Clinical variable	With sexual dysfunction (n=13) n (%)	Without sexual dysfunction (n=88) n (%)	Multivariate statistics [a]	
			Adjusted odds ratio (95% CI)	p-value
Male gender	**13 (100)**	**60 (68.2)**	**--**	**0.02[b]**
Age ≥ 42 years (75th percentile)	3 (23.1)	23 (26.1)	0.85 (0.22-3.39)	0.82
Completed graduate education	8 (61.5)	34 (38.6)	2.56 (0.77-8.48)	0.13
Family history of schizophrenia	3 (23.1)	14 (15.9)	1.60 (0.39-6.60)	0.51
Paranoid subtype	10 (76.9)	75 (85.2)	0.57 (0.14-2.37)	0.44
Onset before 18 years of age	2 (15.4)	25 (28.4)	0.46 (0.10-2.22)	0.33
Past history of catatonia	1 (7.7)	4 (4.5)	1.82 (0.18-18.18)	0.61
Any Axis II diagnosis	1 (7.7)	5 (5.7)	1.38 (0.15-12.86)	0.78
Duration of illness > 5 years	13 (100)	77 (87.5)	--	0.35[b]
DUP more than one year	2 (15.4)	27 (30.7)	0.41 (0.09-1.98)	0.27
Prior poor drug compliance [c]	8 (61.5)	30 (34.1)	3.09 (0.93-10.27)	0.07
SSRI co-medication	0 (0)	8 (9.1)	--	0.59[b]
Valproate co-medication	0 (0)	9 (10.2)	--	0.60[b]
High Caffeine intake [d]	4 (30.8)	20 (22.7)	1.56 (0.43-5.73)	0.50
Smoking ≥ one pack/day	4 (30.8)	13 (14.8)	2.56 (0.68-9.55)	0.16
BPRS score ≥ 38 (75th percentile)	4 (30.8)	21 (23.9)	1.40 (0.39-5.12)	0.61
Childhood trauma [e]	5 (38.5)	43 (48.9)	0.65 (0.20-2.14)	0.48
Recent Trauma [f]	8 (61.5)	30 (34.1)	3.20 (0.95-10.80)	0.06
Cognitive deficits [g]	7 (53.8)	46 (52.3)	1.06 (0.33-3.41)	0.93
Poor premorbid adjustment [h]	**2 (15.4)**	**45 (51.1)**	**0.17 (0.04-0.83)**	**0.03**

[a] Odds ratios adjusted for the effects of serum clozapine levels (ng/ml); [b] Fisher's exact test; [c] Had been off antipsychotics for six months or more in the past; [d] Three or more cups of coffee or tea intake/day; [e] Childhood Traumatic Event Scale total score > 4; [f] Recent Traumatic Event Scale total score > 2; [g] Addenbrooke's Cognitive Examination- Revised total score < 68; [h] Premorbid Adjustment Scale total score > 54; DUP: Duration of Untreated Psychosis SSRI: Selective Serotonin Reuptake Inhibitor; BPRS: Brief Psychiatric Rating Scale.

Table 16: Association between the clinical variables and clozapine related seizures (N=101)

Clinical variable	With seizures (n=9) n (%)	Without seizures (n=92) n (%)	Multivariate statistics [a]	
			Adjusted odds ratio (95% CI)	p-value
Female gender	3 (33.3)	25 (27.2)	1.22 (0.26-5.71)	0.80
Age ≥ 35 years	7 (77.8)	42 (45.7)	4.67 (0.87-25.15)	0.07
Age ≥ 42 years (75th percentile)	**5 (55.6)**	**21 (22.8)**	**6.37 (1.32-30.78)**	**0.02**
Family history of schizophrenia	0 (0)	17 (18.5)	--	0.35[b]
Paranoid subtype	8 (88.9)	77 (83.7)	1.30 (0.15-11.53)	0.81
Onset before 18 years of age	1 (11.1)	26 (28.3)	0.32 (0.04-2.76)	0.30
Past history of catatonia	0 (0)	5 (5.4)	--	1.00[b]
Duration of illness > 5 years	9 (100)	81 (88.0)	--	0.59[b]
DUP more than one year	1 (11.1)	28 (30.4)	0.29 (0.03-2.51)	0.26
Prior poor drug compliance [c]	5 (55.6)	33 (35.9)	2.38 (0.56-10.03)	0.24
Past history of ECT	4 (44.4)	33 (35.9)	1.32 (0.31-5.55)	0.71
SSRI co-medication	0 (0)	8 (8.7)	--	1.00[b]
High Caffeine intake [d]	2 (22.2)	22 (23.9)	1.27 (0.23-7.08)	0.78
Smoking ≥ one pack/day	2 (22.2)	15 (16.3)	1.36 (0.24-7.68)	0.73
BPRS score ≥ 38 (75th percentile)	2 (22.2)	23 (25.0)	0.49 (0.07-3.41)	0.47
Presence of Tardive Dyskinesia	2 (22.2)	9 (9.8)	3.79 (0.63-22.96)	0.15
Recent Trauma [e]	5 (55.6)	33 (35.9)	3.32 (0.72-15.44)	0.13
Cognitive deficits [f]	5 (55.6)	48 (52.2)	1.04 (0.25-4.32)	0.96
Poor premorbid adjustment [g]	4 (44.4)	43 (46.7)	0.83 (0.20-3.49)	0.79

[a] Odds ratios adjusted for the effects of serum clozapine levels (ng/ml); [b] Fisher's exact test; [c] Had been off antipsychotics for six months or more; [d] Three or more cups of coffee or tea intake/day; [e] Recent Traumatic Event Scale total score > 2; [f] Addenbrooke's Cognitive Examination- Revised total score < 68; [g] Premorbid Adjustment Scale total score > 54; DUP: Duration of Untreated Psychosis; ECT: Electro Convulsive Therapy; SSRI: Selective Serotonin Reuptake Inhibitor; BPRS: Brief Psychiatric Rating Scale.

Table 17: Association between the clinical variables and Clozapine related nocturnal enuresis (N= 101)

Clinical variable	With enuresis (n=6) n (%)	Without enuresis (n=95) n (%)	Multivariate statistics[a]	
			Adjusted Odds ratio (95% CI)	p-value
Female gender	2 (33.3)	26 (27.4)	1.32 (0.23-7.67)	0.76
Age ≥ 42 years (75th percentile)	3 (50.0)	23 (24.2)	3.18 (0.59-17.01)	0.18
Family history of schizophrenia	0 (0)	17 (17.9)	--	0.59[b]
Paranoid subtype	5 (83.3)	80 (84.2)	0.93 (0.10-8.58)	0.95
Onset before 18 years of age	2 (33.3)	25 (26.3)	1.40 (0.24-8.12)	0.71
Past history of catatonia	1 (16.7)	4 (4.2)	4.90 (0.43-55.57)	0.20
Duration of illness > 5 years	5 (83.3)	85 (89.5)	0.59 (0.06-5.55)	0.64
DUP more than one year	2 (33.3)	27 (28.4)	1.26 (0.22-7.28)	0.80
SSRI co-medication	0 (0)	8 (8.4)	--	1.00[b]
Valproate co-medication	1 (16.7)	8 (8.4)	2.22 (0.21-23.93)	0.51
High Caffeine intake[c]	1 (16.7)	23 (24.2)	0.63 (0.07-5.79)	0.68
Smoking ≥ one pack/day	1 (16.7)	16 (16.8)	0.98 (0.11-9.01)	0.99
BPRS score ≥ 38 (75th percentile)	**4 (66.7)**	**21 (22.1)**	**7.24 (1.22-42.87)**	**0.03**
Presence of Tardive dyskinesia	1 (16.7)	10 (10.5)	1.74 (0.18-16.69)	0.63
Childhood trauma[d]	3 (50.0)	45 (47.4)	1.11 (0.21-5.78)	0.91
Recent Trauma[e]	1 (16.7)	37 (38.9)	0.31 (0.04-2.81)	0.30
Cognitive deficits[f]	3 (50.0)	50 (52.6)	0.89 (0.17-4.68)	0.89
Poor premorbid adjustment[g]	3 (50.0)	44 (46.3)	1.16 (0.22-6.04)	0.86

[a] Odds ratios adjusted for the effects of serum clozapine levels (ng/ml); [b] Fisher's exact test; [c] Three or more cups of coffee or tea intake/day; [d] Childhood Traumatic Event Scale total score > 4; [e] Recent Traumatic Event Scale total score > 2; [f] Addenbrooke's Cognitive Examination- Revised total score < 68; [g] Premorbid Adjustment Scale total score > 54; DUP: Duration of Untreated Psychosis; SSRI: Selective Serotonin Reuptake Inhibitor; BPRS: Brief Psychiatric Rating Scale.

6.3.3. Clinical predictors of disability and of cognitive dysfunction:

World Health Organization Disability Assessment Scale - II **(**WHODAS-II**)** total scores of our participants varied between zero and 48. Psychopathology of the participants, measured by the BPRS total scores, had significant linear relationship (β= 0.22; SE= 0.10; t= 2.09; p= 0.04) with their WHODAS-II total disability scores. The median value of WHODAS-II total scores of our participants was 15. Participants, who had WHODAS-II total scores more than this threshold of 15, were categorized to have higher disability. Women (Adjusted OR 2.69; 95%CI 1.08-6.70) were significantly more disabled than men, after adjusting for the effects of age and serum clozapine levels.

The median value of the Addenbrooke's Cognitive Examination – Revised (ACE-R) total scores of our participants was 68. Participants, who had ACE-R total scores less than this threshold of 68, were categorized to have cognitive deficits. Participants, who had cognitive deficits were significantly more disabled (Adjusted OR 3.41; 95%CI 1.44-8.10) than others, after adjusting for the effects of age and serum clozapine levels. The 25^{th} percentile of the ACE-R total scores of our participants was 52. Those participants, who had the first quartile ACE-R total scores, were categorized to have severe cognitive deficits. The clinical variables associated with such severe cognitive deficits were presented in Table 18. Non-paranoid subtype of schizophrenia was significantly associated with severe cognitive deficits, as evidenced by ACE-R total scores less than 52.

Table 18: Association between the clinical variables and severe cognitive deficits

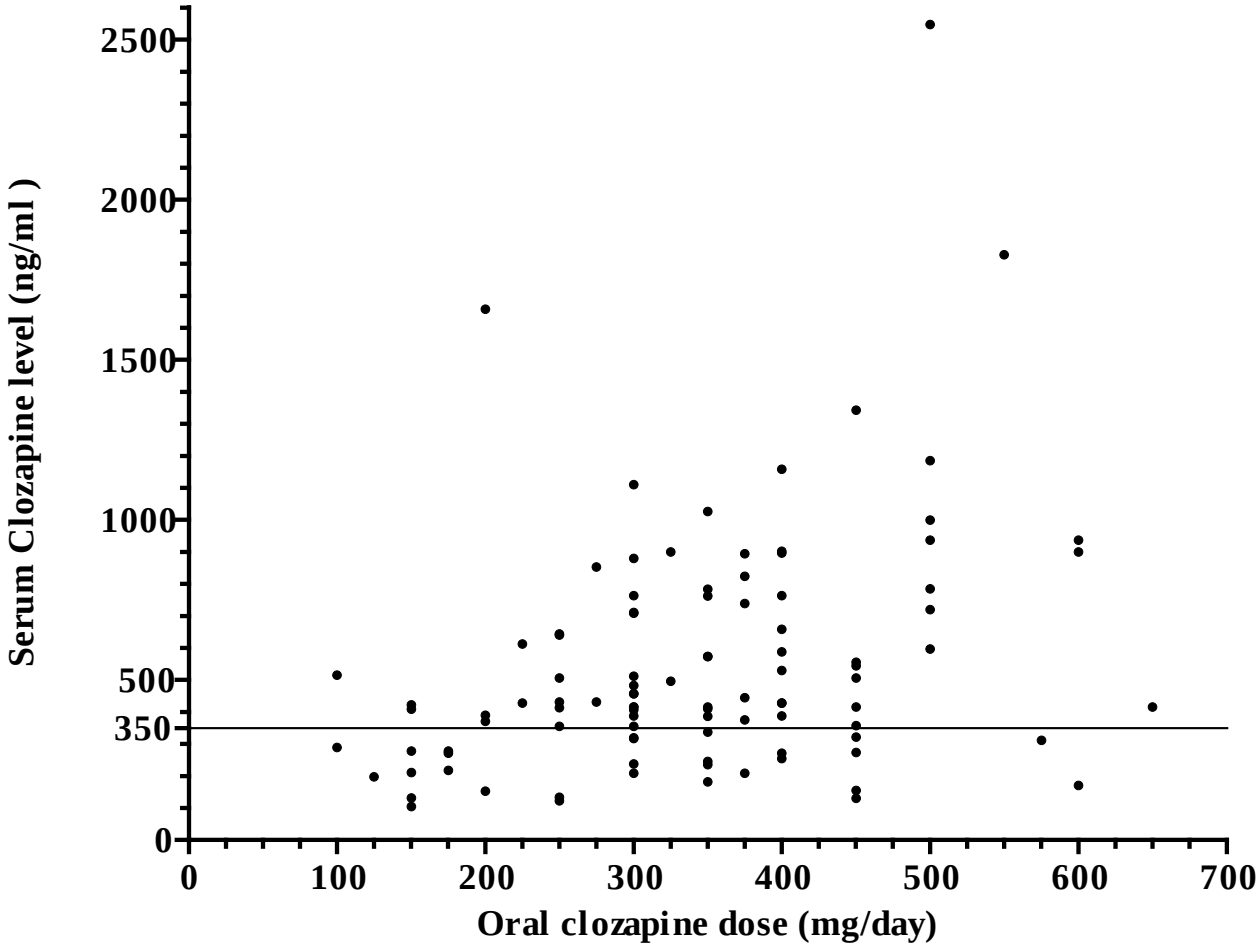
Clinical variable	ACE-R ≥ 52 (n= 79) n (%)	ACE-R < 52 (n= 22) n (%)	Odds ratio (95% CI)	p-value
Female gender	25 (31.6)	3 (13.6)	0.34 (0.09-1.26)	0.11
Had secondary school education	68 (86.1)	22 (100.0)	-	0.12[a]
Family history of schizophrenia	15 (19.0)	2 (9.1)	0.43 (0.09-2.03)	0.28
Paranoid subtype	**70 (88.6)**	**15 (68.2)**	**0.28 (0.09-0.86)**	**0.03**
Onset before 18 years of age	18 (22.8)	9 (40.9)	2.35 (0.86-6.38)	0.09
Past history of catatonia	4 (5.1)	1 (4.5)	0.89 (0.10-8.42)	0.92
Any Axis II diagnosis	5 (6.3)	1 (4.5)	0.71 (0.08-6.37)	0.76
Duration of illness > 5 years	69 (87.3)	21 (95.5)	3.04 (0.37-25.18)	0.30
DUP more than one year	22 (27.8)	7 (31.8)	1.21 (0.44-3.36)	0.72
Prior poor drug compliance [b]	28 (35.4)	10 (45.5)	1.52 (0.58-3.95)	0.39
Past history of ECT	27 (34.2)	10 (45.5)	1.61 (0.62-4.19)	0.33
Clozapine level < 350 ng/ml	24 (30.4)	5 (22.7)	0.67 (0.22-2.04)	0.49
High Caffeine intake [c]	20 (25.3)	4 (18.2)	0.66 (0.20-2.17)	0.49
Smoking ≥ one pack/day	12 (15.2)	5 (22.7)	1.64 (0.51-5.30)	0.41
Excessive sedation on clozapine [d]	61 (77.2)	16 (72.7)	0.79 (0.27-2.31)	0.66
Tardive dyskinesia	11 (13.9)	0 (0)	-	0.12[a]
Recent Trauma [e]	28 (35.4)	10 (45.5)	1.52 (0.58-3.95)	0.39
Poor premorbid adjustment [f]	39 (49.4)	8 (36.4)	0.59 (0.22-1.55)	0.28

[a] Fisher's exact test; [b] Had been off antipsychotics for six months or more in the past; [c] Three or more cups of coffee or tea intake/day; [d] Sleeping more than 9 hours of day; [e] Recent Traumatic Event Scale total score > 2; [f] Premorbid Adjustment Scale total score > 54; ACE-R: Addenbrooke's Cognitive Examination – Revised Scale total score; DUP: Duration of Untreated Psychosis; ECT: Electro Convulsive Therapy.

6.4. Clinical variables predicting serum clozapine levels:

Twenty nine participants (28.7%) had serum clozapine less than 350 ng/ml. Twenty four participants (23.8%) had serum clozapine levels above 750 ng/ml. Mean serum clozapine level/ oral clozapine dose ratio was 1.71 (SD 1.12). There was a 30-fold interindividual variability among the clozapine level/oral dose ratios of our participants, which varied between 0.28 and 8.29. The scatter plot between oral doses of clozapine and serum clozapine levels was presented in Figure 6.

Figure 6: Association between oral Clozapine doses and serum Clozapine levels



6.4.1. Clinical predictors of serum clozapine levels:

The bivariate non-parametric robust regression models for the associations between various hypothesized clinical variables and serum clozapine levels of our participants were presented in Table 19. Oral doses of clozapine and Valproate co-medication were positively associated with serum clozapine levels during bivariate analyses. The results of non-parametric multiple robust regression models evaluating the associations between various clinical variables and serum clozapine levels were presented in Table 20. Oral doses of clozapine and Valproate co-medication were significantly associated with serum clozapine levels, after accounting for the effects of potential confounders. Those participants, who consumed three or more cups of coffee or tea every day, had significantly lower serum clozapine levels, while adjusting for the effects of age, gender and of oral clozapine doses. Age, gender, body weight and smoking were not significantly associated with serum clozapine levels among our participants. Our participants were on the following co-medications, Risperidone (n=10; 9.8%), Haloperidol (n=6; 5.9%), Quetiapine (n=2; 2.0%), Aripiprazole (n=4; 3.9%), Trihexyphenidyl (n=12; 11.8%) and Clonazepam (n=7; 6.9%). None of these co-medications was significantly associated with serum clozapine levels or with clinical responses to clozapine in our sample.

A non-parametric multiple robust regression model ($F = 9.78$; $P < 0.001$), including the multivariate significant clinical variables, to predict the serum clozapine levels of our participants was showed in Table 21. The following equation was derived from this model,

Serum clozapine level (ng/ml) = 194.51 + 0.91 (oral clozapine dose in mg/day) – 130.45 (if high caffeine intake is present) + 247.93 (if Valproate co-medication is present).

Table 19: Bivariate analyses [a] for the clinical variables associated with the serum clozapine levels of the participants (N=101)

Clinical variable	β	SE	T	p
Male gender	-14.87	63.27	-0.24	0.82
Age (years)	-0.55	3.03	-0.18	0.86
Body weight (Kg)	2.41	2.27	1.06	0.29
Body Mass Index (Kg/m^2)	4.57	6.11	0.75	0.46
Current oral dose of clozapine (mg/day)	**0.83**	**0.23**	**3.69**	**<0.001**
Total duration of clozapine (months)	0.75	0.71	1.06	0.29
Current smoking	-50.14	72.21	-0.69	0.49
Total smoking pack years	-3.89	3.94	-0.99	0.33
High caffeine consumption [b]	-63.40	66.68	-0.95	0.34
Duration of illness (years)	-2.93	4.22	-0.69	0.49
Age of onset of illness (years)	1.82	3.95	0.46	0.65
Duration of untreated Psychosis (months)	1.84	2.12	0.87	0.39
Paranoid subtype of schizophrenia	-5.39	77.78	-0.07	0.95
Concurrent Fluoxetine or Sertraline intake	-17.40	105.42	-0.17	0.87
Concurrent Valproate intake	**265.97**	**92.68**	**2.87**	**0.005**

[a] Robust regression models with serum clozapine level (ng/ml) as the dependent variable; [b] three or more cups of coffee or tea intake/day.

Table 20: Multivariate analyses [a] for the clinical variables associated with the serum clozapine levels of the participants (N=101)

Clinical variable	B	SE	T	p
Male gender	-31.63	60.36	-0.52	0.60[b]
Age (years)	-1.12	2.87	-0.39	0.70[c]
Body weight (Kg)	3.63	2.23	1.63	0.11
Body Mass Index (Kg/m^2)	8.75	6.09	1.44	0.15
Current oral dose of clozapine (mg/day)	**0.84**	**0.23**	**3.67**	**<0.001[d]**
Total duration of clozapine (months)	1.13	0.70	1.62	0.11
Current smoking	-121.92	74.65	-1.63	0.11
Total smoking pack years	-5.00	3.86	-1.30	0.20
High caffeine consumption[e]	**-126.84**	**63.21**	**-2.01**	**0.04**
Duration of illness (years)	-5.40	5.28	-1.02	0.31
Age of onset of illness (years)	6.12	5.30	1.15	0.25
Duration of untreated Psychosis (months)	0.34	2.05	0.16	0.87
Paranoid subtype	-4.85	75.10	-0.06	0.95
Concurrent Fluoxetine or Sertraline intake	-46.73	100.33	-0.47	0.64
Concurrent Valproate intake	**263.80**	**91.40**	**2.89**	**0.005**

[a] Multiple robust regression models with serum clozapine level (ng/ml) as the dependent variable adjusted for the effects of age (years), gender (male) and of oral clozapine dose (mg/day); [b] adjusted for the effects of age (years) and of oral clozapine dose (mg/day); [c] adjusted for the effects of gender (male) and of oral clozapine dose (mg/day); [d] adjusted for the effects of age (years) and of gender (male); [e] three or more cups of coffee or tea intake/day.

Table 21: Multivariate model [a] explaining the serum clozapine level of the participants (N=101)

Model predictors	β	95% CI for β	SE	T	p
Constant	194.51	42.14 - 346.89	76.77	2.53	0.01
Current oral dose of clozapine (mg/day)	**0.91**	**0.48 - 1.34**	**0.22**	**4.19**	**<0.001**
High caffeine consumption [b]	**-130.45**	**-249.98 - -10.93**	**60.22**	**-2.17**	**0.03**
Concurrent Valproate intake	**247.93**	**42.14-346.89**	**89.06**	**2.78**	**0.006**

[a] Multiple robust regression model with serum clozapine level (ng/ml) as dependent variable; [b] three or more cups of coffee or tea intake/day

The serum clozapine levels, below 350 ng/ ml, were categorised as inadequate serum clozapine levels (28, 48, 49). The results of multiple logistic regression models investigating the clinical variables associated with inadequate serum clozapine levels were presented in Table 22. Oral doses of clozapine less than 250 mg/day increased the risk of inadequate serum levels by four times (Adjusted OR 4.27; 95% CI 1.47-12.41; p=0.008), after adjusting for the effects of age and gender of the participants. All participants who were on Valproate co-medication had serum clozapine levels above 350ng/ml. A multiple logistic regression model including oral clozapine dose below 250 mg/day, Valproate co-medication and high caffeine intake could predict the inadequate serum clozapine levels correctly in 73.3% of participants. Hosmer Lemeshow test confirmed the goodness of fit of this multivariate model (χ^2= 0.08; df= 2; P= 0.96; Nagelkerke pseudo R^2 = 0.196).

Table 22: Multivariate analyses [a] for the clinical variables associated with inadequate serum clozapine levels (less than 350 ng/ml)

Clinical variable	Level < 350 ng/ml (n=29) n (%)	Level ≥ 350 ng/ml (n=72) n (%)	Adjusted Odds Ratio	95% CI	p
Male gender	21 (72.4)	52 (72.3)	1.12[b]	0.42-3.02	0.82
Age above 40 years	8 (27.6)	19 (26.4)	0.95[c]	0.35-2.58	0.92
Oral dose < 250mg/day	**10 (34.5)**	**8 (11.1)**	**4.27[d]**	**1.47-12.41**	**0.008**
Body weight ≥ 75Kg	4 (13.8)	14 (19.4)	0.50	0.14-1.74	0.28
Current smoking ≥ one pack/day	5 (17.2)	12 (16.7)	1.49	0.41-5.44	0.55
High caffeine consumption [e]	9 (31.0)	15 (20.8)	2.26	0.80-6.36	0.12
Duration of illness > 10 years	18 (62.1)	34 (47.2)	2.11	0.73-6.15	0.17
Age of onset of illness < 18years	5 (17.2)	22 (30.6)	0.40	0.11-1.46	0.17
Duration of clozapine > 2 years	13 (44.8)	41 (56.9)	0.45	0.17-1.18	0.10
Presence of Tardive Dyskinesia	4 (13.8)	7 (9.7)	1.42	0.34-5.91	0.63
Concurrent SSRI intake	3 (10.3)	5 (6.9)	1.62	0.35-7.39	0.53
Concurrent Valproate intake	0 (0)	9 (12.5)	-	-	0.05[f]

[a] Multiple logistic regression models with serum clozapine level below 350 ng/ml as the outcome variable, adjusted for the effects of age (years), gender (male) and of oral clozapine dose (mg/day); [b] adjusted for the effects of age (years) and of oral clozapine dose (mg/day); [c] adjusted for the effects of gender (male) and of oral clozapine dose (mg/day); [d] adjusted for the effects of age (years) and of gender (male); [e] three or more cups of coffee or tea intake/day; [f] Fisher's exact test (two-tailed); SSRI: Selective Serotonin Reuptake Inhibitor

6.4.2. Clinical utility of serum clozapine levels:

The relationship between serum clozapine levels and psychopathology of the participants, as measured by the Brief Psychiatric Rating Scale total scores, was not significant (β= 0.12; SE= 2.21; t= 0.06; P= 0.95), after adjusting for the oral doses of clozapine. Higher serum clozapine levels were associated with worse cognitive functioning (β= -0.011; SE= 0.005; t= -2.07; P= 0.04), as measured by ACE-R total scores, after adjusting for the effects of age and oral clozapine dose. However, this relationship was not significant (β= -0.006; SE= 0.005; t= -1.17; P= 0.25), when psychopathology of the participants was also included in this non-parametric multiple robust regression model. Similarly, serum clozapine levels were not associated (β= 0.006; SE= 0.004; t= 1.68; P= 0.10) with disability, as measured by WHODAS-II total scores, after adjusting for the effects of age, oral clozapine dose and psychopathology of the participants.

Among those, who had serum clozapine levels below 350 ng/ml (n=29), BPRS total scores were significantly associated with worse cognitive functioning, as measured by less ACE-R total scores (β= -0.65; SE= 0.20; t= -3.33; P= 0.003). Serum clozapine levels were not significantly associated with Abnormal Involuntary Movements Scale total scores (β= -0.003; SE= 0.002; t= -1.46; P= 0.15), after adjusting for the effects of age and oral clozapine dose of the participants. Serum clozapine levels above 500 ng/ml significantly increased the risk of having the history of clozapine related seizures (Adjusted OR 5.96; 95% CI 1.06-33.44; P= 0.04), after adjusting for the effects of age and oral clozapine dose of the participants. Other clozapine related adverse effects such as hyper somnolence, excessive salivation, sexual dysfunction, constipation, nausea, nocturnal enuresis and obesity were not significantly associated with serum clozapine levels of the participants.

6.5. Pharmacogenetic associations between *CYP1A2* SNP and response to clozapine:

6.5.1. Allele frequencies of *CYP1A2* Single Nucleotide Polymorphisms*:*

The allele and genotype frequencies of the four SNP were presented in Table 23. The allele frequencies of *CYP1A2*1C* (rs2069514), *CYP1A2*1D* (rs35694136), *CYP1A2*1E* (rs2069526) and *CYP1A2*1F* (rs762551) were consistent with the Hardy Weinberg Equilibrium. The Minor Allele Frequencies of rs2069514, rs35694136, rs2069526 and rs762551 among our participants were 0.109, 0.307, 0.089 and 0.431 respectively.

Table 23: *CYP1A2* SNP allele frequencies among the participants (N=101)

SNP	Allele		Allele frequencies		Genotype frequencies (n)			HWE[a]	p
	1	2	1	2	11	12	22	χ^2, df	
CYP1A2*1C rs2069514	G	A	0.891	0.109	81	18	2	0.68, 1	0.41
CYP1A2*1D rs35694136	T	-	0.693	0.307	48	44	9	0.06, 1	0.81
CYP1A2*1E rs2069526	T	G	0.911	0.089	84	16	1	0.06, 1	0.81
CYP1A2*1F rs762551	A	C	0.569	0.431	37	41	23	2.29, 1	0.08

[a] Goodness of fit with Hardy Weinberg Equilibrium

6.5.2. Associations between *CYP1A2* SNP and response to clozapine:

Our principal outcome definition defined the response to clozapine by the Brief Psychiatric Rating Scale total scores of 35 or less. While employing this outcome definition, the allelic associations between the four SNP in the *CYP1A2* gene and response to treatment with clozapine were showed in Table 24.

Table 24: Allelic associations between *CYP1A2* gene SNP and response to clozapine among the clozapine responders (n=65) and non-responders (n=36)

SNP	Allele		Responder		Non-responder		Allelic Odds ratio [a] (95% CI)	Allelic P
	1	2	1	2	1	2		
CYP1A2*1C rs2069514	G	A	115	15	65	7	0.83 (0.27-2.29)	0.69
CYP1A2*1D rs35694136	T	-	89	41	51	21	0.89 (0.45-1.75)	0.73
CYP1A2*1E rs2069526	T	G	118	12	66	6	0.89 (0.26-2.72)	0.83
CYP1A2*1F rs762551	A	C	79	51	36	36	1.54 (0.83-2.88)	0.14

[a] Calculated with variant allele (2) as the exposure variable and clozapine non-response as the outcome variable.

The associations between the four SNP genotypes and response to clozapine among our participants were presented in Table 25.

Table 25: Associations between *CYP1A2* gene SNP genotypes and response to clozapine among the clozapine responders (n=65) and non-responders (n=36)

SNP	Allele		Responder			Non-responder			CATT[a] χ^2, df	Genotype p
	1	2	11	12	22	11	12	22		
CYP1A2*1C rs2069514	G	A	52	11	2	29	7	0	0.15, 1	0.70
CYP1A2*1D rs35694136	T	-	31	27	7	17	17	2	0.13, 1	0.72
CYP1A2*1E rs2069526	T	G	54	10	1	30	6	0	0.05, 1	0.83
CYP1A2*1F rs762551	A	C	26	27	12	11	14	11	1.87, 1	0.17

[a] Cochran Armitage Test for Trend.

None of the *CYP1A2* alleles and genotypes was significantly associated with treatment response to clozapine. The *CYP1A2*1F* CC genotype did not significantly increase the risk of non-response to clozapine (OR 2.17; 95% CI 0.74-6.38; p= 0.16), when compared to the AA genotype. Multiple logistic regression analyses, adjusting for the effects of multivariate significant clinical variables, confirmed these findings. Further analyses were performed using five more outcome definitions, which were described in section 5.9. Absence of statistically significant associations between *CYP1A2* SNP and treatment response to clozapine were replicated using multiple logistic regression models, which employed these differing outcome definitions for non-response to clozapine.

6.5.3. Clinical utility of CYPA2 genotyping:

Associations between the four *CYP1A2* Single Nucleotide Polymorphisms and all clozapine related adverse effects, listed in Table 9, were not statistically significant (p values > 0.10). Multiple logistic regression models, adjusting for the effects of serum clozapine levels, confirmed these findings. The associations between *CYP1A2*1C* and psychopathology, serum clozapine levels, cognition as well as disability were presented in Table 26. The differences among the median values of these variables between the *CYP1A2*1C* genotypes were not statistically significant.

Table 26: Associations between *CYP1A2*1C* genotypes and serum clozapine levels, psychopathology, disability as well as cognition of the participants (n=101)

Variable	**GG (n = 81) Mean (SD)**	**GA (n = 18) Mean (SD)**	**AA (n = 2) Mean (SD)**	$\chi^{2\,a}$	**p**
Serum level [b]	543.67 (389.96)	580.67 (336.91)	557.00 (455.38)	0.38	0.83
Psychopathology [c]	34.49 (12.18)	36.67 (14.23)	27.00 (2.83)	0.85	0.65
Disability [d]	17.81 (12.96)	14.50 (12.93)	31.00 (7.07)	3.74	0.15
Cognitive status [e]	62.41 (21.19)	68.00 (17.68)	47.50 (31.82)	1.71	0.43

[a] Kruskal Wallis test with two degrees of freedom; [b] Serum clozapine level in ng/ml; [c] Brief Psychiatric Rating Scale total score; [d] World Health Organization Disability Assessment-II Scale total score; [e] Addenbrooke's Cognitive Examination- Revised total score

The associations between *CYP1A2*1D* and BPRS total scores, serum clozapine levels, cognition as well as disability of the participants were presented in Table 27. The associations between *CYP1A2*1E* and psychopathology, serum clozapine levels, cognition as

well as disability status of the participants were presented in Table 28. Table 29 presented the associations between *CYP1A2*1F* and psychopathology, serum clozapine levels, cognition as well as disability of the participants. The differences among the median values of these clinical variables between the *CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F* genotypes were not statistically significant. Appropriate multiple quantile regression models, adjusting for the effects of multivariate significant clinical variables, confirmed these negative findings. Participants, who smoked more than 20 cigarettes a day (n=17), did not differ significantly from other participants in their serum clozapine levels (Kruskal wallis χ^2= 0.39; df= 2; p= 0.82) depending on their *CYP1A2*1F* genotypes. Multiple robust regression analysis, adjusting for the effects of age, oral dose and of body mass index, confirmed this finding.

Table 27: Associations between *CYP1A2*1D* genotypes and serum clozapine levels, psychopathology, disability as well as cognition of the participants (n=101)

Variable	**TT (n = 48)** **Mean (SD)**	**T- (n = 44)** **Mean (SD)**	**-- (n = 9)** **Mean (SD)**	**$\chi^{2\,a}$**	**p**
Serum level [b]	543.56 (322.01)	559.35 (440.77)	544.56 (369.01)	0.22	0.89
Psychopathology [c]	36.17 (14.35)	34.43 (10.96)	28.56 (5.15)	2.58	0.28
Disability [d]	19.54 (14.47)	15.43 (11.23)	16.56 (12.19)	1.65	0.44
Cognition [e]	61.54 (19.70)	64.00 (21.63)	67.11 (23.83)	1.15	0.56

[a] Kruskal Wallis test with two degrees of freedom; [b] Serum clozapine level in ng/ml; [c] Brief Psychiatric Rating Scale total score; [d] World Health Organization Disability Assessment-II Scale total score; [e] Addenbrooke's Cognitive Examination- Revised total score

Table 28: Associations between *CYP1A2*1E* genotypes and serum clozapine levels, psychopathology, disability as well as cognition of the participants (n=101)

Variable	TT (n = 84) Mean (SD)	TG (n = 16) Mean (SD)	GG (n = 1)	$\chi^{2\,a}$	p
Serum level [b]	569.55 (401.33)	471.50 (217.98)	217.00	1.81	0.40
Psychopathology [c]	34.17 (11.31)	38.31 (17.44)	25.00	2.34	0.31
Disability [d]	17.18 (12.89)	20.00 (13.53)	3.00	2.11	0.35
Cognition [e]	62.80 (21.08)	63.38 (19.72)	85.00	1.70	0.43

[a] Kruskal Wallis test with two degrees of freedom; [b] Serum clozapine level in ng/ml; [c] Brief Psychiatric Rating Scale total score; [d] World Health Organization Disability Assessment-II Scale total score; [e] Addenbrooke's Cognitive Examination- Revised total score

Table 29: Associations between *CYP1A2*1F* genotypes and serum clozapine levels, psychopathology, disability as well as cognition of the participants (n=101)

Variable	AA (n = 48) Mean (SD)	AC (n = 44) Mean (SD)	CC (n = 9) Mean (SD)	$\chi^{2\,a}$	p
Serum level [b]	475.22 (260.99)	589.84 (383.27)	601.61 (507.29)	1.49	0.48
Psychopathology [c]	35.59 (15.45)	32.93 (9.27)	36.57 (12.11)	1.69	0.43
Disability [d]	17.65 (14.27)	17.37 (12.29)	17.43 (12.59)	0.04	0.98
Cognition [e]	60.46 (22.05)	66.63 (20.06)	61.09 (19.94)	2.15	0.34

[a] Kruskal Wallis test with two degrees of freedom; [b] Serum clozapine level in ng/ml; [c] Brief Psychiatric Rating Scale total score; [d] World Health Organization Disability Assessment-II Scale total score; [e] Addenbrooke's Cognitive Examination- Revised total score

Participants, who smoked more than 20 cigarettes a day, did not differ significantly from other participants on their clinical responses to clozapine (CATT χ^2= 2.00; df= 1; p= 0.16) depending on their *CYP1A2*1F* genotypes.

6.6. Pharmacogenetic associations between *HTR3A* SNP and response to clozapine:

6.6.1. Allele frequencies of *HTR3A* Single Nucleotide Polymorphisms*:*

Data for the genotypes of *HTR3A* gene SNP rs2276302 were complete. However, repeated Polymerase Chain Reactions of one DNA sample failed to amplify the *HTR3A* gene region flanking rs1062613, probably due to other polymorphisms within the primer binding sites. The allele and genotype frequencies of these two SNP were performed in Table 30. The Minor Allele Frequencies of rs1062613 and of rs2276302 were 0.080 and 0.104, respectively. Allele frequencies of both rs1062613 and rs2276302 were consistent with the Hardy Weinberg Equilibrium.

Table 30: *HTR3A* SNP allele frequencies among the participants

SNP	Allele		Allele frequencies		Genotype frequencies (n)			HWE[a]	p
	1	2	1	2	11	12	22	χ^2, df	
rs1062613 (N=100)	C	T	0.920	0.080	85	14	1	0.24, 1	0.62
rs2276302 (N=101)	A	G	0.896	0.104	81	19	1	0.01, 1	0.92

[a] Goodness of fit with Hardy Weinberg Equilibrium

6.6.2. Associations between *HTR3A* SNP and response to clozapine:

The response to clozapine was initially defined by the Brief Psychiatric Rating Scale total scores of 35 or less. While employing this outcome definition, the allelic associations between the two *HTR3A* gene SNP and response to treatment with clozapine were presented in Table 31.

Table 31: Allelic associations between *HTR3A* gene SNP and response to clozapine

SNP	Allele		Responder		Non-responder		Allelic Odds ratio [a] (95% CI)	Allelic P
	1	2	1	2	1	2		
rs1062613 (N=100)	**C**	**T**	**115**	**15**	**69**	**1**	**9.00 (1.62-50.14)**	**0.01**
rs2276302 (N=101)	**A**	**G**	**111**	**19**	**70**	**2**	**5.99 (1.58-22.71)**	**0.008**

[a] Calculated by variant allele (2) as the exposure variable and clozapine response (defined by BPRS total score ≤ 35) as the outcome variable.

Minor alleles of both rs1062613 and rs2276302 were significantly associated with good clinical responses to clozapine. These allelic associations were statistically significant (p=0.02), after corrections for multiple testing by 100 million max(T) permutations. The associations between the two *HTR3A* SNP genotypes and response to clozapine, defined by BPRS total scores of 35 or less, among our participants were presented in Table 32.

Table 32: Associations between *HTR3A* gene SNP genotypes and response to clozapine

SNP	Allele		Responder			Non-responder			CATT[a] χ^2, df	Genotype p
	1	2	11	12	22	11	12	22		
rs1062613 (N=100)	**C**	**T**	**51**	**13**	**1**	**34**	**1**	**0**	**6.02, 1**	**0.01**
rs2276302 (N=101)	**A**	**G**	**47**	**17**	**1**	**34**	**2**	**0**	**7.04, 1**	**0.008**

[a] Cochran Armitage Test for Trend.

Variant *HTR3A* genotypes were significantly associated with good clinical responses to clozapine. These pharmacogenetic associations were statistically significant for both rs1062613 (p= 0.0258) and for rs2276302 (p= 0.0188), after corrections for multiple testing by 100 million max(T) permutations. They were statistically significant, while assuming either autosomal dominant or additive modes of inheritance. Post-hoc power analyses by Quanto 1.2.4 software (267) revealed that this research had 92.7% and 91.0% power to detect the allelic associations of rs1062613 and rs2276302, respectively.

6.6.3. Clinical utility of *HTR3A* SNP pharmacogenetic associations:

A multiple logistic regression model including the variant genotypes of both *HTR3A* SNP, could explain only 13.8% (Nagelkerke R^2= 0.138) of observed variability among the clinical responses to clozapine in our participants. Associations between the variant genotypes of both *HTR3A* SNP and clozapine related adverse effects, listed in Table 9, were not statistically significant. Multiple logistic regression models, adjusting for the effects of

age, gender and serum clozapine levels, confirmed these negative results. The associations between these *HTR3A* SNP and disability as well as cognition of the participants were presented in Table 33. The differences among the median values of WHODAS-II and ACE-R total scores between the rs1062613 as well as rs2276302 genotypes were not statistically significant.

Table 33: Associations between *HTR3A* SNP genotypes and disability as well as cognition

SNP & Variable	**Wild genotype Mean (SD)**	**Heterozygous variant Mean (SD)**	**Homozygous variant**	**χ^{2} [a]**	**p**
rs1062613	**CC**	**CT**	**TT**		
Disability [b]	18.18 (13.36)	13.93 (10.25)	24.00	1.55	0.46
Cognition [c]	62.58 (21.46)	69.43 (11.01)	45.00	1.98	0.37
rs2276302	**AA**	**AG**	**GG**		
Disability [b]	17.70 (12.96)	16.21 (13.63)	24.00	0.80	0.67
Cognition [c]	63.48 (21.31)	64.05 (15.87)	45.00	1.05	0.59

[a] Kruskal Wallis test with two degrees of freedom; [b] World Health Organization Disability Assessment-II Scale total score; [c] Addenbrooke's Cognitive Examination- Revised total score

6.6.4. Developing a combined clinical and pharmacogenetic model to predict response to clozapine:

Past history of catatonia, smoking, cognitive dysfunction and hypersomnolence were the significant clinical predictors of non-response to clozapine, defined by BPRS total scores of 36 or more, after adjusting for the effects of age, gender and serum clozapine levels. A backward conditional logistic regression model was developed, combining these clinical

predictors and the *HTR3A* variant genotypes. When the clinical predictors and *HTR3A* pharmacogenetic associations were combined, they could explain 38% of variability (Nagelkerke R^2= 0.380) among the clinical responses to clozapine in our participants with Treatment-resistant schizophrenia. Hosmer Lemeshow test confirmed the goodness of fit of this combined model (χ^2 = 6.53; df = 6; p = 0.37). The second step of that backward conditional logistic regression model was presented in Table 34.

Table 34: Combined clinical predictors and *HTR3A* pharmacogenetic association model to predict clinical response to clozapine among the participants

Predictor	**Multivariate analysis [a]**	
	Adjusted Odds ratio (95% CI)	**p-value**
rs2276302 (GA or GG)	**4.91 (1.00-24.09)**	**0.04**
Past history of Catatonia	0.00 (0.00-∞)	0.99
Currently smoking ≥ one pack / day	0.30 (0.08-1.11)	0.07
Excessive sedation [b]	0.28 (0.07-1.12)	0.07
Cognitive dysfunction [c]	**0.33 (0.12-0.90)**	**0.03**

[a] Backward conditional multiple logistic regression model including all significant clinical predictors (past history of catatonia, smoking ≥ one pack / day, excessive sedation and cognitive deficits) and *HTR3A* genetic (rs1062613 and rs2276302 variant genotypes) variables, with good response to clozapine treatment (defined by BPRS total score ≤ 35) as the outcome variable; [b] Sleeping more than 9 hours/day; [c] Addenbrooke's Cognitive Examination- Revised total score < 68 (median value).

6.6.5. Outcome definitions influenced the observed *HTR3A* pharmacogenetic associations:

Analysing the pharmacogenetic associations between *HTR3A* gene single nucleotide polymorphisms and response to clozapine were repeated, while employing five more

outcome definitions, described in section 5.9. The results of our bivariate and multivariate analyses, while employing varying outcome definitions, were presented in Table 35.

Table 35: The influence of outcome definitions on the pharmacogenetic associations between *HTR3A* gene SNP and response to clozapine among the participants

Models [a]		*HTR3A* rs1062613			*HTR3A* rs2276302		
		OR/ AOR	**95% CI**	**p**	**OR/ AOR**	**95% CI**	**p**
Definition 1[b] n [c] = 65	**OR**	**9.33**	**1.17 - 74.31**	**0.04**	**6.51**	**1.42 - 29.95**	**0.02**
	AOR [d]	**28.36**	**1.69 - 476.03**	**0.02**	**6.59**	**1.32 - 32.95**	**0.02**
Definition 2[e] n [c] = 27	**OR**	0.98	0.28 - 3.39	0.98	1.22	0.42 - 3.60	0.71
	AOR [d]	1.12	0.29 - 4.40	0.87	1.07	0.34 - 3.38	0.91
Definition 3[f] n [c] = 53	**OR**	1.95	0.62 - 6.20	0.26	2.51	0.88 - 7.19	0.09
	AOR [d]	2.33	0.62 - 8.74	0.21	2.68	0.84 - 8.51	0.09
Definition 4[g] n [c] = 76	**OR**	5.19	0.65 - 41.76	0.12	-	-	**0.003[h]**
	AOR [d]	9.48	0.80 - 113.08	0.08	-	-	-
Definition 5[i] n [c] = 71	**OR**	1.86	0.49 - 7.15	0.37	1.34	0.44 - 4.09	0.61
	AOR [d]	2.31	0.49 - 10.91	0.29	1.07	0.32 - 3.62	0.91
Definition 6[j] n [c] = 86	**OR**	2.76	0.34 - 22.73	0.35	-	-	**0.03[g]**
	AOR [d]	7.56	0.28 - 200.94	0.23	-	-	-

[a] Variant genotypes as the exposure variable and good response to clozapine treatment as the outcome variable; [b] Clozapine response was defined by BPRS total score ≤ 35; [c] number of clozapine responders; [d] Adjusted for the effects of gender, age of onset of illness (in years), serum clozapine level (in ng/ml), current smoking (more than one pack/day), duration of untreated psychosis (in months), family history of schizophrenia, and of total scores of premorbid adjustment scale, childhood traumatic event scale as well as recent traumatic events scales, by multiple logistic regression analysis; [e] Clozapine response was defined by BPRS total score ≤ 25 (25^{th} percentile) [f] Clozapine response was defined by BPRS total score ≤ 31 (median value); [g] Clozapine response was defined by BPRS total score ≤ 38 (75^{th} percentile); [h] Fisher exact test p value (two tailed), OR could not be calculated, because all participants with variant genotypes had good response to clozapine; [i] Clozapine response was defined by all of the five selected BPRS items, suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization and unusual thought content, scored mild or less; [j] Clozapine response was defined by not more than one of those five BPRS items scored moderate and above; OR = Odds Ratio; AOR = Adjusted Odds Ratio.

Pharmacogenetic association between rs1062613 and response to clozapine was significant, only when employing the outcome definition of Brief Psychiatric Rating Scale total scores of 35 or less. Pharmacogenetic Association between rs2276302 and response to clozapine was significant, while employing only two more outcome definitions,

(i) BPRS total scores of 38 or less (75^{th} percentile),

(ii) Scores of moderate or above severity in not more than one of the five BPRS items rating suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization and unusual thought content (98).

When non-parametric robust regression analyses was performed, with BPRS total scores as the continuous dependent variable, *HTR3A* gene rs1062613 (β = -2.70; SE= 2.51; t= -1.08; p= 0.28) and rs2276302 (β= -3.97; SE= 2.38; t= -1.67; p= 0.10) were not significantly associated with response to clozapine among our participants with Treatment-resistant schizophrenia.

CHAPTER 7

DISCUSSION

This research evaluated the clinical and pharmacogenetic associations predicting response to clozapine among patients with Treatment-resistant schizophrenia. A combined clinical and pharmacogenetic model was developed to predict response to clozapine. A non-parametric equation to predict serum clozapine levels, using clinical proxy measures, was also developed. The importance of varying outcome definitions for response to treatment in schizophrenia, while evaluating the pharmacogenetic associations, was demonstrated.

7.1. Strengths of this research:

This research is the first of its kind from India to evaluate the pharmacogenetics of clozapine. Strengths of these studies include the following,

(i) Unlike the previous studies evaluating the pharmacogenetics of clozapine (22, 57, 63), this research exclusively recruited patients with Treatment-resistant schizophrenia in this research. It did not include patients with schizoaffective disorder, other psychoses and patients with schizophrenia, who had not had two adequate antipsychotic trials in the past before initiating clozapine.

(ii) Refusal rate of the consecutive patients, who satisfied the eligibility criteria, was minimal (3.81%). Among the 105 consecutive patients, who were confirmed to be eligible, only four refused to participate in this research.

(iii) Unlike the previous studies on this topic (19-22), this research collected rich clinical data of our participants by using standard assessment schedules to assess premorbid adjustment, traumatic life events, cognition and disability. Hence, this research was

able to develop a combined clinical and pharmacogenetic model to predict response to clozapine.

(iv) Serum clozapine levels influence the clinical responses to clozapine (47-49). However, many clinical and pharmacogenetic association studies evaluating response to clozapine (13-22) did not estimate serum clozapine levels. As this research estimated the serum clozapine levels of our participants, it could include them in the multivariate models.

(v) Despite the poor replication of the results of pharmacogenetic studies are often explained by varying outcome definitions for treatment responses (61, 62), pharmacogenetic studies of clozapine seldom employ multiple outcome definitions. This research evaluated our pharmacogenetic associations, while employing multiple outcome definitions.

(vi) Our consecutive sampling strategy reduced the possibility of selection bias.

(vii) As three independent investigators, who were blind to each other's findings, carried out the assessments of treatment responses to clozapine, various clinical variables and genotyping, this research minimized the possibility of observer bias. Training of the personnel for the laboratory investigations and the employed clinical assessments was rigorous.

(viii) This research attempted to minimize recall bias on the reported clinical variables by interviewing first-degree relatives of the participants and by verifying their well-documented follow-up medical records.

(ix) Due to high inter-individual variability, serum clozapine levels do not usually follow Gaussian distribution among the clinical samples. Hence, dosing nomograms based on

the linear regression models (28, 29), are often inadequate (31). This research employed a non-parametric multiple robust regression model to predict serum clozapine levels.

(x) This research contributed new data on *CYP1A2* and *HTR3A* allele frequencies among the south Indian population (271).

7.2. Limitations of this research:

The potential limitations of this study include the following,

(i) This research defined the response to clozapine by cross-sectional clinical assessment. It recruited only the participants, who were maintained on stable dosage of clozapine for a minimum duration of 12 weeks, when their treating psychiatrists did not need to change their prescription. Hence, their cross-sectional Brief Psychiatric Rating Scale scores were more indicative of the persistent psychopathology than of any acute fluctuations in their illnesses. As patients with Treatment-resistant schizophrenia may respond to clozapine after a delay of 24-32 weeks (14, 272), short-term longitudinal studies, with less than 24 weeks of follow up, also had similar temporal uncertainties (13, 17). Considering the pragmatic constraints and the paucity of pertinent data in Indian settings, this research determined the response to clozapine among the participants with the cross-sectional BPRS assessment, while employing multiple outcome definitions.

(ii) The cross sectional design precluded longitudinal assessments of psychopathology and hence did not allow for using reduction in psychopathology in defining outcome. However, the validity of such reductions, which employ such relative reductions in rating scale scores, is also debated. As the clinical significance of many statistically

significant reductions in the total scores of rating scales is uncertain (273), this research did not define response to clozapine by any percentage of reduction in the BPRS total scores but defined it by the presence of persistent positive or negative symptoms (265).

(iii) We employed the BPRS for assessing outcomes. While a more comprehensive assessment may have been useful (93), the BPRS is the commonest instrument employed in pharmacogenetic research.

(iv) The cross-sectional nature of this research led to temporal ambiguity. This research could not infer any causal associations for the clinical variables, which were associated with response to clozapine. Cohort studies will be required to establish the direction of associations and causality.

(v) While estimating the serum clozapine levels of our participants, this research did not estimate their serum levels of clozapine metabolite, norclozapine.

7.3. Clinical predictors of response to clozapine:

The results of this research suggest that past history of catatonia, smoking more than one pack/ day, hyper-somnolence and cognitive deficits were significantly associated with non-response to clozapine. This research documented that the clinical predictors varied, depending on the outcome definitions for non-response to clozapine.

7.3.1. Disparities among clinical predictors:

Previous literature have reported female gender (16), earlier age of onset (16, 25), non-paranoid subtype (26), longer duration of illness (23), baseline psychopathology (13, 15, 27), baseline quality of life (27), serum clozapine levels below 350 ng/ml (23) and poor

functioning during the previous year (12, 14) as the potential clinical predictors of non-response to clozapine. Findings on the utility of past history of antipsychotic medication induced extra pyramidal symptoms (15, 23, 134) and of the baseline negative symptoms (15, 25) to predict clinical responses to clozapine were contradictory. Ethnic diversity, population characteristics, lack of structured assessment of clinical variables, differing outcome definitions and causal heterogeneity of response to clozapine may explain the disparities between the reported findings. Results of this research were negative for these potential clinical predictors, but they have brought forth four clinical variables, which could explain 31.9% of observed variability in the treatment response to clozapine among our participants. When this research employed alternative outcome definitions, female gender, high caffeine intake and non-paranoid subtype were also associated with non-response to clozapine. They added evidence that differing outcome definitions could, at least partly, explain the disparities among the previous studies, which evaluated the clinical predictors of response to clozapine.

Schizophrenia is not a single disease, but a clinically heterogeneous disorder, caused by multiple genetic as well as environmental factors (157). Pharmacokinetics and pharmacodynamics of clozapine are complex (274). Hence, any statistical model with limited clinical predictors often explains only a small proportion of observed variability among the responses to clozapine in patients with Treatment-resistant schizophrenia. The disparities among the available literature, which evaluated clinical predictors of response to clozapine, should not be considered contradictory but should be considered complementing each other. Results of this research supported the need for employing multiple outcome definitions to address the clinical heterogeneity of Treatment-resistant schizophrenia and of its response to clozapine.

7.3.2. Catatonia and response to clozapine:

Debate on the nosological status of catatonia, as a sub-type of schizophrenia or as a separate clinical syndrome, remains unresolved. The patients who have both schizophrenia and catatonia may have unique underlying pathophysiology (275). Catatonia could increase the risk for clozapine induced neuroleptic malignant syndrome (276). It might develop during withdrawal from clozapine (277). Unlike the earlier case reports, which stated good response to clozapine in patients with recurrent catatonia (278, 279), all participants with past history of catatonia (n=5) were non-responders to clozapine in this study. The association between past history of catatonia and non-response to clozapine was statistically significant, while employing varying outcome definitions. As the pathophysiology of catatonia is currently poorly understood, results of this research support further studies investigating the relationship between catatonia and response to clozapine as well as the possible underlying neurobiological processes.

7.3.3. Smoking and response to clozapine:

Smoking reduces serum clozapine levels (28). However, available literature on the relationship between smoking and the non-response to clozapine are contradictory (280, 281). Studies associating smoking with non-response to clozapine emphasize its ability to induce Cytochrome P-450 1A2 (CYP1A2) enzyme and to reduce serum clozapine levels (280). Studies associating smoking with good response to clozapine focus on its ability to activate nicotinic acetyl choline receptors (281). Results of this research have documented that, smoking more than 20 cigarettes/ day, increased the risk of non-response to clozapine by five times, after adjusting for the effects of age, gender and serum clozapine levels. This research supports the relationship between smoking and non-response to clozapine in patients with Treatment-resistant schizophrenia. Findings of this research added that such relationship

could not be solely explained by reduced serum clozapine levels. Smoking can be considered as a potentially modifiable risk factor and suggest nicotine deaddiction for all smoking patients with Treatment-resistant schizophrenia to augment their clozapine response. As concerns over abrupt smoking cessation by patients with Treatment-resistant schizophrenia, which may suddenly elevate their serum clozapine levels to toxic range, exist, appropriate titration of clozapine dosage against its serum levels are suggested during smoking cessation (282). There are concerns regarding the weight gain associated with smoking cessation. However, a recent large prospective cohort study confirmed that smoking cessation was associated with less risk for cardio vascular disease events, after accounting for the effects of consequent weight gain (283).

7.3.4. Other potential modifiable risk factors for non-response to clozapine:

Akin to previous studies reporting the ability of cognitive factors discriminating clozapine responders and non-responders (135), this research found cognitive deficits as one of the significant clinical predictor for non-response to clozapine. This research also documented that cognitive dysfunction was significantly associated with higher disability, after adjusting for the effects of age and serum clozapine levels. Appropriate cognitive interventions can be suggested to augmenting the clinical response to clozapine in such disabled patients. Daily consumption of caffeinated beverages is common globally. Findings of this research suggest the need for motivating all patients on clozapine, especially those who have poor response to clozapine, to reduce their caffeine consumption for augmenting their clinical response (280). Despite temporal ambiguity, hyper-somnolence was associated with non-response to clozapine among the participants. Complaints of excessive sedation on clozapine are often overlooked during clinical practice. Such complaints may indicate poor response to clozapine in patients with Treatment-resistant schizophrenia. Appropriate

behavioural management and rationalizing concurrent psychopharmacological agents to reduce excessive sedation can be considered for such patients.

7.4. Clinical predictors of serum clozapine levels:

Identifying clinical predictors, which act as proxy measures of serum clozapine levels, is desired during clinical practice in many Indian settings. Hence, this research examined the clinical predictors of serum clozapine levels among Indian patients with treatment-resistance schizophrenia. Results of this research showed that higher oral doses of clozapine as well as Valproate co-medication were significantly associated with higher serum clozapine levels and that high caffeine intake significantly reduced serum clozapine levels.

7.4.1. Clinical proxy measures for serum clozapine levels:

Previous studies on this topic have reported that oral clozapine dose is the only clinical variable consistently associated with serum clozapine levels, but is insufficient to explain the high inter individual variability of serum levels (28-34). Female gender has been associated with higher clozapine levels (29-31, 33, 35), but this association has been questioned later (34, 136). Increasing age has been associated higher clozapine levels (29, 35) but the evidence to the contrary also exists (30, 33, 34, 136). This study did not find any association between serum clozapine levels and age or gender of our participants. Studies supporting (28, 29) and challenging (30) the association between smoking and lower serum clozapine levels are present. As the prevalence of smoking was low in this sample (18.8%) and this sample did not have any female smokers, due to cultural reasons, this research was probably underpowered to detect any association between smoking and serum clozapine levels of the participants.

Results of this research confirmed the association between Valproate co-medication and higher serum clozapine levels (131, 136, 284). Selective Serotonin Reuptake Inhibitors (SSRI) such as Fluvoxamine (136) and Paroxetine (285) were reported to be associated with higher serum clozapine levels. Eight of the participants, who were on SSRI co-medication, were receiving either Fluoxetine or Sertraline. This research did not find any association between these SSRI co-medications and serum clozapine levels of the participants.

7.4.2. Caffeine consumption and serum clozapine levels:

Available literature regarding the association between caffeine consumption and serum clozapine levels are contradictory. Previous smaller studies have suggested that caffeine either reduces (280) or increases (286, 287) serum clozapine levels, due to competitive inhibition of CYP1A2 hepatic enzyme. Results of this research have clarified that caffeine consumption was significantly associated with lower serum clozapine levels, while accounting for the effects of age, gender and of oral clozapine doses. The pharmacokinetic pathways underlying this observed association between caffeine consumption and lower serum clozapine levels warrant further investigation. As smoking and drinking caffeinated beverages are positively correlated (288), their combined influence over CYP1A2 hepatic enzyme activity needs to be investigated in more detail. Constituents in the caffeinated beverages, other than caffeine, may also induce CYP1A2 enzyme activity and may reduce serum clozapine levels. Daily consumption of three or more cups of coffee has been reported to increase CYP1A2 enzyme activity significantly in Serbs and Swedes (213). Polycyclic aromatic hydrocarbons, which are generated during roasting of coffee beans, may partly explain for this inducing effect (201).

7.4.3. Serum clozapine levels and chronic Schizophrenia:

Higher serum clozapine levels have been reported to be associated with more cognitive impairment in patients with chronic schizophrenia (289), after adjusting for the effects of age, gender and oral dose (290). This research replicated this finding among our participants, but further clarified that this observed association was confounded by the psychopathology of the participants. It documented that the bivariate positive correlation, between psychopathology and serum clozapine levels, was spurious, due to the confounding effect of oral clozapine doses. Patients who had severe psychopathology developed more cognitive deficits, and they required more oral doses of clozapine, producing higher serum clozapine levels. Hence, the findings of this research argue against any direct causal relationship between serum clozapine levels and cognitive impairment in patients with chronic schizophrenia. Similarly, this research suggested that the observed relationship between serum clozapine levels and disability in patients with chronic schizophrenia was also confounded by the psychopathology of the participants. A previous study has hypothesized that patients with tardive dyskinesia would differ in their metabolism of clozapine and would have higher serum clozapine levels (291). Results of this research disproved this hypothesis with appropriate multivariate analyses.

7.4.4. Clinical and research implications:

Prior literature on this topic and results of this research imply the following for clinical settings and for future research,

(i) Serum clozapine levels, above the minimum threshold value of its therapeutic window, maximize clinical responses to clozapine (47-49), reduce the lag time to response (28) and reduce the relapse rates (35) in Treatment-resistant schizophrenia. A non-parametric multiple robust regression equation was developed to predict the

serum clozapine levels by three clinical variables. In clinical settings where routine therapeutic drug monitoring of clozapine is not feasible, this equation can aid the treating psychiatrists during dose adjustments of clozapine therapy.

(ii) Valproate co-medication significantly increased the serum clozapine levels. Results of this research highlight the clinical need for appropriate clozapine dose reductions, while adding Valproate co-medication, to reduce level dependent adverse effects (292).

(iii) Patients all over the world consume caffeinated beverages every day and their importance is often neglected during clinical dose adjustments of clozapine (280). Patients should be advised to inform their psychiatrists, when they make any abrupt changes in their caffeine consumption habits (293). The dosing equation may help the clinical psychiatrists to consider appropriate clozapine dose adjustments in such situations.

(iv) Oral doses of clozapine above 600 mg/day are known to increase the risk of seizures (129). This research identified a threshold value of serum clozapine levels (500 ng/ml), above which, the risk of seizures increased by almost six-fold. If patients, who are on maintenance treatment with clozapine, report serum clozapine levels above 500 ng/ml, appropriate dose reductions or adjustment of co-medications should be considered.

(v) This research identified that the oral clozapine doses below 250mg/day were four times more likely to produce inadequate serum clozapine levels less than 350 ng/ml. Contemporary prescription guidelines suggest that serum clozapine level of 350 ng/ml should be achieved by employing target oral doses ranging from 250 mg/day to 550

mg/day (294). Hence, the minimum clinical dose for clozapine should be 250 mg/day, unless the patients develop intolerable adverse effects on lower doses.

(vi) Due to high inter-individual variability, serum clozapine levels are seldom normally distributed among clinical samples. Hence, clozapine dosing nomograms based on linear regression models (28, 29), are often misleading (31). Clinical psychiatrists and future researchers are advised to pursue dosing equations, developed from appropriate non-parametric regression models.

7.5. Pharmacogenetic associations between *CYP1A2* SNP and response to clozapine:

This research examined the pharmacogenetic associations between four functionally relevant as well as previously investigated Single Nucleotide Polymorphisms in the *CYP1A2* gene and clinical responses to clozapine among patients with Treatment-resistant schizophrenia. The sample size was relatively larger than most of the available studies on this topic (44-46, 65, 66). This research provided new data on the *CYP1A2* allele frequencies in a population of south Indian ethnicity. These allele frequencies are similar to other Asian and sub-Saharan African populations (269, 270, 295, 296). Findings of this research suggested that the presence of two SNP (*CYP1A2*1C* and *CYP1A2*1D*) in the 5' flanking region and two others (*CYP1A2*1E* and *CYP1A2*1F*) in intron one of the *CYP1A2* gene were not associated with clinical responses to clozapine and with serum clozapine levels of our participants. Association between *CYP1A2* gene SNP and tardive dyskinesia in patients with chronic schizophrenia has already been reported. Such pharmacogenetic association was reported to be statistically not significant, after corrections for multiple testing (231). Results of this research confirmed the lack of any significant pharmacogenetic association between these four SNP in *CYP1A2* gene and tardive dyskinesia among our participants.

7.5.1. *CYP1A2*1F* and non-response to clozapine:

Earlier case studies have reported possible association between *CYP1A2*1F* and non-response to clozapine, especially in smokers (44, 45). Small uncontrolled samples and lack of multivariate analyses may explain the findings of these reports, because subsequent larger studies did not find any significant association between *CYP1A2* SNP and clinical response to clozapine (64-66). Findings of this research corroborated the lack of association between *CYP1A2*1F* and non-response to clozapine. *CYP1A2*1F* has been associated with higher induction of CYP1A2 activity by smoking (45) and by heavy caffeine consumption (46). Hence, *CYP1A2*1F* was expected to be associated with lower serum clozapine levels. However, further research did not establish any significant association between this *CYP1A2* SNP and serum clozapine levels (64-66). Results of this research confirmed the lack of association between *CYP1A2*1F* and lower serum clozapine levels among smokers (45) and other participants. Smoking is known to have a major influence over serum clozapine levels (28). However, its relationship with treatment response to clozapine is controversial (280, 281). As this research established that smoking was a significant clinical predictor of non-response to clozapine, poor response to clozapine in smokers can be considered as secondary to smoking rather than to *CYP1A2*1F* (280).

7.5.2. Clinical implications:

Results of this research showed that the pharmacogenetic associations between these four single nucleotide polymorphisms in the *CYP1A2* gene and the clinical responses to clozapine as well as the clozapine related adverse effects were not statistically significant. The associations between these four SNP in the *CYP1A2* gene and the serum clozapine levels, disability status as well as the cognitive functioning of our participants were also statistically not significant. Previous studies have claimed that CYP1A2 genotyping could

have high clinical utility for the patients on clozapine (45). CYP450 pharmacogenetic test chips are being currently marketed (59). However, results of this research have proved the contrary. The findings of this research imply that these four single nucleotide polymorphisms of *CYP1A2* gene do not help to predict the clinical responses to clozapine. Hence, routine screening for them prior to starting clozapine is unwarranted at present.

7.6. Pharmacogenetic associations between *HTR3A* SNP and response to clozapine:

This research examined the pharmacogenetic associations between rs1062613 as well as rs2276302 Single Nucleotide Polymorphisms in *HTR3A* gene and clinical responses as well as adverse effects of clozapine, within the context of differing outcome definitions and clinical predictors. This research demonstrated that pharmacogenetic associations depend heavily on their outcome definitions for treatment response in schizophrenia. Results of this research documented that combined models, including both clinical and pharmacogenetic variables, have better predictive values.

7.6.1. Clinical utility of *HTR3A* SNP pharmacogenetic associations:

Two previous studies did not find any significant pharmacogenetic association between *5HT3A* gene single nucleotide polymorphisms and response to clozapine (20, 63). These studies employed the outcome definition of 20 points improvement in the Global Assessment Scale (103) total scores by retrospective assessment of their participants. However, a recent study has reported that the allelic and genotypic associations between rs1062613 as well as rs2276302 and treatment response to clozapine were nominally significant, while employing the outcome definition of more than 20% reduction from the baseline Brief Psychiatric Rating Scale total scores (22). Only the rs1062613 pharmacogenetic association was significant, after 100,000 permutations (22). Further details of these studies evaluating the pharmacogenetic associations between *HTR3A* gene

polymorphisms and response to clozapine in patients with schizophrenia were presented in Table 1.

This research replicated these pharmacogenetic associations, while employing another outcome definition of BPRS total scores of 35 or less, and confirmed the statistical significance of both associations after 100,000,000 permutations. However, these *HTR3A* gene variant genotypes together could explain only 13.8% of the variability observed in the clinical response to clozapine among our participants. These allelic and genotypic associations were not significant, when other outcome definitions were employed. Moreover, their associations with disability status and cognitive functioning of the participants were also not statistically significant. Clinical significance of statistically significant reductions in the psychiatric rating scale scores are debated in many clinical trials (273). Similarly, claiming evidence for clinically significant pharmacogenetic associations between *HTR3A* gene polymorphisms and responses to clozapine, from these statistically and functionally significant pharmacogenetic associations, becomes questionable.

7.6.2. Clinical heterogeneity and outcome definitions of schizophrenia:

Schizophrenia is not a single disease, but a heterogeneous group of polygenic multi-factorial disorders (142). Searches for genetic variants, which exert large effects on the pathogenesis and on clinical responses of schizophrenia, have not been successful (158). As many candidate gene and Genome Wide Association Studies disregard the importance of collecting rich clinical data from their participants (142), generalizing their statistically significant genetic or pharmacogenetic associations to different clinical contexts remains difficult. There are numerous outcome measures to assess treatment response in schizophrenia (93). Although consensus criteria to define remission in schizophrenia have been proposed, their utility and validity have not been established so far (98). Differing

BPRS based response cut-off scores caused significant variability in the results of many clinical trials in schizophrenia (97). Pharmacogenetic studies often neglect this intricate clinical reality. They evaluate the statistical associations between multiple Single Nucleotide Polymorphisms and response to clozapine or other drug response in schizophrenia, while employing a single arbitrarily chosen outcome definition. Poor replication of pharmacogenetic association signals and the overall inability of pharmacogenetics of schizophrenia to live up to its potential (60), stem from varying outcome definitions and clinical heterogeneity (62). Pharmacogenetic studies of schizophrenia often neglect collecting data of known clinical predictors for treatment responses, such as age of onset of illness, duration of untreated psychosis, family history of schizophrenia, serum clozapine levels, smoking, premorbid adjustment and traumatic life events (16, 280). They hope for technical advances in the fields of molecular and statistical genetics to overcome their research barriers. However, next generation sequencing and computationally intensive statistics may not solve the puzzles, if the basic clinical quandaries of defining and measuring the phenotype, schizophrenia and its clinical response, remain unresolved.

7.6.3. Clinical and research implications:

Results of this research imply that the results of pharmacogenetic studies rely heavily on the clinical predictors and the outcome definitions. Hence, the following clinical and research implications need to be highlighted,

(i) Future pharmacogenetic studies should include known clinical predictors. Pharmacogenetic studies should not underestimate the contribution of reliable clinical variables (16, 280), to predict clinical responses to clozapine. Explanatory pluralism is better than biological reductionism to understand the intricacies of psychiatric disorders (297). Combined clinical and genetic model could explain 38% variability

among the clinical responses to clozapine, while genetic variants alone could explain only 13.8% variability among the participants of this research. Future studies should document these clinical variables, to study the pharmacogenetic associations in their clinical context and to investigate the gene environment interactions.

(ii) There is a need for a consensus on defining outcome in schizophrenia. Such consensus should involve different aspects of schizophrenia (positive, negative, cognitive, disability, quality of life, etc.). The domains to be assessed and the combinations of domains to be used in such definitions have to be considered. There should also be a consensus on the instruments employed to assess these variables and also their thresholds, which define improvement. Such definitions will then need to be employed a priori.

(iii) Unambiguous definition of phenotypes will lead to reliable pharmacogenetic associations. Akin to DSM-5 diagnostic criteria, reliable, if not valid, research criteria are needed to define treatment responses for pharmacogenetic studies of psychiatric disorders.

(iv) Until the consensus on defining outcome in schizophrenia is reached, pharmacogenetic studies need to confirm their association signals by employing multiple outcome definitions. Although a clinically relevant BPRS or other scales' cut-off score should be decided a priori, more analyses with varying response thresholds should be presented (97).

(v) Employing multiple outcome definitions will increase the risk of type I errors. It is possible to address this problem with appropriate statistical corrections for multiple testing (298). However, reporting a multiple testing adjusted statistically significant pharmacogenetic association, which does not hold true, while considering other

possible outcome definitions, is not desired. This research argues for consistent pharmacogenetic associations, which remain significant, while employing multiple clinically relevant outcome definitions.

(vi) Future pharmacogenetic studies should look beyond the statistical significance of their positive association signals. All statistically significant, or even functionally relevant, pharmacogenetic associations need not be clinically significant (273). Akin to many common diseases, pharmacogenetics of schizophrenia deals with common variants and small effect sizes (142). Hence, we should be more realistic, while discussing the clinical implications of pharmacogenetic associations of individual genetic variants (299).

(vii) Clinical heterogeneity of schizophrenia demand syndrome sub-categorization, employing biological variables, to achieve etiological homogeneity (300). Similarly, appropriate biological variables to define treatment response in schizophrenia should be searched for.

Pharmacogenetics of schizophrenia should progress, by developing reliable outcome definition criteria and by future studies evaluating combined models of pharmacogenetic and clinical predictors.

CHAPTER 8

SUMMARY AND CONCLUSION

8.1. Summary of this research:

Despite clozapine's superior clinical efficacy in treatment-resistant schizophrenia (TRS), its adverse effects, need for periodic leukocyte monitoring, cost as well as variable clinical outcomes mandate a clinical need to predict its treatment response. Cytochrome P450 1A2 (CYP1A2) is the principal determinant of metabolism of clozapine. Antagonism of serotonin 5HT3A receptor contributes to the superior clinical efficacy of clozapine. Hence, this research investigated the associations between various clinical variables, four single nucleotide polymorphisms in *CYP1A2* gene (rs2069514, rs35694136, rs2069526 and rs762551) as well as two SNP in *HTR3A* gene (rs1062613 and rs2276302) and treatment responses as well as adverse events of clozapine in patients with TRS.

101 consecutive patients with TRS, on stable doses of clozapine, were recruited into this research. The following clinical assessment instruments were employed: Brief Psychiatric Rating Scale, Abnormal Involuntary Movements Scale, Addenbrooke's Cognitive Examination- Revised, WHO Disability Assessment Scale-II, Childhood and Recent Traumatic Events Scale, and Premorbid Assessment Scale. Serum clozapine levels were determined by using high performance liquid chromatography. The four *CYP1A2* SNPs were genotyped by Polymerase Chain Reaction and restriction fragment length polymorphisms method. The two HTR3A SNPs were genotyped by direct DNA sequencing. Clozapine response was defined by six varying outcome definitions. A case-control design framework was adopted and appropriate multivariate statistics were employed.

Past history of catatonia, smoking, hyper-somnolence and cognitive dysfunction were significantly associated with non-response to clozapine. Outcome definitions of non-response to clozapine influenced its association with the clinical predictors. While employing non-parametric multiple robust regression models, oral clozapine dose, high caffeine consumption and Valproate co-medication were significantly associated with serum clozapine levels. A dosing nomogram was developed including these clinical predictors for serum clozapine levels. Results of this research revealed that the four *CYP1A2* gene SNPs were not significantly associated with clozapine treatment response, adverse effects, serum clozapine levels or with disability status of the participants. Minor alleles of *HTR3A* gene SNPs, rs1062613 and rs2276302, were significantly associated with good clinical response to clozapine, after appropriate corrections for multiple testing. However, these pharmacogenetic associations varied depending on the employed outcome definition for response to clozapine. A pharmacogenetic model including both *HTR3A* gene SNPs could explain only 13.8% of variability observed in the responses to clozapine, while a combined clinical predictors and *HTR3A* pharmacogenetic association model could explain 38% of variability observed in the responses to clozapine among the patients with TRS.

8.2. Conclusions:

Clinical variables are useful to predict response to clozapine and to model a dosing nomogram for serum clozapine levels. Smoking is a potentially modifiable risk factor for non-response to clozapine. Importance of caffeine consumption and Valproate co-medication should be considered during clozapine dose adjustments to enhance its therapeutic response and safety profile. As the four *CYP1A2* gene single nucleotide polymorphisms do not help to predict the clinical response to clozapine, routine screening for them prior to starting clozapine is currently unwarranted. Combined clinical and pharmacogenetic models could

predict responses to clozapine better than *HTR3A* pharmacogenetic associations alone. The results of pharmacogenetic studies in schizophrenia depend heavily on their outcome definitions. This research indicates that future pharmacogenetic studies should evaluate associated clinical variables. It highlights the need for consensus criteria to define treatment outcomes in pharmacogenetic studies of schizophrenia.

Schizophrenia is not a single disease. It is a heterogeneous group of clinical disorders. The inherent heterogeneity within the clinical category of treatment-resistant schizophrenia obscures the search for the clinical and pharmacogenetic predictors for its response to clozapine. Syndrome sub-categorization employing biological variables is desired to achieve the etiological homogeneity of schizophrenia (300). Appropriate biological markers, defining the response to clozapine, have to be identified for developing better prediction models with clinical and pharmacogenetic predictors. Until we identify such biological markers, we need reliable consensus research criteria, which will be analogous to the prevailing diagnostic criteria, to define treatment responses for pharmacogenetic studies of schizophrenia. There should be a consensus on which domains of schizophrenia should be assessed and on which assessment instruments should be employed to assess those domains. After developing such consensus criteria, all pharmacogenetic studies of schizophrenia should employ them a priori to define treatment outcomes.

As explanatory pluralism is the need of the hour in psychiatry (297), it is high time to reduce the wide gulf between the clinical and pharmacogenetic research on this topic. Combining clinical and pharmacogenetic predictors may identify many patients, who are more likely to benefit with clozapine, and may prevent the unnecessary exposure of many potential non-responders to serious adverse effects. Future longitudinal studies, investigating both clinical and pharmacogenetic factors together with consensus outcome definitions, are

desired to predict response to clozapine among patients with treatment-resistant schizophrenia.

Ethnic differences in the serum levels and the clinical responses of clozapine are known to exist among the Asian patients with schizophrenia (67). However, systematic searches of electronic databases have not yielded any Indian studies examining the pharmacogenetic associations of clozapine. This research has provided new data on the *CYP1A2* as well as *5HT3A* allele frequencies, clinical as well as pharmacogenetic predictors of responses to clozapine and on the clinical proxy measures of serum clozapine levels among Indian patients with treatment-resistant schizophrenia. These data may contribute towards improving the management of patients with treatment-resistant schizophrenia in India.

CHAPTER 9

RECOMMENDATIONS

On the basis of the findings of this research, the following are recommended,

(i) Pharmacogenetic studies of schizophrenia need to collect data on pertinent clinical predictors. They should aim to develop combined clinical and pharmacogenetic association models. When we enthusiastically pursue our research for the promise of new discoveries, we should not overlook the inherent clinical heterogeneity of schizophrenia.

(ii) There is a need for reliable consensus research criteria to define treatment responses for future pharmacogenetic studies of schizophrenia. Such consensus criteria should go beyond a narrow focus on positive psychotic symptoms to incorporate global assessments of outcome, including cognition, functional disability and quality of life. Once a consensus on the domains of schizophrenia to be assessed and on the assessment instruments is reached, future pharmacogenetic studies of schizophrenia should employ such consensus criteria a priori to define treatment outcomes.

(iii) Routine screening for *CYP1A2* gene Single Nucleotide Polymorphisms (**1C, *1D, *1E* and **1F*) prior to starting clozapine is currently unwarranted.

(iv) Smoking is a potentially modifiable risk factor for non-response to clozapine. Nicotine deaddiction should be advised for all smoking patients with Treatment-resistant schizophrenia to augment their response to clozapine.

(v) Because of high inter-individual variability of serum clozapine levels, routine therapeutic drug monitoring of clozapine is desired in all clinical settings.

(vi) Clinical psychiatrists, working in the settings where routine therapeutic drug monitoring of clozapine is not feasible, may consider using our non-parametric equation during clozapine dose adjustments.

(vii) Minimum target oral dose for clozapine should be 250 mg/day for the patients with treatment-resistant schizophrenia, unless the patients develop intolerable adverse effects on lower doses.

(viii) If patients, who are on maintenance treatment with clozapine, report serum clozapine levels above 500ng/ml, appropriate dose reductions or adjustment of co-medications should be considered to reduce the risk of clozapine related seizures.

(ix) Clinical psychiatrists need to consider the influence of caffeinated beverages on serum clozapine levels of their patients. The patients should be advised to inform their treating psychiatrists, when they make any abrupt changes in their caffeine habits.

CHAPTER 10

APPENDIX

10.1. List of publications:

The work for this thesis resulted in the following publications,

10.1.1. Rajkumar AP, Chitra C, Bhuvaneshwari S, Poonkuzhali B, Kuruvilla A, Jacob KS. Clinical predictors of response to clozapine in patients with Treatment Resistant Schizophrenia. Psychopharmacol bull. 2011; 44(3): 4. Embase Accession Number: 2012391618

10.1.2. Rajkumar AP, Poonkuzhali B, Kuruvilla A, Jacob M, Jacob KS. Clinical predictors of serum clozapine levels in patients with Treatment Resistant Schizophrenia. Int Clin Psychopharmacol. 2013; 28(1): 50-6. PMID: 23104241

10.1.3. **Rajkumar AP**, Poonkuzhali B, Kuruvilla A, Srivastava A, Jacob M, Jacob KS. Association between *CYP1A2* gene single nucleotide polymorphisms and clinical responses to clozapine in patients with treatment-resistant schizophrenia. Acta Neuropsychiatr. 2013; 25 (1): 2-11. DOI: 10.1111/j.1601-5215.2012.00638.x

10.1.4. Rajkumar AP, Poonkuzhali B, Kuruvilla A, Srivastava A, Jacob M, Jacob KS. Outcome definitions and clinical predictors influence pharmacogenetic associations between HTR3A gene polymorphisms and response to clozapine in patients with schizophrenia. Psychopharmacology (Berl). 2012; 224(3): 441-9. PMID: 22700043

RESEARCH ARTICLE

Key Words: Schizophrenia, clozapine, catatonia, smoking, psychopathology

Clinical Predictors of Response to Clozapine in Patients with Treatment Resistant Schizophrenia

By Rajkumar A.P., Chitra C., Bhuvaneshwari S., Poonkuzhali B., Kuruvilla, A., Jacob, K.S.

ABSTRACT ~ Objectives: *Despite clozapine's superior clinical efficacy in Treatment Resistant Schizophrenia (TRS), its adverse effects, need for periodic leukocyte monitoring, cost and variable clinical outcomes make the therapeutic decision making process difficult and mandate a clinical need to predict its treatment response. Hence, we investigated various clinical variables associated with treatment responses and adverse events of clozapine in TRS.* ***Experimental Design:*** *We assessed socio-demographic and clinical profiles, premorbid adjustment, traumatic life events, cognition, disability, psychopathology and serum clozapine levels of 101 patients with TRS on stable dose of clozapine using the following instruments: Brief Psychiatric Rating Scale, Abnormal Involuntary Movements Scale, Addenbrooke's Cognitive Examination—Revised, WHO Disability Assessment Scale-II, Childhood and Recent Traumatic Events Scale, and Premorbid Assessment Scale. We defined clozapine response a priori, adopted a case-control design framework and employed appropriate multivariate analyses.* ***Principal Observations:*** *Past history of catatonia ($p = 0.005$), smoking more than one pack/day ($p = 0.008$), hyper-somnolence ($p = 0.03$) and cognitive dysfunction ($p = 0.007$) were associated with non-response to clozapine. Outcome definitions of non-response to clozapine influenced its association with clinical predictors.* ***Conclusions:*** *Clinical variables are useful to predict response to clozapine. Smoking can be a potentially modifiable risk factor. Future longitudinal studies, investigating clinical and pharmacogenetic variables together, are desired.* Psychopharmacology Bulletin. *2011;44(3):00–00.*

INTRODUCTION

Clozapine is the drug of choice for the management of Treatment Resistant Schizophrenia (TRS).[1] The advantages of clozapine include its superior clinical

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efficacy, its ability to reduce negative symptoms as well as the risk for suicide[2] and its low propensity to cause movement disorders.[3] Its disadvantages are suboptimal response in 40–70% patients with TRS,[4] high cost, serious adverse events, such as seizures, agranulocytosis, weight gain and dyslipidemia,[5] and the need for periodic monitoring of leukocytes. Therapeutic decision-making process for starting clozapine is difficult and vacillating for many patients, their families and psychiatrists. There is a clinical need to identify the factors associated with response to clozapine in TRS.[6]

Research on the factors associated with clinical outcomes of clozapine in schizophrenia has focussed mainly on genetic[7–10] and other biological markers.[11,12] However, many biological predictors are inconclusive, contradictory or pending replication[9] and their clinical utility is currently doubtful.[13] Clinical variables, such as baseline psychopathology and premorbid functioning have been reported as reliable predictors of clozapine response.[6] Clinical psychiatrists often consider more clinical variables, before discussing the option of clozapine to their patients with TRS.

Most studies evaluating clinical predictors of response to clozapine[14–19] were not specific to TRS and have recruited patients without treatment resistance as well as patients with schizoaffective disorders. They have seldom employed standard instruments to assess premorbid adjustment, traumatic life events, cognition and disability. Many studies have not estimated serum clozapine levels of their participants. Despite ethnic differences in the plasma levels and the clinical responses of clozapine are known to exist,[20] pertinent data on the clinical predictors of response to clozapine in Asian patients remain sparse.[21] Hence, we aimed to investigate the clinical predictors of response to clozapine, exclusively in patients with TRS in India, with structured assessment of various clinical variables and estimation of serum clozapine levels.

MATERIALS AND METHODS

Study Design

We employed a cross-sectional study to investigate our objectives. We used a case control design framework to identify the clinical variables associated with non-response to clozapine.

Setting

We conducted this study in Department of Psychiatry, Christian Medical College (CMC), Vellore, India, a tertiary referral centre for the

management of psychiatric disorders. The hospital has short-term inpatient services, daily outpatient and regular follow-up clinics. Patients with schizophrenia are initially treated with either dopamine antagonists or serotonin dopamine antagonists (SDA). Clozapine is never used as the first line antipsychotic and is reserved for patients with TRS. Leukocyte counts and metabolic parameters are periodically monitored. Detailed medical records of treatment are maintained for all patients. Most of our outpatients with schizophrenia live in the community with their families. Their medications are directly provided by their first degree relatives or spouses, who report any degree of non-adherence to the treating psychiatrists during periodically scheduled follow-up visits.

Recruitment of Participants

We invited all consecutive patients who satisfied the following eligibility criteria: (i) DSM IV-TR diagnosis of schizophrenia (ii) established treatment resistance in the past after failure to respond at least two adequate antipsychotic trials, as documented by treating psychiatrists. An adequate antipsychotic trial was defined by 600 mg chlorpromazine equivalents for duration of at least six weeks with good drug compliance. The two adequate antipsychotic trials included at least one adequate trial with a SDA. (iii) On stable dose regimen of clozapine for at least twelve weeks with good drug compliance during that period. Participants with severe neurological illnesses, intellectual disability and sensory impairment, precluding the assessment, were excluded.

Assessment

We employed the following instruments:

1. *Brief Psychiatric Rating Scale (BPRS)*: BPRS covers a broad range of psychopathology including thought disturbance, emotional withdrawal, anxiety, depression, hostility and suspiciousness. It has good psychometric properties[22] and is used widely in clinical settings.
2. *Abnormal Involuntary Movements Scale (AIMS)*: AIMS is a clinical examination and rating scale developed to measure antipsychotic drug induced dyskinesia.[23]
3. *Addenbrooke's Cognitive Examination (ACE-R)*: ACE-R is a brief cognitive test battery, which incorporates the sub-domains, orientation, attention, memory, verbal fluency, language and visuo-spatial. ACE-R has very good internal consistency, convergent validity and is sensitive to early cognitive dysfunction.[24]

4. *WHO Disability Assessment Scale II (WHODAS II)*: WHODAS II assesses the disability status of physically and psychiatrically ill, by evaluating their functioning in the domains of communication, mobility, self-care, interpersonal, life activities, and participation.[25,26]
5. *Childhood Traumatic Events Scale (CTES)*: CTES briefly assesses six early traumatic experiences prior to the age of seventeen such as bereavement, parental discord, violence, sexual abuse, physical illness and other major life events.[27]
6. *Recent Traumatic Events Scale (RTES)*: RTES assesses seven traumatic experiences within the past three years such as bereavement, divorce, violence, sexual abuse, physical illness, occupational change and other major life events.[27]
7. *Premorbid Assessment Scale (PAS)*: PAS is a widely used rating scale to reliably assess premorbid functioning retrospectively.[28]
8. *Socio-demographic, clinical and treatment profile*: We employed a structured questionnaire to collect socio-demographic, clinical and treatment data. We recorded data about developmental delay, urbanization, caffeine consumption, smoking and anthropometric measures. We collected self reported data on clozapine related adverse effects, using a detailed check list.

ACE-R, CTES, RTES and PAS were translated into the local language, Tamil, and then back translated to English by bilingual health professionals, who focused on content, conceptual, semantic as well as technical equivalence. The final Tamil versions were obtained by consensus among the translators.

Data Collection

Every participant was individually assessed for psychopathology (SB) using BPRS. An independent investigator (CC) employed other instruments and assessed various clinical variables by detailed personal interviews with the participants and their primary care givers. We collected peripheral venous blood sample from all participants, 12 hours after their last clozapine dose. Serum clozapine levels were measured by high performance liquid chromatography with ultraviolet detection.[29] Investigators collecting data on outcome (SB) and exposure variables (CC) remained blind to each other's findings till the completion of the study. The protocol of this study was approved by the Institutional Review Board of CMC, Vellore, India. We provided a fact sheet about the details of this study to all participants. We discussed those details

and obtained written informed consent from the participants and from their first-degree relatives or spouses. We accessed the medical records of all participants with their consent.

Data Analyses

We initially analysed the study variables using descriptive statistics. Many studies have defined the response to clozapine by greater than 20% reduction in the total score of BPRS.[30] However, most clinical psychiatrists do not refer to non-response based on a change on any rating scale, but rather on the presence of persistent positive or negative symptoms.[31] Moreover, due to our cross-sectional study design, we defined the response to clozapine, with the widely employed cross-sectional threshold of having BPRS total score of 35 or less.[30,31] We dichotomously categorized the participants, who had BPRS total scores equal to or less than 35, as clozapine responders. We converted other continuous clinical variables to categorical measures, by splitting them into two categories, using their median values.[32] We calculated the odds ratios (OR) with 95% confidence intervals for various hypothesised clinical predictors. When a clinical variable was absent in either the responder or in the non-responder group, we employed the Fisher's exact test to assess its statistical significance, as odds ratios cannot be calculated. We employed multiple logistic regression analyses to calculate adjusted odds ratios (AOR) accounting for the effects of age, gender and serum clozapine levels. We used Nagelkerke pseudo R^2 statistics to know the proportion of variability explained by such statistical model. We used Hosmer-Lemeshow test to assess goodness of fit of the model. We also performed multiple linear regression analyses with BPRS total scores as the dependent variable, adjusted for the effects of age, gender and serum clozapine levels. We analysed our data using statistical software packages, SPSS 16.0 and STATA 12.0.

RESULTS

Participant Characteristics

We assessed 113 consecutive patients, satisfying the eligibility criteria. We excluded six patients, who were not completely compliant with clozapine, within the past 12 weeks. One patient with severe Parkinson's disease and another with moderate mental retardation were also excluded. Among the 105 patients, confirmed to be eligible, 101 consented to participate, making the response rate as 96.2%. Common

reasons for refusing consent were lack of interest in study objectives and reluctance to provide blood samples. Participants (n = 101) and those who were excluded (n = 12) did not differ significantly on gender (χ^2 = 0.04; df = 1; p = 0.84), age (t = −1.41; df = 111; p = 0.16) and on their duration of illness (t = −1.27; df = 111; p = 0.21). There were 65 (64.4%) clozapine responders and 36 (35.6%) non-responders, who had BPRS total scores, 36 or more.

The majority of the sample were male (n = 73; 72.3%), unemployed (n = 60; 59.4%) and living in urban areas (n = 58; 57.4%). Their mean age was 35.43 years (SD 9.43) and their mean Body Mass Index (BMI) was 24.54 (SD 4.64). They had an average of 11.86 years of education (SD 3.89) and an average monthly family income of Indian Rupees (INR) 4733 (SD 6062) [US ≥ 106.36 (SD 136.22)]. Most of the participants had paranoid subtype (n = 85; 84.2%) of schizophrenia. Their mean duration of illness, age of onset of illness and duration of untreated psychosis were 12.40 years (SD 6.77), 23.07 years (SD 7.22) and 11.21 months (SD 13.38) respectively. Their average duration of antipsychotic drug treatment for schizophrenia was 113.64 months (SD 78.46) and their mean duration of clozapine treatment was 41.61 months (SD 39.58; median 28 months; range 4–174 months). Their mean scores on BPRS, ACE-R, WHODAS-II, CTES, RTES and PAS, were 34.73 (SD 12.45), 63.11 (SD 20.78), 17.49 (SD 12.98), 8.32 (SD 10.48), 5.94 (SD 8.71) and 54.83 (SD 21.29) respectively. Mean daily oral dose of clozapine was 340.84 mg/day (SD 119.04; median 350 mg/day; range 100–650 mg/day). Mean serum clozapine level was 550.53 ng/ml (SD 378.46; median 428 ng/ml).

Clinical Predictors of Response to Clozapine

We present the bivariate analyses for clinical predictors of response to clozapine in Table 1. Past history of catatonia, smoking more than one pack/day, excessive sedation and cognitive deficits were significantly associated with non-response to clozapine. We performed multivariate analyses adjusting for the effects of age, gender as well as serum clozapine levels. We provide those adjusted odds ratios in Table 2, which confirmed the findings of earlier bivariate analyses. A multiple logistic regression model including these four variables could explain 31.9% of variability observed in the response to clozapine (Nagelkerke R^2 = 0.319). Hosmer-Lemeshow test confirmed the goodness of fit of this model (χ^2 = 0.25; df = 5; p = 0.99). These four variables could predict the good clinical response to clozapine correctly in 95.4% of participants.

TABLE 1

CLINICAL VARIABLES ASSOCIATED WITH NON-RESPONSE TO CLOZAPINE AMONG THE RESPONDERS (n = 65) AND NON RESPONDERS (n = 36) DURING BIVARIATE ANALYSES (n = 101)

FACTOR	RESPONDERS n (%)	NON-RESPONDERS n (%)	BIVARIATE STATISTICS ODDS RATIO (95% CI)	p-VALUE
Female gender	15 (23.1)	13 (36.1)	1.88 (0.77–4.60)	**0.16**
Never married/ divorced	39 (60.0)	27 (75.0)	2.00 (0.81–4.93)	**0.13**
Completed school education	57 (87.7)	33 (91.7)	1.54 (0.38–6.23)	**0.54**
Family history of schizophrenia	11 (16.9)	6 (16.7)	0.98 (0.33–2.92)	**0.97**
Paranoid subtype	56 (86.2)	29 (80.6)	0.67 (0.23–1.97)	**0.46**
Onset before 18 years of age	17 (26.2)	10 (27.8)	1.09 (0.44–2.71)	**0.89**
Past history of catatonia	**0 (0)**	**5 (13.9)**	–	**0.005**[a]
Any Axis II diagnosis	3 (4.6)	3 (8.3)	1.88 (0.36–9.83)	**0.46**
Duration of illness >5 years	60 (92.3)	30 (83.3)	0.42 (0.12–1.48)	**0.18**
DUP more than one year	17 (26.2)	12 (33.3)	1.41 (0.58–3.43)	**0.45**
Prior poor drug compliance[b]	25 (38.5)	13 (36.1)	0.90 (0.39–2.10)	**0.82**
Past history of ECT	24 (36.9)	13 (36.1)	0.97 (0.41–2.25)	**0.94**
Clozapine level <350 ng/ml	18 (27.7)	11 (30.6)	1.15 (0.47–2.81)	**0.76**
Clozapine duration >1 year	52 (80.0)	25 (69.4)	0.57 (0.22–1.45)	**0.24**
High Caffeine intake[c]	15 (23.1)	9 (25.0)	1.11 (0.43–2.87)	**0.83**
Smoking ≥ one pack/day	**7 (10.8)**	**10 (27.8)**	**3.19 (1.09–9.30)**	**0.03**
Excessive sedation on clozapine[d]	**45 (69.2)**	**32 (88.9)**	**3.56 (1.11–11.40)**	**0.03**
Sialorrhoea on clozapine	28 (43.1)	19 (52.8)	1.48 (0.65–3.35)	**0.35**
AIMS	5 (7.7)	6 (16.7)	2.40 (0.68–8.51)	**0.18**
Childhood trauma[e]	33 (56.8)	15 (41.7)	0.69 (0.30–1.58)	**0.38**
Recent Trauma[f]	26 (40.0)	12 (33.3)	0.75 (0.32–1.76)	**0.51**
Cognitive deficits[g]	**27 (41.5)**	**26 (72.2)**	**3.66 (1.52–8.83)**	**0.004**
Poor premorbid adjustment[h]	28 (43.1)	19 (52.8)	1.48 (0.65–3.35)	**0.35**

[a]Fisher's exact test, Odds ratios could not be calculated; [b]After initiating antipsychotic therapy, had been off medication for longer than six months duration; [c]Three or more cups of coffee or tea intake/day; [d]more than 9 hours of sleep/day; [e]Childhood Traumatic Event Scale total score >4; [f]Recent Traumatic Event Scale total score >2; [g]Addenbrooke's Cognitive Examination—Revised total score <68; [h]Premorbid Adjustment Scale total score >54; DUP: Duration of Untreated Psychosis; AIMS: Abnormal Involuntary Movements; ECT: Electro Convulsive Therapy.

TABLE 2

Multivariate Analyses[a] of the Clinical Predictors for Non-Response to Clozapine, Employing Multiple Outcome Definitions

Outcome definition for clozapine non-response	Non-responders n (%)	Past history of catatonia[a]	Smoking ≥ one pack/day	Excessive sedation[b]	Cognitive deficits[c]	Paranoid subtype	High caffeine intake[d]	Female gender
BPRS total score >35	36 (35.6)	**p = 0.005**[e]	**5.03 (1.52–16.64) p = 0.008**	**4.25 (1.19–15.20) p = 0.03**	**3.64 (1.43–9.23) p = 0.007**	NS	NS	NS
At least one of the five BPRS items[f] >3	30 (29.7)	**18.16 (1.81–182.38) p = 0.01**	NS	NS	NS	NS	NS	NS
BPRS total score ≥38 (worst quartile)	25 (24.8)	**p = 0.001**[e]	NS	NS	**3.71 (1.28–10.79) p = 0.02**	**0.27 (0.08–0.87) p = 0.03**	NS	NS
At least two of the five BPRS items[f] >3	15 (14.9)	**46.34 (4.51–476.66) p = 0.001**	**15.61 (2.24–108.70) p = 0.006**	NS	NS	NS	**7.28 (1.77–29.98) p = 0.006**	**4.54**[g] **(1.30–15.84) p = 0.02**

NS: statistically not significant (p value ≥ 0.05); [a]Multiple logistic regression models to calculate Adjusted Odds Ratios (95% CI), accounting for the effects of age, gender and serum clozapine levels (ng/ml); [b]more than 9 hours of sleep/day; [c]Addenbrooke's Cognitive Examination—Revised total score <68; [d]Three or more cups of coffee or tea intake/day; [e]Fisher's exact test two tailed p value; [f]Suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization and unusual thought content; [g]Adjusted for the effects of age and serum clozapine levels (ng/ml).

Secondary Analyses

We repeated similar analyses using three more outcome definitions for non-response to clozapine. We present the results of those multivariate analyses in Table 2. The clinical predictors varied, when different outcome definitions were employed. However, past history of catatonia, defined by one or more catatonic symptoms listed in DSM IV-TR, remained as a consistent clinical predictor of non-response, while using all definitions. In order to dispel the concerns over the dichotomization of response to clozapine, we performed multiple linear regression analyses with BPRS total scores as the dependent variable, adjusted for the effects of age, gender and serum clozapine levels. Past history of catatonia ($\beta = 22.75$; SE = 5.16; t = 4.41; p = 0.001), smoking more than one pack/day ($\beta = 5.40$; SE = 2.64; t = 2.05; p = 0.04) and cognitive deficits ($\beta = 7.73$; SE = 2.41; t = 3.20; p = 0.002) were significantly associated with higher BPRS total scores. The clozapine responders and non-responders, who had BPRS total scores, 36 or more, did not differ on their prescription for any psychotropic co-medication ($\chi^2 = 0.34$; df = 1; p = 0.56).

Correlates of Adverse Events

Our participants had the following adverse events, related to clozapine, sialorrhoea (n = 47; 46.5%), nausea or vomiting (n = 21; 20.8%), constipation (n = 21; 20.8%), erectile dysfunction (13 men; 27.7%), dyslipidemia (n = 12; 11.9%), seizures (n = 9; 8.9%) and nocturnal enuresis (n = 6; 5.9%). Fifteen participants (14.9%) were obese (BMI $\geq$ 30) and 23 (22.8%) were overweight (25 $\leq$ BMI $<$ 30). As we never re-challenge patients, who have developed neutropenia on clozapine, none of our participants had past history of neutropenia or agranulocytosis. The following variables were significantly associated with each other, after adjusting for the effects of serum clozapine levels, by multiple logistic regression: age above 42 years and clozapine related seizures (AOR 6.37; 95% CI 1.32–30.78); worst quartile BPRS total scores ($\geq$38) and nocturnal enuresis (AOR 7.24; 95% CI 1.22–42.87); poor premorbid adjustment scores and obesity (AOR 3.80; 95% CI 1.11–12.94).

Correlates of Disability

BPRS total scores ($\beta = 0.22$; SE = 0.10; t = 2.09; p = 0.04) had significant linear relationship with WHODAS-II 12 item total disability scores. The median value of WHODAS-II total scores of our partici-

pants was 15 (range 0–48). Participants, who scored more than that threshold, were categorized to have higher disability. Female gender (AOR 2.69; 95% CI 1.08–6.70) and cognitive deficits (AOR 3.41; 95% CI 1.44–8.10) were significantly associated with higher disability after adjusting for the effects of age and serum clozapine levels.

DISCUSSION

This study investigated the clinical predictors of response to clozapine among patients with TRS. Our results suggest that past history of catatonia, smoking more than one pack/day, hyper-somnolence and cognitive deficits were associated with non-response to clozapine. We documented that the clinical predictors varied, depending on the outcome definitions for non-response to clozapine.

Strengths and Limitations

The strengths of this study include, exclusively recruiting patients with TRS, relatively larger[14–19] sample size, minimal refusal rate, estimation of serum clozapine levels and employing standard instruments to assess premorbid adjustment, traumatic life events, cognition and disability. Consecutive sampling strategy reduced the possibility of selection bias. The independent assessment of exposure and outcome variables by two different investigators minimized potential observer bias. Interviewing primary caregivers of the participants and verifying their follow-up medical records reduced the possibility of recall bias on the reported clinical variables. Clinical predictors of response to clozapine often overlap with clinical predictors of poor prognosis in schizophrenia.[6] Exclusively recruiting patients with TRS made our results more specific.

The potential limitations of this study include the cross-sectional clinical assessment of response to clozapine and the temporal ambiguity. Our participants were on clozapine for a mean duration of 41.61 months. We recruited only the participants, who were maintained on stable dosage of clozapine for a minimum duration 12 weeks, when their treating psychiatrists did not need to change their prescription. Hence, their cross-sectional BPRS scores were more indicative of the persistent psychopathology than of any acute fluctuations in their illnesses. As patients with TRS may respond to clozapine after a delay of 24–32 weeks,[15,33] short-term longitudinal studies, with less than 12 weeks of follow up, also had similar temporal uncertainties.[14,18] Considering the pragmatic constraints and the paucity of pertinent data in resource-poor settings, we determined the response to clozapine

among our participants with the cross-sectional BPRS assessment, employing multiple outcome definitions.

The dichotomous categorization of clinical response to clozapine is debatable. Despite many researchers define the response to clozapine by the reduction in the total scores of BPRS, most clinical psychiatrists prefer to use the discrete clinical category of non-response based on the presence of persistent positive or negative symptoms.[31] Clinical significance of many statistically significant reductions in the total scores of rating scales is uncertain.[34] Hence, we analyzed multiple categorical outcome definitions and BPRS total scores, as dependent variables, by appropriate multiple logistic regression and multiple linear regression models to confirm our findings. Despite the extensive use of BPRS, we should acknowledge that there are more diverse outcome measures to assess the treatment responses in schizophrenia.[35]

Disparities among Clinical Predictors

Female gender,[17] earlier age of onset,[17,21] non-paranoid subtype,[36] longer duration of illness,[11] baseline psychopathology,[14,16,37] baseline quality of life,[37] serum clozapine levels below 350 ng/ml[11] and poor functioning during the previous year[6,15] have already been reported as the potential clinical predictors of non-response to clozapine. Findings on the utility of past history of neuroleptic induced extra pyramidal symptoms[11,16,38] and of baseline negative symptoms[16,21] as clinical predictors were contradictory. Ethnic diversity, population characteristics, lack of structured assessment of clinical variables, differing outcome definitions and causal heterogeneity of response to clozapine may explain the disparities between these findings. Our study was negative for these potential clinical predictors, but it has brought forth four clinical variables, which could explain 31.9% of variability in the treatment response to clozapine. When we employed alternative outcome definitions, female gender, high caffeine intake and non-paranoid subtype were also associated with non-response to clozapine. Our results add evidence that differing outcome definitions can, at least partly, explain the disparities among the previous studies on this topic.

Schizophrenia is not a single disease, but a heterogeneous disorder, caused by multiple genetic as well as environmental factors.[39] Pharmacokinetics and pharmacodynamics of clozapine is complex.[40] Hence, any statistical model with limited clinical or biological predictors often explains only a small proportion of variability among the responses to clozapine in TRS. Differing BPRS based response cut-off thresholds are known to cause significant variability in the results of antipsychotic drug trials.[41] Though a primary BPRS cut-off score,

based on clinical relevance, should be chosen a priori, further analyses with wider range of cut-off scores should be presented to explain more variability in the treatment response.[41] Hence, our results support the need for employing multiple outcome definitions to address the heterogeneity of TRS and of its response to clozapine.

Catatonia and Response to Clozapine

Debate on the nosological status of catatonia, as a sub-type of schizophrenia or as a separate syndrome, remains unresolved. The patients who have both schizophrenia and catatonia may have unique underlying pathophysiology.[42] Reports on catatonia, increasing the risk for clozapine induced neuroleptic malignant syndrome,[43] and on clozapine withdrawal catatonia[44] are available. Unlike the earlier case reports, stating good response to clozapine in patients with recurrent catatonia,[45,46] all participants with past history of catatonia (n = 5) were clozapine non-responders in this study. Investigating the relationship between catatonia and its response to clozapine as well as the possible underlying neurobiological processes are warranted.

Clinical Recommendations for Modifiable Risk Ffactors

Smoking reduces serum clozapine levels.[47] However, available literature on the relationship between smoking and the response to clozapine are contradictory.[48,49] Studies associating smoking with non-response to clozapine emphasize its ability to induce Cytochrome P-450 1 A2 (CYP1 A2) enzyme and to reduce serum clozapine levels.[48] Studies associating smoking with good response to clozapine focus on its ability to activate nicotinic receptors.[49] Our results have documented that, smoking more than 20 cigarettes/day, increased the risk of non-response to clozapine by five times, after adjusting for the effects of age, gender and serum clozapine levels. We infer that the relationship between smoking and non-response to clozapine cannot be solely explained by reduced serum clozapine levels. We highlight smoking as a potentially modifiable risk factor and suggest nicotine deaddiction for all smoking patients with TRS to augment their clozapine response. As concerns over abrupt smoking cessation, which may suddenly elevate the serum clozapine levels to toxic range, exist, we suggest appropriate titration of clozapine dosage against its serum levels during smoking cessation.[50]

Daily consumption of caffeinated beverages is common globally. Our findings suggest the need for motivating all patients on clozapine, especially those who have poor response to clozapine, to reduce their

caffeine consumption, for augmenting their clinical response.[48] Akin to previous studies reporting the ability of cognitive factors discriminating clozapine responders and non-responders,[51] we found cognitive deficits as an important predictor for non-response. We also documented that cognitive dysfunction was associated with higher disability. We suggest augmenting the clinical response to clozapine with appropriate cognitive interventions, for such disabled patients. Despite temporal ambiguity, hyper-somnolence was associated with non-response to clozapine. Complaints of excessive sedation on clozapine are often overlooked during clinical practice. Appropriate behavioural management and adjuvant psychopharmacological agents to reduce excessive sedation should be considered.

CONCLUSIONS

The inherent heterogeneity within the clinical category of TRS obscures the search for the clinical and biological predictors for its response to clozapine. Syndrome sub-categorization employing biological variables is desired to achieve the etiological homogeneity of schizophrenia.[52] Appropriate biological parameters to categorize response to clozapine have to be identified for developing better prediction models with clinical or biological variables. As *explanatory pluralism* is the need of the hour,[53] it is high time to reduce the wide gulf between the clinical and pharmacogenetic research on this topic. Combining clinical and pharmacogenetic predictors may identify more patients, who are most likely to benefit with clozapine, and may prevent the unnecessary exposure of many potential non-responders to serious adverse effects. We suggest future longitudinal studies, investigating both clinical and pharmacogenetic factors together with multiple outcome definitions, to predict response to clozapine in patients with TRS. ✤

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Clinical predictors of serum clozapine levels in patients with treatment-resistant schizophrenia

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Fixed oral doses of clozapine produce up to 45-fold interindividual variability among its serum levels in patients with treatment-resistant schizophrenia. Although the relationship between serum clozapine level and its therapeutic response is uncertain, the presence of a therapeutic window and level-dependent adverse effects require the estimation of serum clozapine levels. As routine therapeutic drug monitoring of clozapine is not feasible in many clinical settings, identification of clinical predictors of serum clozapine levels is desirable. Hence, we aimed to evaluate the clinical variables associated with serum clozapine levels. We assessed the sociodemographic and clinical profiles, cognition, disability and psychopathology of 101 consecutive patients with treatment-resistant schizophrenia on a stable dose of clozapine, using standard assessment schedules. We determined their serum clozapine levels using high-performance liquid chromatography with ultraviolet detection. While employing multivariate robust regression models, oral clozapine dose ($P<0.001$), caffeine intake ($P=0.04$) and Valproate comedication ($P=0.005$) were associated with serum clozapine levels. Serum clozapine levels above 750 ng/ml increased the risk of seizures (odds ratio 5.15; $P=0.03$). Clinical variables are useful to model a dosing nomogram for serum clozapine levels. The importance of caffeine consumption and Valproate comedication should be considered during clozapine dose adjustments to enhance its therapeutic response and safety profile. *Int Clin Psychopharmacol* 28:50–56

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Introduction

Clozapine is the most preferred antipsychotic drug for the management of treatment-resistant schizophrenia (TRS) because of its superior clinical efficacy (Kane *et al.*, 1988), its ability to reduce negative symptoms and its low propensity for movement disorders (Tandon and Fleischhacker, 2005). However, it may lead to a suboptimal response in 40–70% patients (Remington *et al.*, 2005) and serious adverse events such as seizures, agranulocytosis and dyslipidaemia (Henderson *et al.*, 2000). Fixed oral doses of clozapine can produce up to 45-fold interindividual variability in serum levels (Potkin *et al.*, 1994). Many patients with suboptimal therapeutic responses to clozapine have inadequate serum clozapine levels and their responses can be enhanced by ensuring serum clozapine levels above a minimum threshold value (Miller *et al.*, 1994; Potkin *et al.*, 1994; Kronig *et al.*, 1995). Moreover, serum clozapine levels above a maximum threshold value increase the risk for its toxicity (Ulrich *et al.*, 2003) and seizures (Cooper, 1996). Such a therapeutic window and the predisposition for high interindividual variability mandate the estimation of serum clozapine levels, especially in patients who show poor response to treatment with clozapine, who are intolerant to usual therapeutic doses and who require clozapine dose titration above 600 mg/day (Cooper, 1996; Bell *et al.*, 1998).

The lower limit of this therapeutic window for serum clozapine levels is considered between 250 and 420 ng/ml (Miller *et al.*, 1994; Potkin *et al.*, 1994; Kronig *et al.*, 1995; Cooper, 1996; Bell *et al.*, 1998; Perry *et al.*, 1998; Ulrich *et al.*, 2003). The minimum threshold value for serum clozapine level is suggested to be 350 ng/ml (Miller *et al.*, 1994; Kronig *et al.*, 1995; Perry *et al.*, 1998) for acute treatment with clozapine, which can be lower for maintenance therapy (Gaertner *et al.*, 2001). Above this cut-off value, the relationship between serum clozapine level and clinical response to clozapine remains uncertain (Kronig *et al.*, 1995; Cooper, 1996; Wong *et al.*, 2006). Besides, routine therapeutic drug monitoring of clozapine is not feasible in many clinical settings, especially in low-income and middle-income countries (Greenwood-Smith *et al.*, 2003). Hence, identification of clinical predictors, which act as proxy measures of serum clozapine levels, is desirable during everyday clinical practice in such settings (Perry *et al.*, 1998).

Studies examining the clinical factors associated with serum clozapine levels have focused on age, sex or on smoking so far, and their results have not been consistent

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(Haring *et al.*, 1989; Centorrino *et al.*, 1994; Lane *et al.*, 1999; Palego *et al.*, 2002; Rostami-Hodjegan *et al.*, 2004; Tang *et al.*, 2007). Pharmacogenetic studies evaluating the association between serum clozapine levels and single nucleotide polymorphisms of the specific candidate genes, responsible for the metabolism of clozapine, have also been inconclusive (Van der Weide *et al.*, 2003; Kootstra-Ros *et al.*, 2005; Jaquenoud Sirot *et al.*, 2009). Most of these studies did not exclusively recruit patients with TRS and evaluated only a very limited number of variables. They also did not carry out structured assessments of cognition, disability and adverse effects (Haring *et al.*, 1989; Centorrino *et al.*, 1994; Lane *et al.*, 1999; Palego *et al.*, 2002; Van der Weide *et al.*, 2003; Rostami-Hodjegan *et al.*, 2004; Kootstra-Ros *et al.*, 2005; Tang *et al.*, 2007; Jaquenoud Sirot *et al.*, 2009). Despite the presence of ethnic differences in the serum clozapine levels (Ng *et al.*, 2005), there is a dearth of relevant data from Asian countries, where there is a clinical need for proxy measures of serum clozapine levels (Tang *et al.*, 2007). Hence, we aimed to investigate the associations between various clinical variables and serum clozapine levels in patients with TRS in India.

Methods

Study design

We used a cross-sectional study to evaluate the clinical factors associated with serum clozapine levels. We used a case–control design framework to identify the clinical factors associated with inadequate serum clozapine levels below 350 ng/ml.

Setting

We carried out this study at the Department of Psychiatry, Christian Medical College, Vellore, India, a tertiary referral centre for the management of psychiatric disorders. The hospital has short-term inpatient services, daily outpatient and regular follow-up clinics. Patients with schizophrenia are initially treated with either dopamine antagonists or serotonin dopamine antagonists. Clozapine is never used as the first-line antipsychotic drug and is reserved for patients with TRS. Detailed medical records of treatment are maintained for all patients. Leucocyte counts and metabolic parameters of the patients receiving clozapine are monitored periodically. Most of our outpatients with schizophrenia live in the community with their families. Their medications are directly provided by their first-degree relatives or spouses, who report any degree of nonadherence to the treating psychiatrists during periodically scheduled follow-up visits.

Recruitment of participants

We invited all consecutive patients who fulfilled the following eligibility criteria: (i) *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed., Text Revision diagnosis of schizophrenia, (ii) treatment resistance established after failure to respond to at least two adequate antipsychotic trials, as documented by treating psychiatrists. An adequate antipsychotic trial was defined by 600 mg chlorpromazine equivalents for a duration of at least 6 weeks, with good drug compliance. The two adequate antipsychotic trials included at least one adequate trial with an serotonin dopamine antagonist. (iii) On stable dose regimen of clozapine for at least 12 weeks, with good drug compliance during that period. Participants with severe neurological illnesses, intellectual disability and sensory impairment, before the assessment, were excluded.

Serum clozapine assay

Peripheral venous blood samples were collected from all participants by venipuncture 12 h after their last clozapine dose. Serum clozapine levels were measured by deproteinization with diethyl ether and subsequent high-performance liquid chromatography with ultraviolet detection (Wongsinsup *et al.*, 2010). The serum clozapine levels were expressed as ng/ml.

Clinical assessment

We used a structured questionnaire to collect sociodemographic, clinical and treatment data. We recorded data on comorbidity, comedication, caffeine as well as grape juice consumption, smoking and anthropometric measures. We collected self-reported data on clozapine-related adverse effects using a check list of adverse effects. We also used the following instruments: (i) the Brief Psychiatric Rating Scale (BPRS) to assess psychopathology (Overall and Gorham, 1962), (ii) the Abnormal Involuntary Movements Scale (AIMS) to measure neuroleptic induced dyskinesia, if present (Guy, 1976), (iii) Addenbrooke's Cognitive Examination-Revised (ACE-R), a brief cognitive test battery to evaluate cognitive functioning (Mioshi *et al.*, 2006), and (iv) WHO Disability Assessment Scale-II (WHODAS-II), to quantify the disability (WHO, 2001).

The protocol of the study was approved by the Institutional Review Board of Christian Medical College, Vellore, India. We provided a fact sheet about the details of this study to all participants. We discussed these details and obtained written informed consent from the participants and from their first-degree relatives or spouses. We assessed every participant for psychopathology using the BPRS. Another independent investigator, who was blinded to the serum clozapine levels, assessed other clinical variables through detailed personal interviews with the participants and their primary caregivers. She accessed the medical records of all participants with their consent. We followed standard quality control procedures to ensure the accuracy of our data collection, data entry and of the serum clozapine assay.

Data analyses

We initially analysed the study variables using descriptive statistics. We checked whether all continuous variables were normally distributed using one-sample Kolmogorov–Smirnov tests. As the distribution of serum clozapine

levels was nonparametric, we used Spearman's correlation analyses to assess the bivariate correlation between serum clozapine levels and continuous clinical variables. We studied the associations between serum clozapine levels and various hypothesized clinical explanatory variables with robust regression models, using the STATA *rreg* command. Robust regression models are valid, despite the presence of influential outliers and the non-normality of residuals. Ordinary least squares regression was initially performed to compute absolute residuals, which were scaled by the median residual value. After estimating Huber weights and Tukey biweights, iteratively reweighted least squares regression was performed to estimate the regression coefficients. We also determined multiple robust regression statistics to adjust for the effects of age, sex and oral clozapine dose. We developed a multivariate model with all predictors that were significant during bivariate analyses.

Then, we dichotomously categorized the serum clozapine levels into adequate levels and inadequate levels on the basis of the minimum threshold value of 350 ng/ml (Miller *et al.*, 1994; Kronig *et al.*, 1995; Perry *et al.*, 1998). The outcome variable was inadequate serum clozapine level and the predicting variables were the hypothesized clinical variables. We calculated the odds ratios (ORs) with 95% confidence intervals (CIs). We used multiple logistic regression models to calculate adjusted ORs accounting for the effects of age, sex and oral clozapine dose. We used Nagelkerke pseudo R^2 statistics to determine the proportion of variability explained by the model and the Hosmer–Lemeshow test to assess the goodness of fit of the model. We analysed our data using the statistical software packages, SPSS 16.0 (IBM, New York, New York, USA) and STATA 12.1 (StataCorp, College station,Texas, USA).

Sample size estimation

An earlier study has documented a correlation coefficient, between serum clozapine level and oral clozapine dose, as high as 0.7 (Centorrino *et al.*, 1994). We estimated the required sample size with an anticipated correlation coefficient of 0.3, for the clinical variables, 80% power and 5% α error as 79 for a two-sided test.

Results

Sample characteristics

We assessed 113 consecutive patients who fulfilled the eligibility criteria. We excluded six patients, who were not completely compliant with clozapine, within the past 12 weeks. One patient with severe Parkinson's disease and another with moderate mental retardation were also excluded. Among the 105 patients found to be eligible, 101 consented to participate, yielding a participation rate of 96.2%. Common reasons for refusing consent were lack of interest in study objectives and reluctance to provide blood samples. Participants ($n = 101$) and those who were excluded ($n = 12$) did not differ significantly in terms of sex ($\chi^2 = 0.04$; $d.f. = 1$; $P = 0.84$), age ($t = -1.41$; $d.f. = 111$; $P = 0.16$) and duration of illness ($t = -1.27$; $d.f. = 111$; $P = 0.21$). Table 1 presents the sociodemographic and clinical profiles of all participants. The majority of the participants were single or separated men, unemployed and were living in urban areas ($n = 58$; 57.4%). Most of the participants had a chronic continuous course of paranoid schizophrenia. The oral doses of clozapine ranged from 100 to 650 mg/day, with a median value of 350 mg/day. The duration of clozapine treatment ranged from 4 to 174 months, with a median value of 28 months. More than half of the participants (53.5%) had received clozapine for more than 2 years.

Table 1 Sociodemographic and clinical profiles of the participants ($N = 101$)

Characteristics	*N* (%)	Mean (SD)
Male sex	73 (72.3%)	–
Marital status: single/separated	66 (65.3%)	–
Currently unemployed	60 (59.4%)	–
Family history of schizophrenia	17 (16.8%)	–
Paranoid subtype	85 (84.2%)	–
Past history of catatonia	5 (4.9%)	–
Presence of abnormal involuntary movements	11 (10.9%)	–
High caffeine consumption[a]	24 (23.8%)	–
Current smoking	19 (18.8%)	–
Concurrent Fluoxetine or Sertraline intake	8 (7.9%)	–
Concurrent Valproate intake	9 (8.9%)	–
Concurrent Carbamazepine intake	0 (0%)	–
Concurrent oral contraceptive pills	0 (0%)	–
Age (years) (range 20–60)	–	35.4 (9.4)
Number of years of education	–	11.9 (3.9)
Monthly family income (INR)	–	4733 (6062)
BMI	–	24.5 (4.6)
Age of onset of illness (years)	–	23.1 (7.2)
Duration of illness (years)	–	12.4 (6.8)
Duration of untreated psychosis (months)	–	11.2 (13.4)
Total duration of drug treatment (months)	–	113.6 (78.5)
Total duration of clozapine (months)	–	41.6 (39.6)
Current oral dose of clozapine (mg/day)	–	341 (119)
BPRS total score	–	34.7 (12.5)
ACE-R total score	–	63.1 (20.8)
WHODAS-II total score	–	17.5 (13.0)

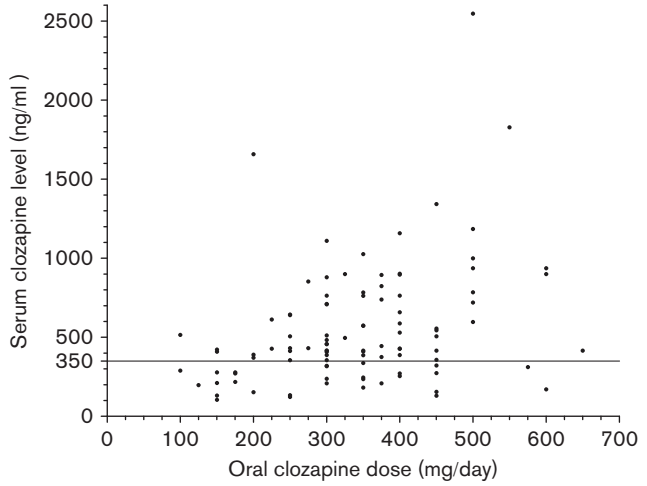
ACE-R, Addenbrooke's Cognitive Examination-Revised; BPRS, Brief Psychiatric Rating Scale; WHODAS-II, WHO Disability Assessment Scale-II.
[a]Three or more cups of coffee or tea intake/day.

Clinical variables associated with serum clozapine levels

Serum clozapine levels of the participants ranged from 104 to 2547 ng/ml, with a median value of 428 ng/ml. The mean serum clozapine level was 550.5 (SD 378.5) ng/ml. Twenty-nine participants (28.7%) had serum clozapine less than 350 ng/ml. Twenty-four participants (23.8%) had serum clozapine levels above 750 ng/ml. The mean serum clozapine level/oral dose ratio was 1.71 (SD 1.12). There was a 30-fold interindividual variability among the participants, with their clozapine level/dose ratio ranging between 0.28 and 8.29. We present the association between oral doses of clozapine and serum clozapine levels in Fig. 1. Table 2 presents the bivariate as well as multivariate analyses for the associations between various clinical variables and serum clozapine levels. Oral doses of

clozapine and Valproate comedication were associated positively with serum clozapine levels. Those who consumed three or more cups of coffee or tea everyday had significantly lower serum clozapine levels. Age, sex, body weight and smoking were not associated with serum clozapine levels in our sample. Our participants were on the following comedications: Risperidone ($n = 10$; 9.8%), Haloperidol ($n = 6$; 5.9%), Quetiapine ($n = 2$; 2.0%), Aripiprazole ($n = 4$; 3.9%), Trihexyphenidyl ($n = 12$; 11.8%) and Clonazepam ($n = 7$; 6.9%). None of these comedications was significantly associated with serum clozapine levels in our sample. Table 3 shows a multiple robust regression model predicting serum clozapine levels ($F = 9.78$; $P < 0.001$). We derived the following equation from this model: serum clozapine level (ng/ml) = 194.51 + 0.91 (oral clozapine dose in mg/day) – 130.45 (if high caffeine intake is present) + 247.93 (if Valproate comedication is present).

Fig. 1



Association between oral clozapine doses and serum clozapine levels.

Multiple logistic regression models examining the clinical variables associated with serum clozapine levels below 350 ng/ml showed that age, sex, body weight, smoking, caffeine intake, duration of illness, age of onset of illness, duration of treatment with clozapine, tardive dyskinesia, fluoxetine or sertraline and Valproate comedication were not significantly associated with serum clozapine levels below 350 ng/ml. Oral doses of clozapine less than 250 mg/day increased the risk of inadequate serum levels by four times (OR 4.27; 95% CI 1.47 – 12.41; $P = 0.008$), after adjusting for the effects of age and sex. All participants who were on Valproate comedication had serum clozapine levels above 350 ng/ml. A multiple logistic regression model including an oral clozapine dose below 250 mg/day, Valproate comedication and high caffeine intake could predict the inadequate serum clozapine levels correctly in 73.3% of participants. The Hosmer–Lemeshow test confirmed the goodness of fit of this model ($\chi^2 = 0.08$; *d.f.* = 2; $P = 0.96$; Nagelkerke pseudo $R^2 = 0.196$).

Clinical utility of serum clozapine levels

The relationship between serum clozapine levels and psychopathology of the participants, as measured by the BPRS total scores, was not significant, after adjusting for the oral dose of clozapine ($\beta = 0.12$; SE = 2.21; $t = 0.06$; $P = 0.95$). Higher serum clozapine levels were associated with worse cognitive functioning, as measured by ACE-R

Table 2 **Factors influencing serum clozapine level of the participants (N=101)**

	Bivariate statistics[a]				Multivariate statistics[b]			
Explanatory variables	β	SE	t	P	β	SE	t	P
Male sex	–14.87	63.27	–0.24	0.82	–31.63	60.36	–0.52	0.60[c]
Age (years)	–0.55	3.03	–0.18	0.86	–1.12	2.87	–0.39	0.70[d]
Body weight (kg)	2.41	2.27	1.06	0.29	3.63	2.23	1.63	0.11
BMI (kg/m^2)	4.57	6.11	0.75	0.46	8.75	6.09	1.44	0.15
Current oral dose of clozapine (mg/day)	**0.83**	**0.23**	**3.69**	**<0.001**	**0.84**	**0.23**	**3.67**	**<0.001[e]**
Total duration of clozapine (months)	0.75	0.71	1.06	0.29	1.13	0.70	1.62	0.11
Current smoking	–50.14	72.21	–0.69	0.49	–121.92	74.65	–1.63	0.11
Total smoking pack years	–3.89	3.94	–0.99	0.33	–5.00	3.86	–1.30	0.20
High caffeine consumption[f]	–63.40	66.68	–0.95	0.34	**–126.84**	**63.21**	**–2.01**	**0.04**
Duration of illness (years)	–2.93	4.22	–0.69	0.49	–5.40	5.28	–1.02	0.31
Age of onset of illness (years)	1.82	3.95	0.46	0.65	6.12	5.30	1.15	0.25
Duration of untreated psychosis (months)	1.84	2.12	0.87	0.39	0.34	2.05	0.16	0.87
Paranoid subtype	–5.39	77.78	–0.07	0.95	–4.85	75.10	–0.06	0.95
Concurrent Fluoxetine or Sertraline intake	–17.40	105.42	–0.17	0.87	–46.73	100.33	–0.47	0.64
Concurrent Valproate intake	**265.97**	**92.68**	**2.87**	**0.005**	**263.80**	**91.40**	**2.89**	**0.005**

Bold numerals represent $P<0.05$.
[a]Robust regression models with serum clozapine level (ng/ml) as the dependent variable.
[b]Multiple robust regression models with serum clozapine level (ng/ml) as the dependent variable adjusted for the effects of age (years), gender (male) and oral clozapine dose (mg/day).
[c]Adjusted for the effects of age (years) and oral clozapine dose (mg/day).
[d]Adjusted for the effects of sex (male) and oral clozapine dose (mg/day).
[e]Adjusted for the effects of age (years) and sex (male).
[f]Three or more cups of coffee or tea intake/day.

Table 3 Multivariate model[a] explaining the serum clozapine level of the participants ($N=101$)

Model predictors	β	95% CI for β	SE	t	P
Constant	194.51	42.14 – 346.89	76.77	2.53	0.01
Current oral dose of clozapine (mg/day)	**0.91**	**0.48–1.34**	**0.22**	**4.19**	**<0.001**
High caffeine consumption[b]	**–130.45**	**–249.98 to –10.93**	**60.22**	**–2.17**	**0.03**
Concurrent Valproate intake	**247.93**	**42.14–346.89**	**89.06**	**2.78**	**0.006**

Bold numerals represent $P<0.05$.
CI, confidence interval.
[a]Multiple robust regression model with serum clozapine level (ng/ml) as the dependent variable.
[b]Three or more cups of coffee or tea intake/day.

total scores, after adjusting for the effects of age and oral clozapine dose ($\beta = -0.011$; SE = 0.005; $t = -2.07$; $P = 0.04$). However, this relationship was not significant when psychopathology of the participants was also included in this model ($\beta = -0.006$; SE = 0.005; $t = -1.17$; $P = 0.25$). Similarly, serum clozapine levels were not associated with disability, as measured by WHODAS-II total scores, after adjusting for the effects of age, oral clozapine dose and psychopathology ($\beta = 0.006$; SE = 0.004; $t = 1.68$; $P = 0.10$). Among those who had serum clozapine levels below 350 ng/ml ($n = 29$), BPRS total scores were significantly associated with worse cognitive outcomes ($\beta = -0.65$; SE = 0.20; $t = -3.33$; $P = 0.003$). Serum clozapine levels were not associated with AIMS total scores after adjusting for the effects of age and oral clozapine dose ($\beta = -0.003$; SE = 0.002; $t = -1.46$; $P = 0.15$). Serum clozapine levels above 750 ng/ml increased the risk of seizures by five times after adjusting for the effects of age and oral clozapine dose (OR 5.15; 95% CI 1.11–23.88; $P = 0.03$). Other adverse effects such as hypersomnolence, sialorrhoea, neutropenia, sexual dysfunction, constipation, nausea, nocturnal enuresis and obesity were not associated with serum clozapine levels.

Discussion

This study examined the clinical predictors of serum clozapine levels among patients with TRS. The strengths of this study include exclusive recruitment of treatment-resistant patients, adequate sample size, minimal refusal rate, structured assessment of clinical variables and the use of nonparametric robust regression models for analyses. Consecutive sampling strategy minimized the possibility of selection bias. An independent assessment of serum clozapine levels and clinical variables by two different investigators reduced the possibility of observer bias. Interview of primary caregivers of the participants and verification of their follow-up medical records reduced the possibility of recall bias on the reported clinical variables. The potential limitations of this study are its cross-sectional design and lack of assessment of serum levels of the clozapine metabolite, norclozapine. The optimum study design for evaluating the lower limit of the therapeutic window for serum clozapine levels is a prospective double-blind trial, where participants are randomly assigned to different groups and are treated with varying oral doses of clozapine, which would yield predefined ranges of serum clozapine levels (VanderZwaag *et al.*, 1996; Hiemke *et al.*, 2011).

Clinical predictors of serum clozapine levels

Our results show that higher oral doses of clozapine as well as Valproate comedication are associated with higher serum clozapine levels and that high caffeine intake reduces serum clozapine levels. Previous studies have reported that the oral clozapine dose is the only clinical variable consistently associated with serum clozapine levels, but is insufficient to explain the high interindividual variability of serum levels (Haring *et al.*, 1989; Centorrino *et al.*, 1994; Perry *et al.*, 1998; Lane *et al.*, 1999; Palego *et al.*, 2002; Rostami-Hodjegan *et al.*, 2004; Tang *et al.*, 2007). Female sex has been associated with higher clozapine levels (Haring *et al.*, 1989; Centorrino *et al.*, 1994; Lane *et al.*, 1999; Ulrich *et al.*, 2003; Tang *et al.*, 2007), but this association has been questioned (Palego *et al.*, 2002, Diaz *et al.*, 2008). Increasing age has been associated with higher clozapine levels (Haring *et al.*, 1989; Ulrich *et al.*, 2003), but evidence to the contrary also exists (Centorrino *et al.*, 1994; Palego *et al.*, 2002; Tang *et al.*, 2007; Diaz *et al.*, 2008). Our study did not find any association between serum clozapine levels and age or sex in our sample. There are studies supporting (Haring *et al.*, 1989; Perry *et al.*, 1998) and challenging (Tang *et al.*, 2007) the association between smoking and lower serum clozapine levels. As the prevalence of smoking was low in our sample (18.8%) and we did not have any female smokers, because of cultural reasons, our study was probably underpowered to detect any association between smoking and serum clozapine levels.

We confirmed the association between Valproate comedication and higher serum clozapine levels (Facciola *et al.*, 1999; Wong *et al.*, 2006; Diaz *et al.*, 2008). The literature available on the association between caffeine consumption and serum clozapine levels is contradictory. Previous smaller studies have suggested that caffeine either reduces (Dratcu *et al.*, 2007) or increases (Hagg *et al.*, 2000; Raaska *et al.*, 2004) serum clozapine levels, because of the competitive inhibition of the CYP1A2 hepatic enzyme. Our results have clarified that caffeine consumption is associated with lower serum clozapine levels. Underlying pharmacokinetic pathways warrant further investigation. As smoking and drinking caffeinated beverages are positively correlated (Rihs *et al.*, 1996), their

combined influence over CYP1A2 hepatic enzyme activity needs to be investigated in more detail. Constituents in the caffeinated beverages, other than caffeine, may also induce CYP1A2 enzyme activity. Selective serotonin reuptake inhibitors (SSRI) such as Fluvoxamine (Diaz *et al.*, 2008) and Paroxetine (Spina *et al.*, 2000) have been reported to be associated with higher serum clozapine levels. Eight of our participants, who were on SSRI comedication, were receiving either Fluoxetine or Sertraline. We did not find any association between these SSRI comedications and serum clozapine levels.

Serum clozapine levels and treatment-resistant schizophrenia

Higher serum clozapine levels have been reported to be associated with greater cognitive impairment in patients with chronic schizophrenia (Adler *et al.*, 2002) after adjusting for the effects of age, sex and oral dose (Rajji *et al.*, 2010). We replicated this finding in our sample, but further clarified that this observed association was confounded by the psychopathology of the participants. We documented that the bivariate positive correlation, between psychopathology and serum clozapine levels, was spurious, because of the confounding effect of oral clozapine doses. Patients who have severe psychopathology develop more cognitive deficits, and they require more oral doses of clozapine, producing higher serum clozapine levels. Hence, we argue against any direct causal relationship between serum clozapine levels and cognitive impairment in patients with chronic schizophrenia. Similarly, our findings suggest that the observed relationship between serum clozapine levels and disability in patients with chronic schizophrenia was also confounded by the psychopathology of the participants. A previous study has hypothesized that patients with tardive dyskinesia would differ in their metabolism of clozapine and would have higher serum clozapine levels (Pollack *et al.*, 1993). Our results disprove this hypothesis with appropriate multivariate analyses.

Clinical recommendations

The literature and our results suggest the following:

(1) Serum clozapine levels, above the minimum threshold value of its therapeutic window, maximize the clinical response to clozapine (Miller *et al.*, 1994; Potkin *et al.*, 1994; Kronig *et al.*, 1995), reduce the lag time to response (Perry *et al.*, 1998) and reduce the relapse rates (Ulrich *et al.*, 2003). We presented an equation to predict serum clozapine levels using three clinical variables. In clinical settings where routine therapeutic drug monitoring of clozapine is not feasible, this equation can aid the treating psychiatrists during dose adjustments of clozapine.
(2) We highlight the clinical need for appropriate clozapine dose reductions while adding Valproate comedication to reduce level-dependent adverse effects (Nielsen *et al.*, 2011).
(3) Patients worldwide consume caffeinated beverages everyday and their importance is often neglected during clinical dose adjustments of clozapine (Dratcu *et al.*, 2007). Patients should be advised to inform their psychiatrists when they make any abrupt changes in their caffeine habits (Carrillo *et al.*, 1998). Our dosing equation may help the psychiatrists to consider appropriate clozapine dose adjustments in such situations.
(4) Oral doses of clozapine above 600 mg/day are known to increase the risk of seizures (Cooper, 1996). We identified a threshold value of serum clozapine levels (750 ng/ml) above which the risk of seizures increases by five-fold. If patients who are on maintenance treatment with clozapine have serum clozapine levels above 750 ng/ml, appropriate dose reductions or adjustment of comedications should be considered.
(5) We found that oral clozapine doses below 250 mg/day were four times more likely to produce inadequate serum clozapine levels (< 350 ng/ml). Hence, we suggest the minimum clinical dose for clozapine should be 250 mg/day unless the patients develop intolerable adverse effects on lower doses.
(6) Because of high interindividual variability, serum clozapine levels do not usually follow Gaussian distributions among clinical samples. Hence, dosing nomograms on the basis of linear regression models (Haring *et al.*, 1989; Perry *et al.*, 1998) are often misleading (Lane *et al.*, 1999). We recommend that clinical psychiatrists and future researchers to pursue dosing equations developed from appropriate non-parametric regression models.

Conclusion

The pharmacokinetics of clozapine is complex. Hence, statistical models with clinical predictors often explain only a fraction of the variability observed in the serum clozapine levels (Perry *et al.*, 1998; Rostami-Hodjegan *et al.*, 2004). We should consider the differing findings of the studies on this topic as not contradictory but as contributing to each other. Routine therapeutic drug monitoring of clozapine is desired in all clinical settings (Ulrich *et al.*, 2003). Until it becomes a reality in resource-poor settings, we should combine the available clinical predictors for serum clozapine levels to aid appropriate clozapine dose adjustments. When we progress towards genome-wide pharmacogenetic studies to explain the interindividual variability of serum clozapine levels, we should not overlook the valuable contribution by the clinical predictors. We recommend that future longitudinal studies investigate both clinical and pharmacogenetic factors together to develop better models for predicting serum clozapine levels.

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carried out the data collection. A.P.R. and K.S.J. analysed the data and wrote the manuscript. All authors were involved in revising the manuscript. The authors are grateful to Dr P. Thangadurai, S.D. Manoranjitham, Dr Binu Susan Mathew, K. Saravanakumar and S. Velvizhi, Christian Medical College, Vellore, for help and support. They thank all participants and their families.

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Conflicts of interest

There are no conflicts of interest.

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Association between *CYP1A2* gene single nucleotide polymorphisms and clinical responses to clozapine in patients with treatment-resistant schizophrenia

Rajkumar AP, Poonkuzhali B, Kuruvilla A, Srivastava A, Jacob M, Jacob KS. Association between *CYP1A2* gene single nucleotide polymorphisms and clinical responses to clozapine in patients with treatment-resistant schizophrenia.

Objectives: Despite clozapine's superior clinical efficacy in treatment-resistant schizophrenia (TRS), its adverse effects, need for periodic leukocyte monitoring, cost and variable clinical outcomes mandate a clinical need to predict its treatment response. Although cytochrome P450 1A2 (CYP1A2) is the principal determinant of metabolism of clozapine, the role of *CYP1A2* gene in the clinical response to clozapine is uncertain. Hence, we investigated its association with treatment responses and adverse events of clozapine in TRS.
Methods: We evaluated four single nucleotide polymorphisms (SNP) in the *CYP1A2* gene, clinical responses and serum clozapine levels in 101 consecutive patients with TRS on stable doses of clozapine. We defined clozapine response *a priori* and investigated allelic and genotypic associations. We assessed the socio-demographic and clinical profiles, premorbid adjustment, traumatic life events, cognition and disability of the participants, using standard assessment schedules for appropriate multivariate analyses.
Results: Our results revealed that *CYP1A2* gene SNP (**1C, *1D, *1E* and **1F*) were not associated with clozapine treatment response, adverse effects, serum clozapine levels or with disability (p values > 0.10).
Conclusions: As *CYP1A2* gene SNP do not help to predict the clinical response to clozapine, routine screening for them prior to start clozapine is currently unwarranted. We suggest future longitudinal genome-wide association studies investigating clinical and pharmacogenetic variables together.

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Keywords: clozapine; cytochrome P-450 CYP1A2; pharmacogenetics; single nucleotide polymorphism

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Significant outcomes

- *CYP1A2* gene SNP (**1C, *1D, *1E* and **1F*) were not associated with treatment response and adverse effects of clozapine in patients with TRS.
- *CYP1A2* gene SNP (**1C, *1D, *1E* and **1F*) were not associated with serum clozapine levels in patients with TRS.

- Routine screening for *CYP1A2* gene SNP prior to start clozapine is currently unwarranted.

Limitations

- Cross-sectional study design.
- Dichotomous categorisation of clinical response to clozapine is debatable.
- Outcome measures to assess the treatment responses in schizophrenia are diverse.

Introduction

Clozapine is the drug of choice for the management of treatment-resistant schizophrenia (TRS) (1). It is a serotonin-(5-HT1A, 5-HT2A, 5-HT2C, 5-HT3A, 5-HT3B, 5-HT6 and 5-HT7) dopamine (D1–D4) antagonist (SDA), which also acts on histaminergic, adrenergic and cholinergic receptors (2). The advantages of clozapine include its superior clinical efficacy, its ability to reduce negative symptoms as well as the risk for suicide (3) and its low propensity to produce movement disorders (4). Its disadvantages are sub-optimal response in 40–70% patients with TRS (5), adverse events such as seizures, agranulocytosis, weight gain and dyslipidaemia (6), high cost and the need for periodic leucocyte monitoring. There is a clinical need to determine factors associated with good response to clozapine in order to predict its clinical outcomes and to prevent unnecessary use in patients who are unlikely to improve with clozapine (7).

Cytochrome P-450 1A2 (CYP1A2), a member of the cytochrome P-450 mixed-function oxidase system, is the principal determinant of clozapine metabolism by N-demethylation and N-oxidation (8). CYP1A2 enzyme activity influences the serum clozapine levels (9–11). Inadequate serum clozapine levels are associated with sub-optimal clinical response to clozapine in many patients (12–14). Hence, we get the impetus to evaluate the association between *CYP1A2* gene polymorphisms and clinical response to clozapine. The *CYP1A2* gene (gene ID: 1544) is located in 15q24.1 and spans 7.8 kilo bases, comprising seven exons, six introns and an enhancer region (15,16). CYP1A2 enzyme activity has been reported to show marked inter-individual variations (up to 60-fold), because of various genetic and environmental factors (17). Genetic factors alone may explain 35–75% of the variations seen in CYP1A2 enzyme activity (16). In addition, marked inter-individual variations (15- to 40-fold) have been also documented in the expression levels of CYP1A2 mRNA and protein (16). At least 33 single nucleotide polymorphisms (SNP) and 17 haplotypes in the *CYP1A2* gene have been identified so far (18). Among these SNP, *CYP1A2*1C* causes decreased enzyme activity *in vivo* and *CYP1A2*1F* leads to higher inducibility of the enzyme (18). *CYP1A2*1D* and *CYP1A2*1E* are relatively more frequent in Asian populations and have been previously investigated for their influence of CYP1A2 enzyme activity (16,18,19). However, the association between these four SNP and clinical responses to clozapine in TRS remains uncertain.

Although evidence for association between response to clozapine and *5HT2A* (20) as well as *5HT3A* (21) gene SNP exists, results of other studies investigating the clinical (22–25), genetic (26–28) and other biological predictors (29,30) are mostly inconclusive, with many of them being contradictory or pending replication. The Royal Dutch Association for the advancement of pharmacy has recently evaluated therapeutic dose recommendations for clozapine based on CYP2D6 genotypes and concluded against any specific recommendations (31). Although non-synonymous coding SNP have not been found to be associated with clozapine treatment response (16,17), case studies have claimed an association between *CYP1A2*1F* SNP and treatment resistance to clozapine (9). Ultra-rapid CYP1A2 activity, because of *CYP1A2*1F* polymorphism, has been hypothesised to yield low serum clozapine levels and poor treatment response (10). *CYP1A2*1F* has also been associated with higher induction of the enzyme by smoking (10) and heavy caffeine consumption (11). However, subsequent studies were negative for the association between *CYP1A2* SNP and serum clozapine levels, as well as clozapine treatment response (32–34). These reports did not exclusively study patients with established TRS and investigated only less than 80 participants (9,32–34). They did not use structured assessment of clinical variables such as premorbid adjustment, traumatic life events, cognition and disability nor did they adjust for these variables. Hence, investigating the association between the *CYP1A2* gene and clinical response to clozapine in a relatively larger sample of patients with TRS, accompanied by structured assessment of clinical variables, is desired.

Aims of the study

Our principal aim is to evaluate the association between four SNP in the *CYP1A2* gene (*CYP1A2*1C, CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F*) and the clinical responses as well as adverse effects to clozapine in patients with TRS, while adjusting for the effects of confounding clinical variables. Our secondary aims include investigating the association between these four SNP and the serum clozapine levels, disability and cognition of patients with TRS.

Materials and methods

Study design

We used a pharmacogenetic association study to investigate the association between these functionally relevant four SNP in the *CYP1A2* gene and clinical responses to clozapine.

Setting

We conducted this study in the Department of Psychiatry, Christian Medical College (CMC), Vellore, India, a tertiary referral centre for the management of psychiatric disorders. The hospital has short-term inpatient services, daily outpatient and regular follow-up clinics. Patients with schizophrenia are initially treated with either dopamine antagonists or serotonin dopamine antagonists (SDA). Clozapine is never used as the first line antipsychotic medication and is reserved for patients with TRS. Standard international guidelines (35,36) are followed to monitor total and differential leukocyte counts of all patients receiving clozapine. Their metabolic parameters are also periodically monitored. Detailed medical records of treatment are maintained for all patients. Most of our outpatients with schizophrenia live in the community with their families. Their medications are directly provided by their first-degree relatives or spouses, who report any degree of non-adherence to the treating psychiatrists during periodically scheduled follow-up visits.

Recruitment of participants

We invited all consecutive patients, who satisfied the following eligibility criteria, into the study: (a) Diagnostic and Statistical Manual of Mental Disorders-IV TR diagnosis of schizophrenia (37), (b) established treatment resistance in the past after failure to respond at least two adequate antipsychotic trials, as documented by treating psychiatrists. An adequate antipsychotic trial was defined by 600 mg chlorpromazine equivalents for a duration of at least 6 weeks with good drug compliance. The two adequate antipsychotic trials included at least one adequate trial with a SDA, (c) on stable dose regimens of clozapine for at least 12 weeks with good drug compliance during that period, (d) origin of South Indian ethnicity. Written informed consent was obtained from the patients and from their first-degree relatives. Patients with severe neurological illnesses, intellectual disability and sensory impairment, precluding the assessment, were excluded.

Clinical assessment

We used the following instruments: (a) Brief Psychiatric Rating Scale (BPRS) to assess treatment response to clozapine (38), (b) Abnormal Involuntary Movements Scale (AIMS) to measure neuroleptic-induced dyskinesia (39), if present, (c) Addenbrooke's Cognitive Examination (ACE-R), a brief cognitive test battery to evaluate cognitive status (40), (d) World Health Organisation Disability Assessment Scale II to quantify the disability (41), (e) Childhood Traumatic Events Scale (CTES) to assess early traumatic experiences before the age of 17 (42), (f) Recent Traumatic Event Scale (RTES) to assess traumatic experiences within the past 3 years (42), (g) Premorbid Adjustment Scale (PAS) to assess premorbid functioning retrospectively (43) and (h) a structured questionnaire to collect sociodemographic, clinical and treatment data. We also recorded data about developmental delays, obstetric complications, urbanisation, recent migrations, smoking, caffeine as well as grape juice consumption and anthropometric measures. ACE-R, CTES, RTES and PAS were translated into the local language, Tamil, and then back translated to English by bilingual health professionals. The final versions were obtained by consensus among the translators who emphasised on content and on conceptual, semantic and technical equivalence.

Serum clozapine assay

Peripheral venous blood samples were collected from all participants by venipuncture, 12 h after their last clozapine dose. Serum clozapine levels were measured by deproteinisation with diethyl ether and subsequent high-performance liquid chromatography with ultra-violet detection (44). The serum clozapine levels were expressed as ng/ml.

CYP1A2 genotyping

Genomic DNA was isolated from whole blood using QIAamp DNA mini Kit (Qiagen GmBH, Hilden, Germany). *CYP1A2*1C, CYP1A2*1D, CYP1A2*1E*

and *CYP1A2*1F* were genotyped using previously published PCR-restriction fragment length polymorphisms method (19). Briefly, 200 ng of genomic DNA was subjected to PCR amplification using appropriate primers (19) and Genei™ Red Dye PCR master mix (Genei, Bangalore, India). Amplified PCR products were subjected to restriction digestion with appropriate enzymes, *Bse*L I, *Nde* I, *Bsu*R I and *Bsp* 120I (Fermentas-Genetix biotech Asia, New Delhi, India) respectively for *CYP1A2*1C, CYP1A2*1D, CYP1A2*1E* and *CYP1A2*1F*. The digested products were separated by gel electrophoresis in a 3% agarose gel. Then, they were identified by the unique patterns, characteristic to their specific genotypes.

Data collection

The protocol of the study was approved by the Institutional Review Board of CMC, Vellore, India. We provided a fact sheet about the details of this study to all participants. We discussed those details and obtained written informed consent from the participants and from their first-degree relatives or spouses. Every participant was individually assessed for psychopathology using BPRS. The participants were examined for tardive dyskinesia, when present, its severity was recorded using the AIMS. Another independent investigator, who was blind to the clozapine response status, used other instruments and assessed various clinical variables by detailed personal interviews with the participants and their primary care givers. She accessed the medical records of all participants with their consent. The principal investigator (A. P. Rajkumar), who was blind to clozapine response status and to the clinical data, carried out *CYP1A2* genotyping of all samples. Hence, separate investigators collected data on outcome variable of clozapine treatment response (S. Bhuvaneshwari), exposure variables of *CYP1A2* genotype (A. P. Rajkumar) and clinical variables (C. Chitra). They ensured that they were blind to each others' findings till the completion of the study. We followed standard quality control procedures to ensure the accuracy of our data collection, data entry and of the *CYP1A2* genotyping.

Statistical analyses

We initially analysed the study variables using descriptive statistics. Many researchers prefer to define the response to clozapine by greater than 20% reduction in the total score of BPRS (45). However, most clinical psychiatrists do not refer to non-response based on a change on any rating scale, but rather on the presence of persistent positive or negative symptoms (46). Moreover, due to our cross-sectional study design, we defined the response to clozapine, with the widely used cross-sectional threshold of having BPRS total score of 35 or less (45,46). We dichotomously categorised the participants, who had BPRS total scores equal to or less than 35, as clozapine responders. We calculated the *CYP1A2* allele frequencies in our sample and checked whether they were in Hardy-Weinberg Equilibrium (HWE). We calculated the allelic odds ratios (ORs) with 95% confidence intervals (CIs). We used the Cochran-Armitage Test for Trend (CATT) to assess statistical significance of the association between *CYP1A2* genotypes and clinical response to clozapine. We used one sample Kolmogorov-Smirnov test to check for the normal distribution of all continuous variables. We compared the means of psychopathology and disability scores and serum clozapine levels between *CYP1A2* genotypes, using the Kruskal-Wallis test. We used appropriate multivariate statistics to adjust for the effects of clinical variables. We estimated the prerequisite sample size and post hoc power using Quanto 1.2.4 software (47). We performed other analyses using the statistical software packages, STATA 12.0 and PLINK v1.07 (48).

Sample size estimation

A previous study using BPRS for the assessment of clinical outcome has reported that 44.3% patients with TRS were clozapine non-responders (49). The minor allele (C) frequency of *CYP1A2*1F* (rs762551) in the Asian population is 0.386 (50). We estimated that the prerequisite sample size to be 34 cases of clozapine non-responders for an unmatched case control study two-sided test, with 5% alpha error, 80% power and with odds ratio (OR) of 2.5. The variant allele (del-T) frequency of *CYP1A2*1D* (rs35694136) is 0.414 (51). We estimated the prerequisite sample size to be 33 cases of clozapine non-responders for an unmatched case control study two-sided test with 5% alpha error, 80% power and with OR of 2.5.

Results

Sample characteristics

We assessed 113 consecutive patients. We excluded six patients, who were not completely compliant with clozapine, within the past 12 weeks. One patient with severe Parkinson's disease and another with moderate intellectual disability were also excluded. Among the 105 patients, confirmed to be eligible, 101 consented to participate, making the response

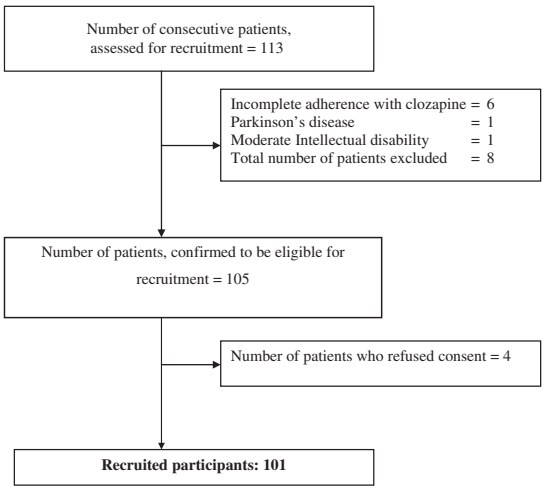

Fig. 1. Flowchart for the recruitment of participants.

rate as 96.2%. Common reasons for refusing consent were lack of interest in study objectives and reluctance to provide blood samples. We present the flowchart for recruitment of the patients as Fig. 1. Participants ($N = 101$) and those who were excluded ($n = 12$) did not differ significantly on gender ($\chi^2 = 0.04$; d.f. $= 1$; $p = 0.84$), age ($t = -1.41$; d.f. $= 111$; $p = 0.16$) and on their duration of illness ($t = -1.27$; d.f. $= 111$; $p = 0.21$). There were 65 (64.4%) clozapine responders and 36 (35.6%) non-responders, who had BPRS total scores, 36 or more.

Table 1 presents the socio-demographic, clinical and treatment profiles of all participants and of the clozapine responders as well as non-responders. The majority of the participants were single or separated men ($n = 66$; 65.3%) who were unemployed ($n = 60$; 59.4%) and living in urban areas ($n = 58$; 57.4%). The duration of clozapine treatment ranged between 4 and 174 months, with a median value of 28 months. More than half of the participants (53.5%) had received clozapine for longer than 2 years. Thirty seven participants (36.6%) had received electroconvulsive therapy in the past. We did not have any participants who were concurrently on carbamazepine or on oral contraceptive pills. The oral doses of clozapine ranged from 100 to 650 mg/day, with a median value of 350 mg/day. Serum clozapine levels of the participants ranged from 104 to 2547 ng/ml, with a median value of 428 ng/ml.

CYP1A2 allele frequencies

Table 2 shows the *CYP1A2* allele frequencies of the four SNP. The allele frequencies of *CYP1A2*1C* (rs2069514), *CYP1A2*1D* (rs35694136), *CYP1A2*1E* (rs2069526) and *CYP1A2*1F* (rs762551) were consistent with the HWE.

Association between *CYP1A2* SNP and clinical responses to clozapine

Table 3 shows the association between the four SNP in the *CYP1A2* gene and response to treatment with clozapine in patients with TRS. None of the *CYP1A2* alleles and genotypes was significantly associated with clozapine treatment response. The *CYP1A2*1F* CC genotype did not significantly increase the risk of clozapine non-response (OR 2.17; 95% CI 0.74–6.38; $p = 0.16$), when compared to the AA genotype. Multiple logistic regression analyses, adjusting for the effects of various clinical variables, including the serum clozapine levels, confirmed these findings. Table 4 presents the association between these four SNP in the *CYP1A2* gene and psychopathology, serum clozapine levels, cognition and disability. The differences among the median values of these variables between the *CYP1A2* genotypes were not statistically significant. Appropriate multiple quantile regression analyses, adjusting for the effects of other clinical variables, including the serum clozapine levels, also confirmed these findings.

Association between *CYP1A2* SNP and adverse effects to clozapine

Our participants had the following adverse effects, related to clozapine: hypersomnolence (n = 77; 76.2%), sialorrhoea (n = 47; 46.5%), nausea or vomiting ($n = 21$; 20.8%), constipation ($n = 21$; 20.8%), erectile dysfunction (13 men; 27.7%), dyslipidaemia ($n = 12$; 11.9%), clozapine-related seizures ($n = 9$; 8.9%), nocturnal enuresis ($n = 6$; 5.9%) and obesity ($n = 15$; 14.9%). As we never re-challenge patients, who have developed neutropenia with clozapine, none of our participants had past history of neutropenia or agranulocytosis. Association between the four CYP1A2 SNP and all adverse effects were not statistically significant (p values > 0.10). Multiple logistic regression analyses, adjusting for the effects of other clinical variables, including the serum clozapine levels, confirmed these findings.

Secondary analyses

We repeated similar analyses using the following three more definitions for non-response to treatment with clozapine: (a) Total score of BPRS 38 and above (worst quartile); (b) At least one the five selected BPRS items for suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganisation and unusual thought content was scored moderate and above; (c) At least two of

Table 1. Socio-demographic, clinical and treatment profiles of the clozapine responders ($n = 65$) and non-responders ($n = 36$)

Characteristics	Sample ($N = 101$) N (%) /mean (SD)	Clozapine responders ($N = 65$) N (%)/mean (SD)	Clozapine non-responders ($N = 36$) N (%)/mean (SD)	$\chi^2/t/U$*	p value
Male gender	73 (72.3)	50 (76.9)	23 (63.9)	0.88	0.35
Age (years)	35.43 (9.43)	35.46 (9.07)	35.36 (10.18)	0.20	0.84
Number of years of education	11.86 (3.89)	11.91 (3.84)	11.78 (4.02)	1112.00	0.68
Monthly family income (INR)	4733 (6062)	5065 (6833)	4135 (4363)	986.50	0.19
Body mass index (kg/m^2)	24.54 (4.64)	24.85 (4.50)	23.99 (4.90)	1.20	0.24
Family history of schizophrenia	17 (16.8)	11 (16.9)	6 (16.7)	0.00	0.97
Age of onset of illness (years)	23.07 (7.22)	22.32 (6.10)	24.42 (8.82)	1076.50	0.51
Duration of illness (years)	12.40 (6.77)	13.14 (7.31)	11.06 (5.51)	1.33	0.19
DUP (months)	11.21 (13.38)	11.37 (14.65)	10.92 (10.92)	1065.00	0.45
Presence of AIMS	11 (10.9)	5 (7.7)	6 (16.7)	1.92	0.17
Paranoid sub-type	85 (84.2)	56 (86.2)	29 (80.6)	0.55	0.46
Past history of catatonia	**5 (4.9)**	**0 (0)**	**5 (13.9)**	—	**0.005**†
Total duration of treatment (months)	113.64 (78.46)	117.62 (85.30)	106.47 (64.82)	0.40	0.69
Total duration of clozapine (months)	41.61 (39.58)	45.83 (42.24)	34.00 (33.47)	991.00	0.20
Oral dose of Clozapine (mg)	**340.84 (119.04)**	**321.92 (101.98)**	**375.00 (140.03)**	**−2.42**	**0.02**
Serum clozapine level (ng/ml)	550.53 (378.46)	503.23 (260.37)	635.93 (523.07)	−1.85	0.07
High caffeine intake‡	24 (23.8)	15 (23.1)	9 (25.0)	0.05	0.83
Smoking $\geq$ one pack/day	**17 (16.8)**	**7 (10.8)**	**10 (27.8)**	**4.79**	**0.03**
BPRS total score	**34.73 (12.45)**	**27.94 (3.79)**	**47.00 (13.27)**	**0.00**	**<0.001**
ACE-R total score	**63.11 (20.78)**	**67.65 (18.64)**	**54.61 (22.77)**	**3.21**	**0.002**
WHODAS-II total score	17.49 (12.98)	16.49 (12.71)	19.28 (13.45)	−1.72	0.09
CTES total score	8.32 (10.48)	8.62 (10.56)	7.78 (10.46)	1101.50	0.62
RTES total score	5.94 (8.71)	5.55 (7.84)	6.64 (10.17)	1156.50	0.92
PAS total score	54.83 (21.29)	53.46 (17.31)	57.31 (27.15)	−0.97	0.34

DUP, duration of untreated psychosis; AIMS, abnormal involuntary movements; BPRS, Brief Psychiatric Rating Scale; ACE-R, Addenbrooke's cognitive examination-revised; WHODAS-II, World Health Organisation Disability Assessment Scale; CTES, Childhood Traumatic Event Scale; RTES, Recent Traumatic Event Scale; PAS, Premorbid Adjustment Scale; INR, Indian rupees. Clozapine non-responders: participants with BPRS total scores 36 and above.

Statistically significant associations with p values < 0.05 are presented in bold.

*Chi square or independent samples t-test or Mann-Whitney U test between responders and non-responders.

†Fisher exact test p value (two tailed).

‡Three or more cups of coffee or tea intake/day.

Table 2. CYP1A2 allele frequencies among the participants ($N = 101$)

SNP	Allele		Allele frequencies		Genotype frequencies (n)			HWE* χ^2, d.f.	p
	1	2	1	2	11	12	22		
*CYP1A2*1C* rs2069514	G	A	0.891	0.109	81	18	2	0.68, 1	0.41
*CYP1A2*1D* rs35694136	T	—	0.693	0.307	48	44	9	0.06, 1	0.81
*CYP1A2*1E* rs2069526	T	G	0.911	0.089	84	16	1	0.06, 1	0.81
*CYP1A2*1F* rs762551 rs762551	A	C	0.569	0.431	37	41	23	2.29, 1	0.08

*Goodness of fit with HWE.

these five selected BPRS items was scored moderate and above. Absence of statistically significant associations between *CYP1A2* SNP and clozapine treatment response were replicated using these differing outcome definitions for non-response to clozapine. Participants who smoked more than 20 cigarettes a day ($n = 17$) did not differ in their serum clozapine levels (Kruskal-Wallis $\chi^2 = 0.39$; d.f. = 2; $p = 0.82$) and on their clinical responses to clozapine (CATT $\chi^2 = 2.00$; d.f. = 1; $p = 0.16$) depending on their *CYP1A2*1F* genotypes. Multivariate analyses adjusting for the effects of age, oral dose and of body mass index confirmed these findings.

Discussion

This study examined the association between four SNP in the *CYP1A2* gene and clinical responses to clozapine among patients with TRS, accompanied by structured assessment of clinical variables. Our sample size is relatively larger than most of the available studies on this topic (9–11,32,33) and

Table 3. Association between *CYP1A2* gene SNP and treatment response to clozapine among the clozapine responders ($n = 65$) and non-responders ($n = 36$)

	Allele		Responder		Non-responder		Allelic OR*		Responder			Non-responder				
SNP	1	2	1	2	1	2	(95% CI)	Allelic *p*	11	12	22	11	12	22	CATT† χ^2, d.f.	Genotype *p*
*CYP1A2*1C* rs2069514	G	A	115	15	65	7	0.83 (0.27–2.29)	0.69	52	11	2	29	7	0	0.15, 1	0.70
*CYP1A2*1D* rs35694136	T	–	89	41	51	21	0.89 (0.45–1.75)	0.73	31	27	7	17	17	2	0.13, 1	0.72
*CYP1A2*1E* rs2069526	T	G	118	12	66	6	0.89 (0.26–2.72)	0.83	54	10	1	30	6	0	0.05, 1	0.83
*CYP1A2*1F* rs762551	A	C	79	51	36	36	1.54 (0.83–2.88)	0.14	26	27	12	11	14	11	1.87, 1	0.17

*Calculated with variant allele (2) as the exposure variable and clozapine non-response as the outcome variable.
†CATT.

Table 4. Association between *CYP1A2* gene SNP and serum clozapine levels, psychopathology, disability as well as cognitive status among the participants with TRS ($N = 101$)

SNP and variables		Wild type mean (SD)	Heterozygous variant mean (SD)	Homozygous variant mean (SD)	χ^{2*}	*p*
*CYP1A2*1C*	Serum level†	543.67 (389.96)	580.67 (336.91)	557.00 (455.38)	0.38	0.83
rs2069514	Psychopathology‡	34.49 (12.18)	36.67 (14.23)	27.00 (2.83)	0.85	0.65
	Disability§	17.81 (12.96)	14.50 (12.93)	31.00 (7.07)	3.74	0.15
	Cognitive status¶	62.41 (21.19)	68.00 (17.68)	47.50 (31.82)	1.71	0.43
*CYP1A2*1D*	Serum level†	543.56 (322.01)	559.35 (440.77)	544.56 (369.01)	0.22	0.89
rs35694136	Psychopathology‡	36.17 (14.35)	34.43 (10.96)	28.56 (5.15)	2.58	0.28
	Disability§	19.54 (14.47)	15.43 (11.23)	16.56 (12.19)	1.65	0.44
	Cognitive status¶	61.54 (19.70)	64.00 (21.63)	67.11(23.83)	1.15	0.56
*CYP1A2*1E*	Serum level†	569.55 (401.33)	471.50 (217.98)	217.00	1.81	0.40
rs2069526	Psychopathology‡	34.17 (11.31)	38.31 (17.44)	25.00	2.34	0.31
	Disability§	17.18 (12.89)	20.00 (13.53)	3.00	2.11	0.35
	Cognitive status¶	62.80 (21.08)	63.38 (19.72)	85.00	1.70	0.43
*CYP1A2*1F*	Serum level†	475.22 (260.99)	589.84 (383.27)	601.61(507.29)	1.49	0.48
rs762551	Psychopathology‡	35.59 (15.45)	32.93 (9.27)	36.57 (12.11)	1.69	0.43
	Disability§	17.65 (14.27)	17.37 (12.29)	17.43 (12.59)	0.04	0.98
	Cognitive status¶	60.46 (22.05)	66.63 (20.06)	61.09 (19.94)	2.15	0.34

*Kruskal-Wallis test with two degrees of freedom.
†Serum clozapine level in ng/ml.
‡BPRS total score.
§World Health Organisation Disability Assessment-II Scale total score.
¶Addenbrooke's cognitive examination-revised total score.

we have exclusively recruited only patients with established treatment resistance. The strengths of this study include minimal refusal rate, access to well-documented medical records, estimations of serum clozapine levels, testing multiple outcome definitions and structured assessments of clinical variables such as premorbid adjustment, traumatic life events, cognition as well as disability. Consecutive sampling strategy reduced the possibility of selection bias. The independent assessments of *CYP1A2* genotypes, clozapine treatment response and clinical variables minimised the possibility of observer bias. We attempted to minimise the recall bias on the reported clinical variables by interviewing one or more first-degree relatives of the participants and by verifying their follow-up medical records.

The potential limitations of this study include the cross-sectional clinical assessment of response to clozapine and its dichotomous categorisation. We recruited only the participants, who were maintained on stable dosage of clozapine for a minimum duration 12 weeks, when their treating psychiatrists did not need to change their prescription. Hence, their cross-sectional BPRS scores were more indicative of their persistent psychopathology than of any acute fluctuations in their illnesses. Although many researchers define the response to clozapine by the reduction in the total scores of BPRS, most clinical psychiatrists prefer to use the discrete clinical category of non-response based on the presence of persistent positive or negative symptoms (46). Clinical significance of many statistically significant reductions in the total scores of psychiatric rating scales remains uncertain (52). Hence, we analysed multiple BPRS-derived categorical outcome definitions, as dependent variables, by appropriate multivariate models to confirm our findings. Despite the extensive use of BPRS, we should acknowledge that there are more diverse outcome measures to assess the treatment responses in schizophrenia (53).

Our findings suggest that the presence of two SNP (*CYP1A2*1C* and *CYP1A2*1D*) in the 5′ flanking region and two others (*CYP1A2*1E* and *CYP1A2*1F*) in intron 1 of the *CYP1A2* gene are

not associated with clinical response to clozapine and with serum clozapine levels. *CYP1A2*1F* has been associated with higher induction of CYP1A2 activity by smoking (10) and by heavy caffeine consumption (11). Earlier case studies have reported possible association between *CYP1A2*1F* and clozapine non-response, especially in smokers (9,10). Small uncontrolled samples and lack of multivariate analyses may explain these reports, because subsequent larger studies did not find any significant association between *CYP1A2* SNP and serum clozapine levels or with clinical response to clozapine (32–34). Associations between *CYP1A2* gene and schizophrenia as well as tardive dyskinesia were also not significant, after corrections for multiple comparisons (54). Our results corroborate the available literature and confirm the lack of association between these four SNP in the *CYP1A2* gene and clinical response to clozapine. Our results do not support the association of *CYP1A2*1F* with low serum clozapine levels in smokers (10). Smoking has been reported to have a major influence over serum clozapine levels (55). Its relationship with clozapine treatment response is, however, controversial (56,57). We may consider that poor clozapine response in smokers may be secondary to smoking rather than to *CYP1A2*1F* (56).

Although early studies have claimed that CYP1A2 genotyping could have high clinical utility for the patients on clozapine (10) and CYP450 pharmacogenetic test chips are being currently marketed (58), our results have proved the contrary. On the basis of our study findings, we conclude that these four *CYP1A2* gene SNP do not help to predict the clinical responses to clozapine. Hence, routine screening for them prior to start clozapine is unwarranted at present. Our study also provides new data on the *CYP1A2* allele frequencies in a population of south Indian ethnicity. These allele frequencies are similar to other Asian and sub-Saharan African populations (50,51,59,60).

Schizophrenia is not a single disease, but a heterogeneous polygenic multi-factorial disorder, caused by multiple common genetic variants (61) as well as environmental factors (62). Searches for rare genetic variants, which exert significant effects on the pathogenesis and on clinical responses of schizophrenia, have not been fruitful (63). The pharmacokinetics and pharmacodynamics of clozapine are also complex (64). Hence, searching for a single gene to explain major variances of the clinical response to clozapine in TRS usually yields negative results. We suggest that future studies to predict treatment responses to clozapine in TRS should spread their nets wide to study multiple candidate genes that may be involved in major pharmacodynamic and pharmacokinetic processes of clozapine. Studies which have moved beyond the traditional focus on neurotransmitters and polymorphisms in genes for associated receptors and transporters have, so far, been more successful in elucidating common genetic variants associated with schizophrenia (61). Hence, we may need genome-wide association studies that use longitudinal assessments of clinical outcomes to better understand the intricacies of clozapine pharmacogenetics. Pharmacogenetic association studies should not underestimate the importance of environmental factors, gene-environment interactions and the utility of clinical variables to predict clinical responses to clozapine (7,22–24). Such studies, investigating both clinical and pharmacogenetic factors together, are called for to make progress towards the goal of identifying patients who are most likely to benefit from clozapine and to prevent unnecessary exposure of non-responders to serious adverse effects of the drug.

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ORIGINAL INVESTIGATION

Outcome definitions and clinical predictors influence pharmacogenetic associations between *HTR3A* gene polymorphisms and response to clozapine in patients with schizophrenia

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Abstract
Rationale Pharmacogenetics of schizophrenia has not yet delivered anticipated clinical dividends. Clinical heterogeneity of schizophrenia contributes to the poor replication of the findings of pharmacogenetic association studies. Functionally important *HTR3A* gene single-nucleotide polymorphisms (SNPs) were reported to be associated with response to clozapine.
Objective The aim of this study was to investigate how the association between HTR3A gene SNP and response to clozapine is influenced by various clinical predictors and by differing outcome definitions in patients with treatment-resistant schizophrenia (TRS).
Methods We recruited 101 consecutive patients with TRS, on stable doses of clozapine, and evaluated their *HTR3A* gene SNP (rs1062613 and rs2276302), psychopathology, and serum clozapine levels. We assessed their socio-demographic and clinical profiles, premorbid adjustment, traumatic events, cognition, and disability using standard assessment schedules. We evaluated their response to clozapine, by employing six differing outcome definitions. We employed appropriate multivariate statistics to calculate allelic and genotypic association, accounting for the effects of various clinical variables.
Results T allele of rs1062613 and G allele of rs2276302 were significantly associated with good clinical response to clozapine (p=0.02). However, varying outcome definitions make these associations inconsistent. rs1062613 and rs2276302 could explain only 13.8 % variability in the responses to clozapine, while combined clinical predictors and *HTR3A* pharmacogenetic association model could explain 38 % variability.
Conclusions We demonstrated that the results of pharmacogenetic studies in schizophrenia depend heavily on their outcome definitions and that combined clinical and pharmacogenetic models have better predictive values. Future pharmacogenetic studies should employ multiple outcome definitions and should evaluate associated clinical variables.

Keywords Clozapine · *HTR3A* gene · Single-nucleotide polymorphism · Pharmacogenetics · Schizophrenia

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Background

Schizophrenia is a complex neuropsychiatric disorder governed by both genetic and environmental factors. Prevalence of treatment resistance among patients with schizophrenia varies from 20 to 60 %, depending on the definitions used (Solanki et al. 2009). Clozapine is the drug of choice for the

management of treatment-resistant schizophrenia (TRS) (Kane et al. 1988) because of its superior clinical efficacy, its ability to reduce suicidal risk (Meltzer et al. 2003), and its low propensity to produce movement disorders (Tandon and Fleischhacker 2005). Its disadvantages are sub-optimal response in 40–70 % patients with TRS (Remington et al. 2005); adverse events such as seizures, agranulocytosis, weight gain, and dyslipidemia (Henderson et al. 2000); high cost; and the need for periodic leukocyte monitoring. Hence, predicting the clinical response and adverse effects of clozapine has been the focus of many pharmacogenetic studies over the past decade (Arranz et al. 2000; Mancama et al. 2002).

Antagonism of type 3 serotonin (5HT3) receptor contributes to the superior clinical efficacy of clozapine (Horacek et al. 2006; Rammes et al. 2009). The 5HT3 antagonist, ondansetron, can augment response to clozapine (Briskin and Curtis 1997). 5HT3A subunits (encoded by *HTR3A* gene on chromosome 11q23.1) are essential for the formation of functional 5HT3 receptors, either as homopentamers or heteropentamers (Davies et al. 1999). Pre-synaptic 5HT3A receptors determine the intracellular Ca^{2+} levels and modulate the release of several neurotransmitters. Post-synaptic 5HT3A receptors mediate fast synaptic neurotransmission and learning (Rammes et al. 2009).

A recent study has reported the association between *5HT3A* gene SNP (rs1062613 and rs2276302) and response to clozapine (Souza et al. 2010). rs1062613 is a missense mutation (Pro16Ser) within the upstream regulatory region of *HTR3A* gene (Niesler et al. 2001). Its minor allele T increases HTR3A expression (Iidaka et al. 2005). rs1062613 modulates neuronal activation in human amygdala (Iidaka et al. 2005) and alters amygdalar reactivity during emotional face processing (Kilpatrick et al. 2011). It is associated with harm avoidance, aggression, anxiety, social desirability (Melke et al. 2003), and with bipolar disorder (Niesler et al. 2001). T/T genotype of rs1062613 is associated with the need for higher daily antipsychotic dosage during maintenance therapy (Ji et al. 2008), and with the time to therapeutic response to antipsychotic medications (Schuhmacher et al. 2009).

Pharmacogenetics of schizophrenia strives to tailor *personalized* treatment with antipsychotic medications. However, it has achieved only limited clinical success so far (Arranz and Kapur 2008; Basile et al. 2002; de Leon et al. 2008). Results of many pharmacogenetic studies of clozapine are either contradictory or pending replication (Hwang et al. 2005; Zhao et al. 2005). Despite poor replication of the results of pharmacogenetic studies, which are often explained by clinical heterogeneity and by varying definitions for treatment responses (Foster et al. 2007), pertinent pharmacogenetic studies seldom employ multiple outcome definitions or investigate rich clinical data (Gutierrez et al. 2002; Souza et al. 2010). Hence, we aimed to investigate the association between *HTR3A* SNP and clinical response to clozapine in patients with TRS, while evaluating the influence of various clinical predictors and multiple outcome definitions.

Methods

Study design

A pharmacogenetic case–control association study was employed.

Setting

We conducted this study in the Department of Psychiatry, Christian Medical College (CMC), Vellore, India. Patients with schizophrenia are initially treated with either dopamine antagonists or serotonin dopamine antagonists (SDA). Clozapine is never used as the first-line antipsychotic and is reserved for patients with TRS. Leukocyte counts and metabolic parameters of the patients receiving clozapine are periodically monitored (Schulte 2006). Most patients with schizophrenia live in the community with their families. Their medications are directly provided by their first-degree relatives, who report any degree of non-adherence to the treating psychiatrists during periodically scheduled follow-up visits.

Recruitment of participants

We invited all consecutive patients who satisfied the following eligibility criteria: (1) DSM IV-TR diagnosis of schizophrenia (APA 2000), (2) treatment resistance had been established, after failure to respond at least two adequate antipsychotic trials, as documented by treating psychiatrists. An adequate antipsychotic trial was defined by 600 mg chlorpromazine equivalents for duration of at least 6 weeks with good drug compliance. Two antipsychotic trials included at least one adequate trial with an SDA, (3) on stable dose regimens of clozapine for at least 12 weeks with good drug compliance during that period, (4) origin of South Indian ethnicity. Patients with severe neurological illnesses, intellectual disability, and sensory impairment, precluding the assessment, were excluded.

Clinical assessment

We employed the following standard instruments: (1) Brief Psychiatric Rating Scale (BPRS) to assess treatment response to clozapine (Overall and Gorham 1962), (2) Addenbrooke's Cognitive Examination (ACE-R), a brief cognitive test battery (Mioshi et al. 2006), (3) WHO Disability

Assessment Scale II (WHODAS II) to quantify disability (WHO 2001), (4) Childhood Traumatic Events Scale (CTES) to assess early traumatic experiences prior to the age of 17 years (Pennebaker and Susman 1988), (5) Recent Traumatic Events Scale (RTES) to assess traumatic experiences within the past 3 years (Pennebaker and Susman 1988), (6) Premorbid Assessment Scale (PAS) to assess premorbid functioning retrospectively (Rabinowitz et al. 2007), and (7) a structured questionnaire to collect sociodemographic and clinical data. We recorded data about developmental delays, obstetric complications, recent migrations, smoking, caffeine consumption, and anthropometric measures. Peripheral venous blood samples were collected from all participants, 12 h after their last clozapine dose. We measured serum clozapine levels by deproteinization and subsequent high-performance liquid chromatography with ultra-violet detection (Wongsinsup et al. 2010).

HTR3A genotyping

Genomic DNA was isolated from whole blood using QIAamp DNA mini-kit (Qiagen-GmBH, Germany). Two hundred nanograms of genomic DNA were subjected to polymerase chain reactions (PCR), using appropriate primer sequences (rs1062613: forward 5′-TACTCCTTGGGGAAACATGG-3′ and reverse 5′-GAGTGTGGGGAGGAGCAAG-3′; rs2276302: forward 5′-TGCTGACCACCTACATCTGG-3′ and reverse 5′-GGTTTGGAGGGTTTCTCCTC-3′) and Genei™ Red-Dye PCR master mix (Genei, Bangalore, India), for amplification of the *HTR3A* gene regions flanking both SNPs. PCR products were subsequently subjected to direct DNA sequencing using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) in an Applied Biosystem 3130 Genetic Analyzer (Applied Biosystems, California, USA). We aligned the sequences and identified SNPs using SeqSacpe Sequence alignment software (Applied Biosystems, California, USA).

Data collection

The protocol of the study was approved by the Institutional Review Board of CMC, Vellore, India. Information sheets about this study were provided to all participants, and written informed consent was obtained from the participants and their first-degree relatives. Every participant was individually assessed for psychopathology using BPRS. Another independent investigator, who was blind to the clozapine response status, employed other instruments and assessed various clinical variables by detailed personal interviews with the participants and their primary caregivers. She accessed the medical records of all participants with their consent. The principal investigator (APR) carried out *HTR3A* genotyping of all samples. Thus, three independent investigators collected data on treatment responses, *HTR3A* genotypes, and clinical variables. They remained blind to each other's findings until the completion of the study.

Outcome definitions

Many researchers prefer to define the response to clozapine by greater than 20 % reduction in the total score of BPRS (Conley et al. 1997). However, most clinical psychiatrists do not refer to non-response based on a change on any rating scale, but rather on the presence of persistent positive or negative symptoms (Buckley et al. 2001). We defined the response to clozapine by six varying outcome definitions: (1) widely employed cross-sectional threshold of having BPRS total score of 35 or less (Buckley et al. 2001; Conley et al. 1997); (2) BPRS total score below 25th percentile; (3) BPRS total score below median value; (4) BPRS total score below 75th percentile; (5) all five proposed remission criteria BPRS items (Andreasen et al. 2005), suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization, and unusual thought content, rated mild or less; and (6) not more than one of these five BPRS items, rated moderate or above.

Statistical analyses

We initially analyzed the study variables using descriptive statistics. We calculated the *HTR3A* allele frequencies and checked whether they were consistent with Hardy–Weinberg Equilibrium. Participants were dichotomously divided as clozapine responders and non-responders. We calculated allelic odds ratios with 95 % confidence intervals. We employed Cochran–Armitage test for trend to assess the association between *HTR3A* genotypes and clinical response to clozapine. We performed permutation-based statistics to confirm observed allelic and genotypic associations. We employed multiple logistic regression models to study these associations, using different outcome definitions, after adjusting for the effects of clinical predictors. We estimated the prerequisite sample size and post-hoc power using Quanto 1.2.4 (Gauderman 2002). We performed other analyses using statistical software, SPSS 16.0, STATA 12.1, and PLINK v1.07 (Purcell et al. 2007).

Sample size estimation

Minor allele frequency (MAF) of rs1062613 is 15.1 % among Asian populations (NCBI-SNP 2011). While assuming that at least 40 % patients respond to clozapine (Souza et al. 2010), we estimated the prerequisite sample size as 44 clozapine responders for an unmatched case control study two-sided test, with 5 % alpha error, 80 % power, and with anticipated odds ratio of 4.0.

Results

Sample characteristics

We assessed 113 consecutive patients and excluded six patients who were not completely compliant with clozapine, within the past 12 weeks. One patient with severe Parkinson's disease and another with moderate intellectual disability were also excluded. Among the 105 eligible patients, 101 consented to participate, making the participation rate as 96.2 %. Participants (n=101) and those excluded (n=12) did not differ significantly on gender (χ^2=0.04; df=1; p=0.84), age (t=−1.41; df=111; p=0.16), or on their duration of illness (t=−1.27; df=111; p=0.21). Majority of participants were male (n=73; 72.3 %) and single or separated (n=66; 65.3 %). Mean age of the participants was 35.4 (SD 9.4) years. Mean duration of illness, age of onset of illness, and duration of untreated psychosis were 12.4 (SD 6.8) years, 23.1 (SD 7.2) years, and 11.2 (SD 13.4) months, respectively. Mean duration of antipsychotic treatment was 113.6 (SD 78.5) months, and mean duration of clozapine treatment was 41.6 (SD 9.6; median 28; range 4–174) months. Average scores on BPRS, ACE-R, WHODAS-II, CTES, RTES, and PAS were 34.73 (SD 12.45), 63.11 (SD 20.78), 17.49 (SD 12.98), 8.32 (SD 10.48; median 4.00), 5.94 (SD 8.71; median 2.00), and 54.83 (SD 21.29), respectively. Mean oral dose of clozapine was 340.84 (SD 119.04; median 350; range 100–650) mg/day. Mean serum clozapine level was 550.53 (SD 378.46; median 428; range 104–2547) ng/ml. We have reported the clinical predictors of response to clozapine among these participants elsewhere (Rajkumar et al. 2012).

HTR3A allele frequencies

Our data for rs2276302 genotypes were complete. However, repeated PCR of one sample failed to amplify the *HTR3A* gene region flanking rs1062613, probably due to other polymorphisms within the primer binding site. MAF of rs1062613 (T) and of rs2276302(G) were 0.08 and 0.10, respectively. Allele frequencies of both rs1062613 (χ^2=0.24; df=1; p=0.62) and rs2276302 (χ^2=0.01; df=1; p=0.92) were consistent with the Hardy–Weinberg Equilibrium.

Association between *HTR3A* SNP and clozapine response

Table 1 shows allelic and genotypic associations between *HTR3A* SNP and response to clozapine, defined by BPRS total scores ≤35. Minor alleles of both rs1062613 and rs2276302 were significantly associated with good clinical responses to clozapine. These allelic associations were statistically significant, after testing both SNP with 100 million max(T) permutations (p=0.02). Variant genotypes were significantly associated with good clinical responses to clozapine, while assuming both autosomal dominant and additive modes of inheritance. Post-hoc power analyses revealed that our study had 92.7 and 91.0 % power for the allelic association of rs1062613 and rs2276302, respectively. Multiple logistic regression model including variant genotypes of both SNP could explain only 13.8 % of variability among the clinical responses to clozapine (Nagelkerke R^2=0.138).

Association between *HTR3A* SNP and adverse effects

Association between variant genotypes of both SNP and common adverse effects related to clozapine (hypersomnolence, sialorrhoea, nausea, vomiting, sexual dysfunction, dyslipidemia, seizures, nocturnal enuresis, and obesity) were not significant, after adjusting for the effects of age, gender, and serum clozapine levels.

Influence of outcome definitions and clinical predictors

Past history of catatonia (p=0.005), smoking (p=0.03), cognitive dysfunction (p=0.004), and hypersomnolence (p=0.03) were the significant clinical predictors of non-response to clozapine (defined by BPRS total scores ≥36), after adjusting for the effects of age, gender, and serum clozapine levels (Rajkumar et al. 2012). Table 2 presents

Table 1 Association between *HTR3A* gene SNP and clinical response to clozapine among the clozapine responders (n=65) and non-responders (n=36)

SNP	Allele		Responder		Non-responder		Odds ratio[a] (95 % CI)	Allelic p	Responder			Non-responder			CATT[b] χ^2, df	Genotype p	Permutated p[c]
	0	1	0	1	0	1			00	01	11	00	01	11			
rs1062613 (N=100)	C	T	115	15	69	1	9.00 (1.62–50.14)	0.01	51	13	1	34	1	0	6.02, 1	0.01	0.014
rs2276302 (N=101)	A	G	111	19	70	2	5.99 (1.58–22.71)	0.008	47	17	1	34	2	0	7.04, 1	0.008	0.013

[a] Calculated by variant allele (1) as the exposure variable and clozapine response (defined by BPRS total score ≤35) as the outcome variable

[b] Cochran–Armitage test for trend

[c] Point wise empirical p value for allelic association, after 100 million permutations

Table 2 Combined clinical predictors and *HTR3A* pharmacogenetic association model to predict clinical response to clozapine among the participants with treatment-resistant schizophrenia

Exposure variable	Multivariate analysis[a]	
	Adjusted odds ratio (95 % CI)	*p* value
rs2276302 (GA or GG)	4.91 (1.00–24.09)	0.04
Past history of Catatonia	0.00 (0.00–∞)	0.99
Currently smoking ≥1 pack/day	0.30 (0.08–1.11)	0.07
Excessive sedation[b]	0.28 (0.07–1.12)	0.07
Cognitive dysfunction[c]	0.33 (0.12–0.90)	0.03

[a] Backward conditional multiple logistic regression model including all significant clinical predictors (past history of catatonia, smoking ≥ one pack per day, excessive sedation, and cognitive deficits) and *HTR3A* genetic (rs1062613 and rs2276302 variant genotypes) variables, with good response to clozapine treatment (defined by BPRS total score ≤35) as the outcome variable

[b] More than 9 h of sleep per day

[c] Addenbrooke's Cognitive Examination-revised total score <68 (median value)

the backward conditional logistic regression model, combining these clinical predictors and *HTR3A* variant genotypes. Combined clinical predictors and *HTR3A* pharmacogenetic association model could explain 38 % of variability among the clinical responses to clozapine (Nagelkerke R^2=0.380). Hosmer–Lemeshow test confirmed the goodness of fit of this model (χ^2=6.53; *df*=6; *p*=0.37).

We present the bivariate and multivariate analyses, while employing six varying outcome definitions, in Table 3. Pharmacogenetic association of rs1062613 was significant only when employing the outcome definition of BPRS total scores ≤35. Association of rs2276302 was significant while employing only two more outcome definitions, (1) BPRS total score ≤38 (75th percentile) and (2) not more than one of the five proposed remission criteria (Andreasen et al. 2005) BPRS items, rated moderate or above. When we performed non-parametric robust regression analyses, using STATA *rreg* command, with BPRS total scores as the dependent variable, rs1062613 (β=−2.70; SE=2.51; *t*=−1.08; *p*=0.28) and rs2276302 (β=−3.97; SE=2.38; *t*=−1.67; *p*=0.10) were not significantly associated. Both rs1062613 (β=−0.50; SE=7.73; *t*=−0.07; *p*=0.95) and rs2276302 (β=−4.17; SE=7.07; *t*=−0.59; *p*=0.56) were not significantly associated with the duration of treatment with clozapine. Association between these SNP and disability as well as cognition were also not significant.

Discussion

We examined the association between *HTR3A* gene SNP and response to clozapine, in the context of differing outcome definitions and clinical predictors. We documented that pharmacogenetic associations depend heavily on their outcome definitions and that combined clinical and pharmacogenetic models have better predictive values.

Strengths and limitations

Strengths of this study include exclusively recruiting patients with TRS, minimal refusal rate, collecting rich clinical data by standard assessment schedules, estimating serum clozapine levels, and employing multiple outcome definitions. Consecutive sampling reduced the possibility of selection bias. Independent assessments of treatment response, clinical variables, and genotyping minimized the possibility of observer bias. We attempted to minimize recall bias on the reported clinical variables by interviewing first-degree relatives of the participants and by verifying their follow-up medical records. This study also contributed new data on *HTR3A* allele frequencies among south Indian population (NCBI-SNP 2011).

Potential limitation of this study was the cross-sectional clinical assessment of response to clozapine. We recruited only the participants who were maintained on stable dosage of clozapine for a minimum duration of 12 weeks, when their treating psychiatrists did not need to change their prescription. Hence, their cross-sectional BPRS scores were more suggestive of their persistent psychopathology than of any acute fluctuations of their illnesses. In the absence of baseline BPRS scores, we inferred the responses to clozapine by assessing the persistent psychopathology of the participants, after receiving adequate trial of clozapine with good compliance. Despite extensive use of BPRS, we should acknowledge that there are diverse outcome measures to assess treatment outcomes in schizophrenia (Burns 2007).

Association between *HTR3A* SNP and clinical responses

Contrary to previous studies (Arranz et al. 2000; Gutierrez et al. 2002), Souza et al. (2010) has reported that association between rs1062613, rs2276302, and response to clozapine was nominally significant, and that only rs1062613 association was significant, after 100,000 permutations. We present further details of these studies evaluating the pharmacogenetic associations between *HTR3A* gene polymorphisms and response to clozapine in patients with schizophrenia in Table 4. We replicated these pharmacogenetic associations, while employing another outcome definition (BPRS total scores ≤35), and confirmed the statistical significance of both associations after 100,000,000 permutations. However, both variant genotypes together could explain only a small proportion of the variability among the clinical responses and their pharmacogenetic associations were not significant, when we employed other outcome

Table 3 The influence of outcome definitions in the association between *HTR3A* gene SNP and clozapine treatment response among the participants with treatment-resistant schizophrenia

Models[a]		rs1062613			rs2276302		
		OR/AOR	95 % CI	*p*	OR/AOR	95 % CI	*p*
Definition 1[b] (n^c=65)	OR	9.33	1.17–74.31	0.04	6.51	1.42–29.95	0.02
	AOR[d]	28.36	1.69–476.03	0.02	6.59	1.32–32.95	0.02
Definition 2[e] (n^c=27)	OR	0.98	0.28–3.39	0.98	1.22	0.42–3.60	0.71
	AOR[d]	1.12	0.29–4.40	0.87	1.07	0.34–3.38	0.91
Definition 3[f] (n^c=53)	OR	1.95	0.62–6.20	0.26	2.51	0.88–7.19	0.09
	AOR[d]	2.33	0.62–8.74	0.21	2.68	0.84–8.51	0.09
Definition 4[g] (n^c=76)	OR	5.19	0.65–41.76	0.12	–	–	0.003[h]
	AOR[d]	9.48	0.80–113.08	0.08	–	–	–
Definition 5[i] (n^c=71)	OR	1.86	0.49–7.15	0.37	1.34	0.44–4.09	0.61
	AOR[d]	2.31	0.49–10.91	0.29	1.07	0.32–3.62	0.91
Definition 6[j] (n^c=86)	OR	2.76	0.34–22.73	0.35	–	–	0.03[g]
	AOR[d]	7.56	0.28–200.94	0.23	–	–	–

OR odds ratio, *AOR* adjusted odds ratio

[a] Variant genotypes as the exposure variable and good response to clozapine treatment as the outcome variable

[b] Clozapine response is defined by BPRS total score ≤35

[c] Number of clozapine responders

[d] Adjusted for the effects of gender, age of onset of illness (in years), serum clozapine level (in nanograms per milliliter), current smoking (more than one pack/day), duration of untreated psychosis (in months), family history of schizophrenia, and of total scores of premorbid adjustment scale, childhood traumatic event scale as well as recent traumatic events scales, by multiple logistic regression analysis

[e] Clozapine response is defined by BPRS total score ≤25 (25th percentile)

[f] Clozapine response is defined by BPRS total score ≤31 (median value)

[g] Clozapine response is defined by BPRS total score ≤38 (75th percentile)

[h] Fisher exact test *p* value (two tailed), OR could not be calculated, because all participants with variant genotypes had good response to clozapine

[i] Clozapine response is defined by all of the five selected BPRS items, suspiciousness, hallucinatory behaviours, grandiosity, conceptual disorganization, and unusual thought content, scored mild or less

[j] Clozapine response is defined by not more than one of those five BPRS items scored moderate and above

Table 4 Previous studies evaluating the pharmacogenetic associations between *HTR3A* gene polymorphisms and response to clozapine in patients with schizophrenia

Study	Participants	*HTR3A* SNPs	Outcome definition	Clinical predictors	Results
Arranz et al. (2000)	200 of British origin with schizophrenia. TRS was not an explicit inclusion criterion.	rs1062613, 1596-A/G	Retrospective assessment using GAS (Endicott et al. 1976). GAS response threshold was not specified.	Not assessed	Allelic *p* for, rs1062613=0.79; 1596-A/G=0.85
Gutierrez et al. (2002)	263 of British origin with DSM-III-R schizophrenia. TRS was not an explicit inclusion criterion.	rs1062613, 1596-A/G	Retrospective assessment for 20 points improvement in GAS scores after a minimum of 3 months treatment.	Not assessed	Allelic *p* for, rs1062613=0.36; 1596-A/G=0.78
Souza et al. (2010)	140 (82 % Caucasians) with DSM-III-R or IV schizophrenia. "Almost all of them were treatment refractory or intolerant to typical antipsychotics"	rs1062613, rs2276302, rs1176713, rs1150226	More than 20 % reduction of BPRS total scores from the baseline.	Not assessed	Both rs2276302 and rs1062613 were nominally significant. Only rs1062613 was significant after 100,000 permutations (p=0.041)

TRS treatment-resistant schizophrenia, *GAS* Global Assessment Scale, *DSM* Diagnostic and Statistical Manual of Mental Disorders, *BPRS* Brief Psychiatric Rating Scale

definitions. Moreover, they were not associated with cognitive functioning or disability. Clinical significance of statistically significant reductions in the psychiatric rating scale scores are debated in many clinical trials (Estellat et al. 2009). Similarly, claiming evidence for clinically significant pharmacogenetic associations between *HTR3A* gene and responses to clozapine, from these statistically and functionally significant associations, becomes questionable.

Clinical heterogeneity and outcome definitions of schizophrenia

Schizophrenia is not a single disease but a heterogeneous polygenic multi-factorial disorder (Stefansson et al. 2009). Searches for genetic variants, which exert large effects on the pathogenesis and on clinical responses of schizophrenia, have not been successful (Prasad et al. 2002). As many candidate gene and Genome Wide Association Studies disregard the importance of collecting rich clinical data (Stefansson et al. 2009), generalizing their statistically significant genetic or pharmacogenetic associations to clinical contexts remains difficult.

There are numerous measures to define outcome in schizophrenia (Burns 2007) and the consensus criteria for remission in schizophrenia have not been validated so far (Andreasen et al. 2005). Differing BPRS-based response cut-off scores caused significant variability in the results of many clinical trials in schizophrenia (Leucht et al. 2007). Pharmacogenetic studies often neglect this intricate clinical reality and evaluate the statistical significance of their associations, while employing single arbitrarily chosen outcome definition. Poor replication of pharmacogenetic association signals and the overall inability of pharmacogenetics of schizophrenia to live up to its potential (Arranz and Kapur 2008) stem from varying outcome definitions and clinical heterogeneity (Foster et al. 2007). Pharmacogenetic studies of schizophrenia often neglect collecting data of known clinical predictors for treatment responses (Dratcu et al. 2007; Lieberman et al. 1994). They hope for technical advances in the fields of molecular and statistical genetics to overcome their research barriers. However, next generation sequencing and computationally intensive statistics may not solve the puzzles, if the basic clinical quandaries of defining and measuring the phenotype, schizophrenia and its clinical response, remain unresolved.

Moving forward

We suggest the following:

1. Pharmacogenetic studies of schizophrenia should confirm their association signals by employing multiple outcome definitions. Although a clinically relevant BPRS or other scales' cut-off score should be decided a priori, more analyses with varying response thresholds should be presented (Leucht et al. 2007).
2. Employing multiple outcome definitions will increase the risk of type I errors. It is possible to address this problem with appropriate statistical corrections for multiple testing (Feise 2002). However, we do not support reporting a multiple testing adjusted statistically significant pharmacogenetic association, which does not hold true, while considering other possible outcome definitions. We argue for consistent pharmacogenetic associations, which remain significant, while employing multiple clinically relevant outcome definitions.
3. Pharmacogenetic studies should include known clinical predictors. As explanatory pluralism is better than biological reductionism (Kendler 2005), combined clinical and pharmacogenetic models hold better predictive value.
4. All statistically significant, or even functionally relevant, pharmacogenetic associations need not be clinically significant (Estellat et al. 2009). Akin to many common diseases, pharmacogenetics of schizophrenia deals with common variants and small effect sizes (Stefansson et al. 2009). Hence, we should be more realistic, while discussing the clinical implications of pharmacogenetic associations of individual genetic variants (Ioannidis et al. 2006).
5. Unambiguous definition of phenotypes will lead to reliable pharmacogenetic associations. Akin to DSM IV-TR diagnostic criteria, we need reliable, if not valid, research criteria to define treatment responses for pharmacogenetic studies of psychiatric disorders.
6. Clinical heterogeneity of schizophrenia demands syndrome sub-categorization, employing biological variables, to achieve etiological homogeneity (Jacob 1994). Similarly, appropriate biological variables to define treatment response in schizophrenia should be searched for.

Pharmacogenetics of schizophrenia should progress by developing reliable outcome definition criteria and by future studies evaluating combined models of pharmacogenetic and clinical predictors.

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10.2. Document to obtain written informed consent:

Title of the study:
Studies on the association between genetic polymorphisms and clozapine drug response in treatment resistant schizophrenia.
Institution:
Christian Medical College & Hospital, Vellore (CMCH)
Nature and purpose of the study:
You are invited to take part in a new research. Genetic variations among people may determine their response and adverse effects to certain medicines. This study aims to investigate the associations between genetic polymorphisms and response to clozapine.
Explanation of procedure to be followed:
Doctors and two research fellows from the department of psychiatry will conduct this study. You will undergo assessment of your psychiatric symptoms, abnormal movements, ability to think, childhood experiences, previous functioning and current disability. You will be asked provide 9 ml blood sample for assessing genetic polymorphisms and serum clozapine levels. Approximately 100 participants will be recruited in this study
Expected duration of involvement:
The assessment will be done in two sessions. One session will last about two hours and another will be around twenty minutes.
Possible benefits of the study:
You will not be charged for this assessment. The information we obtain will help us to assess your problems and genetic polymorphisms. Others may also benefit from the overall conclusions at the end of the study. We are unable to foresee any risks involved in this research.
Confidentiality
The records and all details obtained in this study will remain strictly confidential at all times, but will need to be available to the investigators conducting the study. Your identity will not be revealed. Your personal data will be collected and processed only for the research purposes in connection with the study. You will not be referred to by name or be identified in any report or publication.
Right to withdraw from the study
You are free to leave the study at any time. Your decision for not participating in this study will not cause any loss of benefits or affect your future medical or psychiatric care.
For further queries, you may contact,

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Christian Medical College, Vellore- 632002
Phone: 0416 228 4532
Email: psych1@cmcvellore.ac.in

I/We have read/……...had read out to us, the above information before signing this consent form.

Signature of the Participant:_____
Date: _____/_____/_____
Signatory's Name: _____

Signature of the Investigator: _____
Date: _____/_____/_____
Study Investigator's Name: _____

Signature of the Relative: _____
Date: _____/_____/_____
Name of the Relative: _____

10.3. Structured questionnaire for clinical assessment:

I. Socio Demographic Data:

1. Participant Name:
2. Participant ID:
3. Interview Date:
4. Father/ Husband's name:
5. Gender: Male / Female
6. Age (in years):
7. Marital status: Single/ Married /Widowed/ Separated
8. Residence: Rural/ Urban
9. Accommodation: Own/Rented
10. Education: No formal education/ Primary/Middle/Secondary/ Higher secondary/ Graduate/ Professional
11. Education (Number of years):
12. Occupation: Unemployed/ Labourer/ Skilled/ Professional/Others
13. Total family income per month (In INR):
14. Total number of family members:
15. Average number of cups of coffee/ day:
16. Average number of cups of tea/ day:
17. Average number of cups of grape juice/ day:
18. History of migration: Yes/ No If yes, provide details:

19. Past history of obstetric complications: Yes/ No

20. History of developmental delay during childhood: Yes/ No

II. Clinical profile:

21. Hospital number:

22. ICD-10 diagnostic codes:

23. Family history: Dementia/ psychosis/ mood / seizure/ nil/

Other neuropsychiatry morbidity, specify _____.

24. Suicidal risk: Present/ Absent

25. Past history of catatonic symptoms: Present/ Absent

26. Duration of current episode:

27. Duration of illness:

28. Age at onset of illness:

29. Duration of untreated psychosis:

30. Course of Illness: Continuous/ Episodic

31. If episodic, number of past episodes:

32. Axis I Co-morbidity: Present/ Absent. If present, specify,_____

33. Axis II diagnosis: Present/ Absent. If present, specify,_____

34. Axis III diagnosis: Present/ Absent. If present, specify,_____

35. Height:

36. Weight:

37. Smoking status: [Current smoker/ Past smoker/ Non-smoker]

If yes, pack years:

III. Treatment profile:

DRUG	DURATION	DOSE	ADVERSE EVENTS
Typical Antipsychotics (Specify)_____			
Atypical Antipsychotics (Specify)_____			
Depot Antipsychotics (Specify)_____			
Clozapine			
Lithium (recent level)			
Anti-convulsants (level) (Specify)_____			
Antidepressants (Specify)_____			
Anticholinergics (Specify)_____			
Benzodiazepines (Specify)_____			
Others, (Specify)_____			

38. Total duration of drug treatment:

39. Psychotherapy: Present/ Absent, If present, duration_____

40. Occupational therapy: Present/ Absent; If present, Regular/ Irregular

41. Electro Convulsive Therapy: Yes/ No

42. If yes, How many ECT:

43. Oral contraceptive pills: Yes/ No

44. Number of adequate trials of antipsychotics in the past:

10.4. Clinical assessment instruments:

10.4.1. Brief Psychiatric Rating Scale

10.4.2. Abnormal Involuntary Movements Scale

10.4.3. World Health Organization Disability Assessment Scale – II 12 items version

Scoring Procedure

Please enter the score for the term which best describes the patient's condition 0 = not assessed, 1 = not present, 2 = very mild, 3 = mild, 4 = moderate, 5 = moderately severe, 6 = severe, 7 = extremely severe

1. SOMATIC CONCERN	
Degree of concern over present bodily health. Rate the degree to which physical health is perceived as a problem by the patient, whether complaints have a realistic basis or not.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

2. ANXETY	
Worry, fear, or over-concern for present or future. Rate solely on the basis ot verbal report of patient's own subjective experiences. Do not infer anxiety from physical signs or from neurotic defense mechanisms.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

3. EMOTIONAL WITHDRAWAL	
Deficiency in relating to the interviewer and to the Interviewer situation. Rate only the degree to which the patient gives the impression of tailing to be in emotional contact with other people in the interview situation.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

4. CONCEPTUAL DISORGANIZATION	
Degree to which the thought processes are confused, disconnected, or disorganized. Rate on the basis of integration of the verbal products of the patient; do not rate on the basis of patient's subjective impression of his own level of functioning	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

5. GUILT FEELINGS	
Over-concern or remorse for past behavior. Rate on the basis of the patient's subjective experiences of guilt as evidenced by verbal report with appropriate affect; do not inter guilt feelings from depression, anxiety or neurotic defenses.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

6. TENSION	
Physical and motor manifestations of tension "nervousness', and heightened activation level. Tension should be rated solely on the basis of physical signs and motor behavior and not on the basis of subjective experiences of tension reported by the patient.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

7. MANNERISMS AND POSTURING	
Unusual and unnatural motor behavior, the type of motor behavior which causes certain mental patients to stand out in a crowd of normal people. Rate only abnormality of movements; do not rate simple heightened motor activity here.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

8. GRANDIOSITY	
Exaggerated self-opinion, conviction of unusual ability or powers. Rate only on the basis of patient’s statements about himself or self-in-relation-to-others, not on the basis of his demeanor in the interview situation.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

9. DEPRESSIVE MOOD	
Despondency in mood. sadness. Rate only degree of despondency; do not rate on the basis 0 inferences concerning depression based upon general retardation and somatic complaints.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

10. HOSTILITY	
Animosity, contempt, belligerence, disdain for other people outside the interview situation. Pate solely on the basis of the verbal report of feelings and actions of the patient toward others; do not infer hostility from neurotic defenses, anxiety, nor somatic complaints. (Rate attitude toward interviewer under uncooporativeness").☐	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

11. SUSPICIOUSNESS	
Belief (delusional or otherwise) that others have now, w have had in the past, malicious or discriminatory intent toward the patient. On the basis of verbal report, rate only those suspicions which are currently held whether they concern past or present circumstanes.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

12. HALLUCINATORY BEHAVIOR	
Perceptions without normal external stimulus correspondence. Rate only those experiences which are reported to have occurred within the last week and which are described as distinctly different from the thought and imagery processes of normal people.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

13. MOTOR RETARDATION	
Reduction in energy level evidenced in slowed movements. Rate on the basis of observed behavior of the patient only; do not rate on the basis of patient's subjective impression of own energy level.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

14. UNCOOPERATIVENESS	
Evidence d resistance, unfriendliness, resentment and lack of readiness to cooperate with the interviewer. Rate only on the basis of the patients attitude and responses to the interviewer and the interview situation; do not rate on basis of reported resentment or uncooperativeness outside the interview situation.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

15. UNUSUAL THOUGHT CONTENT	
Unusual, odd, strange or bizarre thought content. Rate here the degree of unusualness, not the degree of disorganizations of thought processes.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

16. BLUNTED AFFECT	
Reduced emotional tone, apparent lack of normal feeling or involvement	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

17. EXCITEMENT	
Heightened emotional tone, agitation. increased reactivity.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

18. DISORIENTATION	
Contusion or lack of proper association for person, place or time.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

Examination Procedure

Either before or after completing the examination procedure, observe the patient unobtrusively at rest (e.g., in the waiting room).
The chair to be used in this examination should be a hard, firm one without arms.

1. Ask the patient whether there is anything in his or her mouth (such as gum or candy) and, if so, to remove it.
2. Ask about the *current* condition of the patient's teeth. Ask if he or she wears dentures. Ask whether teeth or dentures bother the patient *now*.
3. Ask whether the patient notices any movements in his or her mouth, face, hands, or feet. If yes, ask the patient to describe them and to indicate to what extent they *currently* bother the patient or interfere with activities.
4. Have the patient sit in chair with hands on knees, legs slightly apart, and feet flat on floor. (Look at the entire body for movements while the patient is in this position.)
5. Ask the patient to sit with hands hanging unsupported -- if male, between his legs, if female and wearing a dress, hanging over her knees. (Observe hands and other body areas).
6. Ask the patient to open his or her mouth. (Observe the tongue at rest within the mouth.) Do this twice.
7. Ask the patient to protrude his or her tongue. (Observe abnormalities of tongue movement.) Do this twice.
8. Ask the patient to tap his or her thumb with each finger as rapidly as possible for 10 to 15 seconds, first with right hand, then with left hand. (Observe facial and leg movements.)
9. Flex and extend the patient's left and right arms, one at a time.
10. Ask the patient to stand up. (Observe the patient in profile. Observe all body areas again, hips included.)
11. Ask the patient to extend both arms out in front, palms down. (Observe trunk, legs, and mouth.)
12. Have the patient walk a few paces, turn, and walk back to the chair. (Observe hands and gait.) Do this twice.

Patient Information									
Patient		Date	Day	Mth.	Year	Time	Hour	Min
Personal notes									

Scoring Procedure

Complete the examination procedure before making ratings.
For the movement ratings (the first three categories below), rate the highest severity observed.
0 = none, 1 = minimal (may be extreme normal), 2 = mild, 3 = moderate, 4 = severe.
According to the original AIMS instructions, one point is subtracted if movements are seen **only on activation**, but not all investigators follow that convention.

Facial and Oral Movements	
1. Muscles of facial expression, e.g., movements of forehead, eyebrows, periorbital area, cheeks. Include frowning, blinking, grimacing of upper face.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4
2. Lips and perioral area, e.g., puckering, pouting, smacking.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4
3. Jaw, e.g., biting, clenching, chewing, mouth opening, lateral movement.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4
4. Tongue. Rate only increase in movement both in and out of mouth, not inability to sustain movement.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4

Abnormal Involuntary Movement Scale (AIMS)

Extremity Movements	
5. Upper (arms, wrists, hands, fingers). Include movements that are choreic (rapid, objectively purposeless, irregular, spontaneous) or athetoid (slow, irregular, complex, serpentine). Do not include tremor (repetitive, regular, rhythmic movements).	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4
6. Lower (legs, knees, ankles, toes), e.g., lateral knee movement, foot tapping, heel dropping, foot squirming, inversion and eversion of foot.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4

Trunk Movements	
7. Neck, shoulders, hips, e.g., rocking, twisting, squirming, pelvic gyrations. Include diaphragmatic movements.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4

Global Judgements	
8. Severity of abnormal movements. Based on the highest single score on the above items.	☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4
9. Incapacitation due to abnormal movements.	☐ none, normal ☐ minimal ☐ mild ☐ moderate ☐ severe
10. Patient's awareness of abnormal movements.	☐ no awareness ☐ aware, no distress ☐ aware, mild distress ☐ aware, moderate distress ☐ aware, severe distress

Dental status	
11. Current problems with teeth and/or dentures.	☐ no ☐ yes
12. Does patient usually wear dentures?	☐ no ☐ yes

WORLD HEALTH ORGANIZATION

DISABILITY ASSESSMENT SCHEDULE

WHODAS II

Phase 2 Field Trials – Health Services Research
12-Item Interviewer Administered Version
February 2000

SECTION 1. Face Sheet

ITEMS F1- F6 ARE TO BE COMPLETED BY INTERVIEWERS PRIOR TO STARTING EACH INTERVIEW

F1 RESPONDENT I.D . #

☐☐☐ – ☐☐☐ – ☐

CENTRE # - SUBJECT #

F2 INTERVIEWER I.D. #

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CENTRE # - INTERVIEWER #

F3 ASSESSMENT TIME POINT (1, 2, ETC.) ☐

F4 INTERVIEW DATE ___ ___/___ ___/___ ___

month day year

F5 LIVING SITUATION AT TIME OF INTERVIEW (CIRCLE ONLY ONE)

Independent in Community	1
Assisted Living	2
Hospitalized	3

F6 SAMPLE *(CIRCLE ONLY ONE)*

General population	1
Drug related problems	2
Alcohol related problems	3
Mental health problems	4
Physical problems	5
Other (*specify*)	6

_____

SECTION 4. CORE QUESTIONS

H1	How do you rate your overall health in the past 30 days? *Read choices to respondent.*	Very good	Good	Moderate	Bad	Very Bad

SHOW FLASHCARD #2 to participant

In the last 30 days how much difficulty did you have in:

		None	Mild	Moderate	Severe	Extreme /Cannot Do
S1	Standing for long periods such as 30 minutes?	1	2	3	4	5
S2	Taking care of your household responsibilities?	1	2	3	4	5
S3	Learning a new task, for example, learning how to get to a new place?	1	2	3	4	5
S4	How much of a problem did you have joining in community activities (for example, festivities, religious or other activities) in the same way as anyone else can?	1	2	3	4	5
S5	How much have you been emotionally affected by your health problems?	1	2	3	4	5

Continue to next page…

In the last 30 days how much difficulty did you have in:						
		None	Mild	Moderate	Severe	Extreme /Cannot Do
S6	Concentrating on doing something for ten minutes?	1	2	3	4	5
S7	Walking a long distance such as a kilometre [or equivalent]?	1	2	3	4	5
S8	Washing your whole body?	1	2	3	4	5
S9	Getting dressed?	1	2	3	4	5
S10	Dealing with people you do not know?	1	2	3	4	5
S11	Maintaining a friendship?	1	2	3	4	5
S12	Your day to day work?	1	2	3	4	5

		None	Mild	Moderate	Severe	Extreme /Cannot Do
H2	Overall, how much did these difficulties interfere with your life? *Read choices to respondent.*	1	2	3	4	5
H3	Overall, in the past 30 days, how many days were these difficulties present?	RECORD NUMBER OF DAYS ___/___				
H4	In the past 30 days, for how many days were you totally unable to carry out your usual activities or work because of any health condition?	RECORD NUMBER OF DAYS __/__				
H5	In the past 30 days, not counting the days that you were totally unable, for how many days did you cut back or reduce your usual activities or work because of any health condition?	RECORD NUMBER OF DAYS __/__				

This concludes our interview, thank you for participating.

10.4.4. Addenbrooke's Cognitive Examination – ACE-R:

Orientation:

- **Ask what is the :** Day/ Date/ Month/ Year/ Season **(Score 0-5)**
- **Ask which is the** : Building/ Floor/ Town/ state/ country **(Score 0-5)**

Registration:

Tell: "I am going to give three words and I'd like you to repeat me: Lemon, Key, and Ball". After subject repeats, say "Try to remember them because I am going to ask you later". Score only the first trial (repeat three times if necessary)

Register number of trials_____ **(Score 0-3)**

Attention and concentration:

Ask the subject: "Could you take seven away from 100? After the subject responds, ask him/her to take away another seven to a total of five subtractions. If subject makes a mistake carry on and check the subsequent answer (i.e., 93, 84, 77, 70, and 63-score-4)

Stop after five subtractions (93, 86, 79, 72, 65)……. (**Score 0-5)**

Ask: 'could you please spell the word **WORLD** for me? Then ask him/her to spell in backwards……. **(Score 0-5)**

Memory – Recall:

Ask: 'Which three words did I ask you to repeat and remember?"

_____, _____,_____ **(Score 0-3)**

Memory – Anterograde memory:

Tell: I am going to give you a name and an address and I'd like you to repeat after me.

	1st Trial	2nd Trial	3rd Trial
Selvakumar,			
42, Nehru street,			
Gandhi Nagar,			
Vellore.			

We'll be doing that three times, so you have a chance to learn it. I'll be asking you later'. Score only the third trial **(Score 0-7)**

Memory – Retrograde Memory:

- Name of the current chief minister of Tamil Nadu……
- Name of the woman who was the prime Minister of India…….
- Name of the Indian Prime minister assassinated in 1991……
- Name of the current Indian Prime Minister…….....

Verbal Fluency – Letter 'P' and animals

Letters:

Say: I am going to give you an alphabet and I'd like you to generate as many words you can beginning with that letter, but not names of people or places. Are you ready? You've got a minute and the letter is p' **(Score 0-7)**

Animals

Say: Can you name as many animals as possible, beginning with any letter? **(Score 0-7)**

>21= 7; 17-21= 6; 14-16= 5; 11-13= 4; 9-10= 3; 7-8= 2; 5-6= 1; <5=0

Language - Comprehension:

- **Show written command** **(Score 0-1)**

Close Your Eyes

- **Three stage command** **(Score 0-3)**

Take a paper in your hand / Fold the paper / put the paper on the table

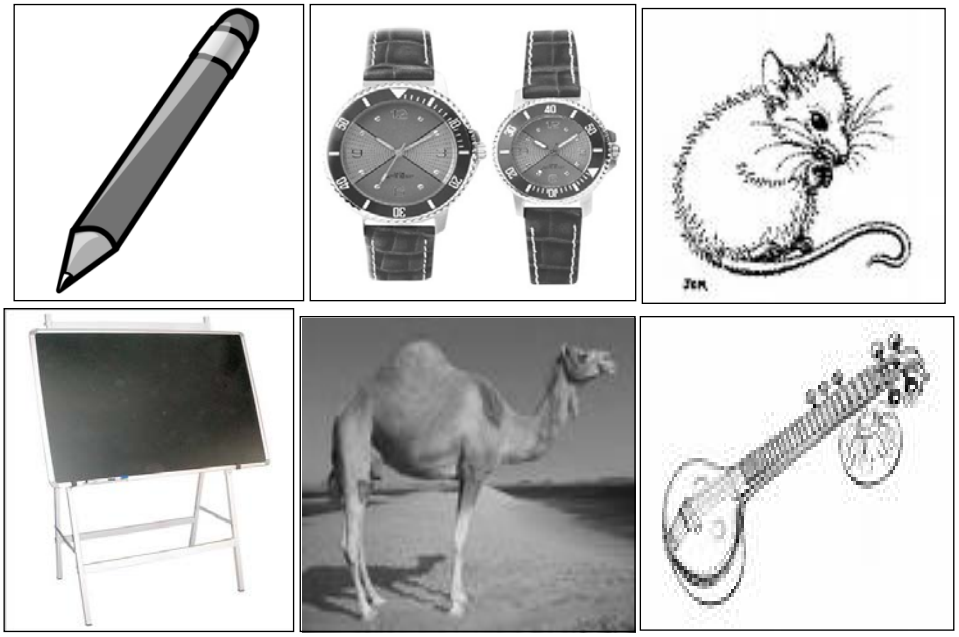
- **Writing**

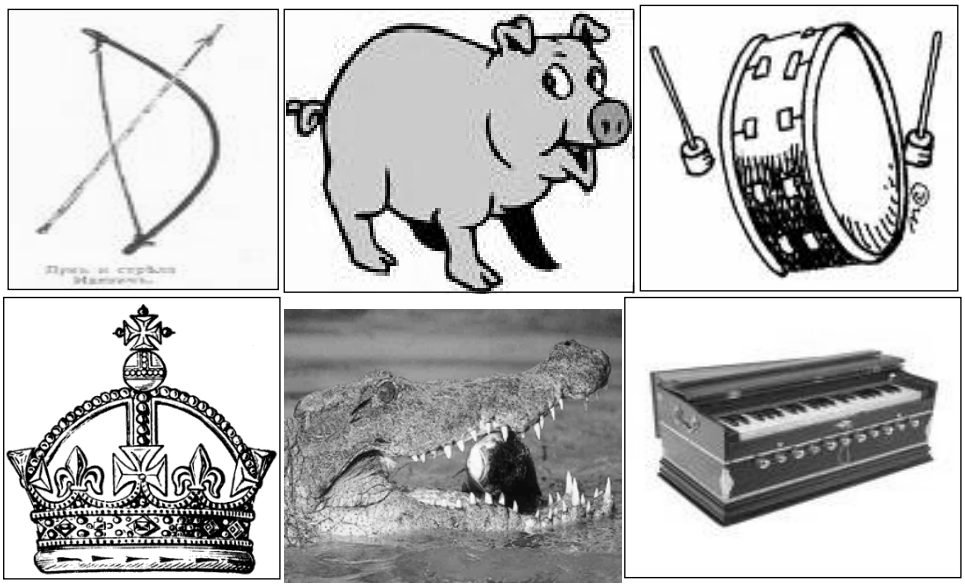
Ask the subject to make up a sentence and write it in the space below: score 1 if sentence contains a subject and a verb. **(Score 0-1)**

Language – Repetition:

- Ask the subject to repeat 'hippopotamus', 'eccentricity', 'unintelligible', and 'statistician'. Score: 2 if all correct: 1 if 3 correct: 0 if 2 or less
- Ask the subject to repeat: 'above beyond and below' **(Score 0-1)**
- Ask the subject to repeat: 'No ifs, ands or buts' **(Score0-1)**

Language – Naming:





Language: comprehension:

Using the pictures above, ask the subjects to: **(Score 0-4)**

Point to the one which is associated with the monarchy _____

Point to the one which is a rodent _____

Point to the one which is found in the desert _____

Point to the one which is associated with warriors _____

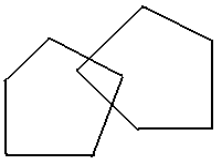
Language: reading:

Ask the subject to read the following words: [score 1 only if all correct] **(Score 0-1)**

SEW	**PINT**	**SOOT**	**DOUGH**	**HEIGHT**

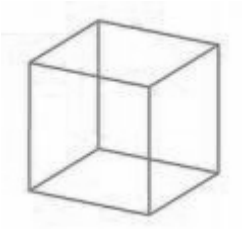
Visuo-spatial Abilities:

- **Overlapping pentagons:** ask the subject to copy this diagram **(Score 0-1)**



- **Wire cube:** ask the subject to copy this drawing

(Score 0-2)



- **Clock:** Ask the subject to draw a clock face with numbers and the hands at ten past five. (For scoring, circle= 1, numbers = 2, hands = 2 if all correct) **(Score 0-5)**

Perceptual abilities:

- Ask the subject to count the dots without pointing them. **(Score 0-4)**
- Ask the subject to identity the letters **(Score 0-4)**

Recall:

Ask "Now tell me what you remember of that name and address we were repeating at the beginning" **(Score 0-7)**

Selvakumar,	
42, Nehru street,	
Gandhi Nagar,	
Vellore.	

Recognition:

This test should be done if subject failed to recall one or more items. If all items were recalled, skip the test and score 5. If only part is recalled start by ticking items recalled in the shadowed column on the right hand side. Then test not recalled items by telling" ok, I'll give you some hints; was the name X, Y or Z?" and so on. Each recognized item scores one point, which is added to the point gained by recalling.

Nallakumar		Selvakumar		Selvakrishnan		Recalled	
24		42		49			
Nehru Road		Patel street		Nehru street			
Chrompet		Gandhi nagar		Saidapet			
Vellore		Nellore		Nagpur			

General Scores	
MMSE	**/30**
ACE-R	**/100**
Sub scores	
Attention and Orientation	**/18**
Memory	**/26**
Fluency	**/14**
Language	**/26**
Visuo-spatial	**/16**

10.4.5. Childhood Traumatic Events Scale

10.4.6. Recent Traumatic Events Scale

10.4.7. Tamil version of Childhood Traumatic Events Scale

10.4.8. Tamil version of Recent Traumatic Events Scale

Childhood Traumatic Events Scale

For the following questions, answer each item that is relevant. Be as honest as you can. Each question refers to any event that you may have experienced **prior to the age of 17**.

1. Prior to the age of 17, did you experience a death of a very close friend or family member?_____ If yes, how old were you?_____

   If yes, how traumatic was this? (using a 7-point scale, where 1 = not at all traumatic, 4 = somewhat traumatic, 7 = extremely traumatic)_____

   If yes, how much did you confide in others about this traumatic experience at the time? (1 = not at all, 7 = a great deal)_____

2. Prior to the age of 17, was there a major upheaval between your parents (such as divorce, separation)?_____ If yes, how old were you?_____

   If yes, how traumatic was this? (where 7 = extremely traumatic)_____

   If yes, how much did you confide in others? (7 = a great deal)_____

3. Prior to the age of 17, did you have a traumatic sexual experience (raped, molested, etc.)?_____ If yes, how old were you?_____

   If yes, how traumatic was this? (7 = extremely traumatic)_____

   If yes, how much did you confide in others? (7 = a great deal)_____

4. Prior to the age of 17, were you the victim of violence (child abuse, mugged or assaulted -- other than sexual)?_____ If yes, how old were you?_____

   If yes, how traumatic was this? (7 = extremely traumatic)_____

   If yes, how much did you confide in others? (7 = a great deal)_____

5. Prior to the age of 17, were you extremely ill or injured?_____ If yes, how old were you?_____

   If yes, how traumatic was this? (7 = extremely traumatic)_____

   If yes, how much did you confide in others? (7 = a great deal)_____

6. Prior to the age of 17, did you experience any other major upheaval that you think may have shaped your life or personality significantly?_____ If yes, how old were you?_____

   If yes, what was the event?_____

   If yes, how traumatic was this? (7 = extremely traumatic)_____

   If yes, how much did you confide in others? (7 = a great deal)_____

Recent Traumatic Events Scale

For the following questions, again answer each item that is relevant and again be as honest as you can. Each question refers to any event that you may have experienced **within the last 3 years**.

1. Within the last 3 years, did you experience a death of a very close friend or family member? _____

   If yes, how traumatic was this? (1 = not at all traumatic, 7 = extremely traumatic)_____

   If yes, how much did you confide in others about the experience at the time? (1 = not at all, 7 = a great deal)_____

2. Within the last 3 years, was there a major upheaval between you and your spouse (such as divorce, separation)?_____

   If yes, how traumatic was this?_____

   If yes, how much did you confide in others?_____

3. Within the last 3 years, did you have a traumatic sexual experience (raped, molested, etc.)?_____

   If yes, how traumatic was this?_____

   If yes, how much did you confide in others?_____

4. Within the last 3 years, were you the victim of violence (other than sexual)?_____

   If yes, how traumatic was this?_____

   If yes, how much did you confide in others?_____

5. Within the last 3 years, were you extremely ill or injured?_____

   If yes, how traumatic was this?_____

   If yes, how much did you confide in others?_____

6. Within the last 3 years, has there been a major change in the kind of work you do (e.g., a new job, promotion, demotion, lateral transfer)?_____

   If yes, how traumatic was this?_____

   If yes, how much did you confide in others?_____

7. Within the last 3 years, did you experience any other major upheaval that you think may have shaped your life or personality significantly?_____

   If yes, what was the event?_____

   If yes, how traumatic was this?_____

   If yes, how much did you confide in others?_____

Foe i j gUt j j py; kdmj hr rp ia cUthf Fk; rkgtq fsp d; msTNfhy;

f otUk; Nfs tpf Sf F, nghUj jkhd c z ikahd t pilfis msp f fTk; xt nthU Nfs tpAk, 17 tajpw F Kd; cq fs; tho tpy; eq fs; mDgt pj j xU rkgtk; njhlhGilajhf ,Uf Fk;

1. 17 tajpw F Kd ghf, xU neUq fpa cwt pdh; myyJ ez ghpd; ,wg ig eq fs; mDgt pj jJ c z lh? Mk; vd py, vej tajpy?
   Mk; vd py, mJ cq fis vt tsT kd mj hr rp f Fs shf fpaJ?
   (7 Gs sp kj pgg l L msit gad gLj Jk; NghJ, 1- xU NghJk; kdmj hr rp ,y iy, 4 – rpwj sT kdmj hr rp, 7 – mj pfg gbahd kdmj hr rp)
   Mk; vd why, ,ij gww vej mst pw F gwh p lk; kdk j pwe J ek g $w pAs s hfs? (1- xU NghJk; ,y iy, 7 – k pf mj pfk)

2. 17 tajpw F Kd ghf, cq fs J ngwNwhh f Sf f pilNa k pFej g pur rp id VNjDk; vOe jjh? (t pthfuj J, jd pahf g phe J tho jy; Nghd wit)
   Mk; vd py, vej tajpy?
   Mk; vd py, mJ cq fis vt tsT kd mj hr rp f Fs shf fpaJ?
   (7 Gs sp kj pgg l L msit gad gLj Jk; NghJ, 1- xU NghJk; kdmj hr rp ,y iy, 4 – rpwj sT kdmj hr rp, 7 – mj pfg gbahd kdmj hr rp)
   Mk; vd why, ,ij gww vej mst pw F gwh p lk; kdk j pwe J ek g $w pAs s hfs? (1- xU NghJk; ,y iy, 7 – k pf mj pfk)

3. 17 tajpw F Kd G, eq fs; ghy pa pay; td K iwa p id rej p j j pUf f pwh fsh? (fwgo pgG, ghy pay;, njhe juT, NtW gy) Mk; vd py, vej tajpy?
   Mk; vd py, mJ cq fis vt tsT kd mj hr rp f Fs shf fpaJ?
   (7 Gs sp kj pgg l L msit gad gLj Jk; NghJ, 1- xU NghJk; kdmj hr rp ,y iy, 4 – rpwj sT kdmj hr rp, 7 – mj pfg gbahd kdmj hr rp)

Mk; vdwhy;, ,ij gwwp vej mstpwF gpwhplk; kdkjpweJ ekgp $wpAs;shfs?(1- xU NghJk; ,y;iy,7 – kpf mjpfk)

4. 17 tajpwF KdG, eqfs; ghypapay; myyhj tdKiwfF gypahfpapUffwhfsh?(nfhLikfs;, Vkhwwgg Ljy;, jhffggLjy;)

Mk; vdpy;, vej tajpy;?

Mk; vdpy;, mJ cqfis vt;tsT kdmjphrrpfFs;shffpaJ?

(7 Gs;sp kjpggl;L msit gadgLj;Jk; NghJ, 1- xU NghJk; kdmjphrrp ,y;iy, 4 – rpwpjsT kdmjphrrp, 7 – mjpfggbahd kdmjphrrp)

Mk; vdwhy;, ,ij gwwp vej mstpwF gpwhplk; kdkjpweJ ekgp $wpAs;shfs?(1- xU NghJk; ,y;iy,7 – kpf mjpfk)

5. 17 tajpwF KdG, eqfs; kpf nfhba Neha;thag;lJ myyJ fhaggl;lJ cz;lh? Mk; vdpy;, vej tajpy;?

Mk; vdpy;, mJ cqfis vt;tsT kdmjphrrpfFs;shffpaJ?

(7 Gs;sp kjpggl;L msit gadgLj;Jk; NghJ, 1- xU NghJk; kdmjphrrp ,y;iy, 4 – rpwpjsT kdmjphrrp, 7 – mjpfggbahd kdmjphrrp)

Mk; vdwhy;, ,ij gwwp vej mstpwF gpwhplk; kdkjpweJ ekgp $wpAs;shfs?(1- xU NghJk; ,y;iy,7 – kpf mjpfk)

6. 17 tajpwF KdG, cqfs; thoffifiaNah myyJ Fzhjpraj;ijNah Fwpggplkgb khwwf;$ba msT nghpa rkgtqfs; VNjDk; elejjh?

Mk; vdpy;, vej tajpy;? Mk; vdpy;, mJ vd;d rkgtk;?

Mk; vdpy;, mJ cqfis vt;tsT kdmjphrrpfFs;shffpaJ?

(7 Gs;sp kjpggl;L msit gadgLj;Jk; NghJ, 1- xU NghJk; kdmjphrrp ,y;iy, 4 – rpwpjsT kdmjphrrp, 7 – mjpfggbahd kdmjphrrp)

Mk; vdwhy;, ,ij gwwp vej mstpwF gpwhplk; kdkjpweJ ekgp $wpAs;shfs?(1- xU NghJk; ,y;iy,7 – kpf mjpfk)

rkg fhyj;jpy; kdmjpHrrpia cUthf;Fk; rkgtq;fspd; msTNfhy;

fotUk; Nfstpfsf;F, nghUj;jkhd czikahd tpilfis mspf;fTk; xtnthU Nfstpak;, flej 3 tUlq;fSf;Fs; cq;fs; thotpy; eq;fs; mDgtpj;j xU rkgtk; njhlhGilajhf ,Uf;Fk;.

1. 17 tajpw;F Kd;ghf, xU neUq;fpa cwtpdh; myyJ ez;ghpd; ,wg;ig eq;fs; mDgtpj;jJ czlh? Mk; vdpy;, vej tajpy?
   Mk; vdpy;, mJ cq;fis vt;tsT kdmjpHrrpf;Fs; shf;fpaJ?
   (7 Gs;sp kjpgg;lL msit gad;gLj;Jk; NghJ, 1- xU NghJk; kdmjpHrrp ,yiy, 4 – rpwpjsT kdmjpHrrp, 7 – mjpfgg;bahd kdmjpHrrp)
   Mk; vdwhy;, ,ij gwwp vej mstpw;F gpwpLk; kdkjpwe;J ekgp $wpAs;shfs? (1- xU NghJk; ,yiy, 7 – kpf mjpfk;)

2. cq;fsJ ngwNwhh;fSf;fpilNa kpFej gurrpid VNjDk; vOe;jh? (tpthfuj;J, jdpahf gphpe;J thoy; Nghdwit)
   Mk; vdpy;, vej tajpy?
   Mk; vdpy;, mJ cq;fis vt;tsT kdmjpHrrpf;Fs; shf;fpaJ?
   (7 Gs;sp kjpgg;lL msit gad;gLj;Jk; NghJ, 1- xU NghJk; kdmjpHrrp ,yiy, 4 – rpwpjsT kdmjpHrrp, 7 – mjpfgg;bahd kdmjpHrrp)
   Mk; vdwhy;, ,ij gwwp vej mstpw;F gpwpLk; kdkjpwe;J ekgp $wpAs;shfs? (1- xU NghJk; ,yiy, 7 – kpf mjpfk;)

3. eq;fs; ghypapay; td;Kiwapid rejpj;jpUf;fwhfsh? (fwgopgG, ghypay;, njhe;juT, NtWgy) Mk; vdpy;, vej tajpy?
   Mk; vdpy;, mJ cq;fis vt;tsT kdmjpHrrpf;Fs; shf;fpaJ?
   (7 Gs;sp kjpgg;lL msit gad;gLj;Jk; NghJ, 1- xU NghJk; kdmjpHrrp ,yiy, 4 – rpwpjsT kdmjpHrrp, 7 – mjpfgg;bahd kdmjpHrrp)

Mk; vd;wh;y;, ;i j gww;p ve;j ms;t;wF gw;wp;l;k; kdk;j;we;J ek;gp $w;As;sh;fs;? (1- xU NghJk; ,y;i y;, 7 – k;f mj;fk;)

4. e;q;fs; ghy;a;ay; my;yh;j td;Kiwf;F gy;ah;f;a;Uf;fwh;fsh? (nfhLikfs;, Vkhwwgg;Ljy;, jh;f;fgg;Ljy;) Mk; vd;y;, ve;j taj;y;?

   Mk; vd;y;, mJ cq;fis vt;tsT kdmj;h;r;r;f;Fs;sh;f;f;aJ?

   (7 Gs;s;p kj;gg;l;L ms;it gad;gLj;Jk; NghJ, 1- xU NghJk; kdmj;h;r;r;p ,y;i y;, 4 – r;wj;sT kdmj;h;r;r;p, 7 – mj;fgg;bah;d kdmj;h;r;r;p)

   Mk; vd;wh;y;, ;i j gww;p ve;j ms;t;wF gw;wp;l;k; kdk;j;we;J ek;gp $w;As;sh;fs;? (1- xU NghJk; ,y;i y;, 7 – k;f mj;fk;)

5. e;q;fs; k;f nfhba Neha;tha;gl;lJ my;yJ fhag;gl;lJ cz;lh? Mk; vd;y;, ve;j taj;y;?

   Mk; vd;y;, mJ cq;fis vt;tsT kdmj;h;r;r;f;Fs;sh;f;f;aJ?

   (7 Gs;s;p kj;gg;l;L ms;it gad;gLj;Jk; NghJ, 1- xU NghJk; kdmj;h;r;r;p ,y;i y;, 4 – r;wj;sT kdmj;h;r;r;p, 7 – mj;fgg;bah;d kdmj;h;r;r;p)

   Mk; vd;wh;y;, ;i j gww;p ve;j ms;t;wF gw;wp;l;k; kdk;j;we;J ek;gp $w;As;sh;fs;? (1- xU NghJk; ,y;i y;, 7 – k;f mj;fk;)

6. f;lej %d;W Mz;LfS f;Fs;, cq;fs; Nt;iya;y; Vjh;tJ ngh;a; khw;wk; Vw;gl;Ls;sjh? (c . jh. Gj;a Nt;iy, gj;t;p cah;T, gj;t;p ,wf;fk;, g;z;p khw;wk;) Mk; vd;y;, ve;j taj;y;?

   Mk; vd;y;, mJ cq;fis vt;tsT kdmj;h;r;r;f;Fs;sh;f;f;aJ?

   (7 Gs;s;p kj;gg;l;L ms;it gad;gLj;Jk; NghJ, 1- xU NghJk; kdmj;h;r;r;p ,y;i y;, 4 – r;wj;sT kdmj;h;r;r;p, 7 – mj;fgg;bah;d kdmj;h;r;r;p)

   Mk; vd;wh;y;, ;i j gww;p ve;j ms;t;wF gw;wp;l;k; kdk;j;we;J ek;gp $w;As;sh;fs;? (1- xU NghJk; ,y;i y;, 7 – k;f mj;fk;)

7. cq;fs; thof;ifiaNah myyJ Fzhj;raj;ijNah Fwpg;gpLk;gb khwwf;$ba msT ngha; rkgtq;fs; VNjDk; elejjh?

Mk; vd;y, vej taj;y? Mk; vd;y, mJ vd;d rkgtk?

Mk; vd;y, mJ cq;fis vt;tsT kdmj;hrr;f;Fs;shf;fpaJ?

(7 Gs;sp kj;gg;l;L msit gad;gLj;Jk; NghJ, 1- xU NghJk; kdmj;hrr; ,yiy, 4 – rpwpjsT kdmj;hrr;, 7 – mj;fgg;bahd kdmj;hrr;)

Mk; vdwhy;, ,ij gwwp vej mstpw;F gpwhp;lk; kdkj;wpe;J ekgp $wpAs;shf;fs? (1- xU NghJk; ,yiy, 7 – kpf mj;fk;)

CHAPTER 11

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