Genetic Risk Factors for the Lack of Response to Clinical Treatment in Mental Disorders: an Approach from Pharmacogenetics

Marina Mitjans Niubó

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Doctoral Thesis presented by

Marina Mitjans Niubó

Directed by Dr. Bárbara Arias

Assistant Lecturer of the Unit of Anthropology

Department of Animal Biology

University of Barcelona

Doctoral program of Biomedicine

Department of Animal Biology – Faculty of Biology

Bárbara Arias Sampériz

Director

Cristóbal Gastó Ferrer

Tutor

Marina Mitjans Niubó

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"Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning"

Albert Einstein

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List of Genes mentioned in the thesis

Gene	Chromosome position	Description
ABCB1	7q21.12	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ACCN1	17q12	Acid-sensing (proton-gated) ion channel 2
ADCY2	5p15.3	Adenylate cyclase 2 (brain)
ADRA2A	10q25.2	Adrenoceptor alpha 2A
ANK3	10q21	Ankyrin 3, node of Ranvier (ankyrin G)
APOE	19q13.2	Apolipoprotein E
AKT1	14q32.32	V-akt murine thymoma viral oncogene homolog 1
BAG-1	9p12	BCL2-associated athanogene
BDNF	11p13	Brain-derived neurotrophic factor
BMP7	20q13	Bone morphogenic protein 7
CACNA1C	12p13.3	Calcium channel, voltage-dependent, L type, alpha 1C subunit
CDH17	8q22.1	Cadherin 17, LI cadherin (liver-intestine)
CNR1	6q14-q15	Cannabinoid receptor 1 (brain)
CNR2	1p36.11	Cannabinoid receptor 2
COMT	22q11.21	Catechol-O-methyltransferase
CREB1	2q34	cAMP responsive element binding protein 1
CRHR1	17q12-q22	Corticotropin releasing receptor 1 gene
CRHR2	7p14.3	Corticotropin releasing receptor 2 gene
CYP2C19	10q24	Cytochrome P450, family 2, subfamily C, polypeptide 19
CYP2D6	22q13.1	Cytochrome P450, family 2, subfamily D, polypeptide 6
DAOA	13q33.2; 13q34	D-amino acid oxidase activator
DGKH	13q14.11	Diacylglycerol kinase, eta
DRD1	5q35.1	Dopamine receptor D1
DRD2	11q23	Dopamine receptor D2
DRD3	3q13.3	Dopamine receptor D3
DRD4	11p15.5	Dopamine receptor D4
DISC1	1q42.1	Disrupted in schizophrenia 1
DTNBP1	6p22.3	Dystrobrevin binding protein 1
FAAH	1p35-p34	Fatty acid amide hydrolase gene
FKBP5	6p21.31	FK506 binding protein 5
GABRB2	5q34	Gamma-aminobutyric acid (GABA) A receptor, beta 2
GADL1	3p24.1-p23	Glutamate decarboxylase-like 1
GFRA2	8p21.3	GDNF family receptor alpha 2

GNB3	12p13	Guanine nucleotide binding protein (G protein), beta polypeptide 3
GRIA2	4q32.1	Glutamate receptor, ionotropic, AMPA 2
GRIK2	6q16.3	Glutamate receptor, ionotropic, kainate 2
GRIK5	19q13.2	Glutamate receptor, ionotropic, kainate 5
GRIN2B	12p12	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B
GPX1	3p21.3	Glutathione peroxidase 1
GRIN1	9q34.3	Glutamate receptor, ionotropic, N-methyl D-aspartate 1
GRIN2	10q11.22	G protein regulated inducer of neurite outgrowth 2
GRIN2B	12p12	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B
GSK3B	3q13.3	Glycogen synthase kinase 3 beta
HRH1	3p25	Histamine receptor H1
HRH2	5q35.2	Histamine receptor H2
HTR1A	5q11.2-q13	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled
HTR2A	13q14-q21	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled
IL11	19q13.3-q13.4	Interleukin 11
INPP1	2q32	Inositol polyphosphate-1-phosphatase
IMPA1	8q21.13-q21.3	Inositol(myo)-1(or 4)-monophosphatase 1
IMPA2	18p11.2	Inositol(myo)-1(or 4)-monophosphatase 2
ITPKC	19q13.1	Inositol-trisphosphate 3-kinase C
MARCKS	6q22.2	Myristoylated alanine-rich protein kinase C substrate
MNSOD	6q25.3	Superoxide dismutase 2, mitochondrial
MTHFR	1p36.3	Methylenetetrahydrofolate reductase (NAD(P)H)
NCAN	19p12	Neurocan
NR3C1	5q31.3	Nuclear receptor subfamily 3, group C, member 1
NRG1	8p12	Neuregulin 1
NRGN	11q24	Neurogranin (protein kinase C substrate, RC3)
NRXN1	2p16.3	Neurexin 1
NTRK2	9q22.1	Neurotrophic tyrosine kinase, receptor, type 2
ODZ4	11q14.1	Teneurin transmembrane protein 4
OXT	20p13	Oxytocin/neurophysin I prepropeptide
PLCG1	20q12-q13.1	Phospholipase C, gamma 1
RORA	15q22.2	RAR-related orphan receptor A
SLC4A10	2q24.2	Solute carrier family 4, sodium bicarbonate transporter, member 10
SLC6A3	5p15.3	Solute carrier family 6 (neurotransmitter transporter), member 3

SLC6A4	17q11.2	Solute carrier family 6 (neurotransmitter transporter), member 4
TRANK1	3p22.2	Tetratricopeptide repeat and ankyrin repeat containing 1
TCF4	18q21.1	Transcription factor 4
UBE3C	7q36.3	Ubiquitin protein ligase E3C
UST	6q25.1	Uronyl-2-sulfotransferase
VIPR2	7q36.3	Vasoactive intestinal peptide receptor 2
ZNF804A	2q32.1	Zinc finger protein 804A

1. Introduction

"To wrest from nature the secrets which have perplexed philosophers in all ages,
to track to their sources the causes of disease,
to correlate the vast stores of knowledge,
that they may be quickly available for the prevention and cure of disease
-these are our ambitions."

-- Sir William Osler --

Introduction

Following World Health Organization (WHO), mental health is defined as a state of well-being in which every individual realizes his or her own potential, can cope with the normal stresses of life, can work productively and fruitfully, and is able to make a contribution to her or his community. In consequence, a mental disorder will be characterized by alterations in thinking, mood, and/or behaviour that are associated with distress and/or impaired functioning. Mental disorders contribute to a host of problems that may include disability, pain, or death causing a major impact on a person's wellbeing and interfering with their daily functioning (at home, work and socially) and adversely affect quality of life.

WHO reports have shown that one in four people in the world will be affected by mental or neurological disorders at some point in their lives. Around 450 million people currently suffer from such conditions, placing mental disorders among the leading causes of ill-health and disability worldwide. Treatments are available, but nearly two-thirds of people with a known mental disorder never seek help from a health professional. Stigma, discrimination and neglect prevent care and treatment from reaching people with mental disorders (WHO).

The total cost of brain disorders (mental and neurologic disorders) in Europe in 2010 was almost €800 billion. Drugs, visits to doctors and hospitalizations — the direct health-care costs — make up 37% of the bill. A further 23% is spent on direct non-medical costs, including informal care, social services and nursing homes. The remainder (40%) is sucked away by indirect costs, such as lost productivity as a result of time off work or early retirement. Specifically, mood disorders and psychotic disorders account for more than a quarter of these costs (Smith, 2011) (Figure 1).

The prevalence and cost of brain disorders are going to increase because of increasing life expectancy, in particular, neurodegenerative disorders, stroke,

depression, and anxiety. Increased focus on research strategies, prevention, and care is necessary to reduce the future cost of brain disorders (Olesen et al., 2012).

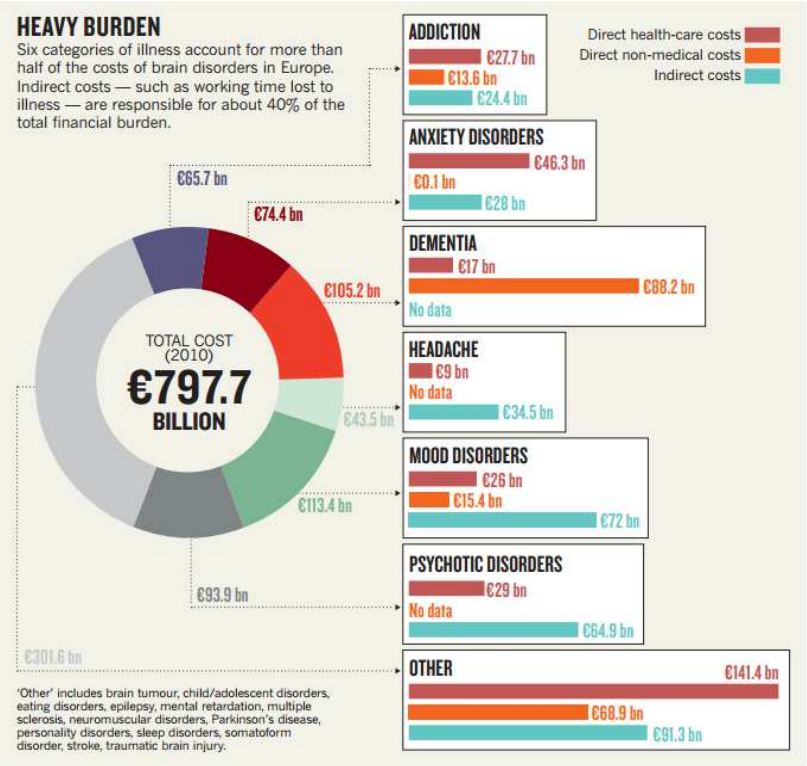


Figure 1. Total cost of brain disorders in Europe in 2010 (Smith, 2011).

Moreover, we cannot forget the impact of mental disorders in families. Family members are often the primary caregivers of people with mental disorders. They provide emotional and physical support, and often have to bear the financial expenses associated with mental health treatment and care. It is estimated that one in four families has at least one member with a mental disorder. In addition to the obvious distress of seeing a loved-one disabled by the consequences of a mental disorder, family members are also exposed to the stigma and discrimination associated with mental ill health. The extent of the burden of mental disorders on family members is difficult to assess and quantify, and is consequently often ignored. However, it does have a significant impact on the family's quality of life.

1.1. Mental Disorders

The existing model for understanding mental health and mental disorders emphasizes the interaction of social, environmental, and genetic factors throughout the lifespan. Mental disorders, like most diseases that affect humans, are part of the group of diseases known as genetically complex diseases, in which both genetic and environmental factors have a role in their aetiology.

The genetic component of these diseases has been identified from studies in families, twins or adopted children. Complex diseases, despite having a genetic basis, do not conform to the classic Mendelian inheritance pattern. In general, the sensitivity threshold model is considered one of the most useful for explaining how the disease is transmitted. This model assumes that the "disease susceptibility" variable is distributed continuously in the population, so that only those individuals who surpass a certain threshold manifest the disorder. It is hypothesized that a number of minor effect genes are involved in the origin of this complex heredity, whose expression can be modulated by many environmental factors (Falconer, 1981) (Figure 2).

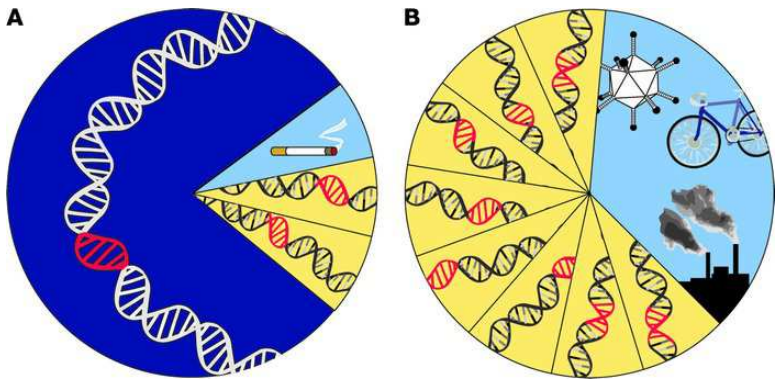


Figure 2. Genetic and environmental contributions to monogenic and complex disorders. (A) Monogenic disease. A variant in a single gene is the primary determinant of a monogenic disease or trait, responsible for most of the disease risk or trait variation (dark blue sector), with possible minor contributions of modifier genes (yellow sectors) or environment (light blue sector). (B) Complex disease. Many variants of small effect (yellow sectors) contribute to disease risk or trait variation, along with many environmental factors (blue sector). Adapted from (Manolio et al., 2008).

1.1.1. Major Depressive Disorder

Major depressive disorder (MDD) is a commonly occurring, serious, recurrent disorder linked to diminished role functioning and quality of life, medical morbidity, and mortality (Spijker et al., 2004; Ustun et al., 2004). The WHO has ranked depression as the fourth leading cause of disability worldwide and projects that, by 2020, it will be the second most important cause of disability, preceded only by cardiovascular disease (Murray and Lopez, 1996).

The peak risk period for onset of MDD across all countries ranges from mid to late adolescence to the early 40s (Zisook et al., 2007). Lifetime prevalence estimates of MDD vary widely across countries, with prevalence ranging from 1.5 to 19.0% (Kessler and Bromet, 2013). The wide variability in lifetime prevalence estimates of MDD is presumably due to a combination of substantive (genetic vulnerability and environmental risk factors) and measurement (cultural differences in the acceptance and meaning of items, and the psychometric properties of the instruments) and study-design factors (Bromet et al., 2011).

Depression is approximately twice as prevalent in women as it is in men (Piccineli and Wilkinson, 2000). While the average gender difference points to more universal genetic, neurohormonal, or psychobiological gender-linked antecedents of depression (Kuehner, 2003), cross-national variation in the gender ratio of depression suggests that social conditions also have a strong association with this diagnosis (Weissman et al., 1996). Hence, most current research accepts that gender differences in depression are the result of a variable interplay among biological, psychological and social factors (Kuehner, 2003; Hopcroft and Bradley, 2007).

MDD diagnose is often complicated due to the difficulty of defining different symptoms and the syndrome of behaviours and feelings in certain life situations and the broad clinical variability present in depressive pictures. Similarly, we should not forget that there are no biological, biochemical or brain morphology markers that allow an unequivocal diagnosis of the disease. Due to this absence of external markers, the diagnosis is necessarily psychopathological and clinical (Peralta and Cuesta, 2002). In this sense, MDD is diagnosed based on diagnostic classification systems such as *Diagnostic and Statistical Manual of Mental*

Disorders (5th ed.; *DSM–V*; American Psychiatric Association, 2013 and previous editions) (Table 1). This classification is based on criteria developed and revised over the past three decades by the American Psychiatric Association (APA) being the most widely accepted nomenclature used by clinicians and researchers for the classification of mental disorders.

Major Depressive Disorder

Diagnostic Criteria

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.
Note: Do not include symptoms that are clearly attributable to another medical condition.

1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, hopeless) or observation made by others (e.g., appears tearful). (**Note:** In children and adolescents, can be irritable mood).
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. (**Note:** In children, consider failure to make expected weight gain).
4. Insomnia or hypersomnia nearly every day.
5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down.
6. Fatigue or loss of energy nearly every day.
7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
9. Recurrent thoughts of death (not just fear or dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
C. The episode is not attributable to the physiological effects of a substance or to another medical condition.

Note: Criteria A-C represent a major depressive episode.
Note: Responses to a significant loss (e.g., bereavement, financial ruin, losses from a natural disaster, a serious medical illness or disability) may include the feelings of intense sadness, rumination about the loss, insomnia, poor appetite, and weight loss noted in Criteria A, which may resemble a depressive episode. Although such symptoms may be understandable or considered appropriate to the loss, the presence of a major depressive episode in addition to the normal response to a significant loss should also be carefully considered. This decision inevitably requires the exercise of clinical judgment based on the individual's history and the cultural norms for the expression of distress in the context of loss.

D. The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.
E. There has never been a manic episode or a hypomanic episode.
Note: This exclusion does not apply if all the manic-like or hypomanic-like episodes are substance-induced or are attributable to the physiological effects of another medical condition.

Table 1. Diagnostic Criteria for Major Depressive Disorder based on DSM-V (American Psychiatric Association, 2013).

According to this categorical diagnosis, MDD is characterized by the presence of a depressed mood (hypothymia) and/or loss of interest and diminished pleasure in daily life activities (anhedonia). These symptoms, which become established and persist over time, interfere seriously with the daily life of the patient and tend to be accompanied by somatic or psychological changes and abnormalities in different biological functions. These symptoms include diminished appetite and weight loss, reduced activity of the individual, sleep disorders such as hypersomnia or early awakening, agitation or generalized inhibition of movement, and decreased libido or loss of libido (Paykel, 1992). Changes in cognitive functions that reduce the capacity to think, concentrate or make decisions are also often exhibited in depressive patients. Pessimistic thinking is also common, often including feelings of guilt and inferiority, ideas of hopelessness, and recurrent thoughts of death or suicide. Suicidal ideation should be kept in mind and suicide attempts are common in people affected by MDD, with more than 15% of patients ending their lives by suicide (Goodwin and Jamison, 1990).

1.1.1.1. Current Biological Hypotheses

The following biological hypotheses provide representative approaches toward understanding depression and antidepressant action.

Altered neurotransmission pathways hypothesis

As antidepressant drug treatments are designed to target monoaminergic neurotransmission, this represents the basis for the so-called catecholamine (Schildkraut, 1965) and serotonin (Coppen, 1967) hypotheses of affective disorders. These hypotheses posit that antidepressants act by increasing the extracellular concentration and function of monoamine transmitters in the brain (Nutt, 2002) and, in consequence, that mood disorders are caused by altered production, release, turnover or function of monoamine transmitters or by altered function of their receptors.

However, there is a growing consensus that altered monoaminergic transmission is not sufficient to explain the aetiology of depressive disorders (Hirschfeld, 2000) and that currently used antidepressants instead are modulating other neurochemical systems that have a more fundamental role in the disease (Heninger et al., 1996).

In this sense, it is known that gamma-aminobutyric acid (GABA)ergic transmission is vital for the control of stress and impaired by chronic stress, the most important vulnerability factor of MDD. Currently used antidepressants, which are designed to augment monoaminergic transmission, have in common that they ultimately serve to enhance GABAergic transmission. GABAergic excitation of immature neurons in the dentate gyrus has been identified as a key mechanism that provides trophic support and controls the dendritic maturation and survival of neurons, a process that serves as a molecular and cellular substrate of antidepressant action. Moreover, comparatively modest deficits in GABAergic transmission are sufficient to cause most of the cellular, behavioural, cognitive and pharmacological sequelae expected of an animal model of MDD (Luscher et al., 2011).

Other neurotransmitter systems such as the endocannabinoid (eCB) have also involved in the aetiology of depression (Domschke et al., 2008). Physiological actions of eCB system in the central nervous system (CNS) are mediated by the activation of a specific cannabinoid receptor, the CB1 receptor (Matsuda et al., 1990) located in the limbic system and in the brain areas related to stress response, such as the central amygdala and the paraventricular nucleus of the hypothalamus (Herkenham, 1991). It has been shown that changes in the functional activity of this system can cause altered activity in other neuromodulatory systems as well as imbalance in the primary GABA/glutamate control system (Rodriguez de Fonseca et al., 2005). Moreover, eCB system could activate the hypothalamic-pituitary-adrenal (HPA) axis (Weidenfeld et al., 1994), the neuroendocrine system involved in the responses to emotional stress.

Neuroendocrine hypothesis

Another etiological hypothesis, supported by a large body of epidemiological evidence, proposes that stress is a major vulnerability factor for mood disorders (Kendler et al., 1999; Gilbertson et al., 2002; Gold and Chrousos, 2002). This evidence includes dysregulation of the HPA axis function. The pattern of HPA axis system dysregulation in depression showing atypical responses to dexamethasone, higher baseline cortisol values and an overactive response to psychological stressors suggests abnormalities within the axis's negative feedback system and corticotrophin releasing hormone (CRH) production but intact pituitary and

adrenal sensitivity (Nemeroff, 1996; Nemeroff, 1998; Holsboer, 2000; Binder and Nemeroff, 2010). The dysregulation of the HPA axis could be the responsible system of abnormalities found in hippocampus or prefrontal cortex of depressed patients. Raised levels of circulating cortisol activate brain receptors stimulating gene transcription and protein synthesis. Although this may have a beneficial effect in the short term, enabling the brain to cope with smaller amounts of stress, persistent hypercortisolaemia in chronic stress can cause neuronal damage.

Neurotrophic hypothesis

An extension of the stress hypothesis puts forward that depressive disorders are caused by inadequate trophic support of neurons and impaired neural plasticity (Manji et al., 2001a; Duman and Monteggia, 2006; Pittenger and Duman, 2008). Evidence from studies showing altered neurotransmitter system function and HPA abnormalities have found the crucial link with neurotrophic factors such as the brain-derived neurotrophic factor (BDNF). The hippocampus is rich in BDNF which plays a major role in neuronal growth, survival and maturation as well as arborization and synaptic plasticity in the adult brain. Stress suppresses BDNF synthesis in the hippocampus and antidepressant drugs increase its synthesis and signalling in the hippocampus and prefrontal cortex (PFC) (Nestler et al., 2002; Shimizu et al., 2003). In depressed patients serum BDNF concentrations are low, correlating with the severity of the depression and increase with antidepressant drugs or electroconvulsive treatment (Shimizu et al., 2003; Piccinni et al., 2009). Stress is associated with reduced BDNF concentrations which further impair neuronal survival. The decrease in BDNF concentrations may be due to the reduction in hippocampal neuronal tissue, as well as a direct effect of hypercortisolaemia; decreased activity in monoaminergic neurotransmission or other noxious factors may also be responsible.

Inflammatory hypothesis

Finally, but not less important, immunological mechanisms have also been implicated in the complex pathophysiology of MDD. Proinflammmatory cytokines (signalling molecules of the immune system) elicit sickness behaviour (fatigue and lethargy) and symptoms of anxiety/depression. Depressive illness is a recognised adverse event in patients receiving treatment with interferon. Severe depression is

associated with immune activation and in particular with raised cytokine concentrations. Raised proinflammatory cytokines are associated with peripheral tryptophan (serotonin precursor) depletion, may influence noradrenergic activity and they stimulate the HPA axis. Such neurotransmitter and neuroendocrine changes may be interpreted by the brain as stressors and potentiate the activation of the HPA axis. It has been suggested that impaired glucocorticoid receptor function may be related to chronic exposure to inflammatory cytokines associated with chronic physical illness or chronic stress and this may explain to some extent the comorbidity of depression and chronic physical illness. Glucocorticoid resistance in turn may cause further increase in inflammation. More research with robust and consistent methodology is needed to establish the importance of the observed immune system changes in the pathogenesis of depression (Iwata et al., 2013).

1.1.1.2. Environmental and Genetic Risk Factors

Environmental risk factors

Environmental risk factors for MDD can be either proximal or distal. Proximal factors precede depression relatively shortly before its onset. Negative life events, such as death of a close relative, assault, serious marital problems, and divorce/breakup are associated with increased depression (Kendler et al., 1995).

Distal factors, on the other hand, are less temporally close to the appearance of depression but nevertheless contribute to vulnerability. Early childhood trauma, such as loss of a parent before adolescence, child neglect, physical, emotional or sexual abuse, are all linked to increased risk for adult depression (Patten, 1991; Nanni et al., 2012; Lindert et al., 2014). Moreover, early traumatic experiences like child abuse have been described as one of the most important environmental risk factors leading to the onset of MDD in adults (Kendler et al., 1993; Kessler and Magee, 1993; Kendler et al., 1999; Kendler et al., 2004). Evidence from neurobiology and epidemiology suggests that disruptive adverse events that occur during an individual's development can cause persistent cerebral dysfunction (Heim and Nemeroff, 2002; Anda et al., 2006).

Genetic risk factors

Broadly defined, heritability (h^2) indicates the proportion of the total phenotype variability that is explained by genetic factors. The estimated h^2 for MDD is around 37% (95% Confidence Intervals 31-42) (Sullivan et al., 2000). First-degree relatives of MDD patients have a 2- to 3-fold increased risk to develop MDD compared to the general population (Levinson, 2006).

Since the first case-control study linking MDD and genetic variability (Beckman et al., 1978), a large number of candidate gene studies of MDD have been published, but few susceptibility genes have been recognized and replicated. These inconsistent results may be due to methodological differences between studies, such as the study design, study population, diagnosis of MDD or even the lack of statistical power due to a small sample size (Mitjans and Arias, 2012).

A meta-analysis of genetic association studies in MDD was recently conducted in which 20 polymorphisms in 18 genes were analyzed. Five of these genes showed a statistically significant association with MDD (*APOE, GNB3, MTHFR, SLC6A3* and *SLC6A4*) (Lopez-Leon et al., 2008).

One of the most recent methodologies used in the search for genetic risk factors in complex diseases is based on genome-wide association studies (GWAS). This methodology is based on genotyping arrays or microarrays that allow the variability of the human genome (up to a million genetic markers in a subject in a single test) to be traced in order to assess the hypothesis of common disease-common variant without the need to conduct a hypothesis-guided study of the aetiology of the disease. In this sense, several GWAS for MDD have been published but they have been unsuccessful in identifying significant individual genetic variants (Sullivan et al., 2009; Lewis et al., 2010; Muglia et al., 2010; Rietschel et al., 2010; Kohli et al., 2011; Shi et al., 2011; Shyn et al., 2011; Wray et al., 2012; Hek et al., 2013; Major Depressive Disorder Working Group of the Psychiatric et al., 2013). These negative findings have led to speculation that depression is particularly heterogeneous both clinically and etiologically, which could dramatically reduce statistical power to identify causal loci (Craddock et al., 2008). Moreover, the failure of GWAS to identify specific polymorphisms for MDD may be further attributed to the fact that environmental exposures differ and have varying

effects among individuals with different genetic background (Zannas and Binder, 2014).

Gene - environment interaction

It has been shown that environmental effects are not independent from genetic individual profile. Gene-environment interaction constitutes a mechanism of gene-environment interplay, which basically means that there are genetically influenced individual differences in the sensitivity to specific environmental features (Van Os and Sham, 2003). With respect to MDD, a paradigmatic study was published in 2003 by Caspi's team. The study showed that individuals carrying the short allele (S) of the 5-HTTLPR polymorphism (*SLC6A4* gene) had experienced stressful life events (SLEs) in childhood and youth, and presented more depressive symptoms, depressive episodes and suicidal behaviour at age 26 (Caspi et al., 2003). Although these results have been widely replicated by several studies both in depression and depressive symptomatology (Lenze et al., 2005; Nakatani et al., 2005; Zalsman et al., 2006; Cervilla et al., 2007; Aguilera et al., 2009), meta-analyses do not clarify the effect that the interaction between *SLC6A4* and SLEs has on the risk for MDD or depressive symptomatology (Brown and Harris, 2008; Uher and McGuffin, 2008; Munafo et al., 2009; Risch et al., 2009; Karg et al., 2011).

FKBP5 gene, a candidate gene belonging to the stress hormone system, seems also to play a key role in modulating the impact of childhood abuse and the risk of the emergence of MDD or depressive symptoms in adulthood (Appel et al., 2011; Zimmermann et al., 2011; Zannas and Binder, 2014).

Gene-environment interactions on depressive symptoms have also been reported for other genes, such as the gene encoding the glucocorticoid receptor (*NR3C1*) (Bet et al., 2009), and brain-derived neurotrophic factor (*BDNF*) (Kim et al., 2007a; Aguilera et al., 2009; Gatt et al., 2009).

1.1.1.3. Treatment Approaches

The treatment of MDD is based in a widely range of efficacy treatments which include the pharmacology therapy, the psychotherapy and the electroconvulsive therapy (ECT). Currently, the most common approach from the Health Care System of a depressive episode is pharmacological treatment with antidepressants (Spigset and Martensson, 1999).

The selective inhibitors of serotonin reuptake (SSRI) antidepressants are the first line treatment in depression. The **SSRIs** —fluoxetine, fluvoxamine, paroxetine, sertraline, citalopram (CIT) and escitalopram— are safer and present fewer side effects compared to other types of antidepressants. SSRIs act by inhibiting presynaptic serotonin reuptake thus increasing the concentration of serotonin in the synaptic cleft and down-regulating the postsynaptic serotonin receptor expression. All SSRIs available nowadays present a different pattern of selectivity to the reuptake, being the CIT and the escitalopram the most selective. Moreover, the SSRIs have direct mechanisms of action that affect a greater or lesser extent other neurotransmitter systems (Hyttel, 1977; Maitre et al., 1982; Koe et al., 1983; Thomas et al., 1987). Since CIT is one of the drugs of interest in the present thesis, its mechanism of action will be explained in more detail in the "Pharmacogenetics of SSRIs (citalopram)" section.

Second line antidepressant treatments include tricyclic antidepressants (TCAs) monoamine oxidase inhibitors (MAOIs) and dual antidepressants (SNRIs). **TCAs** are characterized by inhibiting reuptake of norepinephrine, serotonin, and to a lesser extent, dopamine (DA). TCAs also block histaminergic H1 receptors, muscarinic M1 and postsynaptic adrenergic α1, causing side effects such as dry mouth, blurred vision, orthostatic hypotension, dizziness, etc. (Vallejo, 2005; Zeigler, 2006). The mechanism of **MAOIs** action involves the inhibition of the enzyme monoamine oxidase (MAO), which breaks down monoaminergic neurotransmitters such as serotonin, DA and norepinephrine. Using MAOIs requires a strict diet because of dangerous (or even deadly) interactions with foods — such as certain cheeses, pickles and wines — and some medications including birth control pills, decongestants and certain herbal supplements. **SNRIs** are reuptake inhibitors of serotonin and norepinephrine, which include venlafaxine and fluoxetine among others. Pharmacological profile depends on dosage; when

used at low doses its effects are comparable to that of SSRIs, in medium doses, it blocks reuptake of both serotonin and norepinephrine, and in high doses, it also blocks DA reuptake. Finally, other antidepressants such as Nefazodone (SARI), Mirtazapine (NaSSA) or Bupropion (NDRI) are less frequently prescribed.

Other lines of treatment include psychotherapy and ECT. There are a variety of **psychotherapies** available to treat symptoms of MDD such as cognitive behavioural therapy, interpersonal therapy or problem solving therapy. Psychotherapy may be the treatment of choice in some cases because patients prefer not to take medication or because there are personal nature problems that could improve simply with an appropriate psychotherapeutic intervention. Psychotherapy can be very effective as a sole treatment modality in patients with a mild to moderate depression (Persons et al., 1996). In severe depression, a combination of pharmacotherapy and psychotherapy can give better results than a single therapy (Thase et al., 1997).

ECT is a procedure in which electric currents are passed through the brain, intentionally triggering a brief seizure. ECT seems to cause changes in brain chemistry that can quickly reverse symptoms of certain mental illnesses. It is recommended as a treatment of choice for patients with severe MDD (ex. psychotic or catatonic depression, presence of suicidal thoughts) that is not responsive to pharmacological interventions and/or psychotherapeutic, particularly in those who have significant functional impairment or have not responded to numerous medication trials (Zornberg and Pope, 1993).

1.1.2. Bipolar Disorder

Bipolar Disorder (BD) is a serious mental illness, with a worldwide prevalence of 2-5% of the population (Merikangas et al., 2011). BD imposes a great burden on both patients and their families, and approximately 10-20% of patients commit suicide over the course of their illness (Rihmer and Kiss, 2002). The WHO classifies BD as one of the top 10 leading causes of the global burden of disease for the age group of 15-44-year-old people. Despite the devastating impact of BD on the lives of millions, there is still a dearth of knowledge concerning its aetiology and pathophysiology.

It is classically characterized by intermittent recurrent episodes of mania (**Type I**) or hypomania (**Type II**) interspersed with episodes of depression (Figure 3), which affect thought, perception, emotion and social behaviour. The disturbance of mood in BD is episodic and recurrent, cycling at varying intervals from one mood state to another. It is typically accompanied by reckless and impulsive behaviour, psychotic symptoms (e.g., delusions, hallucinations, and disorganized thinking), and cognitive disturbances.

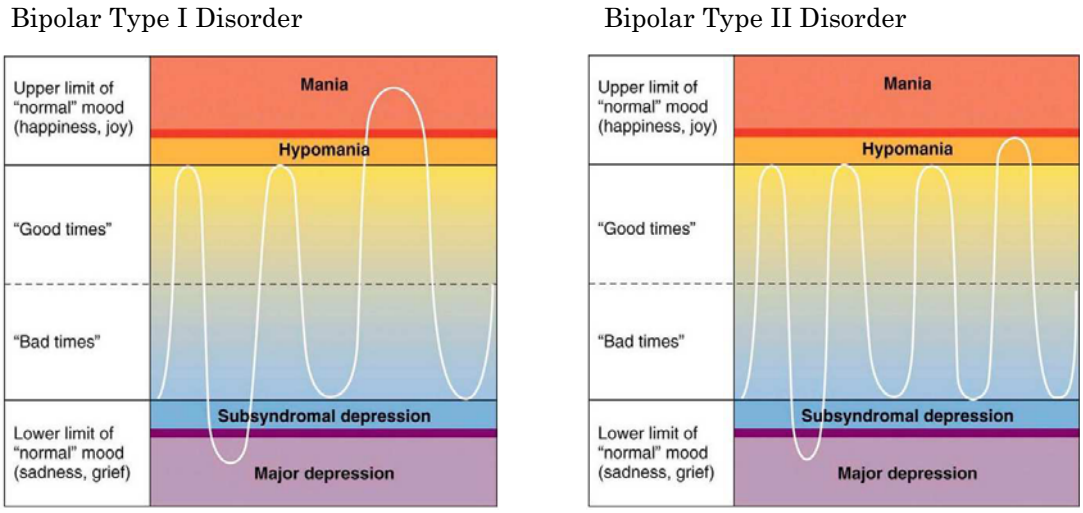


Figure 3. Representation of the pattern of mood in Bipolar Type I and II Disorders (Adapted from www.clevelandclinicmeded.com).

Classic mood elevation in BD is characterized by euphoria and excitement. In practice, the predominant mood is often irritability rather than euphoria. In addition to mood elevation, the symptoms of mania include inflated self-esteem,

decreased need for sleep, pressured and often loud speech, flight of ideas, distractibility, and increased goal-directed behaviour often focused on pleasurable activities that have a high potential for becoming reckless and self-destructive. Hypomania is a lesser form of mania, that is, mania minus the grossly impaired judgment that results in damaging, irresponsible behaviour (e.g., excessive and indiscriminate sexual activity, spending, or travelling without heed to their consequences). Diagnostic criteria for BD states as described in the DSM-V (American Psychiatric Association, 2013) (Table 2).

Bipolar I Disorder

A. Criteria have been met for at least one episode (Criteria A-D under "Manic Episode" above).
B. The occurrence of the manic and major depressive disorder(s) is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified or unspecified schizophrenia spectrum and other psychotic disorder.

Bipolar II Disorder

Criteria have been met for at least one hypomanic episode (Criteria A-F under "Hypomanic Episode" above) and at least one major depressive episode (Criteria A-C under "Major Depressive Episode" above).

A. There has never been a manic episode.
B. The occurrence of the hypomanic episode(s) and major depressive episode(s) is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified or unspecified schizophrenia spectrum and other psychotic disorder.
C. The symptoms of depression or the unpredictability caused by frequent alternation between periods of depression and hypomania causes clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Table 2. Diagnostic Criteria for Bipolar Type I and II Disorder based on DSM-V (American Psychiatric Association, 2013).

The first lifetime manifestation of BD is typically a major depressive episode, usually occurring during late adolescence or early adulthood. The first episode of mania or hypomania might not occur until several years later, and until that time a diagnosis of BD cannot be made. It is uncommon for the first manic episode to occur after age 30 years, although onset after age 60 years has been reported (Carey, 2010).

There is a large amount of variation in how often patients suffer mood episodes. Some patients have discrete episodes that occur rarely (for example, no more than one episode per year) with full recovery in between, others experience episodes more often, and some may fail to fully recover between episodes. A subset of patients suffers from **rapid-cycling**, which is defined as the experience of at least four syndromal depressive, manic, hypomanic or mixed episodes within a 12-month

period (Barrios et al., 2001). Ultra-rapid and ultra-ultra-rapid cycling variants have also been identified, in which mood fluctuates markedly from week to week or even within the course of a single day (Kramlinger and Post, 1996).

1.1.2.1. Current Biological Hypotheses

BD symptoms manifesting as emotional, cognitive, behavioural, autonomic, neuroendocrine, immune, and circadian disturbances better correspond to the dysfunction of interconnected brain networks (Langan and McDonald, 2009; McIntyre et al., 2009; Strakowski et al., 2012). As symptomatolgy shows an integrated perspective of abnormalities in biological pathways is needed in order to understand the aetiology of BD. These biological hypotheses will be briefly reported at the next section.

Neuroendocrine hypothesis

Alterations in HPA axis function in BD, as well as in MDD, have been well substantiated (Taylor and MacQueen, 2006). An increase of the release of corticotrophin releasing factor (CRF) contributes to greater adrenocorticotropic hormone (ACTH) secretion and a subsequent elevation of circulating glucocorticoids (Taylor and MacQueen, 2006). These disturbances are most likely attributable to deficits in cortico-limbic regulation in BD, with consequent amygdala over-activity, and a compromised hippocampal regulatory role (Drevets et al., 2008). Moreover, glucocorticoid receptors (GR) appear to have diminished sensitivity in mood disorders, possibly due to elevation in inflammatory cytokines, thereby disrupting physiological feedback regulation on the HPA axis and immune system (Tsigos and Chrousos, 2002; Pace et al., 2007; Soderlund et al., 2011).

Circadian dysfunction

Evidence indicates a relationship between BD and circadian dysregulation. Circadian disturbances have been described during mania, depression, in the euthymic state, and in healthy relatives of BD patients (Milhiet et al., 2011; Milhiet et al., 2014). Actigraphic evidence and polysomnography studies have detected higher density of REM sleep, greater variability in sleep patterns, longer sleep latency and duration, lower sleep efficiency, greater number of arousals, fragmented sleep, and reduced daily activity, both in actively ill and remitted

bipolar patients compared to healthy controls (Jones et al., 2005; Milhiet et al., 2011; Milhiet et al., 2014; Rock et al., 2014). Moreover, eveningness has a significant correlation with important clinical manifestations of bipolar illness, including intensity of depression, rapid mood swings, anxiety, substance abuse, a greater sensitivity to sleep reduction, daytime lethargy, and reduction in melatonin levels (Milhiet et al., 2011; Gonzalez, 2014; Milhiet et al., 2014).

Immunological disturbances

Several limbic and paralimbic areas implicated in the pathophysiology of BD have an important role in the regulation of autonomic and immune function (Ramirez-Amaya and Bermudez-Rattoni, 1999; Pacheco-Lopez et al., 2005; Maletic and Raison, 2009; Irwin and Cole, 2011). Moreover, several studies and two recent meta-analyses have reported elevated levels of peripheral inflammatory cytokines in bipolar depressed and manic patients compared with healthy controls (O'Brien et al., 2006; Kim et al., 2007b; Brietzke et al., 2009; Hope et al., 2009; Modabbernia et al., 2013; Munkholm et al., 2013a; Munkholm et al., 2013b). Overall, the data suggest that successful treatment leading to a euthymic state may reverse inflammation and normalize peripheral levels of inflammatory mediators (Kim et al., 2007b; Guloksuz et al., 2010; Modabbernia et al., 2013). Inflammatory cytokines are a known cause of diminished sensitivity of glucocorticoid and insulin receptors (Tsigos and Chrousos, 2002). Furthermore, increased peripheral inflammation has been associated with numerous symptoms of mood disorders, such as malaise, fatigue, anhedonia, impairment of concentration, anxiety, irritability, social disconnection, hopelessness, suicidal ideation, bodily aches, and disturbance in sleep and appetite (Alesci et al., 2005; Raison et al., 2006; Eisenberger et al., 2010; Janelidze et al., 2011; Felger and Miller, 2012).

Changes in neuroplasticity and neurotrophin signalling

The role of BDNF in mood disorders has received more attention than other members of the neurotrophin family. It is involved in neuronal maturation, differentiation and survival, synaptic plasticity, and long-term memory consolidation (Grande et al., 2010). Furthermore compelling preclinical evidence suggests that BDNF plays an important role in regulating the release of serotonin, glutamate, and GABA, as well as in slow-wave sleep modulation (Shaltiel et al.,

2007; Faraguna et al., 2008). Evidence suggests that stress and excessive glucocorticoid signalling may interfere with hippocampal neurogenesis in the context of BD (Schloesser et al., 2009). Individuals endowed with at risk alleles of the BDNF gene may have compromised ability to normalize HPA axis activity, thereby adding to mood-disorder pathology (Schule et al., 2006). In addition to its role in regulating the neuroplastic processes, BDNF also acts as a resilience factor, assisting the maturation and differentiation of the nerve cell progenitors (Duman and Monteggia, 2006).

Alterations in GABA, glutamate and monoamine neurotransmission

The role of monoamine disturbances in BD was indirectly suggested by studies in MDD. A study that included a mix of major depressive and bipolar depressed patients noted an association between elevated cerebrospinal fluid (CSF) levels of 3-methoxy-4- hydroxyphenylglycol (MHPG), an orepinephrine metabolite, and agitation and anxiety in depressed patients (Redmond et al., 1986). Additionally, studies reported diminished immunoreactivity of locus coeruleus processes and decreased CSF MHPG in suicidal bipolar subjects compared with controls (Sher et al., 2006; Wiste et al., 2008). A recent review utilized cumulative pharmacological and imaging evidence to put forth the hypothesis of dopaminergic dysfunction in BD. This idea posits that excessive dopaminergic activity in the course of mania precipitates DA receptor down-regulation, which subsequently triggers a transition into a depressed state (Berk et al., 2007). Moreover, studies linking the severity of bipolar symptoms to tardive dyskinesia, even in the absence of pharmacotherapy, lend further support to claims of DA dysfunction in this disease state (van Rossum et al., 2009). Unfortunately, definitive and more direct and consistent evidence implicating monoamines in the aetiology of BD are still unavailable.

Relatively few studies have focused on abnormalities of GABA transmission in BD. Recent studies have reported significantly increased GABA platelet uptake in bipolar depressed patients and decreased GABA uptake during mania (Daniele et al., 2012). By contrast, glutamate platelet uptake was increased in the course of manic episodes relative to healthy controls. Altered platelet GABA and glutamate uptake correlated with the severity of depression and mania, respectively, as measured by standardized scales (Daniele et al., 2012). Overall, multiple, consistent, and convergent evidence from genetic, post-mortem, biochemical, and

imaging studies points to a principal role of glutamatergic dysregulation in the etiopathogenesis of BD. Moreover, evidence links aberrant glial–neuron interactions and endocrine dysregulation with alterations in glutamatergic transmission.

Changes in the intracellular signalling cascades

It is becoming increasingly evident that current mood-stabilizing agents have actions that extend beyond binding to neuronal membrane surface receptors. Therapeutic actions of psychotropic drugs used in the treatment of BD most likely rely on an interface with intracellular signalling cascades and eventual enduring changes in gene expression, accompanied by alterations in neurotransmission and neuroplasticity. Better understanding of intracellular signalling cascades may therefore provide valuable insights into the underlying causes of BD and subsequently to more effective treatment strategies. The phosphoinositide-3-kinase (PI3K)/AKT pathway is a general signal transduction pathway for growth factors, including BDNF. Increased activity in the GSK3 pathway supports apoptosis. Attenuation of GSK-3 activity enhances neuroplasticity and cellular resilience. This pathway is also involved in circadian regulation (Carter, 2007b; Carter, 2007a). Interestingly, manipulation of the GSK3 pathway produces both antimanic and antidepressant effects. Many agents with mood-stabilizing properties, such as lithium (Li), valproate, and atypical antipsychotics, directly and indirectly modulate the PI3K, GSK3, and Wnt signalling pathways, the very same ones implicated in the genetic studies of BD.

1.1.2.2. Environmental and Genetic Risk Factors

Environmental risk factors

Data concerning the effects of environmental factors on BD remains very scarce, although several environmental factors have been identified as potentially involved in this disorder. These factors include early childhood trauma, stressful life events, virus infections, cannabis use, obstetric complications, and even very distant environmental factors, such as solar cycles (Etain et al., 2008).

Genetic risk factors

Heritability, as calculated in recent twin studies, is estimated at about 85% (Bienvenu et al., 2011) suggesting a substantial involvement of genetic factors in the development of the disease. Family studies have demonstrated that the relative risk in the first-degree relatives is seven-fold greater than the risk in general population, indicating that the genetic component is very important in the development of BD (Szczepankiewicz, 2013).

Despite the abundance of genetic findings, the results have often been inconsistent and not replicated for many candidate genes/single nucleotide polymorphisms (SNPs). The most consistent associations have been observed for several genes: serotonin transporter gene *(SLC6A4)*, brain-derived neurotrophic factor (*BDNF)*, D-amino acid oxidase activator (*DAOA*), dysbindin (*DTNBP1)*, neuroregulin (*NRG1)*, disrupted in schizophrenia 1 (*DISC1)*, DA receptor D4 (*DRD4*) (Seifuddin et al., 2012; Szczepankiewicz, 2013).

Since the first GWAS of BD in 2008 (Baum et al., 2008), a handful of risk loci have been identified for association with BD through some larger GWAS: *ZNF804A*, *ANK3*, *NCAN, CACNA1C*, *ODZ4*, *ADCY2* and *TRANK1* (Wellcome Trust Case Control, 2007; Ferreira et al., 2008; Sklar et al., 2008; Cichon et al., 2011; Psychiatric, 2011; Chen et al., 2013; Muhleisen et al., 2014). The biological function of these genes and the hypothetical relevance in the aetiology of BP are still under investigation.

Gene - environment interaction

Compared to both MDD and schizophrenia (SCZ), gene-environment interactions in BD have been understudied. Only a single published study has reported that people with BD who carried Met alleles at the BDNF Val66Met polymorphism were more likely to develop depressive episodes following stressful life events than Val allele homozygotes (Hosang et al., 2010).

1.1.2.3. Treatment Approaches

Treatment of BD conventionally focuses on acute stabilization, in which the goal is to bring a patient with mania or depression to a symptomatic recovery with

euthymic (stable) mood. Effective pharmacological treatment of BD requires treatment of depressive and manic/hypomanic episodes together with long-term treatment to prevent future episodes, both syndromal and sub-syndromal. In recent years the importance of long-term treatment (that is, maintenance treatment) has been emphasized by several guidelines. The need for maintenance treatment is supported by the desire to prevent the costs of future episodes, that is, the intangible suffering to patients and their families and the economic burden of direct and indirect costs.

The types of medications generally used to treat BD include mood stabilizers, atypical antipsychotics, and antidepressants. Mood stabilizers are usually the first choice to treat BD playing the most important role in the treatment of the disorder. A mood stabilizer can be defined as a drug that, if used as monotherapy, i) acts therapeutically in mania or/and depression, ii) acts prophylactically against manic or/and depressive episodes, and iii) does not worsen any therapeutic or prophylactic aspect of the illness outline above (Rybakowski, 2007).

In this sense, **Li** is a mood-stabilizing drug that has been used effectively in the treatment of BD, as well as other mood disorders, for over 60 years. The discovery of Li's efficacy as a mood-stabilizing agent revolutionized the treatment of patients with BD, and after three decades of use in North America, Li continues to be the mainstay of treatment for this disorder, both for the acute manic phase and as prophylaxis for recurrent manic and depressive episodes (Goodwin and Jamison, 1990; Baldessarini et al., 1999). Li has many molecular targets but it is not yet known which are necessary for its therapeutic effect. Li reduces neuronal excitability by modulating action-dependent sodium channels and excitatory neurotransmission, and also affects second-messenger systems, and may have neuroprotective or even neurotrophic effects (Manji et al., 2001b). Since Li is one of the drugs of interest in the present thesis, its mechanism of action will be explained in detail in the "Pharmacogenetics of Lithium" section.

Anticonvulsants are also used as mood stabilizers in less extent and include Valproate, Lamotrigine and Carbamazepine. **Valproate** inhibits neuronal sodium channels and glutamate release and, like lithium, acts on second-messenger systems and induces the expression of neuroprotective genes and proteins. It is used in the treatment of acute mania and mixed episodes (where it may be superior

to other agents), especially if symptoms have responded before. **Lamotrigine** has some efficacy as an acute treatment for less severe bipolar depression. **Carbamazepine** is not advocated as a first-line therapy but may have some efficacy either or alone or in combination with other medications in treatment-resistant cases (Seddon and Nutt, 2007). However, anticonvulsant medications have a warning from the Food and Drug Administration (FDA) because their use may increase the risk of suicidal thoughts and behaviours. People taking anticonvulsant medications should be monitored closely for new or worsening symptoms of depression, suicidal thoughts or behaviour, or any unusual changes in mood or behaviour.

Antipsychotic drugs will often be the appropriate short-term clinical treatment in manic episodes (see "Current Treatment" section of Schizophrenia for details about antipsychotic drugs). On the other hand, the treatment of bipolar depression is usually due by antidepressants. However, some antidepressants increase the likelihood of "switching" to mania in depressed bipolar patients, when used as monotherapy or with mood stabilizers (Calabrese et al., 1999; Post et al., 2006). The selection of which antipsychotic agent and which antidepressant agent usage in each patient is based on psychiatrist criteria relying upon clinical features and the use of other drugs by the patient. Antipsychotic and antidepressant agents will often be the appropriate short-term clinical treatment, although a drug with better long-term evidence of efficacy such as Li might be preferred when continued drug therapy is planned.

ECT is also recommended as a treatment of choice in BD for patients with severe depression, mania and mixed affective states, in highly suicidal patients, in those presenting with catatonia, and in those with treatment refractory illness (Thirthalli et al., 2012).

There is strong evidence for the benefits of **psychological interventions** in reducing the likelihood of relapse (particularly depressive episodes). Educational techniques, empowering the patient to take responsibility for the management of their illness, have been shown to reduce relapse and improve social functioning and employment. Cognitive therapy is aimed at improving skills in managing stress and symptoms, and in identifying early warning signs of impending relapse (Scott et al., 2006).

1.1.3. Schizophrenia

SCZ is a severe mental disorder that involves disturbances in the most basic functions that give a healthy person the feeling of individuality, uniqueness and self-direction. Behaviour may be seriously disturbed during some phases of the disorder, leading to adverse social consequences.

SCZ is characterized by a multiplicity of symptoms arising from almost all domains of mental functions, e.g. perception, emotion, reasoning, motor activity and language. These symptoms vary between patients, creating diverse symptoms profiles. The symptoms can include experiencing false perceptions (hallucinations), having false beliefs of control or danger (delusions), expressing disorganized speech and behaviour (avolition), exhibiting blunted affect, being unable to find pleasure in activities or in the company of others (anhedonia/asociality), poverty of speech and thought (alogia) and impaired cognitive functioning (specially, impaired working memory and attention).

The symptoms of SCZ fall into three broad categories:

- **Positive symptoms** are the presence of certain phenomena that reflects an excess or distortion of normal function (e.g. hallucinations and delusions).
- **Negative symptoms** are the absence of certain functions or aspects that reflect a diminution or loss of normal functioning (e.g. flattening of affect, apathy, poverty of speech, anhedonia, and social withdrawal).
- **Cognitive symptoms** are the disorganization cluster including alogia, attentional impairment, positive formal thought disorder and bizarre behaviour (Bilder et al., 1985).

The prevalence of SCZ is thought to be about 1% of the population around the world (Jablensky, 2000). The disorder is considered to be one of the top ten causes of long-term disability worldwide. No gender differences in SCZ has been described, however the age of onset differs significantly between men and women: it appears earlier in men (in the early 20s) compared with women (in the mid-to-late 20s) (Shtasel et al., 1992; Szymanski et al., 1995).

Although several biological abnormalities have been reproduced (e.g. abnormally large ventricles, abnormal DA concentration, and altered P300) they are not

sensitive enough (usually seen only in 40-50% of patients) or not specific enough (seen in 30% of first degree relatives and 10% of otherwise normal controls) to be of diagnostic usefulness (Allen et al., 2009), thus SCZ is diagnosed based on DSM-V criteria (American Psychiatric Association, 2013) (Table 3).

Schizophrenia

Diagnostic Criteria

A. Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated). At least one of these must be (1), (2), or (3):

   1. Delusions.
   2. Hallucinations.
   3. Disorganized speech (e.g., frequent derailment or incoherence).
   4. Grossly disorganized or catatonic behavior.
   5. Negative symptoms (i.e., diminished emotional expression or avolition).

B. For a significant portion of the time since the onset of the disturbance, level of functioning in one or more major areas, such as work, interpersonal relations, or self-care, is markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, there is failure to achieve expected level of interpersonal, academic, or occupational functioning).
C. Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and many include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or by two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).
D. Schizoaffective disorder and depressive or bipolar disorder with psychotic features have been ruled out because either 1) no major depressive or manic episodes have occurred concurrently with the active-phase symptoms, or 2) if mood episodes have occurred during active-phase symptoms, they have been present for a minority of the total duration of the active and residual periods of the illness.
E. The disturbance is not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication) or another medical condition.
F. If there is a history of autism spectrum disorder or a communication disorder of childhood onset, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations, in addition to the other required symptoms of schizophrenia, are also present for at least 1 month (or less if successfully treated).

Table 3. Diagnostic Criteria for Schizophrenia based on DSM-V (American Psychiatric Association, 2013).

1.1.3.1. Current Biological Hypotheses

Neurotransmitter abnormalities

The DA hypothesis is the oldest and most established of the SCZ hypotheses. In its simplest form this hypothesis proposes that DA neurotransmission is hyperactive in SCZ (Carlsson and Lindqvist, 1963; Carlsson, 1988). This hypothesis

was principally based on two pharmacological observations. First, DA agonists, such as amphetamine, can induce psychotic symptoms in control subjects and exacerbate psychosis in individuals with SCZ (Snyder, 1974). Second, antipsychotic drugs block central DA receptors, and their effectiveness in terms of therapeutic dose correlates with the blockade of DA D2 receptors (Seeman et al., 1976). Given the predominant localization of DA terminals and D2 receptors in subcortical regions such as the striatum and the nucleus accumbens, the classical DA hypothesis of SCZ focused on subcortical regions.

Over the years, the increasing awareness of the importance of both enduring negative symptoms and cognitive symptoms in SCZ, as well as of their resistance to D2 receptor antagonism, led to a reformulation of the classical DA hypothesis. In this sense, it has been suggested that there is an imbalance in DA with hyperactive subcortical mesolimbic projections (resulting in hyperstimulation of D2 receptors and positive symptoms) and hypoactive mesocortical DA projections to the prefrontal cortex (PFC) (resulting in hypostimulation of D1 receptors, negative symptoms, and cognitive impairment) (Deutch et al., 1990; Kolachana et al., 1995; Karreman and Moghaddam, 1996; Wilkinson, 1997; Tzschentke, 2001). Based on these observations, Weinberger (Weinberger, 1987) proposed that both arms of the DA imbalance model might be related, insofar as a deficiency in mesocortical DA function might lead to disinhibition of mesolimbic DA activity. In this context it has been suggested that tonic dopaminergic activity may actually be decreased, whereas the phasic response to stimuli such as stress may be exaggerated (Grace, 1991).

As opposed to dopaminergic models, glutamatergic models view SCZ as resulting from dysfunction converging at glutamatergic synapses, More specifically, it has been proposed that SCZ may be related to deficient glutamate-mediated excitatory neurotransmission via N-methyl d-aspartate (NMDA) receptors (Olney and Farber, 1995b; Olney and Farber, 1995a; Moghaddam, 2003). This theory is supported, firstly, by clinical observations of psychotic symptoms triggered by the NMDA antagonists phencyclidine (PCP) and ketamine (Javitt and Zukin, 1991). Secondly, post-mortem studies of schizophrenic patients have reported reduced expression of glutamate receptors, and especially of the NMDA receptor subunit, in a variety of brain regions, notably the prefrontal cortex and the hippocampus (Harrison et al., 2003). However, such findings have not been consistently replicated (Lewis and

Gonzalez-Burgos, 2006). Thirdly, several genes that have been associated with an increased risk for SCZ can influence the function of modulatory sites on the NMDA receptor or intracellular-receptor interacting proteins that link glutamate receptors to signal transduction pathways (Neuregulin 1 gene (*NRG1*) and the NMDA receptors Erb4 and GRM3) (Harrison and Owen, 2003). Finally, glutamate neurons regulate the function of other neurons that have been strongly implicated in the pathophysiology of SCZ, including GABAergic interneurons, whose morphology has been shown to be altered in SCZ (Lewis, 2000; Lewis et al., 2005) and DA neurons, which are the target of antipsychotic drugs.

The immune hypothesis

The immune hypothesis proposes that SCZ often involves pre- or perinatal exposure to adverse factors that produce a latent immune vulnerability (Kinney et al., 2010. Many epidemiological and clinical studies show the role of various infectious agents as risk factors for SCZ with overlap to other psychoses (see (Muller, 2014) for review). This hypothesis is supported by findings of high levels of immune markers in the blood of schizophrenic patients (Hope et al., 2009). High levels of immune markers have also been associated with having more severe psychotic symptoms (Hope et al., 2009). Moreover, recent GWAS identified seven significant loci in SCZ, with the strongest association in the extended major histocompatibility complex region (MHC) on chromosome 6 which have essential roles for both CNS and immune (Purcell et al., 2009; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

HPA axis dysfunction

An enhanced response to stress mediated by activation of the HPA axis is thought to play an important role in the onset, exacerbation, and relapse of schizophrenia. Several lines of evidence suggest a link between HPA activity and psychosis (Walker et al., 2008). First, illnesses associated with elevated cortisol (eg, Cushing syndrome) and the administration of corticosteroids can induce psychotic symptoms. Second, patients with psychotic disorders manifest increased baseline cortisol and adenocorticotropic hormone levels, increased cortisol response to a pharmacologic challenge and possibly also abnormalities in glucocorticoid receptors (van Winkel et al., 2008). Third, there may be a synergistic relation between

activation of the HPA axis and activation of dopaminergic circuits implicated in psychosis. Although the exact mechanisms remain to be elucidated, evidence suggests that glucocorticoid secretion may increase dopamine activity in certain brain regions (Moghaddam, 2002; Czyrak et al., 2003; Dallman et al., 2004), in particular the mesolimbic system (Marinelli et al., 2006). Fourth, factors implicated in the aetiology of SCZ, especially prenatal factors, can contribute to HPA dysregulation (Kofman, 2002).

Neurodevelopmental hypothesis

The neurodevelopmental hypothesis of SCZ suggests that the disruption of early brain development increases the risk of later developing SCZ (Weinberger, 1987; Murray and Lewis, 1988; Weinberger and Lipska, 1995). This hypothesis focuses attention on critical periods of early brain development, in which genetic and environmental factors could account for neurodevelopmental abnormalities. In recent decades evidence from neuroimaging, neuroanatomical and neurochemical studies has provided support for this hypothesis.

These neurodevelopmental abnormalities (developing in utero as early as the late first or early second trimester for some individuals, and thereafter for others) have been suggested to lead to dysfunction of specific neural networks that would account for premorbid signs and symptoms observed in individuals who go on to develop SCZ. In adolescence, excessive elimination of synapses and loss of plasticity (sometimes due to the exposure to stressful environmental factors) may account for the emergence of symptoms (Keshavan and Hogarty, 1999; Fatemi and Folsom, 2009). Some environmental and genetic factors that could play a role in these neurodevelopmental abnormalities will be commented below in the "Risk Factors for schizophrenia" section.

1.1.3.2. Environmental and Genetic Risk Factors

Environmental risk factors

Growing up in an urban environment, immigration, bulling, childhood maltreatment, use of cannabis in adolescence and perinatal events (hypoxia, maternal infection, stress and malnutrition) have been associated with increased risk of developing SCZ (Tandon et al., 2009; van Os and Kapur, 2009). Advanced

paternal age is also an important "environmental" risk factor (Malaspina et al., 2001; Torrey et al., 2009).

Genetic risk factors

Family, twin and adoption studies have shown evidence that inherited genetic factors influence the susceptibility to develop this mental illness (Riley et al., 2003). The risk of developing SCZ in family members increases with the degree of biological relatedness to the patient — greater risks are associated with higher levels of shared genes (Gottesman, 1991). Twin studies have reported heritability estimates of 60-80% for SCZ, making clear the substantial genetic contribution to the disorder (Cardno and Gottesman, 2000; Lichtenstein et al., 2009).

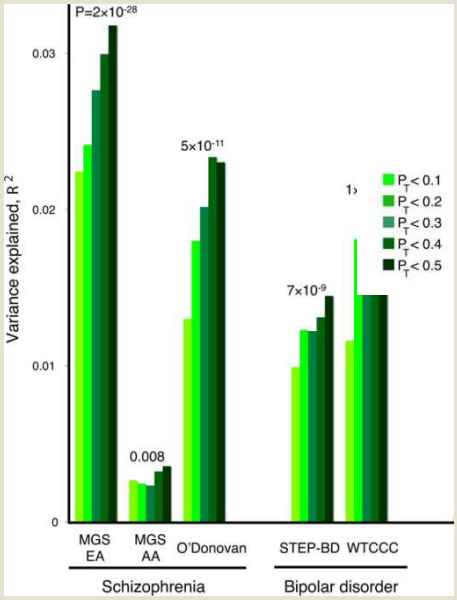
The research done during the last two decades has provided several interesting candidate genes including dystrobrevin binding protein 1 (*DTNBP1)* (Straub et al., 2002), neuregulin 1 (*NRG1*) (Stefansson et al., 2002), D-amino acid oxidase activator (*DAOA*) (Chiesa et al., 2011) and disrupted in schizophrenia 1 (*DISC1*) (Thomson et al., 2013). Unfortunately, they have not been consistently replicated across or within a population (Sanders et al., 2008).

Current GWAS have revealed evidence for genetic susceptibility loci in SCZ, but as in MDD and BD, they have failed to replicate the association of certain candidate genes previously identified by the classical association studies. The rs1344706 in the zinc finger protein 804A *(ZNF804A)* gene was the first SNP to reach genome-wide significance for SCZ (O'Donovan et al., 2008). From then, other GWAS have shown genome-wide significant loci (such as the MHC region, *ZNF804A, ANK3*, *NRGN* and *TCF4)* and CNVs (such as 22q11.21 deletion, 1q21.1 deletion, *NRXN*1 deletion, 3q29 deletion, *VIPR2* duplication, 15q13.2 deletion, and 16p11.2 duplication), all consistently detected as being enriched in cases (Purcell et al., 2009; Stefansson et al., 2009; Shi et al., 2011; Ripke et al., 2013). Interestingly, the most recent GWAS from the Schizophrenia Working Group of the Psychiatric Genomics Consortium has identified 108 loci that meet genome-wide significance, 83 of which have not been previously reported. Associations were enriched among genes expressed in brain, providing biological plausibility for the findings. Many findings have the potential to provide entirely new insights into aetiology, but associations at *DRD2* gene and several genes involved in glutamatergic

neurotransmission highlight molecules of known and potential therapeutic relevance to SCZ, and are consistent with leading pathophysiological hypotheses. Independent of genes expressed in brain, associations were enriched among genes expressed in tissues that have important roles in immunity, providing support for the speculated link between the immune system and SCZ (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Box 1. Polygenic Risk Score: beyond the GWAS

Recent molecular genetic evidence points to a substantial polygenic component to the risk of SCZ that involves a large number of common risk alleles of very small effect. The first empirical test of the polygenic hypothesis of SCZ by the International Schizophrenia Consortium (ISC) used its GWAs (discovery data set) to define a large set of very-small-effect common variants as score alleles with increasingly liberal association significance thresholds (Purcell et al., 2009). With the set of score alleles, the ISC generated an aggregate risk score for each individual in independent target GWAs data sets of SCZ. Aggregate risk scores in schizophrenic patients were found to be significantly higher than in controls. ISC concluded that thousands of common polygenic variants with very small individual effects collectively explain approximately one-third of the total variation in genetic liability to SCZ (Purcell et al., 2009). Interestingly, they showed that this component also contributes to the risk of BD, supporting the suggestive overlaps in the genetic architecture of different mental illnesses (Cross-Disorder Group of the Psychiatric Genomics, 2013).



The largest molecular study of SCZ had been recently published replicating this substantial polygenic component to the risk of SCZ showing that about 7% of the total variation in the liability scale for SCZ is explained by the polygenic component (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Several authors have subsequently explored whether such a polygenic effect might be associated not only with the disease but also with disease-relevant phenotypes (Derks et al., 2012; van Scheltinga et al., 2013; Walton et al., 2013; Papiol, Mitjans et al., 2014).

Gene - environment interaction

The first reported specific gene–environment interaction for a psychotic disorder involved a functional polymorphism in the catechol-O-methyltransferase (*COMT*) gene (Val158Met) (Caspi et al., 2005). Caspi and colleagues reported an interaction between the COMT Val158Met polymorphism and cannabis use. Specifically, the Val allele moderated the risk of developing psychotic symptoms at age 26 following cannabis use in adolescence (Caspi et al., 2005). The interaction between COMT and cannabis was replicated in other studies with psychotic features (Henquet et al., 2006; Estrada et al., 2011). However, other studies did not replicate the interaction (Zammit et al., 2007; Kantrowitz et al., 2009; Zammit et al., 2011; De Sousa et al., 2013). Interestingly, a recent three-way interaction among the COMT genotype Val alleles, childhood maltreatment, and adolescent cannabis use in the aetiology of psychotic experiences was reported and replicated (Vinkers et al., 2013; Alemany et al., 2014).

Another genetic polymorphism, the rs2494732 in the *AKT1* gene, has been identified that moderate the effects of cannabis use in the development of psychosis: carriers of the C/C genotype were most likely to develop psychotic illness after smoking cannabis (van Winkel, 2011; Di Forti et al., 2012).

A group of Danish researchers focused on another established environmental risk factor for SCZ: exposure to virus infection *in utero*. They identified two polymorphisms (rs1805539 and rs1806205) in the *GRIN2B* gene that significantly interacted with maternal positivity for the herpes simplex virus-2 in the risk of developing SCZ (Demontis et al., 2011).

1.1.3.3. Treatment Approaches

Antipsychotic drugs are used to treat SCZ and SCZ-related disorders. They are traditionally classified into two major groups: typical antipsychotics, with strong affinities for DA receptors among others, and atypical antipsychotics, with multitarget profiles (Miyamoto et al., 2005).

Typical antipsychotics were discovered in the 1950s and are also known as traditional or first-generation antipsychotics (FGA) (e.g. haloperidol and chlorpromazine). They possess high affinity for and act as full antagonists at D_2

receptors. This antagonism makes FGA very effective for treating the positive symptoms of SCZ (hallucinations, delusions, thought disorder and disorganized behaviour). However, they are associated with extrapyramidal symptoms (EPS), some reversible (Parkinsonism, acute dystonic reactions and akathisia) and some long-lasting (tardive dyskinesia and dystonia). Moreover, they are less efficacious in the treatment of negative symptomatology.

On the other hand, **atypical antipsychotics** were introduced in the past 15 years and are also known as second generation antipsychotics (SGA) (e.g. clozapine (CLZ), risperidone, olanzapine, quetiapine, aripripazole, ziprasidone). In addition to DA receptor antagonism, atypical antipsychotics have been shown to affect a number of other receptor systems, including serotonin, adrenergic, histamine and cholinergic receptors (Figure 4). Among these targets, the D2 and 5-HT2A antagonism is thought to be underlying the relief of the main psychotic symptomatology.

TABLE 1. RELATIVE RECEPTOR AFFINITIES OF ATYPICAL ANTIPSYCHOTIC DRUGS

Drug	D_1	D_2	D_3	D_4	α_1	α_2	H_1	ACh	5-HT_1	5-HT_2	5-HT_{2A}
Clozapine	++	+	?	+	+++	+++	+++	++	+	++	+
Risperidone	+	+++	?	+	+++	+++	+	–	+	–	+++
Olanzapine	++	+++	+	+	++	+	+++	+	–	++	++
Quetiapine	+	++	+	–	+++	+	+++	–	–	+	+++
Aripiprazole	+	++++	?	?	–	–	–	–	++	?	+++
Ziprasidone	++	+++	++	?	++	–	+	–	++	?	+

D=dopamine; α=alpha-adrenergic; H=histamine; ACh=acetylcholine; 5-HT=5-hydroxytryptamine (serotonin); ++++=very high affinity; +++=high affinity; ++=moderate affinity; +=low affinity; –=negligible affinity; ?=unknown affinity.

Figure 4. Receptor affinities of atypical antipsychotic drugs. Adapted from (Ananth et al., 2004).

In this sense, the D2 and, in general, DA antagonism will related to the treatment of positive symptomatology, while the 5-HT2A and other 5-HT receptor antagonism will be related to treatment of negative symptoms (poverty of thought, blunted affect and social withdrawal). Although SGAs are characterized by a lower induction of EPS, they tend to induce a high incidence of metabolic side-effects (weight gain, increased triglycerides and cholesterol) probably related to the mechanism of action involving 5-HT. Since CLZ is one of the drug of interest in the

present thesis, its mechanism of action will be explained in detail in the "Pharmacogenetics of Atypical Antipsychotics (Clozapine)" section.

Psychotherapy can be used as an adjunct to a good medication plan, which can help maintain the individual on their medication, learn needed social skills, and support the person's weekly goals and activities in their community.

ECT is also recommended for schizophrenic patients who have not responded to antipsychotic medication, patients presenting catatonia, and highly suicidal patients.

"If it were not for the great variability among individuals medicine might as well be a science and not an art"

-- Sir William Osler, 1892 --

1.2. Pharmacogenetics in Psychiatry

Severe mental illness represents a huge burden to society, reflecting the limited efficacy of current drug treatments. Although the progress in development of pharmacological treatments is one of the great successes of modern psychiatry, it should not be forgotten that a very high percentage of patients do not receive and/or seek the proper treatment for their disease.

Individual differences in clinical response to psychotropic drugs have long been recognized as a fundamental problem in the treatment of the seriously mentally ill patient. This variability in individual response ranges from patients who experience complete symptom remission to a subset of patients often describes as "treatment refractory", as well as a marked variability in susceptibility to adverse drug effects. A prior identification of the patients who will respond well to a particular psychotropic drug, or be at a higher risk for development of adverse side effects, has the potential to help clinicians to avoid lengthy ineffective medication trials and to limit patient's exposure to drug side effects. Moreover, enhanced predictability of treatment response early in the course of patient's illness may result in enhanced patient compliance and willingness to seek treatment rapidly upon symptom exacerbation or recurrence (Lerer, 2002).

Psychotropic drug response is a complex trait, likely to be influenced by a number of genetic variables in conjunction with clinical, demographic and environmental factors which lead to highly heterogeneous clinical response among individuals (Gupta et al., 2006) (Figure 5). Delineating the role of such variables can play an important role in predicting appropriate treatment regime for patients.

In this sense, the overall objective of pharmacogenetics is to determine the genetic basis of the variability in drug efficacy and safety, and to use this information to benefit the patient detecting *a priori* those patients that could not respond to a drug and/or present drug side effects. However, pharmacogenetic

studies were aimed not only at the discovery of clinically useful predictors but also at untangling pathophysiology and shedding light on mechanisms of drug action.

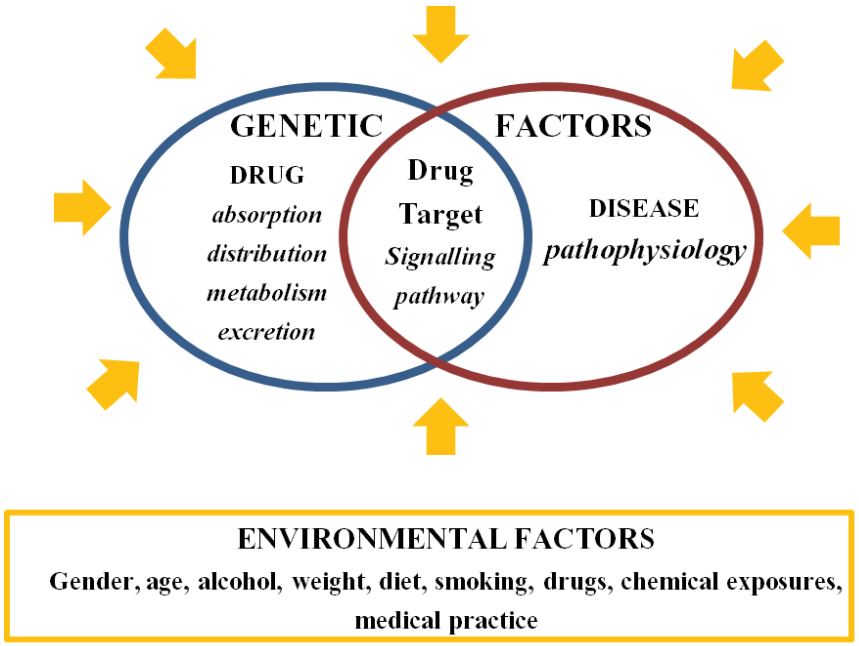


Figure 5. Variability in drug response: a genetics and environmental interplay (Adapted from (Gorwood and Hamon, 2006).

The first approximations to the pharmacogenetic concept were incorporated by Archibald Garrod at the beginning of the last century. Garrod was probably the first to realize that genetic factors could be the causes of chemical transformations to different drugs in humans, developing the concept of "chemical individuality" (Garrod, 1902; Garrod, 1909). Later, in 1957, Motulsky conceptualized that inheritance might explain many individual differences in the efficacy and toxicology of drugs (Motulsky, 1957). It was not until 1959 that Vogel coined the term of "pharmacogenetics" (Vogel, 1959). In 1962, the first monographic on pharmacogenetics was published by Werner Kallow called "Pharmacogenetics – Heredity and the response to drugs" (Kalow, 1962).

The completion of the sequence of the human genome in 2001 (Lander et al., 2001; Venteret al., 2001), and the emergence of new tools to interrogate the entire genome have accelerated interest in studying the relevance of variation across the genome in psychotropic treatment response. In this sense, two different terms coexist: pharmacogenetics and pharmacogenomics. Although some experts use both

terms interchangeably, pharmacogenetics is considered as the study of specific SNPs at specific genes with known functions that could plausibly be linked to drug response. On the other hand, pharmacogenomics studies the whole human genome, their products, interindividual variation, and intraindividual variation in expression and function and how they could predict treatment response.

Two basic areas of study exist within pharmacogenetics: pharmacokinetics and pharmacodynamics. Pharmacokinetics studies the variability that exists in the distribution processes of the drug and/or its metabolites to the target molecule. Pharmacodynamics, on the other hand, focuses on the study of the mechanisms of drug action in the target molecule.

Pharmacokinetics

Pharmacokinetics studies the variability that exists in the process of drug distribution to the target molecule, including absorption, distribution, metabolism and excretion of the drug (ADME) (Figure 6).

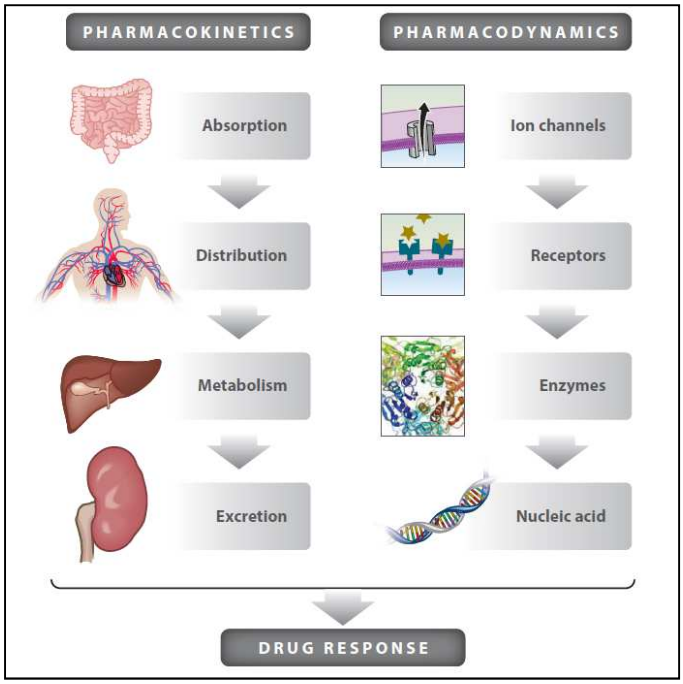


Figure 6. The pharmacokinetic and pharmacodynamic elements responsible for determining variability in drug response (Adapted from (Pirmohamed, 2014)).

Most drugs used in clinical practice that act on the CNS are extensively metabolized in the liver by enzymes of the cytochrome P-450 (CYP) system

(Kawanishi et al., 2000; Ozaki, 2004; Arranz et al., 2011). It has been described twelve families of CYP-450, being CYP1A2, CYP2C19, CYP2D6 and CYP3A4 enzymes, the responsible for the metabolism of virtually all psychotropic drugs currently used in clinical practice. The pharmacokinetic phenotype, which is the activity of CYP450 enzymes, strongly influences the sensitivity or response to medication because of different elimination, concentration, and biotransformation rates (Kawanishi et al., 2000). These pharmacogenetic phenotypes are genetically determined and show great variation between different individuals classifying them into four phenotypic groups: i) normal or extensive metabolisers (EMs) who have normal to high metabolic activity; (ii) poor metabolisers (PMs) who have low to absent metabolic activity; (iii) intermediate metabolisers (IMs) also have impaired metabolic function, which is greater than PMs but less than EMs; and (iv) ultrarapid metabolisers (UMs) who have extreme metabolic activity leading to rapid metabolism and excretion of drugs. As there is a lower metabolic clearance rate of substrates in PMs, they have higher risk of toxicity from medications. On the contrary, UMs who have a high clearance of substrates may be under-dosed with medications leading to treatment failure. Thus, alterations at this level could be related to the lack of response but most importantly to the emergence of side effects (Figure 7). There are considerable differences in the frequencies of these classes across ethnic groups (de Leon et al., 2006).

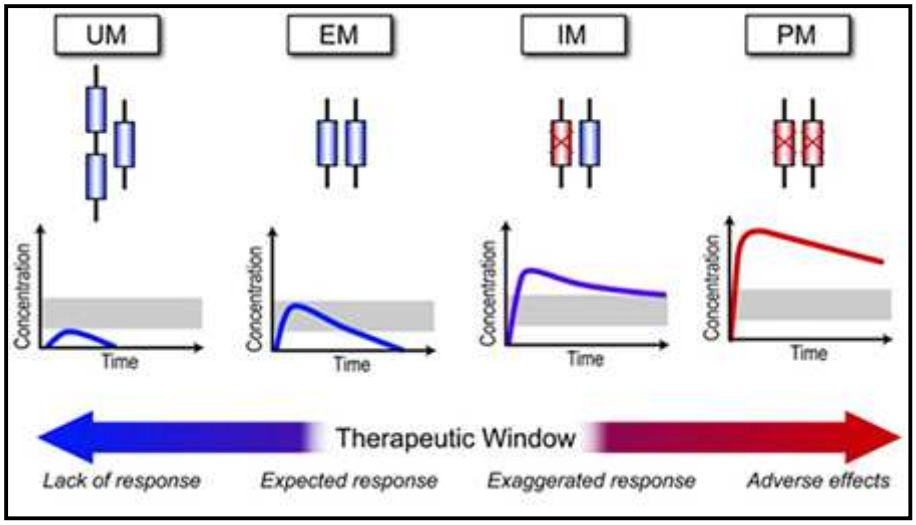


Figure 7. Drug metabolism groups classified by metabolism rates (Adapted from http://www.gbhealthwatch.com)

Pharmacodynamics

Pharmacodynamics, on the other hand, studies the mechanism of drug action (Figure 6). Interindividual differences in the genes that codify for proteins in which the drug directly acts (receptors or enzymes) and their up and downstream signal pathways could explain different levels of drug response. The therapeutic targets (receptors, transporters, enzymes), which contribute to the pharmacodynamics of the drug response, not only are important in the regulation of neurotransmitter systems but also, directly or indirectly, modify the development and plasticity of the neuronal circuits involved in the pharmacological effects.

Most drugs interact with specific target proteins to exert their pharmacological effects, such as receptors, enzymes, or proteins involved in signal transduction, cell cycle control, or many other cellular events. Molecular studies have revealed that many of the genes encoding for these drug targets exhibit genetic variability, which in many cases alters their sensitivity to specific medications.

Accordingly, the therapeutic effect or the toxicity of a specific drug will depend on, in part, the functional and structural expression of a high number of genes related to both pharmacokinetics and pharmacodynamics. Figure 8 illustrates the potential consequences of administering the same dose of a medication to individuals with different drug-metabolism genotypes and different drug-receptor genotypes (Evans and Relling, 1999).

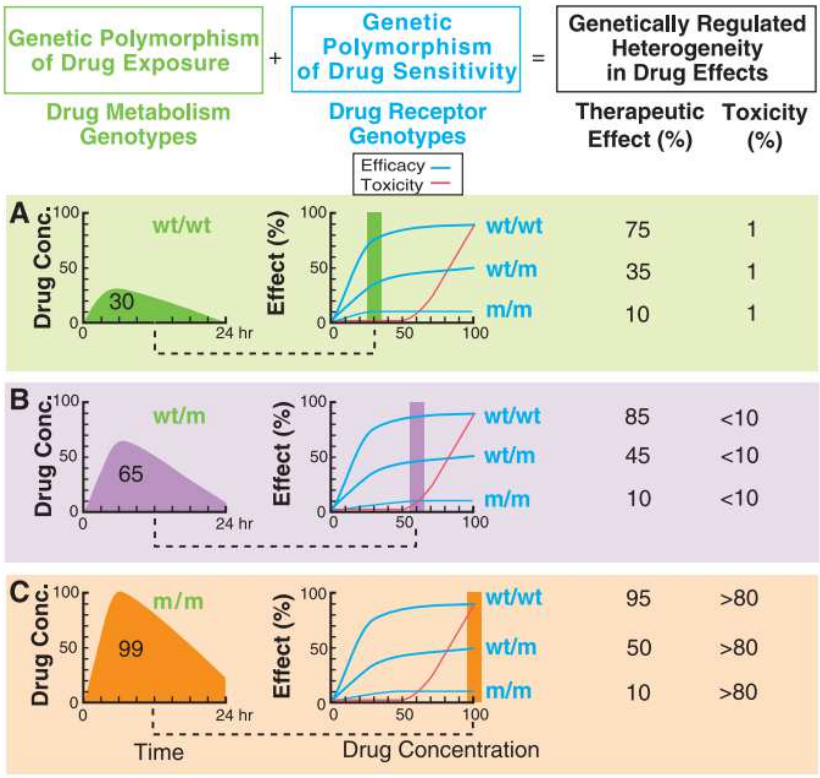


Figure 8. Polygenic determinants of drug effects (Evans and Relling, 1999). Active drug concentrations in systemic circulation are determined by the individual's drug-metabolism genotype (green lettering), with (**A**) homozygous wild type (wt/wt) patients converting 70% of a dose to the inactive metabolite, leaving 30% to exert an effect on the target receptor. (**B**) For the patient with heterozygous (wt/m) drug-metabolism genotype, 35% is inactivated, whereas (**C**) the patient with homozygous mutant (m/m) drug metabolism inactivates only 1% of the dose by the polymorphic pathway, yielding the three drug concentration-time curves. Pharmacological effects are further influenced by different genotypes of the drug receptor (blue lettering), which have different sensitivity to the medication, as depicted by the curves of drug concentration versus effects (middle). Patients with a wt/wt receptor genotype exhibit a greater effect at any given drug concentration in comparison to those with a wt/m receptor genotype, whereas those with m/m receptor genotypes are relatively refractory to drug effects at any plasma drug concentration. These two genetic polymorphisms (in drug metabolism and drug receptors) yield nine different theoretical patterns of drug effects (right).

1.2.1. Designing a pharmacogenetic study

In the context of pharmacogenetics, the usual approach is to examine the active treatment arm of a clinical trial and divide subjects in the treatment arm into those with a negative or no response and those with a positive response to the drug. Some pharmacogenetic studies aim to identify genetic variability related to the presence of drug side-effects. In this context, treated patients will be divided into those presenting side-effects and those who do not present.

The genetic variability studied in pharmacogenetic studies is mainly based on common variation, which occurs with a frequency of at least 1% of the population. This common variation includes single nucleotide polymorphisms (SNPs) (Figure 9), insertions or deletions (INDEL) and variable number tandem repeats (VNTR). SNPs are the variation most studied by pharmacogenetic studies. Variations in the genetic makeup can understandably alter the structures and functions of proteins and play a major role in the pharmacokinetics and pharmacodynamics of psychotropic drugs.

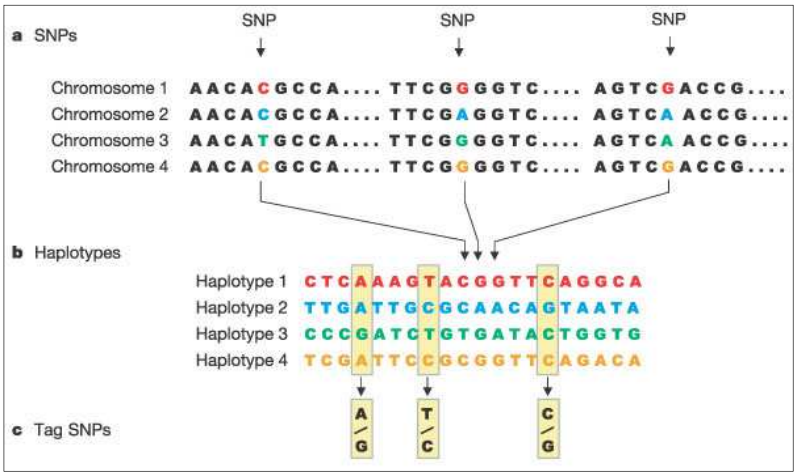


Figure 9. Genetic variability studied in pharmacogenetic studies (a) Single nucleotide polymorphisms (SNPs) (b) Adjacent SNPs that are inherited together are compiled into haplotypes (c) Tag SNPs within haplotypes are identified that uniquely identify those haplotypes. Adapted from (The International HapMap Consortium, 2003).

Genetic linkage is the phenomenon whereby alleles at loci close together on the same chromosome tend to be inherited together. SNPs that are linked together are said to be in linkage disequilibrium (LD) because they are not randomly inherited. A haplotype is a set of closely linked alleles or polymorphisms that are inherited

together and often they give better predictions of drug response than SNPs, since they give information about a gene segment not only a nucleotide position (Figure 9).

The successful identification of all genes in the human genome and the development of a systematic catalogue of SNPs in the human population have revealed an increasingly comprehensive view of all common polymorphisms associated with genes that are of pharmacologic and toxicologic interest (Genome Browser: http://genome.ucsc.edu; National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov).

The relationship between genotype and phenotype is often complex, as most diseases and drug response traits are polygenic. The phenotypic consequence of a specific SNP is often subtle and depends on environmental factors such as age, gender, diet, co-medications and social habits such as alcohol and tobacco use (Evans and Relling, 1999).

Candidate gene studies are perhaps still the most widely used methodology in assessing genetic determinants of drug response. They focus on SNPs in candidate genes, which are generally selected based on known biological, physiological, or functional relevance to the phenotype of interest (Figure 10). In the case of pharmacogenetic studies, a candidate gene could be this related to the drug disposition, pharmacological action of the drug or disease pathogenesis. Candidate gene studies are relatively cheap and quick to perform. The pharmacogenetic studies presented in this thesis are based on this kind of approaches.

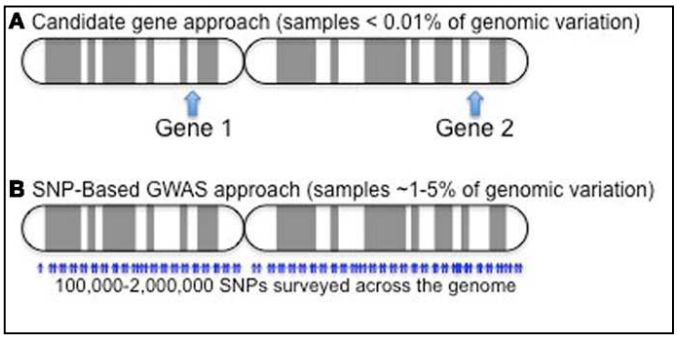


Figure 10. Candidate gene *vs.* GWAS approach. (A) Candidate gene approaches have only involved a few variants in one to several dozen genes (B) GWAS samples a much larger component of the genome. Adapted from (Weiler and Drumm, 2013).

GWAS have been increasingly used over the past five years to identify pharmacogenetic predictors of response scanning the entire genome for common genetic variation (Figure 10). A GWAS rapidly interrogates hundreds of thousands of SNPs for association in large populations (Manolio, 2010) without bias imposed by pre-existing models and provide the opportunity to identify novel genes, regulatory loci, and pathways not previously considered (Figure 11).

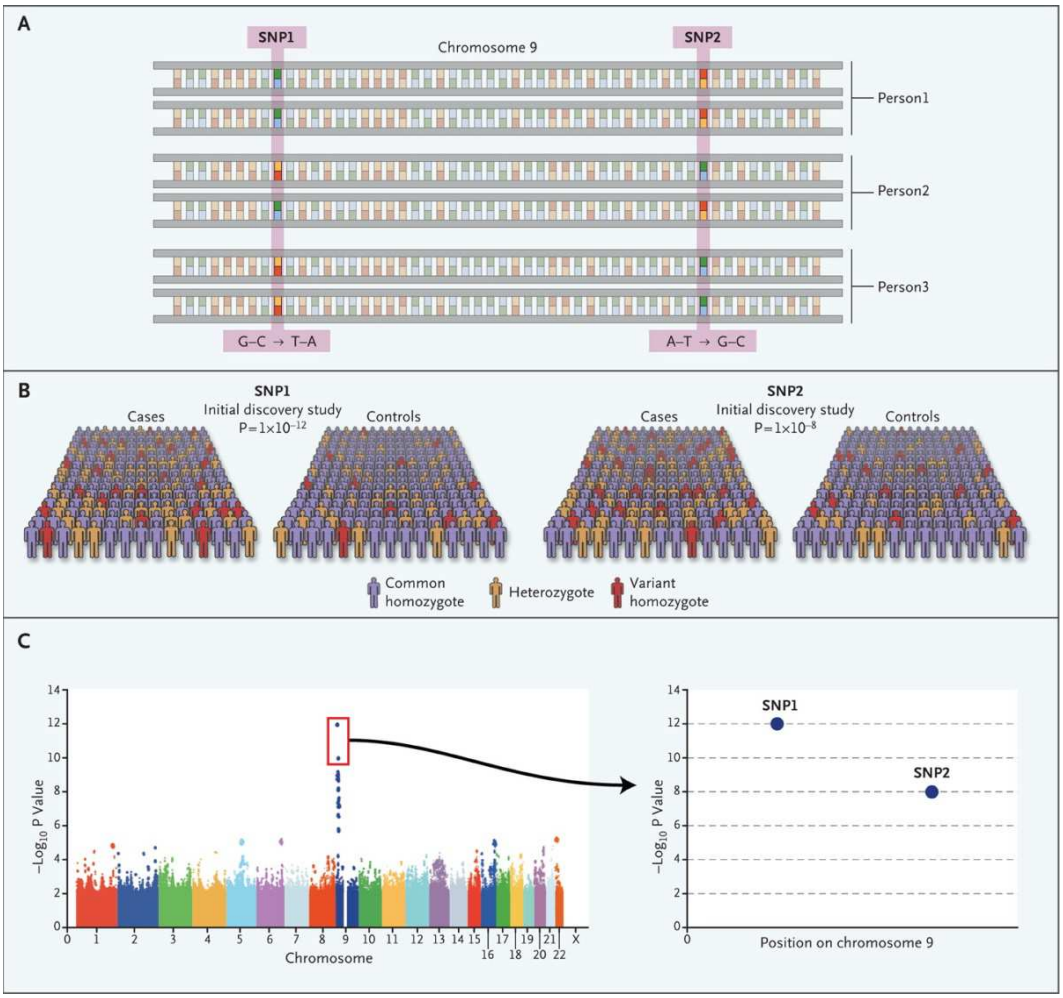


Figure 11. A GWAS design. Panel A depicts a small locus on chromosome 9, and thus a very small fragment of the genome. In Panel B, the strength of association between each SNP and disease is calculated on the basis of the prevalence of each SNP in cases and controls. In this example, SNPs 1 and 2 on chromosome 9 are associated with disease, with p-values of 10^{-12} and 10^{-8}, respectively. The Manhattan plot from Panel C shows the p-values for all genotyped SNPs that have survived a quality-control screen, with each chromosome shown in a different colour. Adapted from (Manolio, 2010).

In the last few years, a large amount of effort has been directed to the search of genetic predictors of drug efficacy in mental disorders. In this emergent discipline a number of papers have reported positive associations between gene variants and response to psychotropic drugs. However, there has been a lack of reproducibility

between the studies carried out so far. This lack of inconclusive findings might depend on several factors making difficult to understand the whole impact of those results. Among these factors is the diverse criteria used by different studies in the definition of drug response, sample size investigated and/or population stratification. These factors are discussed in detail in the "Discussion and Conclusions" section.

Next sections will be focused on the main results from pharmacogenetic studies that have been carried out on the drugs of interest in the present thesis (CIT, Li and CLZ), including pharmacokinetic and pharmacodynamic candidate gene studies and GWAS.

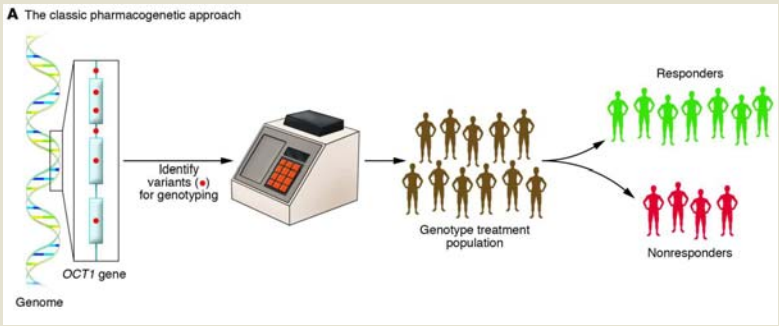
Box 2. Designing a pharmacogenetic candidate gene study: a context for the present thesis.

To design a valid pharmacogenetic candidate gene study two key elements are necessary:

i) an explicit and consistent definition of the drug response or side effect phenotype,

ii) knowledge of the candidate genes with a relevant in the mechanism of action of the drug (Pickar and Rubinow, 2001) or in the aetiology of the disease for which the drug is used to treat.

To study the pharmacogenetic hypothesis and to establish the fundaments for the clinical application, the clinical assays should be design with hypothesis design prior. In this sense, clinical association studies genotype SNPs in clinically relevant populations, compare the allele frequency of each SNP in the responder vs. non-responder groups or patients presenting side effect vs. those do not and establish a potential link between specific alleles and the selected drug response or side effect phenotype.



The classical pharmacogenetic approach. Adapted from (Reitman and Schadt, 2007).

1.2.2. Pharmacogenetics of SSRIs (citalopram)

Antidepressant medications are an important treatment for moderate-to-severe MDD in adults, but 30-50% of patients do not respond to their first antidepressant medication (Singh et al., 2013). Furthermore, relapse after response but not remission is common, making remission the aim in clinical care (Nierenberg and Wright, 1999; Zajecka, 2003). However, only around 40% of patients appear to remit with commonly used antidepressants (Thase et al., 2010).

The observation of clustering of antidepressant response in relatives of affected individuals gave rise to the hypothesis of a genetic contribution of common genetic variations to antidepressant efficacy, since recently they were estimated to explain 42% of individual differences without including rare variants (Tansey et al., 2013).

Since the drug of interest in this thesis in MDD pharmacogenetic studies is CIT, a SSRI, this section mainly focuses on pharmacogenetic studies based specifically on this type of antidepressants.

Mechanism of action of SSRIs

The monoamine theory of depression postulates that depression might be caused by a decrease in serotonergic and noradrenergic neurotransmission. In this sense, SSRIs exert their antidepressant effect by blocking the neuronal serotonin transporter and increasing the availability of extracellular serotonin (5-HT) (Figure 12). However, several weeks of treatment with antidepressants are necessary before their full therapeutic effect becomes clinically apparent (Asberg et al., 1986). This initial delay in clinical response to antidepressant treatment could be the result of secondary neurobiological adaptive mechanisms enhanced by the blockade of the serotonin transporter. The somatodendritic 5-HT1A autoreceptors are believed to be intimately related to this antidepressant delay effect. SSRIs markedly increase the 5-HT concentration near 5-HT containing cells in the midbrain raphe nuclei, which leads to an activation of the 5-HT1A autoreceptors that inhibit the firing and reduce the 5-HT release in the forebrain (Artigas et al., 1996). This sequence of events is thought to be responsible for the slow onset of action of SSRIs, which often require 3–4 weeks before clinical effects become evident (Blier et al., 1987). The effect of long-term administration of SSRIs may be expected to induce desensitization of 5-HT1A autoreceptors, and this would

gradually reinforce serotonergic neurotransmission leading to a pharmacological response in patients (Blier and de Montigny, 1998).

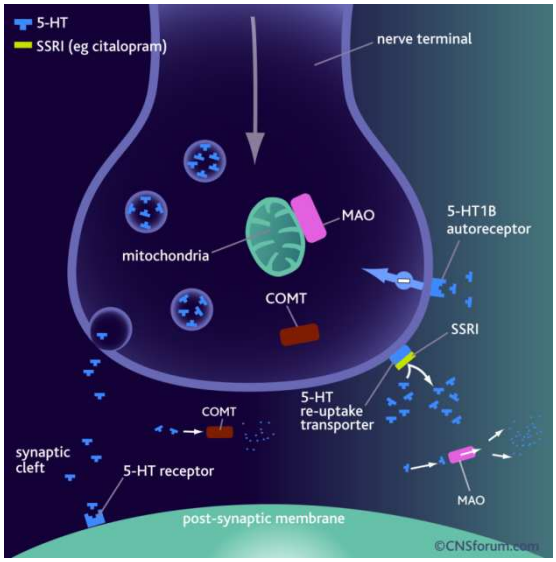


Figure 12. Mechanism of action of SSRIs (https://www.cnsforum.com).

Pharmacokinetic studies

CYP450 enzyme superfamily and P-glycoprotein (P-gp) (coded by the *ABCB1* gene) represent the most investigated among genes involved in antidepressant pharmacokinetics.

CYP450 enzymes are involved in SSRI oxidation and reduction, thus regulating drug plasma levels. The main isoforms involved in SSRI metabolism are CYP2D6, CYP2C9, CYP2C19 and CYP3A4. A commercially available pharmacogenetic test has been clinically approved by the FDA to test for the CYP2D6 and CYP2C19 genetic variants (de LJ, 2006). However, a large number of studies have examined the relationship between variation in these genes and treatment response to antidepressants and the results have been decidedly mixed (Zandi and Judy, 2010). In 2005, a Spanish study analyzing the role of CYP2C19 gene in CIT efficacy was published (Arias et al., 2005). No significant effect was found. Recently, the largest study from the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) (1877 subjects treated with CIT) replicate the lack of association between variation in *CYP2C19,* and also *CYP2D6* either efficacy or tolerability (Peters et al., 2008).

Moreover, a recent review of existing studies found little overall evidence of an association between these two *CYP450* genes and antidepressant response, calling into question the clinical utility of testing for these variants (Thakur et al., 2007). The commercially available pharmacogenetic test and its potential use in clinic practice are discussed in the "Discussion and Conclusions" section.

P-gp is pivotal in regulating the access of lipophilic drugs into the brain (Figure 13). P-gp is an ATP-dependent drug efflux pump for xenobiotic compounds that limit uptake and accumulation of some lipophylic drugs, as psychotropic ones, into key organs such as the brain. Several SSRIs (CIT, fluoxetine, fluvoxamine, paroxetine, sertraline and escitalopram) are substrates of P-gp (O'Brien et al., 2012). Some *ABCB1* SNPs, which were demonstrated to alter P-gp expression and/or function, have been reported to be associated with SSRI response (Horstmann and Binder, 2009; Fabbri et al., 2014).

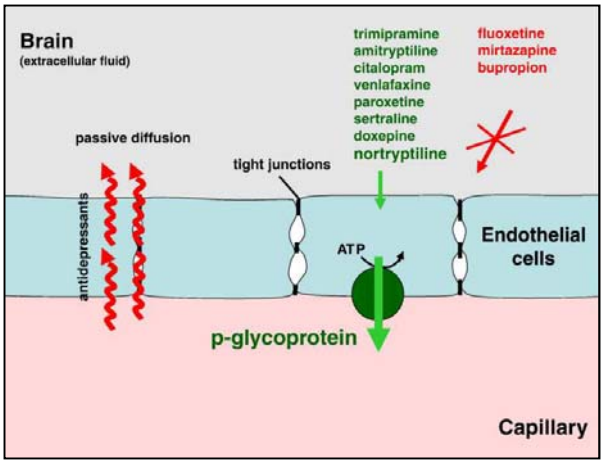


Figure 13. Schematic representation of the p-glycoprotein function. P-glycoprotein sits at the luminal membrane of the capillaries which are part of the blood brain barrier. Psychotropic drugs, including antidepressants are lipophylic and enter the central nervous system via passive diffusion. Antidepressants substrate of p-glycoprotein (1) are actively pumped back into the capillary under consumption of ATP, while non-substrates (2) accumulate in the extra-cellular fluid. Adapted from (Horstmann and Binder, 2009).

Pharmacodynamic studies

The serotonin transporter gene (*SLC6A4*) has been the most investigated in the field of SSRI pharmacodynamics, since it represents the main target of this class of antidepressants. Several polymorphisms have been described in this gene, but the majority of studies have focused on a common functional polymorphism in the 5'

promoter region of *SLC6A4*, referred to as the serotonin (5-HT) transporter gene-linked polymorphic region (5-HTTLPR). It consists in an insertion/deletion which produces a short (S) allele that is 44 base pair shorter than the long (L) allele, having the S variant less transcriptional activity and lower serotonin uptake than the L variant (Heils et al., 1996).

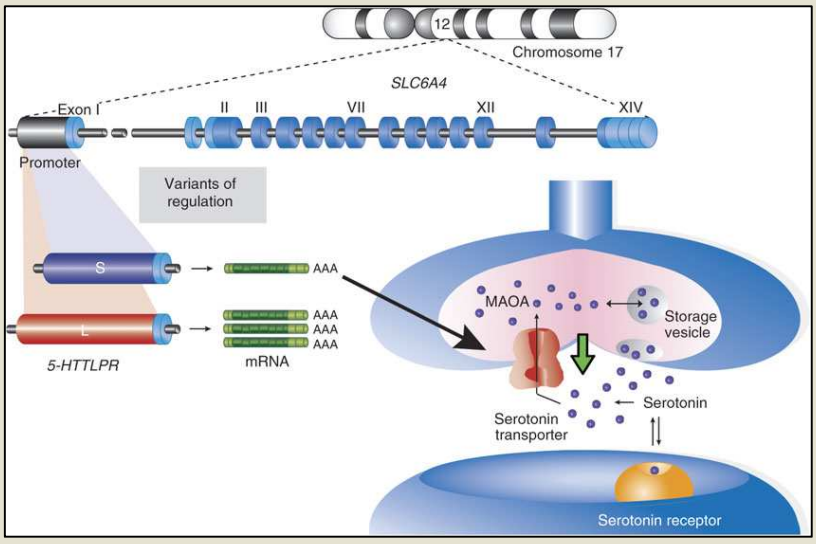
A large number of studies have investigated the role of 5-HTTLPR polymorphism and antidepressant response showing contradictory results (Smeraldi et al., 1998; Pollock et al., 2000; Zanardi et al., 2000; Serretti et al., 2001b; Rausch et al., 2002; Arias et al., 2003). Although some meta-analyses have been carried out in order to clarify the results, they are still quite controversial (Box 2). Initially a meta-analysis of 15 studies revealed a highly significant association between subjects homozygous for the short promoter and a worse remission or response rate (Serretti et al., 2007). Later, in a meta-analysis of 28 studies involving 5408 participants, no such association could be detected (Taylor et al., 2010). More recently, a meta-analysis of 33 studies suggests that the L allele of *5*-HTTLPR polymorphism is a predictor of better SSRI response in Caucasian populations, while in Asian populations the same allele may be associated with poorer SSRI outcome (Porcelli et al., 2012) (see (Serretti and Kato, 2008; Fabbri et al., 2013; Fabbri et al., 2014) for extensive reviews).

Among serotonin (5-HT) receptors, the most established candidate genes for involvement in SSRI efficacy are *HTR1A* and *HTR2A*. The rs6295 (1019C/G) polymorphism in the upstream regulatory region of *HTR1A* has received particular attention since the G allele causes an upregulation of the gene expression (Lemonde et al., 2003). A combined effect between this variant and the S allele of the 5-HTTLPR was found to increase the risk for no remission (Arias et al., 2005). Another variant that was hypothesized to modulate SSRI efficacy is HTR1A rs1800042, since it is in linkage disequilibrium (LD) with rs6295. However, contradictory results have been reported in both cases (Fabbri et al., 2014).

Regarding 5-HT2A receptor, significant association was detected between the A allele of *HTR2A* rs7997012 and treatment response to CIT in a study from the STAR*D (McMahon et al., 2006). A study with a sample from Genome-based Therapeutic Drugs for Depression (GENDEP) showed association with rs9316233 and rs2224721 with antidepressant response (Uher et al., 2009). Horstmann and

collaborators tried to replicate these previous studies in a sample from the Munich Antidepressant Response Signature (MARS) project, finding association with rs17288723 and rs2770297 (Horstmann et al., 2010). These studies indicate that 5-HT2A receptor appears to be a crucial element in the response to SSRI. However, negative findings have also been reported by studies focused on only few *HTR2A* variants (Fabbri et al., 2014) and a meta-analysis (Niitsu et al., 2013).

Box 3. The controversial effect of *SLC6A4* gene in antidepressant response.



The short (S) *5-HTTLPR* variant (purple) of the 5-HTT gene (*SLC6A4*) produces significantly less 5-HTT mRNA and protein, as indicated by the green arrow, than the long (L) variant (red), leading to higher concentrations of serotonin in the synaptic cleft. MAOA, monoamine oxidase A; SSRI, selective serotonin reuptake inhibitor (Canli and Lesch, 2007).

Meta-analysis	N of Studies	Sample size	Result
Serretti et al., 2007	15	1435	SS genotype → lower rates of response and remission.
Taylor et al., 2010	28	5408	No effect of 5-HTTLPR in response to antidepressant.
Porcelli et al., 2012	33 (19 Caucasians / 11 Asians)		L allele → better SSRI response/remission in Caucasians (worse response in Asians).

Although the SSRI efficacy is mediated by serotonergic system, other neurotransmission systems as the dopaminergic, noradrenergic and eCB, as well as

second messenger systems have been also explored because of their possible implication with the antidepressant mechanism of action. Some interesting findings have been reported, however, none of them has been consistently replicated in subsequent studies.

As an example, COMT is a highly polymorphic gene, but the greatest part of studies was focused on the functional Val108/158Met. This polymorphism influences activity of the COMT enzyme (high activity in Val/Val, intermediate activity in Val/Met, and low activity in Met/Met genotype (Lachman et al., 1996). This polymorphism was associated with AD treatment. Particularly Met/Met genotype has been associated both with better (Baune et al., 2008; Benedetti et al., 2009; Tsai et al., 2009) and worse response (Szegedi et al., 2005; Arias et al., 2006).

Because of the evidence of the involvement of the HPA axis in MDD susceptibility and pathogenesis, gens within the HPA axis represent plausible candidates for differential antidepressant response. Polymorphisms in the *FKBP5* gene have been associated with differential response to antidepressant therapy (Lekman et al., 2008; Binder, 2009). In fact, Binder and colleagues characterized a significantly faster response to SSRIs, TCAs, and mirtazapine in patients with MDD who were TT homozygous for the *FKBP5* rs1360780 compared with the C allele carriers (Binder, 2009). However, these results have not been replicated in other studies (Papiol et al., 2007).

GWAS

Three independent samples have been used to investigate the impact of genetic variants across all the genome on antidepressant efficacy: the STAR*D (Garriock et al., 2010), the GENDEP (Uher et al., 2010) and MARS (Ising et al., 2009). These studies are different in their design, genotyping platforms, sample size, and outcome measures (Laje and McMahon, 2011) (table 4). STAR*D and GENDEP have been performed on cohorts with substantial part that was treated with CIT monotherapy (STAR*D) and escitalopram or nortriptyline (GENDEP). The MARS is based, instead, in a cohort whom patients were treated with antidepressants according to the choice of their physicians. Although none of the studies have reported findings with a genome-wide significance, some “top hits” have been identified.

The study from the STAR*D identified three suggestive associations with antidepressant response and remission: the ubiquitin protein ligase E3C (*UBE3C*) gene, the bone morphogenic protein 7 (*BMP7*) gene, and the RAR-related orphan receptor alpha (*RORA*) gene (Garriock et al., 2010).

The MARS results included a marker in the Cadherin-17 (*CDH17*) gene that was associated with early partial response (Ising et al., 2009).

In the GENDEP study instead, on a genome-wide significance level, the gene coding uronyl 2-sulphotransferase (*UST*) was associated with nortriptyline response. On a suggestive level, the interleukin 11 (*IL11*) gene was associated with escitalopram response, and two intergenic regions on chromosome 1 and 10 were found to be linked with response to both medications (Uher et al., 2010).

Recently, a GWAS meta-analysis has been performed in order to increase the sample including the three main GWAS performed so far (GENDEP MARS and STAR-D Investigators, 2013). No individual association met a genome-wide threshold for statistical in the whole sample. The study provided as top finding an intergenic region on chromosome 5 with no evidence of transcription so far, only when the analysis was focused on a relatively homogenous subsample (STAR*D and GENDEP patients treated with escitalopram).

Study	Sample (N)	ADs	Gene	Marker	Phenotype
STAR*D (Garriock et al., 2010)	1948	CIT	*UBE3C*	rs6966038	Rp and Rm
			BMP7	rs6127921	Rp and Rm
MARS (Ising et al., 2009)	700 + 832 (STAR*D)	Different ADs (5 weeks)	*CDH17*	rs6989947	Early partial Rp
GENDEP (Uher et al., 2010)	706	Escitalopram Nortryptiline (12 weeks)	*UST*	rs1126757	Rp to nortriptyline
			IL11	rs2500535	Rp to escitalopram

Table 4. Characteristics and main results for the three published GWAS in antidepressant response. CIT: citalopram; Ads: Antidepressants; Rp: Response; Rm: Remission.

1.2.2. Pharmacogenetics of lithium

Li has been the standard pharmacological treatment for BD over the last 50 years (Lenox et al., 1998; Goodwin and Ghaemi, 1999; Lenox and Hahn, 2000). It is still considered the first-line treatment by its proven efficacy in both acute and maintenance phases (Geddes et al., 2004; Nivoli et al., 2010). Although Li presents a high success rate with approximately 70% to 80% of patients showing full or partial response (Mamdani et al., 2004), the adequate response may range from an excellent response in 24-45%, to a complete lack of response in 10-30% of patients (Peselow et al., 1994; Kulhara et al., 1999).

It is hypothesized that Li-responders may be a genetically distinct phenotype. This hypothesis has been supported by studies showing that i) Li-response appears to be a stable trait, ii) responders are more likely to have a family history of BD than Li non-responders, iii) there are better rates of Li prophylaxis in concordant BD twin pairs than in discordant pairs, and iv) Li response appears to 'breed-true' in affected families (McCarthy et al., 2010).

Mechanism of lithium action

By virtue of its prophylactic properties, Li is thought to target the underlying pathophysiology of the disease, yet the precise molecular mechanism for this therapeutic action remains elusive. This therapeutic action of Li in BD appears not to result from an effect at a single target site, but rather as the culmination of an integrated re-orchestration of a complex concert of events which effectively adjusts neuronal activity at multiple levels (including changes in genetic expression) affecting in a last term processes such as synaptic function, neuroplasticity and neuroprotection.

The main proposed mechanisms of action of Li include:

- **"The myo-inositol depletion hypothesis".** Li inhibits the activity of two enzymes of the phosphatidyl inositol (PI) intracellular signalling, the inositol monophosphatase (IMPase) and inositol polyphosphate 1-phosphatase (IPPase) (Berridge and Irvine, 1989; Gould et al., 2004) (Figure 14). The inactivation of these enzymes causes a reduction in the amount of myo-inositol, ultimately inhibiting this signalling pathway.

- **"The GSK3 inhibition hypothesis"**. This hypothesis is based on Li effects on cell survival through the inhibition of GSK3ß (Klein and Melton, 1996) (Figure 14). Li acts in the same manner as the Wnt pathway to inhibit GSK3ß, leading to the translocation of ß-catenin to the cell nucleus where it becomes part of complexes that regulate the transcription of genetic components involved in cell survival ((Williams and Harwood, 2000; Jope and Bijur, 2002).

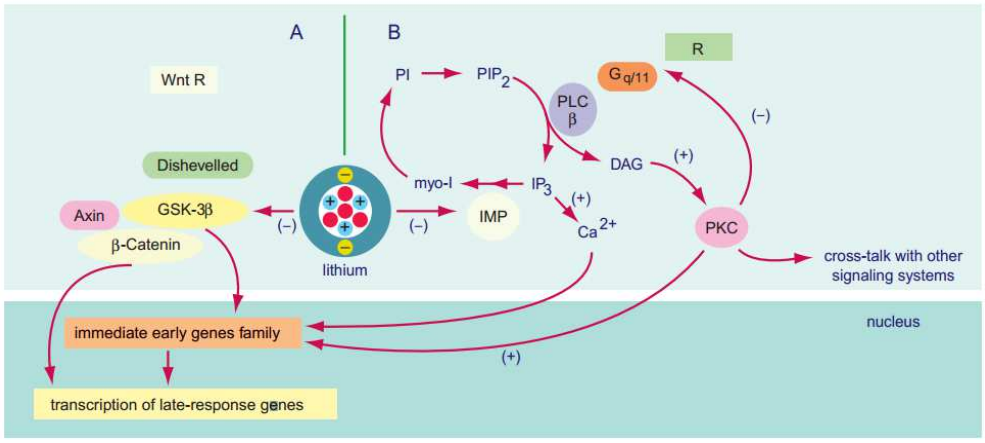


Figure 14. Mechanism of action of lithium. A) Li effects on cell survival through the inhibition of GSK3ß, B) Li inhibition of enzymes involved in the phosphatidyl inositol (PI) intracellular signaling (http://www.thedollblog.com).

- **"The neurotransmitter hypothesis"**, which is based on the fact that responses of BD patients to drugs other than mood stabilizers that act at specific neuroreceptors have suggested that bipolar symptoms arise from excessive dopaminergic and glutamatergic transmission, reduced cholinergic transmission and disturbed serotonergic transmission.

Pharmacodynamic studies

Pharmacogenetics of mood stabilizers such Li has been less studied than the pharmacogenetics of antidepressants or antipsychotics. In reference to Li, and as far as we know, no pharmacogenetic studies have been reported on the pharmacokinetics field. Published studies have instead concentrated on pharmacodynamic factors. The majority of data have been gathered from candidate

gene studies, in which the candidates were selected on the basis of neurobiology of BD and the mechanism of action of Li.

The effect on the PI pathway has long been considered the most important mechanism of therapeutic action of Li in BD. In this sense, a large number of studies have investigated its role in Li response. Genetic variability at *INPP1* gene has been associated with Li response: C937A (Steen et al., 1998) and rs2064721 (Bremer et al., 2007), finding not replicated in independent samples (Steen et al., 1998; Michelon et al., 2006). Two trends for association were found between two polymorphisms of the *IMPA2* gene and good response to Li in BD patients (Dimitrova et al., 2005). Studies on other genes connected with the PI system, such as *IMPA1* and *DGKH* genes, did not find any associations with Li response (Steen et al., 1996; Bremer et al., 2007; Manchia et al., 2009; Squassina et al., 2009).

As mentioned above, the inhibition of *GSK3β*, the enzyme involved in neuroprotection as well as in the circadian cycle, may play an important role in the mechanism of Li action in BD (Gould and Manji, 2005). An association between a functional polymorphism of the *GSK3B* gene and Li response was reported (Benedetti et al., 2005), but this was not confirmed in two other studies (Michelon et al., 2006; Szczepankiewicz et al., 2006; Bremer et al., 2007).

Among neurotransmitters, the serotonergic system has long been implicated in the neurobiology of BD and the mechanism of Li action (Muller-Oerlinghausen, 1985). The results regarding 5-HTTLPR (*SLC6A4*) and Li pharmacotherapy are inconsistent and often contradictory. Reports that the long version of 5-HTTLPR was associated with a worse response to Li (Serretti et al., 2004) have not been replicated (Michelon et al., 2006; Manchia et al., 2009), and some studies have reported opposite findings (Serretti et al., 2001a; Rybakowski et al., 2005a).

Since *BDNF* Val66Met polymorphism has been implicated in BD (Neves-Pereira et al., 2002; Sklar et al., 2002; Lohoff et al., 2005), some studies tried to investigate its role in Li response demonstrating an association of this polymorphism with Li response (Rybakowski et al., 2005b; Dmitrzak-Weglarz et al., 2008). However, this association was not confirmed in populations other than Caucasian (Masui et al., 2006; Michelon et al., 2006).

In 2007, Rybakowski and colleagues found and a significant interaction between the *BDNF* Val66Met and the *SLC6A4* 5-HTTLPR polymorphism in Li response: patients with the s allele and the *BDNF* Val/Val genotype were significantly more frequent in the poor responder group as compared with excellent and/or partial responders to Li (Rybakowski et al., 2007).

GWAS

The first GWAS in Li response was conducted through the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD) initiative in which 458 patients with BD were treated with Li and followed prospectively for 2 years (Perlis et al., 2009). The authors found a region of special interest on chromosome 4q32 spanning a *GRIA2* gene, coding for a glutamate *AMPA* receptor (Perlis et al., 2009).

In another GWAS on Li-treated Sardinian patients with BD, the strongest association was shown for a SNP of the amiloride-sensitive cation channel 1 neuronal (*ACCN1*) gene, located on chromosome 17q12, encoding a cation channel with high affinity for sodium, and permeable to Li (Squassina et al., 2011).

Following an initiative by the International Group for the Study of Lithium-Treated Patient and the Unit on the Genetic Basis of Mood and Anxiety Disorders at the National Institute of Mental Health, Li researchers from around the world have formed the Consortium on Lithium Genetics (ConLiGen) (Box 3). The aim of the ConLiGen was to establish the largest sample to date for GWAS of Li response in BD (Schulze et al., 2010). This sample currently compromises more than 2000 patients characterized for response to Li treatment. The first genetic results of the ConLiGen initiative including 1200 patients was the GWAS top hit ($p=1.52 \times 10^{-6}$) for the *SLC4A10* gene coding solute carrier family 4, sodium bicarbonate transporter, member 10, which belongs to a family of sodium-coupled bicarbonate transporters (Schulze, 2012). Recently, based on 218 cases of Han Chinese or Japanese ancestry, an attempt was made to replicate the results of the study from Chen and colleagues, in which genetic variations in *GADL1* gene were associated with the response to Li maintenance treatment for bipolar I disorder in patients of Han Chinese descent (Chen et al., 2014). However, no association was found between *GADL1* gene and Li response in the ConLiGen sample (Hou et al., 2014).

Box 4. Consortium on Lithium Genetics (ConLiGen)

The ConLiGen project is a highly significant step in the genetic research of Li response. ConLiGen was created as an international multicenter cooperation investigating the genetic basis of Li response (Schulze et al., 2010) aiming to identify genetic determinants of response to Li treatment in BD, as well as genetic determinants of adverse events emerging during Li treatment (e.g. weight gain, hypothyroidism, tremor).

Pharmacogenetic research needs to be based on collaborative efforts allowing the collection of **large samples** adequately powered to detect small to moderate effect sizes, as well as with **stringent and uniform phenotypic definition**.

In this context, ConLiGen project aimed at performing a GWAS in the largest sample to date of Li treated patients. Moreover, a stringent phenotype definition of response is one of the hallmarks of the ConLiGen project. Treatment response is a complex construct that requires researchers to make judgments about adequacy of treatment and tolerability as well as assess changes in episode frequency or symptom severity. In many cases this information must be assessed retrospectively, with the inherent limitations associated with recall bias, missing information, or the fact that the treatment has not followed a strict research protocol. One scale that incorporates such data is an 11-point scale developed by Martin Alda and colleagues (Grof et al., 2002), which is the one used by ConLiGen project. Alda scale allows for either a categorical assessment (i.e. below or above some cut-off point) or a dimensional assessment of Li response. All the patients included in the ConLiGen project have been evaluated for their Li response using this scale, thus, obtaining a homogenous and comparable phenotype between all the samples included.

Our contribution into ConLiGen: four investigators from the Hospital Clínic of Barcelona (Dr. Vieta, Dr. Colom, Dr. Benabarre and Dr. Jiménez) and two investigators from the University of Barcelona (Dr. Arias and M. Mitjans) have participated in the ConLiGen project providing 75 samples with BD evaluated for Li response by the Alda scale.

ConLi+Gen
The International Consortium on Lithium Genetics

1.2.3. Pharmacogenetics of Atypical antipsychotics (clozapine)

Pharmacogenetic studies in SCZ mainly use several classes of antipsychotics. Since the drug of interest in this thesis is also CLZ, this section focuses only on these pharmacogenetic studies based specifically on this drug.

CLZ, an atypical antipsychotic, is widely used in the treatment of SCZ, being more effective than traditional antipsychotics for patients with poor response or resistance to treatment (Malhotra, 2001). In addition, CLZ is an effective treatment for SCZ accompanied by persistent suicidal or self-injurious behaviour. However, due to increased risk of agranulocytosis, a severe adverse drug reaction occurring in up to 1% of treated individuals, its use in clinical practice is reserved for those who do not respond well to or cannot tolerate other antipsychotics (Alvir et al., 1993).

Mechanism of clozapine action

The therapeutic efficacy of CLZ in SCZ is mainly mediated through antagonism of the DA type 2 (D2) and the serotonin type 2A (5-HT2A) receptors. CLZ also acts as an antagonist at adrenergic, cholinergic, histaminergic and other dopaminergic and serotonergic receptors (Figure 15).

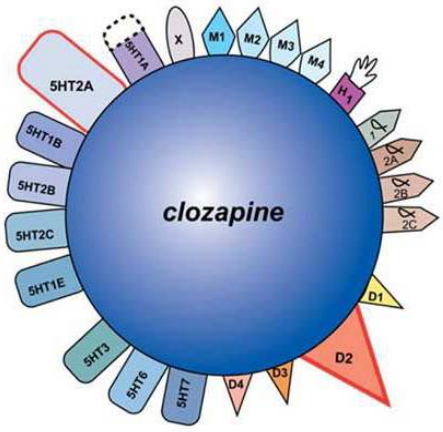


Figure 15. Mechanism of action of clozapine. Adapted from Stahl's Essential Psychopharmacology Online (http://stahlonline.cambridge.org/common_home.jsf).

Pharmacokinetic studies

CLZ depends mainly on CYP1A2 enzyme for its metabolic clearance. It has been reported that UMs for CYP1A2 showed low plasma levels of CLZ and subsequently non-response to CLZ treatment (Eap et al., 2004). However, these results have not been replicated so far (van der Weide et al., 2003). Since CLZ is metabolized minimally by CYP2D6 enzyme, some studies tried to identify genetic variability at the *CYP2D6* gene but no associations were found between polymorphisms in this gene and response to CLZ (Arranz et al., 1995; Jaquenoud Sirot et al., 2009; Kohlrausch et al., 2009).

Studies investigating the role of P-gp protein in CLZ response showed that the 3435(T) and 2677(T) variants of the *ABCB1* gene correlated with higher serum concentrations of CLZ and those patients carrying these alleles required lower doses of CLZ to obtain the same clinical effect as patients without this variant (Consoli et al., 2009; Jaquenoud Sirot et al., 2009).

Pharmacodynamic studies

Since dysregulation of the dopaminergic system was among the first pathological findings observed in SCZ, and CLZ is a high-affinity antagonist of DA receptors, initial studies focused on the relationship between them and the response to CLZ.

Two studies analyzed D1 receptor gene (*DRD1*) and found a significant association with CLZ response in African Americans (Hwang et al., 2007) and Caucasians (Potkin et al., 2003), but another study was unable to replicate this association (Hwang et al., 2011). Regarding studies with D2 receptor gene (*DRD2*) polymorphisms, they produced contradictory results. Positive results were found (Hwang et al., 2005; Hwang et al., 2006) but other studies did not corroborate them (Arranz et al., 1998a; Reynolds et al., 2005; Hwang et al., 2011). A meta-analysis confirmed the importance of genetic variability at D3 receptor gene (*DRD3*), in which significant differences were found when the DRD3 Ser9 allele or the Ser/Ser genotype were compared between responders and non-responders to CLZ in a sample of 233 schizophrenic patients (Jonsson et al., 2003). However, a more recent meta-analysis with a much larger sample size (n=758) reported a negative but consistent trend for the *DRD3* Ser9 allele and poor CLZ response (Hwang et al.,

2010). Two studies found significant associations between polymorphisms in D4 receptor gene (*DRD4*) and response to CLZ (Zhao et al., 2005; Hwang et al., 2012). However, most studies were unable to detect this significant association (Kerwin et al., 1994; Rao et al., 1994; Shaikh et al., 1995; Rietschel et al., 1996; Kohn et al., 1997; Kaiser et al., 2000). Recently, Xu and colleagues (Xu et al., 2010b) found a haplotype combination of genetic variants in the DA transporter gene (*SLC6A3*) significantly associated with response to CLZ, but in a previous study this association was not observed (Szekeres et al., 2004).

Genetic variability at the serotonergic system has also been analyzed in treatment response to CLZ, since CLZ displays affinities for serotonin receptors (5-HT2A, 5-HT2C, 5-HT3A, 5-HT3B and 5-HT6), which have been hypothesized to mediate, at least partially, their therapeutic action (Arranz et al., 1998b; Gutierrez et al., 2002; Meltzer and Massey, 2011). In general, it appears that the associations between the *HTR2A* gene and CLZ response are the strongest. Several studies have yielded weakly positive results and a meta-analysis of all of the published studies of the two *HTR2A* polymorphisms, T102C and His452Tyr, and CLZ response, found an association between these polymorphisms and poor response to medication (Arranz et al., 1998b). Interestingly, T102C polymorphism is a silent substitution; however it appears to be in linkage identity with a promoter region polymorphism that may influence *HTR2A* gene transcription.

An interesting pharmacogenetic study in schizophrenic patients evaluated the relationship between CLZ response and pharmacogenetic variation in multiple candidate genes including α-adrenergic receptors, DA receptors, serotonin receptors, histamine receptors and the serotonin transporter (Arranz et al., 2000). Arranz and colleagues found a combination of six polymorphisms that provided 76.7% success in predicting CLZ response. Although the authors suggested that this result would lead to a simple predictive test for CLZ response, these data have thus far not been replicated so far (Malhotra et al., 2004).

BDNF plays a role in modulation of major neurotransmitter systems including the dopaminergic and serotonergic systems (Tyler et al., 2002; Russo-Neustadt, 2003; Gratacos et al., 2007), which are the targets of CLZ. Based on this evidence, numerous studies investigated the association between the *BDNF* Val66Met polymorphism and treatment response to CLZ, however the results are

inconsistent (Hong et al., 2003; Xu et al., 2010a; Zai et al., 2012; Zhang et al., 2013).

Studies involving other genes such as *COMT*, *DTNBP1*, *GFRA2*, *NRXN1*, and *OXT* showed significant associations with CLZ response (Kohlrausch, 2013). In contrast, other genes such as the *ADRA2A*, *GPX1*, *GRIN1*, *GRIN2*, *GRIN2B, HRH1*, *HRH2* and *MNSOD* were investigated and no significant associations were observed (Kohlrausch, 2013). As these studies are mostly unique, confirmation of the results is necessary.

GWAS

No specific GWAS studies of CLZ response have been published due to the difficulty to achieve large samples to achieve truly significant results. However, few GWAS have been conducted using samples from the CATIE study (Clinical Antipsychotic Trials of Intervention Effectiveness, n=750) (Lieberman et al., 2005) in which the treatment was based on several classes of antipsychotics, either typical or atypical. Candidate genes that were previously found to be associated with individual response to CLZ treatment in candidate gene studies did not show statistical significance in these GWAS.

1.3. Exploring genetic variability in psychotropic response

1.3.2. Citalopram response in MDD

1.3.2.1. Candidate genes at endocannabinoid system: *CNR1, CNR2, FAAH*

The eCB system consists of i) the endogenous cannabinoids, ii) the cannabinoid (CB) receptors and iii) the proteins involved in the regulation and metabolism of the endogenous cannabinoids. Two main endogenous cannabinoids are known, namely 2-arachidonoylglycerol (2-AG) and anandamide (AEA). Both molecules are considered bioactive lipids produced in the CNS and the peripheral organs. The endogenous cannabinoids act mainly on two different receptors, CB receptor type 1 (CB1) and 2 (CB2), which belong to the G-protein coupled receptor family and signal through G0/I family of G-proteins (Devance et al., 1998; Matsuda et al., 1990). CB1 receptors are localized primarily in the CNS (Katona and Freund, 2012). Signal transduction through CB1 receptor is shown in figure 16.

The activation of CB1 provokes i) the inhibition of neuronal depolarisation, ii) the decrease in generation of the action potential, iii) the decrease in the release of excitatory and inhibitory neurotransmitters, and hence iv) the reduction of the impulse propagation. Additionally, other receptors, such as those for monoamines or opioids, located in the same neurons that CB1 receptors (Figure 16), may share common mechanisms, thus setting the scene for interactions between the different modulator systems (Ashton and Moore, 2011).

The CB2 receptor is extensively expressed throughout the immune system (Howlett et al., 2002). However, It has been reported that this receptor are present also in the brain (Van Sickle et al., 2005), but its function in the brain is still known.

The degradation of endogenous cannabinoids is achieved by distinct hydrolytic enzymes, fatty-acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) which degrade AEA and 2-AG, respectively (Hill and Patel, 2013).

The eCB system is widely distributed throughout the CNS modulating many vital functions such as those associated with consciousness (cognition, learning, memory, perception, mood, sleep, pain, appetite, reward, motivation) and many

that do not normally reach consciousness (motor control, cardiovascular regulation, endocrine activity, metabolism, immune reactions).

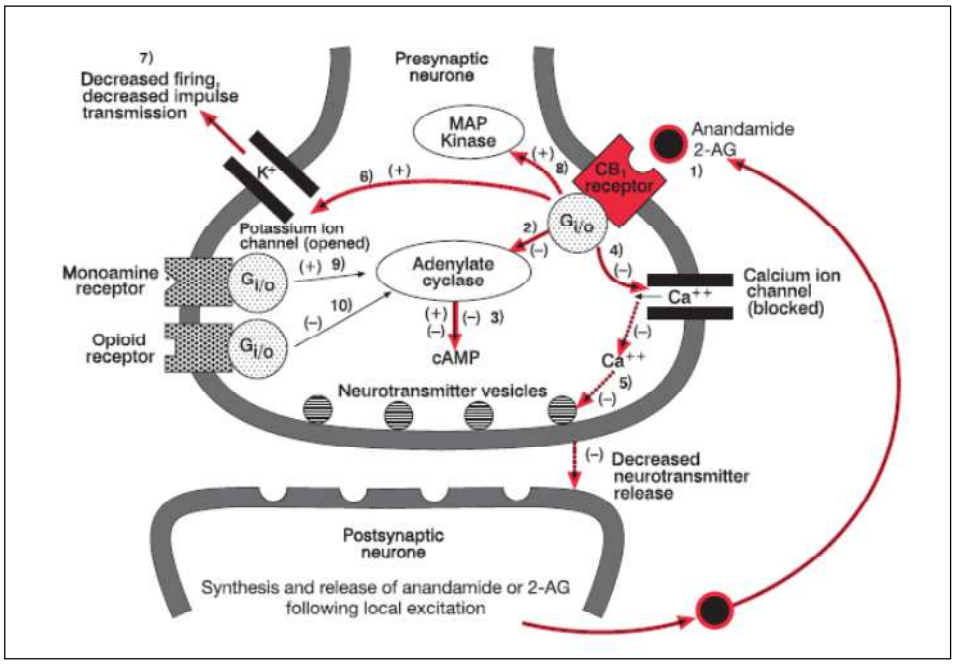


Figure 16. Signal transduction mechanisms mediated by CB1 receptors (Ashton and Moore, 2011).

Some evidence suggests that the eCB system could play an important role in the control of the emotions (Valverde, 2005). Moreover, it has been implicated in physiological processes altered in the MDD, such as motivation, anxiety, cognitive and vegetative functions (Hill and Gorzalka, 2005; Mangleri and Piomelli, 2007). This evidence indicates the implication of the eCB system to the aetiology of MDD.

CB1 receptors and the enzymes involved in the synthesis and degradation of endogenous cannabinoids are located along the neuroanatomical structures and circuits involved in depression, including the prefrontal cortex, hippocampus, amygdala, hypothalamus and the forebrain monoaminergic circuits (Herkenham, 1991) (Figure 17).

From animal models it has been shown that blockade of the eCB system, either pharmacologically or by transgenic animal models, is a risk factor in the pathogenesis of depression and anxiety disorders (Haller et al., 2002; Martin et al., 2002). CB1 knockout mice presented a similar phenotype resembling of depressive symptomatology (Hill and Gorzalka, 2005).

Studies in depressive patients revealed a reduction of endogenous cannabinoids concentrations that, additionally, correlates with the duration of the depressive episode (Gobbi et al., 2005; Hill et al., 2005; Miler et al., 2005). Moreover, a decrease in CB1 receptor density in grey matter glial cells has been reported in the *post mortem* brains of patients with MDD (Hungund et al., 2004). Clinical trials with CB1 receptor antagonists, rimonabant, which is a drug for the treatment of obesity, revealed significantly more anxiety and depression in patients taking rimonabant compared with those with placebo (Nissen et al., 2008).

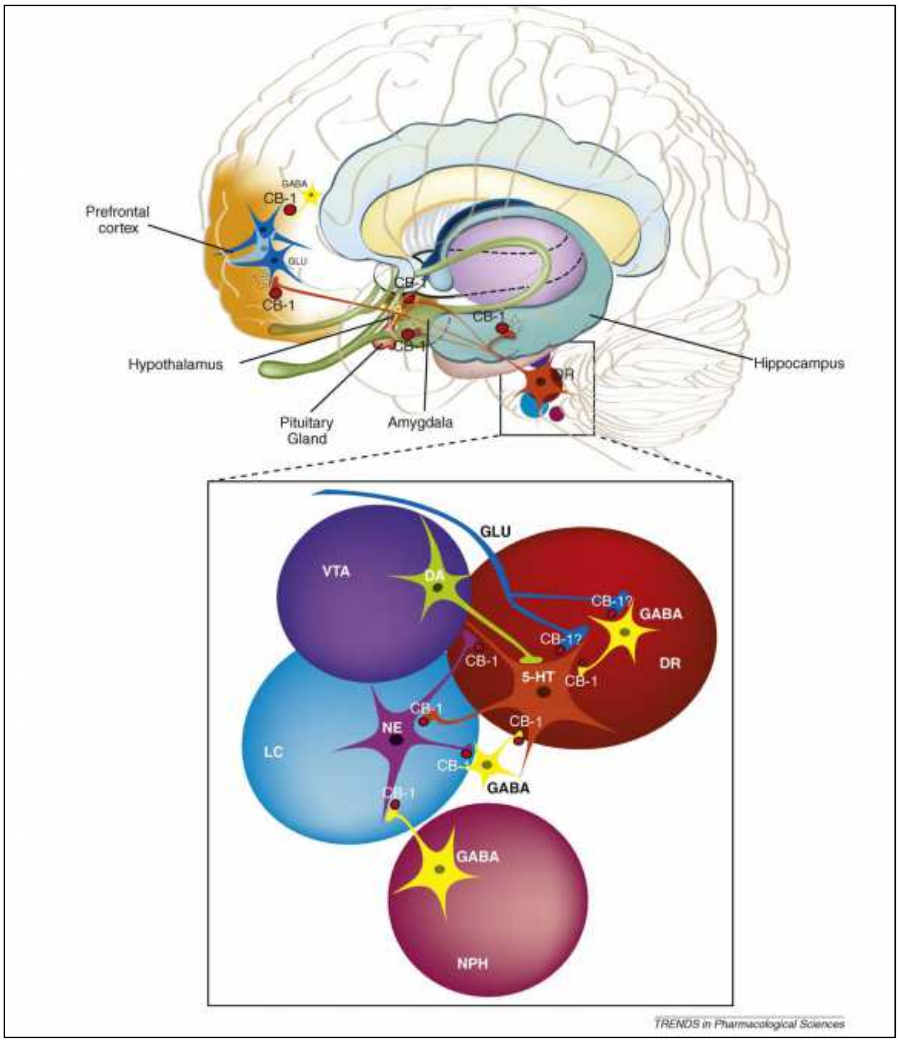


Figure 17. Representation of CB1 receptors localized in regions involved in the mood control (Hill et al., 2009).

In reference to genetic association studies focused in CB1 gene (*CNR1*) and depression, a microsatellite polymorphism containing a variable number of AAT triplet repeat in the promoter region of the gene has been analyzed. The first study carried out in this polymorphism did not find association between the length of the AAT triplet repeat and depression in a Taiwanese sample (Tsai et al., 2001). On the contrary, the study performed by Barrero and colleagues (Barrero et al., 2005) suggest the association between two long alleles with more than 16 repeats and lower susceptibility for depression in Parkinson's disease.

It has been reported that proximal negative life events interact with the minor T allele of rs7766029 in the *CNR1* gene in predicting depression (Juhasz et al., 2009).

Another silent polymorphism in *CNR1* gene (rs1049353) has been examined in relation to the risk for depression. Monteleone and colleagues found a genetic association between MDD and the A allele (Monteleone et al., 2010). Recently, Agrawal and colleagues showed that female individuals who experienced physical abuse in childhood were more likely to develop symptoms of anhedonia if they had rs1049353 GG genotype compared with AG and AA (Agrawal et al., 2012).

Regarding *FAAH* gene, the rs324420 polymorphism produces a replacement of A for C at position 385 (A385C) which results in a change at position 129 in protein from proline to threonine (P129T) (Sipe et al., 2002). It has been reported that human T-lymphocytes with an AA genotype have approximately 50% of the FAAH protein and enzymatic activity compared to cells from individuals with CC genotype (Chiang et al., 2004). Carriers of *FAAH* 385A have higher concentrations of FAAH substrates in plasma than CC homozygotes (Sipe et al., 2010), which supports the biochemical data. Strong evidence from animal studies indicates that elevated levels of AEA and FAAH inhibition produce antidepressant-like effects (Petrosino and Di Marzo, 2010). Moreover, a genotypic association was found between AC heterozygous for this polymorphism and depression (Monteleone et al., 2010).

With respect the involvement of eCB signalling in response to antidepressant, there is also evidence that this treatment induces alterations in some components of the system. In this sense, a study based on animal models showed that chronic treatment with tricyclic antidepressants induces an increase in the density of CB1

receptors in the hippocampus that contributes to the ability of these drugs to suppress activation of the HPA axis induced by stress (Hill et al., 2006). Another study with rodents showed that repeated administration of fluoxetine, an SSRI, induced a decrease in the expression of *CNR1* gene in several brain regions (Oliva et al., 2005).

Finally, Domschke and colleagues found that individuals with the G allele at *CNR1* rs1049353 had increased risk for antidepressant treatment resistance in females, particularly in those with high comorbid anxiety (Domschke et al., 2008).

Taking into account the evidence of both the involvement of eCB system in MDD and the interaction between eCB system and serotonergic system, which is the main target of SSRIs, genetic variability at genes related to eCB system can be considered as appropriate candidate genes to study in reference to antidepressant treatment response in MDD.

1.3.2. Lithium response in Bipolar Disorder

1.3.2.1. Candidate genes at the Phosphoinositide and GSK3β pathways

Phosphoinositide pathway: INPP1, IMPA1, IMPA2, ITPKC, PLCG1, MARCKS.

As it has been reported in the section about pharmacodynamics of BD, at therapeutic concentrations Li acts at the PI pathway, inhibiting the activity of IMPase and INPPase, which cause a reduction in the amount of free inositol available (Figure 18). In this sense, the inositol depletion hypothesis suggests that Li exerts its therapeutic actions by depleting free inositol, and thus dampening the activation of downstream signalling pathways in neurons (Berridge, 1989).

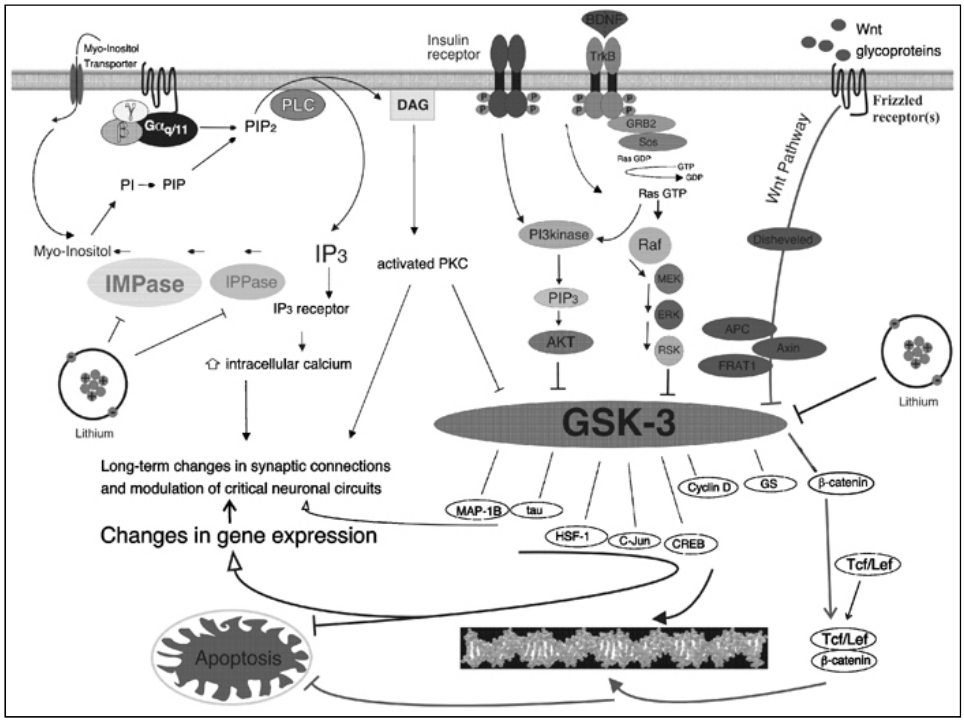


Figure 18. Representation of the mechanism of action of lithium. Adapted from (Gould et al., 2004).

IMPase and IPPase are enzymes involved in recycling and *de novo* synthesis of inositol, which is a necessary component of a primary intracellular signalling pathway, the phosphoinostol signalling pathway. Many extracellular receptors are coupled to the G protein, Gq/11, which, through activation of phospholipase C (PLC), mediates the hydrolysis of phospholipase phosphoinositide 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP3). DAG

activates protein kinase C (PKC). IP3 binds to the IP3 receptor that also functions as a calcium channel in the cell. This interaction results in the release of intracellular calcium reservoirs from the endoplasmic reticulum; calcium is an activator of many enzymes, and plays a prominent role in many cellular signalling events. IP3 is recycled back to PIP2 by the enzymes IMPase and IPPase. This recycling is necessary to maintain phosphoinositol-mediated signalling in cell types where inositol is not freely available (Gould et al., 2004) (Figure 18).

Several studies have investigated the role of gene variants encoding elements of the inositol pathway in BD and Li response.

A study investigated the role of common variants of *INPP1* gene, which encodes for INPPase, in Li response. Steen and collaborators found an association for the C937A variant and response to Li in a Norwegian sample but not in an independent Israeli sample (Steen et al., 1998). This finding was not supported in an independent sample (Michelon et al., 2006).

IMPA1 and *IMPA2* genes encode for human IMPases, which are inhibited by Li. *IMPA1 gene* has been studied in two articles reporting lack of association with Li response (Steen et al., 1998; Bremer et al., 2007). *IMPA2* gene is located in a region previously linked to BD (Stine et al., 1995). Two trends for association were found between two polymorphisms of the *IMPA2* gene and good response to Li in BD patients (Dimitrova et al., 2005).

Other genes involved in the PI system have been studied in relation to Li response. The gene encoding for PLC (*PLCG1*), a key enzyme involved in G protein mediated signals and in the inositol pathway, has been investigated in several studies. An association for a dinucleotide repeat and Li response was reported (Turecki et al., 1998) and replicated in a subsequent study (Lovlie et al., 2001). The analysis of other markers of *PLCG1* gene did not support a major role for this gene in Li response (Ftouhi-Paquin et al., 2001).

The myristoylated alanine-rich C-kinase substrate (MARCKS) is phosphorylated following the activation of PKC and is involved in the neuroplasticity of neurons. Genetic variability at *MARCKS* has not been found associated with Li response (Bremer et al., 2007).

GSK3β pathway: GSK3B, GSK3A, CREB1

Li has also been shown to inhibit GSK3β, which results in the translocation of β-catenin to the nucleus where it then becomes part of transcription complexes that induce the activation of components involved in cell survival (Detera-Wadleigh and Akula, 2011) (Figure 18). GSK3β has also been linked to the modulation of several other transcription factors, such as, Myc, CREB and AP-1 (Jope and Bijur, 2002).

Genetic studies have explored *GSK3B* and related genes in patients with mood disorders. Associations that have been identified include an increase in copy number variations affecting *GSK3B* gene locus in BD (Lachman et al., 2007), and *GSK3B* polymorphisms linked to the age of onset of BD and MDD (Benedetti et al., 2004a; Benedetti et al., 2004b; Benedetti et al., 2005; Szczepankiewicz et al., 2006; Saus et al., 2010). Interestingly, genetic variability at this gene has been associated to increased impulsivity in bipolar patients (Jimenez et al., 2014). Moreover, a functional polymorphism of the *GSK3B* gene has been associated with Li response (Benedetti et al., 2005), but this was not confirmed in two other studies (Michelon et al., 2006; Szczepankiewicz et al., 2006). Mamdani and colleagues found an association between BD and Li response and two polymorphisms of *CREB1* gene (Mamdani et al., 2008). In connection with this, it has been recently showed that alterations in phosphorylated CREB (pCREB) signalling may constitute an endophenotype of Li-responsive BD (Alda et al., 2013).

1.3.2.2. Candidate genes at HPA system: *FKBP5, CRHR1, CRHR2*

The glucocorticoid cortisol, which is released from the adrenal gland, is the final product of the HPA axis, which comprises the hypothalamus, pituitary gland and adrenal cortices. Neurosecretory cells within the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the circulatory system of the pituitary. This causes release of ACTH from the anterior lobe of the pituitary, which leads to cortisol release from the adrenals. Cortisol has numerous cellular effects, which are mediated via the glucocorticod receptor (GR) and the mineralocorticoid receptor (MR) (Schloesser et al., 2012) (Figure 19).

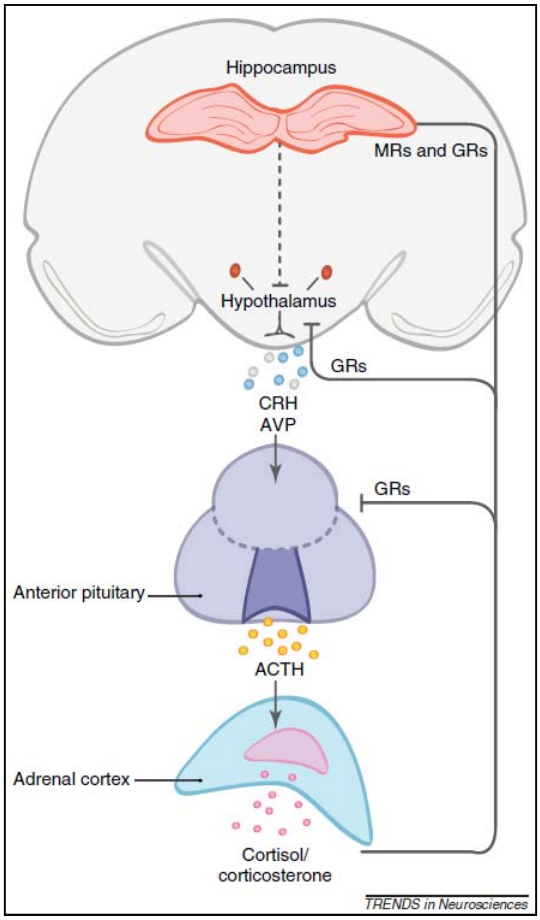


Figure 19. The Hypothalamic-pituitary-adrenal (HPA) axis (Adapted from (Schloesser et al., 2012).

The HPA axis is a major system involved in stress response. Chronic dysregulation of this axis is known to occur in several psychiatric disorders, including BD. Pituitary gland volume has found decreased in BD patients as compared to controls, finding consistent with pituitary hypoactivity in response to HPA stimulation in patients with BD (Sassi et al., 2001). Moreover, HPA axis dysfunction was also suggested to play critical role in the switch from mania to depression in most of ultra-rapid cycling bipolar patients (Juckel et al., 2000).

The activity of the GR is regulated by the *FKBP5* (FK506 binding protein 5) gene. The binding of *FKBP5* to the GR complex decreases the affinity of cortisol binding, followed by a deficient receptor nuclear translocation, and therefore reduces GR sensitivity (Binder, 2009). Menke and colleagues suggested that depressed patients carrying the *FKBP5* rs1360780 allele T exhibit significant GR resistance compared with healthy controls (Menke et al., 2013). Moreover, regard

to suicide, *FKBP5* gene expression was reduced in the amygdala of suicide victims (Perez-Ortiz et al., 2013) and analysis of haplotypes of the *FKBP5* gene in suicidal patients showed an association in previous studies (Roy et al., 2010; Supriyanto et al., 2011).

Other genes related to HPA axis, such as corticotropin releasing receptor 1 (*CRHR1*) and 2 (*CRHR2*), which stimulate the pituitary gland, have been fully investigated in MDD, however, some studies have investigated its role in BD. CRHR1 has been associated with dimensions of BD, such as excitement and psychotic dimensions (Leszczynska-Rodziewicz et al., 2012; 2013). Another study provided evidence for an association between *CRHR2* SNPs and increased suicidal behaviour in people with BD (De Luca et al., 2007).

Preclinical studies suggest that Li may modulate GR expression in different brain areas: hippocampus and paraventricular nucleus of the hypothalamus (Semba et al., 2000). BAG-1, a cochaperone protein involved in GR function, was one of the several genes identified in microarray studies which expression was upregulated by chronic Li administration (Zhou et al., 2005), suggesting that GR-related biological pathways may be involved in the Li action. Interestingly, it has been demonstrated that Li leads to a significant activation of the HPA system in patients with MDD (Bschor et al., 2011).

Since a dysfunction of the HPA axis in BD has been reported by several studies (Taylor and MacQueen, 2006), and it has been reported that Li can modulate HPA system, genes related to HPA regulation could be candidate genes for the study of variability to Li response in BD patients.

1.3.2.3. Candidate genes at Glutamatergic system: *GRIA2, GABRB2, GRIK2, GRIK5*

Glutamate is an excitatory neurotransmitter that is involved in different neural processes including neuronal development, synaptic plasticity and neuronal toxicity (Goff and Coyle, 2001). Glutamate is the major mediator of excitatory synaptic transmission in the mammalian brain (Orrego and Villanueva, 1993). Under normal conditions, glutamate plays a prominent role in synaptic plasticity,

learning, and memory, but in pathophysiological conditions it is known to be a potent neuronal excitotoxin, triggering either rapid or delayed neurotoxicity.

Glutamate mediates its action by the activation of ionotropic (ligand-gated ion channels) and metabotropic (G protein-coupled) receptors. Three subclasses of ionotropic glutamate (iGlu) receptors are known: i) NMDA, ii) alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and iii) kainate receptors (Hollmann and Heinemann, 1994). The metabotropic glutamate (mGlu) receptors consist of at least eight different subtypes classified into three groups based on their sequence homology, pharmacological profile and coupling to intracellular transduction pathways (Pin and Duvoisin, 1995) (Table5).

Ionotropic			**Metabotropic**		
NMDA	**AMPA**	**Kainate**	**Group I**	**Group II**	**Group III**
NR1 (GRIN1)	GluR1 (GRIA1)	GluR5 (GRIA5)	mGluR1 (GRM1)	mGluR3 (GRM3)	mGluR5 (GRM5)
NR2A (GRIN2A)	GluR2 (GRIA2)	GluR6 (GRIA6)	mGluR2 (GRM2)	mGluR4 (GRM4)	mGluR6 (GRM6)
NR2B (GRIN2B)	GluR3 (GRIA3)	GluR7 (GRIA7)			mGluR7 (GRM7)
NR2C (GRIN2C)	GluR4 (GRIA4)	KA1 (GRIK4)			mGluR8 (GRM8)
NR2D (GRIN2D)		KA2 (GRIK5)			
NR3A (GRIN3A)					
NR3B (GRIN3B)					

Table 5. Classification of glutamate receptors and their subunits.

The potential role of the glutamatergic system in the pathophysiology and treatment of mood disorders has recently been investigated in earnest, but the available evidence suggests that abnormal activity of the glutamatergic system is likely to contribute to the impairments in synaptic and neural plasticity that are observed in patients with severe or recurrent mood disorders (Sanacora et al., 2008).

In this sense, Li has been shown to normalize excessive glutamatergic neurotransmission by increasing glutamate reuptake as well as by modulating the phosphorylation of glutamate receptor subunits (Gray and McEwen, 2013). Despite this evidence, relatively few studies have been conducted between genes related to glutamatergic system and Li response.

Recent human genetic studies have identified *GRIK2* (which encodes for GluR6, a kainate receptor implicated in synaptic plasticity) as a potential BD

susceptibility gene (Buervenich et al., 2003). Genetic linkage of BD to chromosome 6q21 has been demonstrated in several studies, and genome-wide significant linkage was recently established by meta-analysis (Dick et al., 2003; Schulze et al., 2004; McQueen et al., 2005; Schumacher et al., 2005).

The gene encoding the NR2B receptor subunit (*GRIN2B*) is localized in the chromosomal region 12p12 previously linked to BD (Faraone et al., 2004). Its altered at the protein level has been observed in BD (Scarr et al., 2003); however, it was not confirmed by another study (Martucci et al., 2006). Based on this previous evidence, a recent study tried to investigate genetic variability in the *GRIN2B* gene and its association with response to Li treatment but it failed to find any association (Szczepankiewicz et al., 2009b).

Since all the evidence of the involvement of glutamatergic neurotransmission in the aetiology of BD and also the effect of Li to glutamatergic system, genes related to the activity of glutamatergic system are considered a good candidate genes for analyzing its involvement in Li response.

1.3.3. Clozapine response in Schizophrenia

1.3.3.1. Candidate genes at HPA system: *FKBP5*, *NR3C1*

It has been shown that antipsychotics, especially atypical ones such as CLZ, may suppress HPA activity by reducing ACTH and cortisol secretion in patients with SCZ (Zhang et al., 2005; Walker et al., 2008).

Elevated baseline cortisol secretion has been detected in schizophrenic patients, especially in drug naive patients (Abel et al., 1996; Ryan et al., 2003; Ryan et al., 2004a; Ryan et al., 2004b; Spelman et al., 2007; Kale et al., 2010). Furthermore, when patients are withdrawn from atypical antipsychotics, cortisol levels rise in correlation with negative symptoms (Zhang et al., 2005). In this sense, atypical antipsychotics have the potential to dampen HPA activity, which may partially explain their therapeutic action (Walker et al., 2008).

As far as we know, the study included in the present thesis is the first in investigating the association between the HPA axis and CLZ response. In this sense, and based on previous evidence which indicate that CLZ modulate HPA axis, genes related to HPA axis could be good candidates for CLZ response. The FKBP5 protein is of special interest since it modulates HPA axis reactivity via glucocorticoid receptor (NR3C1) sensitivity and signalling (Binder, 2009), and it has been analyzed in several pharmacogenetic studies mainly focused in MDD (Binder 2014).

Candidate gene at Neurotrophic Factors: *BDNF*, *NTRK2.*

Mounting evidence has demonstrated that BDNF is involved in the pathophysiology of SCZ. Recently, Zhang and colleagues have shown that BDNF levels were significantly lower in drug-free patients with SCZ (Zhang et al., 2012a). Lee and colleagues have also demonstrated that BDNF levels decreased significantly in unmedicated schizophrenic patients and elevated after successful antipsychotic treatment which parallel symptom improvement of the patients (Lee et al., 2011). Furthermore, post-mortem studies have shown that BDNF levels were significantly lower in prefrontal cortex of schizophrenic patients (Weickert et al., 2003; Issa et al., 2010).

BDNF functions through its high-affinity receptor, neurotrophic tyrosine kinase receptor 2 (NTRK2) (Squinto et al., 1991). NTRK2 has also been found decreased in post-mortem schizophrenic subjects (Weickert et al., 2005). More interestingly, some previous studies have also demonstrated that BDNF and NTRK2 levels were both decreased in the brain tissue of schizophrenic patients (Hashimoto et al., 2005; Ray et al., 2014). This evidence suggests that dysfunction of BDNF and its receptor may be involved in the pathophysiology underlying SCZ.

Since the involvement of neurotrophic factors in SCZ, some studies tried to investigate the role of *BDNF* polymorphisms and treatment response. The *BDNF* Val66Met polymorphism (rs6265) is a frequently studied SNP, with a G to A substitution resulting in a valine to methionine substitution at codon 66. This alters intracellular trafficking and packaging of proBDNF, leading to reduced synaptic plasticity (Egan et al., 2003). Some studies investigated the association between the *BDNF* Val66Met polymorphism and treatment response to CLZ, however the results are inconsistent (Hong et al., 2003; Xu et al., 2010a; Zai et al., 2012; Zhang et al., 2013).

As to *NTRK2* gene polymorphisms, previous studies have shown that *NTRK2* gene polymorphisms rs2769605, rs1387923, and rs1565445 were associated with mood disorders or antidepressant response (Bremer et al., 2007; Smith et al., 2009; Li et al., 2013). However, no literature reported the association between those three *NTRK2* gene polymorphisms or other SNPs at the *NTRK2* gene and neither SCZ or CLZ response.

Taking all together, it seems that neurotrophic factors play an important role in SCZ and several studies reported association between genetic variability at *BDNF* gene and antipsychotic response (Krebs et al., 2000; Hong et al., 2003; Xu et al., 2010a). However, these results are not fully replicated. In this sense, it should be of interest the replication of these previous associations between *BDNF* gene and CLZ response and explore the putative role of the *NTRK2* gene in this phenotype.

2. Hypothesis and Objectives

Based on the background mentioned in the Introduction, the main hypothesis that guides the present thesis is:

Main hypothesis: Clinical response to the pharmacological treatment in psychiatric disorders is considered a complex trait. We hypothesize that lack of response to psychotropic drugs will be associated to genetic variability at genes coding for proteins involved directly or indirectly in the mechanism of action of these drugs.

The specific **subhypotheses** drawn from the main hypothesis are:

Hypothesis 1: Genetic variability at genes of the endocannabinoid system will be associated to the lack of clinical response and/or remission to citalopram treatment in Major Depressive patients.

Hypothesis 2: Genetic variability at genes related to phosphoinositide (PI), glycogen synthetase kinase-3 (GSK3), hypothalamic-pituitary-adrenal (HPA) and glutamatergic pathways will be associated to the lack of clinical response to lithium in Bipolar Disorder patients.

Hypothesis 3: Genetic variability at genes related to neurotrophic factors and HPA axis will be associated to the lack of clinical response to clozapine in Schizophrenic patients.

In relation to **hypothesis 1** the following objectives were established:

Objective 1:

To analyse genetic variability at genes which codify for CB1 and CB2 receptors (*CNR1* and *CNR2*) and the FAAH enzyme (*FAAH*) of the endocannabinoid system in a sample of 154 patients with Major Depressive Disorder evaluated for the clinical response to citalopram.

To investigate the role of this genetic variability as risk factors for transversal clinical no-response (4^{th} weeks) and no-remission (12^{th} weeks) to citalopram treatment.

To investigate the role of this genetic variability as a risk factor for longitudinal clinical response to citalopram treatment evaluated along 12 week follow-up.

Objective 2:

To further analyze the genetic variability at *CNR1* gene of the endocannabinoid system in a sample of 155 patients with Major Depressive Disorder evaluated for the clinical response to citalopram.

To investigate the role of genetic variability at the *CNR1* gene as a risk factor for transversal clinical no-response (4^{th} weeks) and no-remission (12^{th} weeks) to citalopram treatment.

To investigate the role of this genetic variability at *CNR1* gene as a risk factor for longitudinal clinical response to citalopram treatment evaluated along 12 week follow-up.

In relation to **hypothesis 2** the following objective was established:

Objective 3:

To analyse genetic variability at genes related to PI (*INPP1, MARCKS, IMPA1, IMPA2, ITPKC, PLCG1*), GSK3 (*CREB1, GSK3B, GSK3A),* HPA (*FKBP5, CRHR2, CRHR1*) and glutamatergic (*GRIA2, GABRB2, GRIK2, GRIK5*) pathways in a sample of 131 patients with Bipolar Disorder evaluated retrospectively for their response to lithium treatment.

To investigate the role of this genetic variability as a risk factor for the lack of clinical response to lithium treatment.

In relation to **hypothesis 3** the following objective was established:

Objective 4:

To analyse genetic variability at genes related to HPA axis (*FKBP5, NR3C1*) and neurotrophic factors (*BDNF, NTRK2)* in a sample of 591 patients with Schizophrenia treated by clozapine and evaluated retrospectively for their response.

To investigate the role of this genetic variability as a risk factor for the lack of clinical response to clozapine treatment.

3. Supervisor's Report on Articles

Dr. Bárbara Arias
Anthropology Unit, Department of Animal Biology
Faculty of Biology

Supervisor's Report on Articles

The doctoral thesis "**Genetic Risk Factors for the Lack of Response to Clinical Treatment in Mental Disorders: an Approach from Pharmacogenetics**" is based on the original results obtained by Marina Mitjans Niubó. These results have been published or have been submitted to international peer reviewed journals. The impact factors of these journals demonstrate the quality of the research conducted, and are as following:

1. **An approach from the endocannabinoid (eCB) system to 12-week clinical response to citalopram treatment: the role of the *CNR1*, *CNR2* and *FAAH* genes,** published in *Journal of Psychopharmacology.* This multidisciplinary journal is devoted to the publication of preclinical and clinical aspects of psychopharmacology providing an essential forum of the effects of drugs on human behaviour, and the mechanisms underlying these effects. It is indexed in Journal Citation Reports (Social Sciences Edition) with a current impact factor of 3.396 and classified in the first quartile of the area of Pharmacology and Pharmacy (ranking: 60/256).

2. **Screening genetic variability at the *CNR1* gene in both major depression etiology and clinical response to citalopram treatment,** published in *Psychopharmacology.* This is an international journal that covers the broad topic of elucidating mechanisms by which drugs affects behaviour. It is indexed in Journal Citation Reports (Social Sciences Edition) with a current impact factor of 3.988 and classified in the first quartile of the area of Psychiatry (ranking: 30/163).

Universitat de Barcelona

Dr. Bárbara Arias
Anthropology Unit, Department of Animal Biology
Faculty of Biology

3. **Exploring genetic variability at PI, GSK3, HPA and glutamatergic pathways in lithium response: association with *IMPA2*, *INPP1* and *GSK3B* genes,** submitted for publication to *Journal of Clinical Psychopharmacology* that is a leading publication in psychopharmacology, offering a wide range of articles reporting on clinical trials and studies, side effects, drug interactions, overdose management, pharmacogenetics, pharmacokinetics, and psychiatric effects of non-psychiatric drugs. It is indexed in Journal Citation Reports (Social Sciences Edition) with a current impact factor of 3.761 and classified in the first quartile of the area of Pharmacology and Pharmacy (ranking: 47/256).

4. **Hypothalamic-pituitary-adrenal system, neurotrophic factors and clozapine response: association with *FKBP5* and *NTRK2* genes,** submitted for publication to *Pharmacogenetics and genomics.* This multidisciplinary journal publishes articles related to genetic determinants in response to drugs and other chemicals in humans and animals. It is indexed in Journal Citation Reports (Social Sciences Edition) with a current impact factor of 3.450 and classified in the first quartile of the area of Pharmacology and Pharmacy (ranking: 56/256).

Accordingly, I confirm the quality of the published and submitted articles.

Dr. Bárbara Arias

Barcelona, November 10th 2014

4. Publications

An approach from the endocannabinoid (eCB) system to 12-week clinical response to citalopram treatment: the role of the *CNR1*, *CNR2* and *FAAH* genes.

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Genetic variability in the endocannabinoid system and 12-week clinical response to citalopram treatment: the role of the CNR1, CNR2 and FAAH genes

Marina Mitjans[1], Cristóbal Gastó[2,3], Rosa Catalán[2,3], Lourdes Fañanás[1,3] and Bárbara Arias[1,3]

Abstract

First line treatment of major depression is based on selective serotonin re-uptake inhibitors (SSRIs) that enhance serotonergic neurotransmission by blocking the serotonin transporter. However, clinical response is a complex phenomenon in which other systems such as the endocannabinoid system could be involved. Given the evidence for the role of the endocannabinoid system in the pathogenesis of depression as well as in the mediation of antidepressant drug effects, the aim of this study was to analyze genetic variability in the endocannabinoid system genes (CNR1, CNR2 and FAAH genes) and their role in clinical response (at week 4) and remission (at week 12) in SSRI (citalopram) treatment in a sample of 154 depressive outpatients, all of Spanish origin. All patients were treated with citalopram and followed over 12 weeks. Severity of depressive symptomatology was evaluated by means of the 21-item Hamilton Depression Rating Score (HDRS). No differences were found in any of the genotype distributions according to response or remission. The longitudinal study showed that (i) the CNR1 rs1049353-GG genotype conferred a better response to citalopram treatment in the subgroup of male patients and (ii) G allele carriers (CNR2 rs2501431) presented higher HDRS scores in the follow-up than AA homozygous allele carriers. Our results seem to suggest the involvement of CNR1 and CNR2 genes in clinical responses to citalopram treatment.

Keywords

Major depression, pharmacogenetics, endocannabinoid system, SSRI, molecular variation

Introduction

Major depressive disorder has been described as a clinically heterogeneous disease that results from the interplay of multiple genes interacting with environmental factors such as early stressful life events (Caspi et al., 2003). Treatment of major depression is principally based on selective serotonin re-uptake inhibitors (SSRIs) that enhance serotonergic neurotransmission by blocking the serotonin transporter. However, clinical response to drug treatment in depression is a highly complex biological phenomenon in which several factors are involved, some of them genetic (Klengel and Binder, 2011; Uher, 2011). SSRIs were developed as drugs with high selectivity for the target molecule, the serotonin transporter, and have constituted one of the most important advances in the pharmacological treatment of depression since the late 80s (Martin et al., 1997). Although SSRIs exert their action by basically modifying the serotonergic system, previous studies have shown the modulating action of this system on other neurotransmission systems such as the dopaminergic or glutamatergic systems (Arias et al., 2006, 2009; Domschke et al., 2008; Drago et al., 2011; Kato and Serretti, 2010; Porcelli et al., 2011).

In addition, recent evidence suggests that other systems like the endocannabinoid system can also have a role in modulating the serotonergic system (Horstmann and Binder, 2009). In this sense, the endocannabinoid system is expressed in both the brain and at the periphery. It consists of two cannabinoid receptors (CB1 and CB2), their natural ligands and specific enzymes for their biosynthesis and inactivation. It has recently been suggested that the endocannabinoid system may be implicated in the pathophysiology of depression (Hill and Gorzalka, 2005a). This is supported by evidence showing that the cannabinoid receptors and enzymes involved in the synthesis and degradation of endocannabinoid ligands are highly expressed in the neuroanatomical structures and circuits involved in depression, including the prefrontal cortex, hippocampus, amygdala, hypothalamus and forebrain monoaminergic circuits (Herkenham, 1991). Moreover, experimental data showed that the blockade of the endocannabinoid system is a risk factor in the pathogenesis of depression as well as anxiety disorders (Hill and Gorzalka, 2005c; Martin et al., 2002).

The CB1 receptor is coded by the CNR1 gene located on chromosome 6 (6q14-15). It is considered the most abundant G

[1]Unitat d'Antropologia, Universitat de Barcelona, Barcelona, Spain
[2]Centre de Salut Mental Esquerre de l'Eixample, Institut d'Investigació Biomedica Agustı Pi i Sunyer (IDIBAPS), Barcelona, Spain
[3]Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Barcelona, Spain

Corresponding author:
Bárbara Arias, Unitat d'Antropologia, Departament Biologia Animal, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 2on pis, 08028 Barcelona, Spain
Email: barbara.arias@ub.edu

protein-coupled receptor expressed in the CNS of mammalian brain (Herkenham, 1991) and, to a lesser extent, in peripheral tissues (Galiegue et al., 1995; Kumar et al., 2001). The CB1 receptor is mainly located in GABAergic and glutamatergic neurons suggesting a modulating role of synapses of a different nature (Rodriguez de Fonseca et al., 2005). Several studies demonstrate the role of CB1 receptors in the modulation of monoaminergic neurotransmission such as the serotonergic one (Bambico et al., 2007; Gobbi et al., 2005). CB1 receptors are present and function on 5-HT terminals (Balazsa et al., 2008) and human genetic studies have provided evidence for interaction among the CB1 receptor gene, the serotonin receptor gene (SERT), and anxiety (Lazary et al., 2009, 2011). Also, a study of Hill et al. (2006) shows that the expression of CB1 receptor in the hippocampus and the hypothalamus is up-regulated by chronic tricyclic antidepressant treatment which exerts part of its antidepressant action on the serotonergic system.

A recent study shows that CNR1 rs1049353 A allele also confers increased risk for neuroticism and depression especially in haplotypic combination (Juhasz et al., 2009). In addition, a decrease of CB1 receptor density has been detected in grey matter glial cells in post mortem brains of patients with major depression (Koethe et al., 2007). In this context, a meta-analysis has reported the anxiogenic and depressive effects when the CB1 receptor is blocked by antagonist-like rimonabant, a drug for obesity treatment (Christensen et al., 2007). Although, on the contrary, other studies have showed the potential indication of CB1 receptor antagonists in the treatment of depressive symptomatology (Witkin et al., 2005).

On the other hand, the CB2 receptor is basically highly expressed in the periphery and the presence of this receptor has been recently demonstrated in neurons of the brainstem and cerebellum (Onaivi, 2006; Suarez et al., 2008). The CB2 receptor is encoded by the CNR2 gene located on chromosome 1 (1p36.1). Animal and clinical studies have provided evidence of the participation of CB2 receptor in mood disorders. Recent results based on mice models with a genetically-modified CB2 receptor have suggested that this receptor is involved in the regulation of emotional behaviour (Ortega-Alvaro et al., 2011; Racz et al., 2008a, 2008b).

The CB1 and CB2 receptors are activated by endogenous ligands such as anandamide and 2-arachidonylglycerol (2-AG). Hydrolysis of endogenous ligands is controlled by two enzyme systems, fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) and monoacylglycerols lipase (Dinh et al., 2002). The FAAH is coded by the FAAH gene located on chromosome 1 (1p35-34). It has been reported by experimental data that the administration of CB1 receptor agonists, endogenous cannabinoid re-uptake inhibitors or inhibitors of the FAAH enzyme resulted in antidepressant-like effects and in an increased efficacy of fluoxetine in animal models (Adamczyk et al., 2008; Gobbi et al., 2005; Hill and Gorzalka, 2005b).

Taking into account the strong evidence of the role of the endocannabinoid system in the pathogenesis of depression as well as in the mediation of antidepressant drug effects, the aim of this study was to analyze genetic variability in the CB1 receptor gene (CNR1 rs1049353 G/A), CB2 receptor gene (CNR2 rs2501431 G/A) and fatty acid amide hydrolase gene (FAAH rs324420 C/A) and their role on clinical response (four weeks) and clinical remission (12 weeks) after SSRI (citalopram) treatment in major depression.

Methods and materials

Sample

The sample consisted of 154 depressive outpatients (122 females and 32 males; mean age: 39.5±12.19 years) from the Centre de Salud Mental of the Hospital Clinic de Barcelona who were recruited between 1999 and 2002 and followed during at least 12 weeks by experienced psychiatrists. All patients suffered an active episode of major depression diagnosed following DSM-IV-TR criteria (APA, 1994) at the time of inclusion in the study. All cases were diagnosed using the Spanish version of the Structured Clinical Interview (SCID-I) (Spitzer et al., 1990). Detailed data about the severity of clinical features was collected from the medical records of the patients, and data on the presence of melancholic features (n=49 (33.3%)), psychotic symptoms (n=27 (18.5%)), seasonal pattern (n=69 (47.6%)) or previous suicide attempts (n=24 (16.7%)) was also collected (Arias et al., 2009). No patients with bipolar I or II disorder were included in this sample. Patients with drug abuse and dependence, mental retardation or with a medical disease that impairs evaluation have been excluded from the study.

All patients were treated with citalopram (20–40 mg/day). Patients were initially evaluated for the severity of their symptoms using the 21-item Hamilton Rating Scale for Depression (HDRS) (mean initial HDRS: 24.72±4.74). A new HDRS was assessed for all patients every four weeks until the completion of the follow-up at week 12. A positive clinical response to citalopram treatment was considered when a decrease of at least 50% in the baseline score was observed at week 4 (Baumann et al., 1996). Remission for the index episode was considered when HDRS scores were equal or under seven by the end of week 12 (Frank et al., 1991). Plasma levels of citalopram were determined at week 6 using high performance liquid chromatography (HPLC) (Olesen and Linnet, 1996).

All participants were of Spanish ancestry (Caucasian), thereby reducing the possibility of confounding genetic differences by population stratification (Freedman et al., 2004). Ethical approval was obtained from local research ethic committees. Patients provided written informed consent before inclusion in the study.

Selection of gene variants and genetic analysis

Genetic variants were selected according to their role in depression or antidepressant response based on previous publications. Firstly, the genetic variant CNR1 rs1049353 has been reported to confer an increased risk of antidepressant treatment resistance, particularly in female patients (Domschke et al., 2008). Secondly, the CNR2 rs2501431 variant was selected because this polymorphism has been reported to be associated with risk for major depression in Japanese population. (Onaivi et al., 2008). Thus, we hypothesize that the polymorphism could also have an involvement in the treatment response. Finally, the FAAH-rs324420 variant has functional effects producing a 50% reduced activity of the FAAH enzyme

(Chiang et al., 2004). Animal models show that inhibition of the FAAH enzyme has antidepressant effects (Gobbi et al., 2005).

Genomic DNA was extracted from blood samples using a conventional phenol-chloroform extraction protocol. The rs1049353 (CNR1 gene), rs2501431 (CNR2 gene) and rs324420 (FAAH gene) polymorphisms were analyzed using Applied Biosystems (AB) Taqman technology (Applied Biosystems, Foster City, California, USA). All the probes for genotyping were ordered through the TaqMan® SNP Genotyping assays AB assay-on-demand service. The final volume of the polymerase chain reaction (PCR) was 5 mL, which contained 10 ng of genomic DNA, 2.5 mL of TaqMan Master Mix, and 0.125 mL of 40x genotyping assay. The cycling parameters were as follows: 95°C for 10 min followed by 40 cycles of denaturation at 92°C for 15 sec and annealing/extension at 60°C for 1 min. Polymerase chain reaction plates were read on an ABI PRISM 7900HT instrument with SDS v2.1 software (Applied Biosystems).

Genotype determinations were performed blind to clinical condition. A randomized 10% of the individuals were retested for their genotypes to confirm the pattern reproducibility. Table 1 shows the final genetic sample. Of the total sample, 90.25% was successfully genotyped for the CNR1 rs2501431 polymorphism, the 96.1% for the CNR2 rs2501431 polymorphism and the 95.45% for the FAAH rs324420 polymorphism.

Statistical analysis

Hardy-Weinberg equilibrium for genotype frequencies in patients sample were calculated using chi-square tests (Epi Info v3.5.1; Dean et al., 1991). Simple chi-squared tests of independence were performed to confirm the presence or absence of allele or genotype associations. Odds ratios (OR) with 95% confidence intervals (CI) were estimated for the effects of high-risk genotypes. The combined study group had an 80% power (95% CI) to detect OR equal or greater than 2.93–2.98 for No-RP or No-RM according to the allele frequencies of the different polymorphisms analyzed in our sample (Cohen, 1988).

The longitudinal study based on HDRS change scores during the 12 weeks of citalopram treatment was performed using analysis of variance (ANOVA) with repeated measures (genotype and gender as fixed factor, time point as a repeated measure and a number of covariates: see below). Covariates were included in these multivariable ANOVA models if they showed an impact on treatment response (4 weeks). All data were processed using SPSS 17.0 (SPSS for Windows; SPSS Inc., Chicago, Illinois, USA).

Results

Sociodemographic and clinical measures

The genotype distribution of all single nucleotide polymorphisms (SNP) was found to be in Hardy-Weinberg equilibrium in the overall sample (rs1049353: χ^2=0.76, *df*=2, *P*=0.68; 2501431: χ^2=0.14, *df*=2 *P*=0.93; rs324420: χ^2=0.09, *df*=2, *P*=0.95).

In our sample, 101 patients (65.6%) were considered responders (RP) and 53 (34.4%) were classified as no-responders (No-RP) according to the decrease of their HDRS scores at week 4. Considering the remission criteria at week 12, 99 patients (64.7%) were classified as remitters (RM) and 54 (35.3%) as no-remitters (No-RM).

Table 1. Genotype and allele distribution of the CNR1, CNR2 and FAAH polymorphisms in major depression patients according to treatment response at week 4 and remission at week 12.

	rs1049353 (CNR1)						rs2501431 (CNR2)						rs324420 (FAAH)					
	N	Genotypes (%)			Alleles (%)		*N*	Genotypes (%)			Alleles (%)		*N*	Genotypes (%)			Alleles (%)	
		G/G	G/A	A/A	G	A		A/A	A/G	G/G	A	G		C/C	C/A	A/A	C	A
Response (week 4)																		
Yes	88	47 (53.4)	38 (43.2)	3 (3.4)	132 (75)	44 (25)	96	34 (35.4)	43 (44.8)	19 (19.8)	111 (57.8)	81 (42.2)	95	57 (60)	30 (31.6)	8 (8.4)	144 (75.8)	46 (24.2)
No	51	28 (54.9)	20 (39.2)	3 (5.9)	76 (74.5)	26 (25.5)	52	15 (28.8)	29 (55.8)	8 (15.4)	59 (56.7)	45 (43.3)	52	26 (50)	24 (46.2)	2 (3.8)	76 (73.1)	28 (26.9)
		χ^2=0.593, *df*= 2			χ^2=0.01, *df*=1			χ^2=1.634, *df*=2			χ^2=0.03, *df*=1			χ^2=3.572, *df*=2		χ^2=0.26, *df*=1	
		P=0.744			*P*=0.93			*P*=0.442			*P*=0.86			*P*= 0.168		*P*=0.71	
Remission (week 12)																		
Yes	87	48 (55.2)	35 (40.2)	4 (4.6)	131 (75.3)	43 (24.7)	95	33 (34.7)	48 (50.5)	14 (14.7)	114 (60)	76 (40)	94	52 (55.3)	36 (38.3)	6 (6.4)	140 (74.5)	48 (25.5)
No	51	27 (52.9)	22 (43.1)	2 (3.9)	76 (74.5)	26 (25.5)	52	16 (30.8)	24 (46.2)	12 (23.1)	56 (53.9)	48 (46.1)	52	30 (57.7)	18 (34.6)	4 (7.7)	78 (75)	26 (25)
		χ^2=0.129, *df*=2			χ^2=0.02, *df*=1			χ^2=1.611, *df*=2			χ^2=1.043, *df*=1			χ^2=0.240, *df*=2		χ^2=0.01, *df*=1	
		P=0.938			*P*=0.885			*P*=0.447			*P*=0.31			*P*=0.887		*P*=0.97	

Table 2. Distribution of sociodemographic variables and clinical features according to RP/No-RP and RM/No-RM.

	RP	No-RP	RM	No-RM
Sex (men)	23 (23.3%)	9 (16.6%)	23 (22.7%)	9 (17%)
Mean age (SD)	39.5 (12.3)	40 (12)	38.8 (12.3)	41.5 (11.9)
Mean age at onset (SD)	31.5 (10.9)	31.3 (11.2)	31.2 (11.2)	31.7 (10.7)
Presence of melancholic symptoms	28 (28.3%)	22 (42.3%)	29 (29.9%)	21 (39.6%)
Presence of suicide attempts	12 (12.5%)	13 (25%)*	9 (9.5%)	16 (30%)**
Presence of seasonal pattern	46 (47.5%)	25 (52%)	49 (51.6%)	22 (41.5%)
Presence of psychotic symptoms	16 (16.3%)	11 (21%)	14 (14.5%)	13 (24.5%)
Citalopram levels at week 6 (SD)	61.5 (33.3)	47 (31)***	59 (33)	54 (34)

RP: responders; No-RP: no-responders; RM: remitters; No-RM: no-remitters; SD: standard deviation.
*Significant differences between RP/No-RP according to presence of suicide attempts (χ^2=3.75, *df*=1, *P*=0.046). **Significant differences between RM/No-RM according to presence of suicide attempts (χ^2=10.2, *df*= 1, *P*=0.002). ***Significant differences between RP/No-RP according to CIT levels at 6th week (F=4.72, *df*=1, *P*= 0.032).

We compared RP vs No-RP and RM vs No-RM according to sociodemographical variables (sex and age) and clinical features (age at onset, presence of anxiety, melancholic symptoms, suicide attempts, seasonal pattern, psychotic symptoms and citalopram levels at the sixth week) (Table 2). These variables were selected on the basis of previous literature that showed their possible influence on the evolution of response to antidepressant treatment (Arranz and Kapur, 2008; Nierenberg, 2003).

Our result showed that the presence of suicide attempts was associated with No-RP at week 4 (χ^2=3.75, *df*=1, *P*=0.046) and No-RM at week 12 (χ^2=10.204, *df*= 1, *P*=0.002). Also, our results showed that lower citalopram levels at week 6 were associated with No-RP at week 4 (*F*=4.72, *df*=1, *P*= 0.032). Therefore, these variables were considered as covariates in multivariable ANOVA procedures.

Pharmacogenetics of Response (week 4) and Remission (Week 12)

As described in Table 1, we tested whether there was any difference in genotype and allele distribution according to citalopram response at week 4 and remission at week 12. We did not find any statistical difference when comparing response/no response or remission/no remission for rs1049353 (CNR1), 2501431 (CNR2) and rs324420 (FAAH) polymorphisms.

Pharmacogenetics of the longitudinal study

We performed a two-way repeated measure ANOVA on HDRS scores to evaluate the effect of the rs1049353 polymorphism (CNR1 gene) on the 12-week clinical follow-up of the patients treated with citalopram. Our results showed that rs1049353-GG carriers presented a better response to antidepressant treatment compared to the rs1049353-A allele carriers ($F_{(2.78,\ 270.4)}$= 2.914, *P*=0.038) (Figure 1(a)). Stratification for gender revealed that this effect is originated by the subgroup of male patients ($F_{(2.78,\ 270.4)}$=5.85, *P*=0.001) that showed a better outcome in response to citalopram treatment (Figure 1(b)).

When we consider the rs2501431 polymorphism (CNR2 gene), we detected a significant decrease of HDRS scores along time ($F_{(2.74,\ 284.98)}$=137.262, *P*<0.001) and a significant effect of genotype ($F_{(1,\ 104)}$=11.432, *P*=0.001) but a non significant effect of genotype x time interaction ($F_{(2.74,\ 284.98)}$=0.412, *P*=0.72). Thus, we observed significant effect of genotype, indicating that the homozygous AA presented higher scores on the HDRS scale than carriers of the G allele along the follow up (Figure 2).

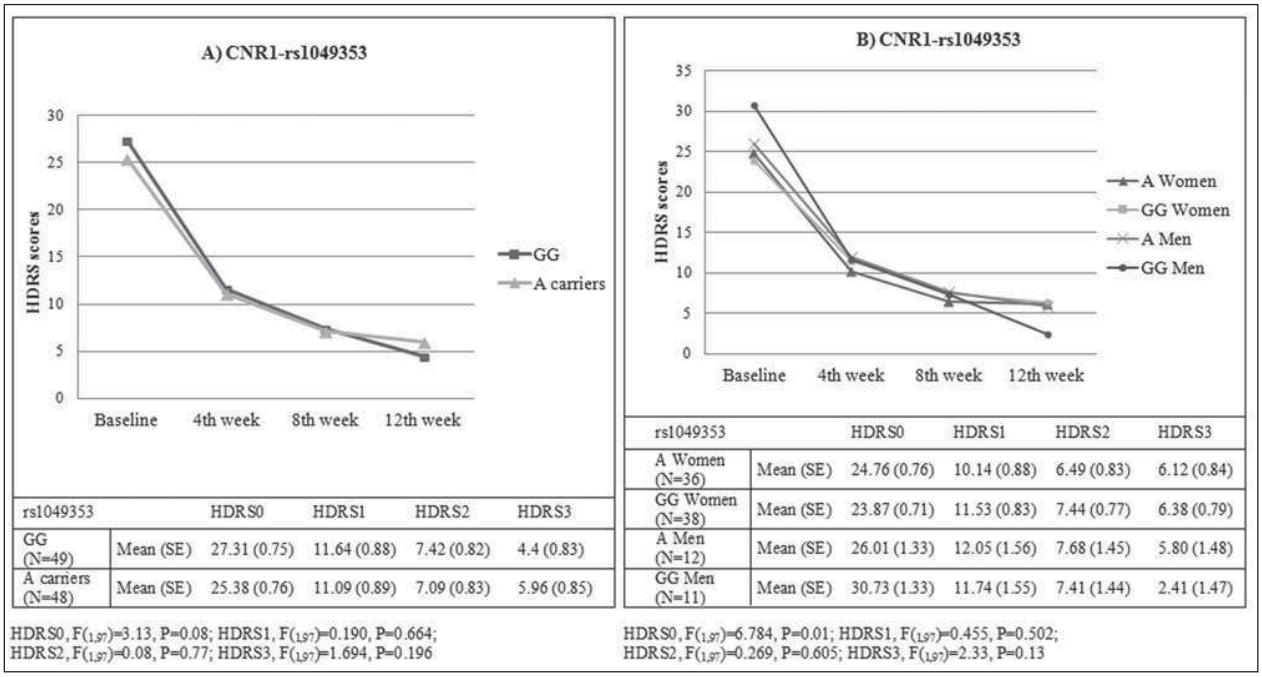
Finally, when we analyzed the effect of the rs324420 polymorphism in the FAAH gene we did not find any significant influence of this polymorphism in the outcome of the depressive episode treated with citalopram (data not shown).

Discussion

Our results showed that genetic variability in endocannabinoid receptors could play a role in the understanding of clinical response. Specifically, molecular variation at CNR1 gene seems to differentiate response to citalopram according to sex and the results on CNR2 gene showed a possible involvement of this gene in the severity of the disease.

The analyses of response and remission criteria according to clinical features have shown an effect of suicide attempts on both lack of response at week 4 and remission at week 12. In this sense, it has been proposed that a history of suicide attempts could be a correlate of severe depressive disorder and that suicide attempters could represent a particular subtype of subjects suffering from major depressive disorder (Gilmer et al., 2008; Zisook et al., 2007). Particularly, it has been previously reported that depressive patients with a history of suicidal attempts presented, among other features, a worse response to antidepressant as it has been shown by our study (Claassen et al., 2007; Forman et al., 2004; Hansen et al., 2003; Roy, 1993).

With respect to the effect of the rs1049353 polymorphism (CNR1 gene) on the 12-week clinical follow-up, our results showed that rs1049353-GG presented a better response to antidepressant treatment compared to the rs1049353-A allele carriers. This effect was stronger when the sample was stratified for gender, revealing that GG-men showed better outcome in response to citalopram treatment than A-carrier men or all women. However, it has recently reported that being an rs1049353-G carrier confers an increased risk of resistance to antidepressant treatment, particularly in female patients with major depression and high comorbid anxiety (Domschke et al., 2008). In this sense, it seems that the CNR1 gene is involved in the antidepressant response; however, the contradictory results related to the rs1049353 polymorphism



rs1049353		HDRS0	HDRS1	HDRS2	HDRS3
GG (N=49)	Mean (SE)	27.31 (0.75)	11.64 (0.88)	7.42 (0.82)	4.4 (0.83)
A carriers (N=48)	Mean (SE)	25.38 (0.76)	11.09 (0.89)	7.09 (0.83)	5.96 (0.85)

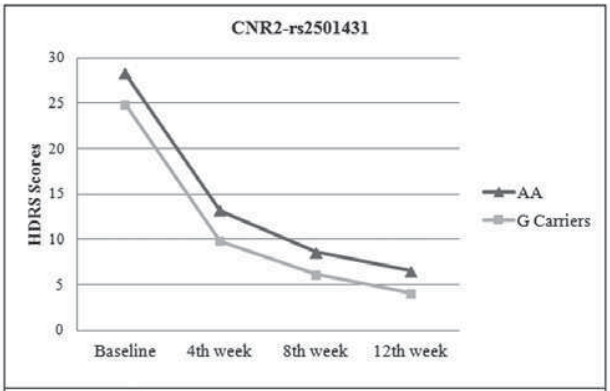
HDRS0, $F_{(1,97)}$=3.13, P=0.08; HDRS1, $F_{(1,97)}$=0.190, P=0.664; HDRS2, $F_{(1,97)}$=0.08, P=0.77; HDRS3, $F_{(1,97)}$=1.694, P=0.196

rs1049353		HDRS0	HDRS1	HDRS2	HDRS3
A Women (N=36)	Mean (SE)	24.76 (0.76)	10.14 (0.88)	6.49 (0.83)	6.12 (0.84)
GG Women (N=38)	Mean (SE)	23.87 (0.71)	11.53 (0.83)	7.44 (0.77)	6.38 (0.79)
A Men (N=12)	Mean (SE)	26.01 (1.33)	12.05 (1.56)	7.68 (1.45)	5.80 (1.48)
GG Men (N=11)	Mean (SE)	30.73 (1.33)	11.74 (1.55)	7.41 (1.44)	2.41 (1.47)

HDRS0, $F_{(1,97)}$=6.784, P=0.01; HDRS1, $F_{(1,97)}$=0.455, P=0.502; HDRS2, $F_{(1,97)}$=0.269, P=0.605; HDRS3, $F_{(1,97)}$=2.33, P=0.13

Figure 1. Genotype distribution of the CNR1 rs1049353 polymorphism according to the different follow-ups based on citalopram treatment; (a) rs1049353-GG carriers presented a better response to antidepressant treatment compared to the rs1049353-A allele carriers (F(2.78, 270.4)= 2.914, P=0.038); (b) this effect originates in the subgroup of male patients that showed a better outcome in response to citalopram treatment (F(2.78, 270.4)=5.85, P=0.001).

ANOVA: analysis of variance; HDRS: Hamilton Rating Scale for Depression; SE: standard error. Data given shows the HDRS scores during the follow-up in relation to genotypes. Results of two-way repeated-measures ANOVA were corrected for suicide attempts and plasma levels of citalopram at sixth week.



rs2501431		HDRS0	HDRS1	HDRS2	HDRS3
AA (N=40)	Mean (SE)	28.27 (0.78)	13.19 (0.89)	8.62 (0.85)	6.55 (0.87)
G Carriers (N=70)	Mean (SE)	24.86 (0.68)	9.83 (0.78)	6.16 (0.74)	4.10 (0.76)

HDRS0, $F_{(1,104)}$=10.79, P=0.001; HDRS1, $F_{(1,104)}$=8.012, P=0.006; HDRS2, $F_{(1,104)}$=4.701, P=0.032; HDRS3, $F_{(1,104)}$=4.438, P=0.038

Figure 2. Genotype distribution of the CNR2 2501431 polymorphism according to the different follow-ups based on citalopram treatment. Variability in CNR2 rs2501431 showed a differential outcome of the depressive episode treated with citalopram (F(1, 104)=11.432, P=0.001).

ANOVA: analysis of variance; HDRS: Hamilton Rating Scale for Depression; SE: standard error. Data given shows the HDRS scores during the follow-up in relation to genotypes. Results of two-way repeated-measures ANOVA were corrected for suicide attempts and plasma levels of citalopram at sixth week.

will require replication and have to be interpreted with caution. Moreover, it is unclear the role that it would play in relation to differential gender response. In this sense, the results referring to differential response mediated by gender still remain controversial (Serretti et al., 2008; Vermeiden et al., 2010). It might be hypothesized that gender differences in the response could also reflect the differences that are found in the etiology of major depression as physiological and epidemiological studies have shown (Biver et al., 1996; Kendler et al., 2001; Legato, 2010; Nishizawa et al., 1997; Weissman et al., 1996;).

As the CB2 receptor has been recently shown to be expressed in CNS, a possible role in brain disorders has still to be established. Thus, no studies have been published in relation to the CNR2 gene and response to antidepressant treatment. However, a recent study found an association between a genetic variant of the rs2501432 polymorphism in the CNR2 gene and increased risk for depression in the Japanese population (Onaivi et al., 2008). These results suggest study of this gene in both depressive illness and response to antidepressants.

The results of the longitudinal study did not show an influence of the polymorphism analyzed in the CNR2 gene on the response to treatment. However, we observed a significant effect of genotype, indicating that homozygous AA carriers presented higher scores on the HDRS scale than carriers of the G allele during the follow-up. This result shows that AA homozygous carriers present a more severe type of depression than G carriers. Thus, this gene appears to be more associated with severity of outcome of the disease than with response to treatment.

Both CNR1 rs1049353 and CNR2 rs2501431 are synonymous in not altering amino acid residues. The rs1049353 has an A to G change at the third position of codon 453 Thr (National Center for Biotechnology Information (NCBI) Protein Database: NP_057167.2) while in rs2501431 there is an A to G change at the third position of codon 155 Gly (NCBI Protein Database: NM_001841.2). Although synonymous SNPs have often been described as silent or unable to affect functional changes, recent reports indicate that there are several mechanisms by which synonymous mutations could bring about such changes. These studies pointed out the value of analyzing a priori silent polymorphism suggesting that altered translation kinetics of a defined mRNA due to synonymous codon substitutions might drive the in vivo folding of the same polypeptide chain into different conformations (Komar, 2007; Sauna et al., 2007). These may have important implications in biology and in the diagnosis and treatment of human diseases. Alternatively, these polymorphisms might not constitute the actual causative variant, but rather reflect association of other polymorphisms in linkage disequilibrium with this locus.

The FAAH-rs324420 predicts a substitution of threonine for highly-conserved proline residue (129 P/T). Expression studies have shown that individuals carrying this polymorphism may have approximately half of the enzymatic activity of FAAH (Chiang et al., 2004). This reduction in the activity of FAAH might increase levels of the endogenous cannabinoids AEA and 2-AG, thereby increasing the activity of the endocannabinoid system. Animal models show that the inhibition of the FAAH enzyme has antidepressant effects (Gobbi et al., 2005) but there are no studies relating the FAAH gene to response and remission to antidepressant treatment. Moreover, a recent case-control study in the Caucasian population did not find any significant difference between the genotype and allele frequencies of this polymorphism between patients with major depression and healthy controls (Monteleone et al., 2010). Following this line of investigation, our results do not suggest that this polymorphism has a role in the response and remission with citalopram treatment.

Our study has some limitations. Firstly, the relatively small size of our pharmacogenetic sample limits the power to detect small differences. However, we have enough power to detect small–medium size effects. Moreover, we investigated only one SNP of each gene (CNR1, CNR2 and FAAH) and the possible functional effects of the markers are still under investigation. We consider that multiple testing corrections are likely to be excessively exclusive in the context of the present study since the selection of the genetic polymorphisms, the sample size and the analyses performed had a directional hypothesis based on previous findings (Cardon and Bell, 2001). However, it should be taken into account that when we consider correction for multiple testing based on the false discovery rate (Benjamini and Hochberg, 1995), most of our significant results do not survive the correction. Secondly, we have not controlled for a possible placebo effect at response time (fourth week). However, the 12-week longitudinal analysis could overcome, in part, this limitation.

In conclusion, our results suggest a role of the endocannabinoid system in antidepressant response. However, further studies will be needed in order to analyze in depth the molecular variability associated with endocannabinoid genes in larger samples. New data could help to improve knowledge about the treatment response to antidepressants and also the etiology of major depression.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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Dr. Bárbara Arias
Anthropology Unit, Department of Animal Biology
Faculty of Biology

Supervisor's report on the contribution of the PhD applicant to the article.

Dr. Bárbara Arias Sampériz, assistant lecturer at the Department of Animal Biology of the Faculty of Biology, University of Barcelona and supervisor of the present doctoral thesis by Marina Mitjans, hereby certifies that the participation of the PhD applicant in the article "An approach from the endocannabinoid (eCB) system to 12-week clinical response to citalopram treatment: the role of the *CNR1*, *CNR2* and *FAAH* genes." included the following tasks:

- Participation in the conception and design of the study.
- Laboratory tasks.
- Statistical analysis and interpretation of data.
- First drafting of the manuscript.
- Critical revision of the article for intellectual content.

Dr. Bárbara Arias

Barcelona, November 10th 2014

Screening genetic variability at the *CNR1* gene in both major depression etiology and clinical response to citalopram treatment.

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ORIGINAL INVESTIGATION

Screening genetic variability at the *CNR1* gene in both major depression etiology and clinical response to citalopram treatment

Marina Mitjans · Alessandro Serretti · Chiara Fabbri · Cristóbal Gastó · Rosa Catalán · Lourdes Fañanás · Bárbara Arias

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Abstract
Rationale The endocannabinoid system has been implicated in the pathogenesis of major depression (MD) as well as in the mediation of antidepressant drug effects.
Objectives To analyze *CNR1* gene variants in MD and clinical response to citalopram (selective serotonin re-uptake inhibitors [SSRI]).
Methods The role of *CNR1* gene (rs806368, rs1049353, rs806371, rs806377 and rs1535255) was investigated in 319 outpatients with MD and 150 healthy individuals. A subsample of 155 depressive patients were treated with citalopram and evaluated for response (fourth week) and remission (12th week) by the 21-item Hamilton Depression Rating Scale (HDRS).
Results We observed a higher frequency of rs806371 G carriers in MD patients with both presence of melancholia (p=0.018) and psychotic symptoms (p=0.007) than in controls. Haplotype frequency distributions between MD sample and controls showed a significant difference for Block 1 (rs806368–rs1049353–rs806371) (p=0.008). This haplotype finding was consistent when we compared controls with MD subsample stratified by melancholia (p=0.0009) and psychotic symptoms (p=0.014). The TT homozygous of the rs806368 and rs806371 presented more risk of no Remission than the C carriers (p=0.008 and 0.012, respectively). Haplotype frequency distributions according to Remission status showed a significant difference for Block 1 (p=0.032). Also, we observed significant effect of time–sex–genotype interaction for the rs806368, showing that the C carrier men presented a better response to antidepressant treatment throughout the follow-up than TT homozygous men and women group (p=0.026).
Conclusions These results suggest an effect of *CNR1* gene in the etiology of MD and clinical response to citalopram.

Keywords Endocannabinoid system · Major depression · Clinical response · Selective serotonin reuptake inhibitors

M. Mitjans · L. Fañanás · B. Arias (✉)
Unitat d'Antropologia, Departament de Biologia Animal, Facultat de Biologia, Universitat de Barcelona/Institut de Biomedicina de la Universitat de Barcelona (IBUB), Av. Diagonal, 643 2on pis, 08028 Barcelona, Spain
e-mail: barbara.arias@ub.edu

M. Mitjans · C. Gastó · R. Catalán · L. Fañanás · B. Arias
Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Madrid, Spain

A. Serretti · C. Fabbri
Institute of Psychiatry, University of Bologna, Bologna, Italy

C. Gastó · R. Catalán
Centre de Salut Mental Esquerre de l'Eixample, Hospital Clínic i Provincial de Barcelona, Villaroel, 170, 08036 Barcelona, Spain

C. Gastó · R. Catalán
Institut d'Investigació Biomedica Agustı Pi i Sunyer (IDIBAPS), Barcelona, Spain

Introduction

Major depression (MD) is a common disease caused by a complex interaction of a large number of genetic and non-genetic factors, each of them with a relatively small

contribution to the disorder (Caspi et al. 2003). Treatment of MD is principally based on selective serotonin re-uptake inhibitors (SSRI) that enhanced serotonergic neurotransmission by blocking the serotonin transporter. However, clinical response to drug treatment in depression is a highly complex biological phenomenon in which several factors are involved, some of them genetic (Rasmussen-Torvik and McAlpine 2007; Klengel and Binder 2011; Uher 2011).

Recently, the endocannabinoid system has been implicated in the pathogenesis of depression and anxiety, the mediation of antidepressant drug effects in animal models and the neurobiology of emotion processing in healthy volunteers (Domschke et al. 2008). Physiological actions of endocannabinoid system in the central nervous system (CNS) are mediated by the activation of a specific cannabinoid receptor, the CB1 receptor (Matsuda et al. 1990). This receptor is coded by the *CNR1* gene located on chromosome 6 (6q14–15). It is considered the most abundant G protein-coupled receptor expressed in the CNS of mammalian brain, being present in the limbic system and in the brain areas related to stress response, such as the central amygdala and the paraventricular nucleus (PVN) of the hypothalamus (Herkenham 1991). In addition, changes in the functional activity of the endocannabinoid system can cause altered activity in other neuromodulatory systems as well as imbalance in the primary GABA/glutamate control system (Rodriguez de Fonseca et al. 2005). Moreover, endocannabinoid system could activate the hypothalamic–pituitary–adrenal (HPA) axis (Weidenfeld et al. 1994), the neuroendocrine system involved in the responses to emotional stress.

Experimental data provide evidence that blocking the endocannabinoid system is a risk factor in the pathogenesis of depression as well as in anxiety disorders (Martin et al. 2002; Hill and Gorzalka 2005a). The administration of CB1 receptor agonist or endogenous cannabinoid re-uptake inhibitors results in antidepressant-like effects and increases efficacy of the antidepressant fluoxetine in experimental animal models (Gobbi et al. 2005; Hill and Gorzalka 2005a; Adamczyk et al. 2008).

In line with that, patients diagnosed with depression are found to have a reduced levels of circulating endocannabinoids (Hill et al. 2009). Moreover, a decreased in CB1 receptor density in grey matter glial cells was found in the post mortem brains of patients with MD (Koethe et al. 2007). Furthermore, an up-regulation of CB1 receptors was observed in the prefrontal cortex of subjects with MD who died by suicide (Hungund et al. 2004).

The involvement of CB1 receptors in regulating mood is further supported by evidences showing that the CB1 receptor antagonist, rimonabant, administered to humans for weight loss and obesity-related metabolic disorders has been shown to increase the risk of depressed mood disorder and anxiety along the treatment even though when the presence of depressed mood was an exclusion criteria in the study (Christensen et al. 2007). Moreover, a genetic study described the association between the polymorphism rs1049353 at the *CNR1* gene and major depressive individuals when comparing with healthy controls showing an odds ratio (OR) of 2.46 for the contribution of the A allele to the probability of having MD (Monteleone et al. 2010).

Recent studies show the link between endocannabinoid system and antidepressant treatment. It has recently been suggested that the expression of CB1 receptor in the hippocampus and the hypothalamus is up regulated by chronic tricyclic antidepressant treatment (Hill et al. 2006). Furthermore, Domschke and colleagues (Domschke et al. 2008) found that individuals with G allele at rs1049353 had increased risk for antidepressant treatment resistance, particularly in females with comorbid anxiety. In contrast, we described that rs1049353 GG men presented better response along the follow-up than A carrier men or the women group (Mitjans et al. 2012).

According to these previous results, which seem to indicate a possible role of *CNR1* gene (rs1049353 polymorphism) in both MD (Monteleone et al. 2010) and pharmacogenetics (Domschke et al. 2008; Mitjans et al. 2012), the aims of this study are therefore to investigate the role of several genetic variability at the *CNR1* gene (rs806368, rs1049353, rs806371, rs806377 and rs1535255) as a risk factor for (a) MD and severity clinical features associated with the disease (b) response to citalopram (CIT) treatment.

Materials and methods

Total sample

The MD sample consisted of 319 depressive outpatients (227 females and 92 males; mean age 46.38 years, SD= 15.08 age of onset 38.29 years, SD=14.92) from the Centre de Salut Mental of the Hospital Clinic de Barcelona. All patients suffered an active episode of MD diagnosed following the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) at the time of inclusion in the study. All cases were diagnosed using the Spanish version of the Structures Clinical Interview (SCID-I) (Spitzer et al. 1990). Detailed data about severity clinical features such as presence of melancholic features (n=151 (50.3 %)), psychotic symptoms (n=75 (25.1 %)) or previous suicide attempts (n=55 (18.6 %)) were also collected (Arias et al. 2009). No patients with bipolar I or II disorder were included in this sample. Patients with drug abuse and dependence, mental retardation or with a medical disease that impairs evaluation have been excluded from the study.

A control sample consisting of 150 healthy individuals (71 females and 79 males; mean age 42.1 years, SD=10.3) with

no personal history of mental illness was included in the study. The Spanish version of the 28-item General Health Questionnaire (Goldberg and Hillier 1979) was used to assess their current mental condition.

All the individuals included in the study were of Spanish origin as stated through the birthplace of their four grandparents, thereby reducing the possibility of confounding genetic differences by population stratification (Freedman et al. 2004).

Ethnical approval was obtained from Spanish local research ethic committees. All patients and controls provided a complete written informed consent before inclusion in the study. All procedures were carried out according to the Declaration of Helsinki.

Pharmacogenetic subsample

A subsample of 155 patients out of the total depressive sample (120 females and 35 males) was followed for a pharmacogenetic study. All patients were treated with CIT and followed along 12 weeks by experienced psychiatrists. Patients were initially evaluated for the severity of their symptoms using 21-item Hamilton Depression Rating Scale (HDRS) (mean initial HDRS 24.72, SD=4.74). A new HDRS was assessed to all patients every 4 weeks until completion of the follow-up at week 12. Clinical response to CIT treatment was considered when a decreased of at least 50 % in the baseline HDRS score was observed at the fourth week (Baumann et al. 1996). Remission for the index episode was considered when HDRS scores were equal or under 7 by the end of 12th week (Frank et al. 1991). Plasma levels of CIT were determined at sixth week using high-performance liquid chromatography (Olesen and Linnet 1996).

All patients were treated with CIT at standard therapeutic doses (mean initial dose 26.39 mg/day; range 20–40 mg/day). Before their inclusion in the study, a 2-week wash-out was carried out with those patients who were being treated with different drugs. In case it was necessary, low dose concomitant treatments with drug such as neuroleptics (10 % of the sample) or benzodiazepines at bedtime (55.4 % of the sample) were allowed. The presence and intensity of side effects was assessed by using the UKU scale (Lingjaerde et al. 1987) at the end of the fourth week of pharmacological treatment.

Ethnical approval was obtained from local research ethic committees. Patients provided written informed consent before inclusion in the study.

Genetic analysis

A total of five single nucleotide polymorphisms (SNPs) located at the *CNR1* gene were selected according to previous literature: rs806368 (T/C), rs1049353 (G/A), rs806371 (T/G), rs806377 (T/C) and rs1535255 (T/G). Genomic DNA was extracted from blood samples using a standard phenol–chloroform extraction protocol. All the polymorphisms were successfully assayed using Sequenom MassArray technology (Tang et al. 1999).

Statistical analysis

The Hardy–Weinberg equilibrium for genotype frequencies in all samples was calculated using chi-square tests with EpiInfo v.3.5.1 (Dean et al. 1991).

Simple chi-square tests of independence were performed to confirm the presence or absence of allele or genotype associations. OR with 95 % confidence intervals (CI) were estimated for the effects of high-risk genotypes. The combined case–control study (MD vs. Controls) had an 80 % power (95 % CI) to detect OR equal or greater than 2.21 for disease according to the minimum allele frequencies of the different polymorphisms analyzed in our sample. In reference to the pharmacogenetic sample, the minimum detectable OR for no-response or no-remission will be equal or greater than 3.1 or 2.99, respectively (Cohen 1988). Bonferroni correction was conservatively applied for multiple analyses in single polymorphism analyses (p=0.01 (=0.05/5 variations)).

Haploview 3.2 (Barret et al. 2005; Barrett et al. 2005) was used to generate a linkage disequilibrium map and to test for Hardy–Weinberg equilibrium in the haplotype analysis. The ‘R’ software (http://www.r-project.org) was used to calculate haplotype frequencies and to include covariates (see “Pharmacogenetic study” section) in the analysis for quantitative traits by the “haplo.stat” package (Schaid et al. 2002). Rare haplotypes less frequent than 1 % were excluded from the analyses. The global significance of the results for haplotype analyses was estimated using permutation (50,000 permutations) to confirm the asymptotic p values.

In the pharmacogenetic subsample the genetic variant effects on HDRS change scores over 12 weeks of CIT treatment was performed using analysis of variance (ANOVA) with repeated measures (genotype and gender as fixed factor, time point as a repeated measure and a number of covariates; see “Pharmacogenetic study” section). These analyses were processed using SPSS 17.00 (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Because the SNP rs1049353 (G/A) was analyzed in our previous study (Mitjans et al. 2012) according to response and remission status in the subsample of major depressive patients treated with CIT (n=155), this polymorphism was only considered in the analyses that include the total sample of MD and controls. It has been also included when haplotype analyses were performed in all case–control design analyses.

Results

Total sample (major depression and control samples)

Genotype distribution of all SNPs was found to be in Hardy–Weinberg equilibrium in the control sample (rs806368 χ^2=0.35, *df*=2, *p*=0.839; rs1049353 χ^2<0.01, *df*=2, *p*=1; rs806371 χ^2=0.4, *df*=2, *p*=0.819; rs806377 χ^2=0.88, *df*=2, *p*=0.644; rs1535255 χ^2=0.1, *df*=2, *p*=0.95) as well as in the MD sample (rs806368 χ^2=0.85, *df*=2, *p*=0.652; rs1049353 χ^2=0.19, *df*=2, *p*=0.91; rs806371 χ^2=0.08, *df*=2, *p*=0.959; rs806377 χ^2=2.08, *df*=2, *p*=0.354; rs1535255 χ^2<0.01, *df*=2, *p*=1). Genotypic and allelic frequencies in patients and controls are shown in Table 1.

Allele and genotype frequencies for the five SNPs analyzed did not significantly differ between MD patients and the control sample (see Table 1 for details).

Also, we did not find any significant difference when we compared the allele and genotype distribution according to clinical features (presence of melancholic features, psychotic symptoms and suicide attempts) in the major depressive sample (data not shown).

However, genotype and allele frequencies of the rs806371 significantly differed between the control sample and those patients that present MD with melancholia (*n*=151) (genotype: χ^2=6.42, *df*=2, *p*=0.04; allele: χ^2=5.97, *df*=1, *p*=0.014). We observed a higher frequency of G carriers in patients with presence of melancholia than in healthy subjects (χ^2=5.59, *df*=1, *p*=0.018; OR=1.83 95 % CI [1.07–3.15]). After multiple correction adjustment these results were no longer significant. Similar results were found when we compare genotype and allele frequencies between the control sample and depressive patients with psychotic symptoms (*n*=75) (genotype: χ^2=8.56, *df*=2, *p*=0.01; allele: χ^2=7.89, *df*=1, *p*=0.004), showing G carriers presented increased risk of 2.22 for suffering MD with psychotic symptoms compared to healthy subjects (χ^2=6.96, *df*=1, *p*=0.008; OR=2.22 95 % CI [1.17–4.22]).

Haplotype analysis has shown the existence of linkage disequilibrium among rs806368–rs1049353–rs806371 (Block 1: *D′*=0.907, r^2=0.557) and rs806377–rs1535255 (Block 2: *D′*=0.938, r^2=0.173) in the MD sample. As we detected the same results for the control sample (Block 1:

Table 1 Genotype and allele distribution of the analyzed polymorphisms of *CNR1* gene for MD patients and the MD samples stratified according to clinical features (melancholia, psychotic symptoms and suicide attempts) vs. control group

Polymorphism			C (%)	MD (%)	*p*-value	MD-Mel (%)	*p*-value	MD-Psy (%)	*p*-value	MD-Suic (%)	*p*-value
rs806368	Genotypes	TT	83 (56.1)	179 (57.4)	0.214	83 (56.4)	0.482	36 (48.6)	0.447	135 (57.7)	0.13
		TC	53 (35.8)	120 (38.4)		57 (38.8)		33 (44.6)		91 (38.9)	
		CC	12 (8.1)	13 (4.2)		7 (4.8)		5 (6.8)		8 (3.4)	
	Alleles	T	219 (74)	478 (76.6)	0.387	223 (75.9)	0.601	105 (70.9)	0.496	361 (77.1)	0.321
		C	77 (26)	146 (23.4)		71 (24.1)		43 (29.1)		107 (22.9)	
rs1049353	Genotypes	GG	84(56.4)	182 (58.1)	0.905	81 (55.1)	0.954	41 (55.4)	0.975	132 (56.4)	0.988
		GA	56 (37.6)	111 (35.5)		56 (38.1)		28 (37.8)		87 (37.2)	
		AA	9 (6)	20 (6.4)		10 (6.8)		5 (6.8)		15 (6.4)	
	Alleles	G	224 (75.2)	475 (75.9)	0.814	218 (74.1)	0.775	110 (74.3)	0.846	351 (75)	0.958
		A	74 (24.8)	151 (24.1)		76 (25.9)		38 (25.7)		117 (25)	
rs806371	Genotypes	TT	114 (76.5)	214 (68.4)	0.120	94 (64)	0.04	44 (59.4)	0.01*	164 (70.1)	0.319
		TG	34 (22.8)	91 (29.1)		49 (33.3)		27 (36.5)		66 (28.2)	
		GG	1 (0.7)	8 (2.5)		4 (2.7)		3 (4.1)		4 (1.7)	
	Alleles	T	262 (88)	519 (82.9)	0.05	237 (80.6)	0.014	115 (77.7)	0.004*	394 (84.2)	0.151
		G	36 (12)	107 (17.1)		57 (19.4)		33 (22.3)		74 (15.8)	
rs806377	Genotypes	TT	50 (33.6)	89 (28.4)	0.421	40 (27.2)	0.415	19 (25.7)	0.487	66 (28.2)	0.421
		TC	65 (43.6)	138 (44.1)		66 (44.9)		36 (48.6)		103 (44)	
		CC	34 (22.8)	86 (27.5)		41 (27.9)		19 (25.7)		65 (27.8)	
	Alleles	T	165 (55.4)	316 (50.5)	0.164	146 (49.7)	0.164	74 (50)	0.284	235 (50.2)	0.163
		C	133 (44.6)	310 (49.5)		148 (50.3)		74 (50)		233 (49.8)	
rs1535255	Genotypes	TT	97 (65.1)	208 (66.5)	0.946	94 (63.9)	0.827	48 (64.9)	0.965	154 (65.8)	0.946
		TG	47 (31.5)	94 (30)		46 (31.3)		23 (31.1)		71 (30.3)	
		GG	5 (3.4)	11 (3.5)		7 (4.8)		3 (4)		9 (3.9)	
	Alleles	T	241 (81)	510 (81.5)	0.827	234 (79.6)	0.695	119 (80.4)	0.906	379 (81)	0.969
		G	57 (19)	116 (18.5)		60 (20.4)		29 (19.6)		89 (19)	

C controls, *MD-Mel* major depression with melancholia, *MD-Psy* major depression with psychotic symptoms, *MD-Suic* major depression with suicide attempts

*Significant *p*-values after Bonferroni correction: *p*=0.01; (see text to comparisons of allele carriers)

D'=0.957; r^2=0.36; Block 2: D'=1.0, r^2=0.191), Fig. 1 shows linkage disequilibrium for the whole sample (MD+ controls).

Comparisons of the haplotype frequency distributions between MD and control samples showed a significant difference for Block 1 (Global-stat=13.76, *df*=4, simulated p=0.0078), showing a lower haplotype frequency for the C–G–T haplotype (rs806368–rs1049353–rs806371) in the MD sample (7.5 %) than in the control sample (14.3 %) (simulated p=0.001) (see Table 2 for haplotype frequencies details).

We did not find any significant difference when we compared the haplotype frequency distributions according to clinical features (presence of melancholic features, psychotic symptoms and suicide attempts) in the major depressive sample either for Block 1 or Block 2 (data not shown).

When patients were stratified according to clinical features and compared to the control subjects, we detect a significant association between the haplotype Block 1 and melancholia (Global-stat=17.537, *df*=4, simulated p=0.0009) and psychotic symptoms (Global-stat=12.003, *df*=4, simulated p=0.014) (see Table 2 for haplotype frequencies details).

Pharmacogenetic study

In the pharmacogenetic subsample, 95 patients (64.6 %) were considered Responders (Rp) and 52 (35.4 %) were classified as no-Responders (No-Rp) according to the decrease of their HDRS scores at fourth week. Considering the remission criteria at 12th week, 96 patients (65.3 %) were classified as Remitters (Rm) and 51 (34.7 %) as no-Remitters (No-Rm).

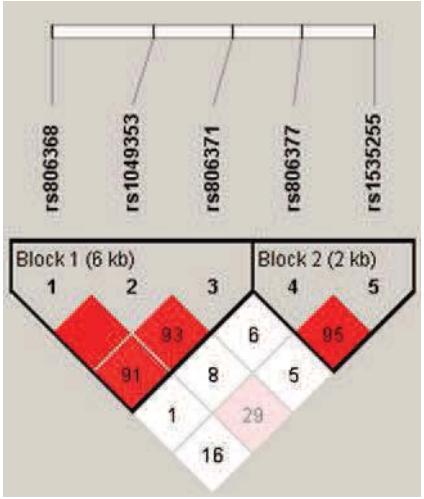


Fig. 1 Linkage disequilibrium among markers in the whole Spanish sample (Block 1: D'=0.918, r^2=0.485; Block 2: D'=0.95, r^2=0.18)

Concerning response at the fourth week, we did not observe any significant difference in genotype or allele distribution of any polymorphism between Rp/no-Rp (Table 3). However, we observed significant differences when we consider Remission status. There was significant differences for the rs806368 allele and genotype distribution according to Remission [genotype: χ^2=7.07, *df*=2, p=0.029; allele: χ^2=5.27, *df*=1, p=0.021], however these results did not survive multiple correction. Carriers analyses showed that TT homozygous presented almost 2.7 times more risk of no-remission than the C carriers (χ^2=6.94, *df*=1, p=0.008; OR=2.64 95 % CI [1.20–5.89]). Furthermore, we observed the same significant difference for rs806371 (genotype: χ^2=6.18, *df*=2, p=0.045; allele: χ^2=5.74, *df*=1, p=0.016). The TT homozygous of the rs806371 presented 2.8 times more risk of no-remission than the G carriers (χ^2=6.18, *df*=1, p=0.012; OR=2.8 95 % CI [1.14–7.01]).

We considered the presence of suicide attempts and CIT levels at the sixth week as covariates in the response and remission haplotype analyses and in the longitudinal study because of their implication in response treatment. In a previous study with the same sample, the presence of suicide attempts was associated with no response at fourth week (χ^2=3.75, *df*=1, p=0.046) and no remission at 12th week (χ^2=10.204, *df*=1, p=0.002). Also, lower CIT levels at sixth week were associated with no response at fourth week (F=4.72, *df*=1, p=0.032) (Mitjans et al. 2012).

Haplotype analyses for the two analyzed blocks did not yield a significant association with response at fourth week (Table 4). However, haplotype analysis showed a significant association between the haplotype Block 1 and remission (Global-stat=10.503, *df*=4, simulated p=0.029) (see Table 4 for haplotype frequencies details).

We performed a longitudinal study through a two-way repeated-measures ANOVA on HDRS scores to evaluate the effect of different polymorphisms on the 12-week clinical outcome of the patients treated with CIT. The longitudinal study showed no effects of the rs806371, rs806377 and rs1535255 polymorphisms on the 12-week clinical outcome. We found a significant effect of rs1049353 and rs806368. The rs1049353 effect had been reported in our previous study showing that individuals with GG genotype presented better response along the follow-up than A carriers (Mitjans et al. 2012). The longitudinal study of rs806368 showed that there was a significant decrease of HDRS scores over 12 weeks ($F_{(2.76,\ 284.98)}$=138.539, p< 0.001), a significant effects of time–sex interaction ($F_{(2.76,\ 284.98)}$=6.85, p<0.001), time–genotype interaction ($F_{(2.76,\ 284.98)}$=4.987, p=0.003) and a time–sex–genotype interaction ($F_{(2.76,\ 284.98)}$=3.233, p=0.026). So, we observed significant effect of time–genotype interaction, showing that the C carriers presented a better response to antidepressant treatment throughout the follow up than TT homozygous.

Table 2 CNR1 markers haplotype distributions in the control and the MD samples

Haplotype	Haplotype frequencies (hf)	Control:MD (hf)	*p* value	Sim *p* value	Global score statistics
Block 1					
TGT	0.507	0.487:0.516	0.430	0.428	Global-stat=13.76, *df*=4
CGG	0.145	0.117:0.158	0.08	0.08	*p*=0.008
TAT	0.241	0.248:0.238	0.746	0.757	Global sim *p*=0.0078*
CGT	0.097	0.143:0.075	0.001	0.001*	
Block 2					
TT	0.476	0.446:0.490	0.216	0.215	Global-stat=3.039, *df*=3
CT	0.351	0.362:0.346	0.595	0.592	*p*=0.3855
CG	0.169	0.191:0.158	0.215	0.215	Global sim *p*=0.378
		Controls:MD melancholia (hf)			
Block 1					
TGT	0.490	0.487:0.492	0.925	0.932	Global-stat=17.537, *df*=4
CGG	0.151	0.117:0.186	0.014	0.016*	*p*=0.0015
TAT	0.253	0.248:0.258	0.776	0.753	Global sim *p*=0.0009*
CGT	0.099	0.143:0.055	<0.001	<0.001*	
Block 2					
TT	0.472	0.446:0.490	0.214	0.235	Global-stat=2.64, *df*=3
CT	0.346	0.362:0.329	0.383	0.38	*p*=0.449
CG	0.179	0.191:0.167	0.454	0.464	Global sim *p*=0.446
		Controls:MD psychotic (hf)			
Block 1					
TGT	0.474	0.487:0.445	0.38	0.374	Global-stat=12.003, *df*=4
CGG	0.149	0.117:0.215	0.005	0.005*	*p*=0.017
TAT	0.251	0.248:0.257	0.846	0.867	Global sim *p*=0.014*
CGT	0.120	0.143:0.075	0.033	0.038*	
Block 2					
TT	0.461	0.446:0.500	0.305	0.317	Global-stat=1.075, *df*=3
CT	0.351	0.362:0.331	0.514	0.514	*p*=0.5842
CG	0.184	0.191:0.169	0.551	0.564	Global sim *p*=0.588
		Controls:MD suicide (hf)			
Block 1					
TGT	0.503	0.487:0.512	0.503	0.512	Global-stat=9.153, *df*=4
CGG	0.135	0.117:0.146	0.219	0.258	*p*=0.057
TAT	0.247	0.248:0.246	0.968	0.95	Global sim *p*=0.054
CGT	0.105	0.143:0.082	0.007	0.007*	
Block 2					
TT	0.474	0.446:0.491	0.225	0.219	Global-stat=3.086, *df*=3
CT	0.349	0.362:0.342	0.522	0.528	*p*=0.378
CG	0.172	0.191:0.160	0.284	0.286	Global sim *p*=0.404

Haplotype distributions in the control and the MD samples stratified according to clinical features (melancholia, psychotic symptoms and suicide attempts) are also shown

sim simulated

*Significant *p* values after permutation procedures.

Stratification for gender revealed that this effect is originated by the subgroup of male patients (Fig. 2).

Discussion

We have conducted an association study in which we have analyzed the genetic variability at *CNR1* gene as a genetic risk factor for MD as well as for no response to clinical treatment with SSRIs.

When analysing the SNPs variability at the *CNR1* gene (rs806368, rs1049353, rs806371, rs806377 and rs1535255), the results of the case–control association study did not show any genetic influence of this variability on the overall risk to suffer MD. However, the haplotype analysis showed that Block 1 C–G–T combination (rs806368–rs1049353–

Table 3 Genotype and allele distribution of the analyzed polymorphisms of *CNR1* gene in response (fourth week) and remission (12th week) status

Polymorphism			Rp (%)	N-Rp (%)	*p*-value	Rm (%)	N-Rm (%)	*p*-value
rs806368	Genotypes	TT	56 (59)	29 (55.8)	0.881	48 (50)	37 (72.6)	0.029
		TC	35 (36.8)	20 (38.4)		43 (44.8)	12 (23.5)	
		CC	4 (4.2)	3 (5.8)		5 (5.2)	2 (3.9)	
	Alleles	T	147 (77.4)	78 (75)	0.646	139 (72.4)	86 (84.3)	0.021
		C	43 (22.6)	26 (25)		53 (27.6)	16 (15.7)	
rs1049353	Genotypes	GG	52 (54.7)	29 (55.8)	0.979	55 (57.3)	26 (51)	0.765
		GA	38 (40)	20 (38.4)		36 (37.5)	22 (43.1)	
		AA	5 (5.3)	3 (5.8)		5 (5.2)	3 (5.9)	
	Alleles	G	142 (74.7)	78 (75)	0.96	146 (76)	74 (72.5)	0.511
		A	48 (25.3)	26 (25)		46 (24)	28 (27.5)	
rs806371	Genotypes	TT	66 (69.5)	36 (69.2)	0.742	60 (62.5)	42 (82.3)	0.045
		TG	25 (26.3)	15 (28.9)		32 (33.3)	8 (15.7)	
		GG	4 (4.2)	1(1.9)		4 (4.2)	1 (2)	
	Alleles	T	157 (82.6)	87 (83.6)	0.823	152 (79.2)	92 (90.2)	0.016
		G	33 (17.4)	17 (16.4)		40 (20.8)	10 (9.8)	
rs806377	Genotypes	TT	35 (36.8)	15 (28.8)	0.140	28 (29.2)	22 (43.1)	0.188
		TC	30 (31.6)	25 (48.1)		37 (38.5)	18 (35.3)	
		CC	30 (31.6)	12 (23.1)		31 (32.3)	11 (21.6)	
	Alleles	T	100 (52.6)	55 (52.9)	0.966	93 (48.4)	62 (60.8)	0.043
		C	90 (47.4)	49 (47.1)		99 (51.6)	40 (39.2)	
rs1535255	Genotypes	TT	60 (63.1)	34 (65.4)	0.373	59 (62.1)	35 (67.3)	0.795
		TG	28 (29.5)	17 (32.7)		30 (31.6)	15 (28.8)	
		GG	7 (7.4)	1 (1.9)		6 (6.3)	2 (3.9)	
	Alleles	T	148 (77.9)	85 (81.7)	0.438	148 (77.9)	85 (81.7)	0.438
		G	42 (22.1)	19 (18.3)		42 (22.1)	19 (18.3)	

Rp responders, *N-Rp* no responders, *Rm* remitters, *N-Rm* no remitters (see text to comparisons of allele carriers)

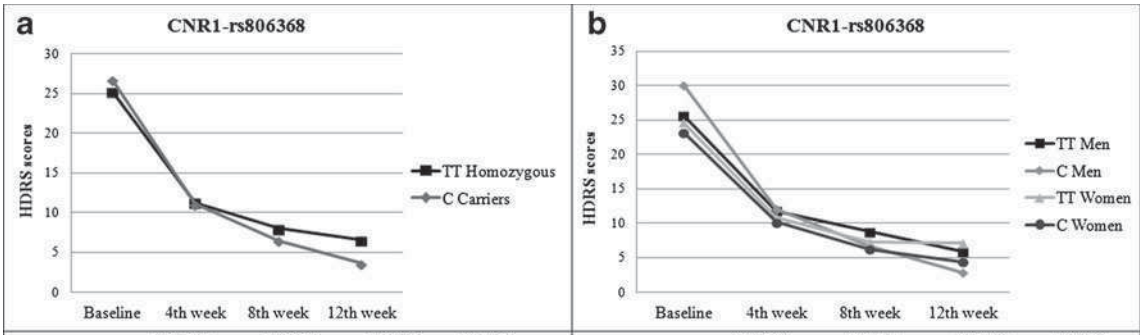
rs806371) is associated with an increased risk for MD. These results are in line with a previous study by Monteleone and colleagues (2010) that associated the *CNR1* gene with depression.

Table 4 CNR1 markers haplotype distributions in response (fourth week) and remission (12th week) status

Haplotype	Haplotype frequencies (hf)	Rp:N-Rp (hf)	*p* value	Sim *p* value	Global score statistics
Block 1					
TGT	0.508	0.513:0.5	0.842	0.846	Global-stat=0.451, *df*=4
CGG	0.155	0.157:0.153	0.963	0.962	*p*=0.978
TAT	0.242	0.243:0.239	0.853	0.854	Global sim *p*=0.975
CGT	0.079	0.069:0.096	0.526	0.533	
Block 2					
TT	0.473	0.473:0.471	0.721	0.727	Global-stat=3.49, *df*=2
CT	0.319	0.305:0.346	0.108	0.114	*p*=0.173
CG	0.207	0.221:0.182	0.19	0.197	Global sim *p*=0.185
		Rm:N-Rm (hf)			
Block 1					
TGT	0.529	0.477:0.567	0.091	0.096	Global-stat=10.5, *df*=4
CGG	0.118	0.193:0.086	0.007	0.006*	*p*=0.032
TAT	0.238	0.226:0.268	0.24	0.245	Global sim *p*=0.029*
CGT	0.097	0.085:0.067	0.153	0.146	
Block 2					
TT	0.472	0.510:0.403	0.082	0.086	Global-stat=3.21, *df*=2
CT	0.319	0.268:0.413	0.133	0.142	*p*=0.20
CG	0.207	0.221:0.182	0.552	0.542	Global sim *p*=0.21

Rp responders, *N-Rp* no responders, *Rm* remitters, *N-Rm* no remitters, *sim* simulated

*Significant *p* values after permutation procedures



	HDRS0 Mean (SD)	HDRS1 Mean (SD)	HDRS2 Mean (SD)	HDRS3 Mean (SD)
TT Homozygous (N=63)	25.21 (0.77)	11.25 (0.88)	7.98 (0.84)	6.49 (0.83)
C Carriers (N=45)	26.72 (0.71)	11.09 (0.81)	6.45 (0.77)	3.62 (0.77)

HDRS0, $F_{(3,105)}$=2.11, P=0.103; HDRS1, $F_{(3,103)}$=2.21, P=0.091; HDRS2, $F_{(3,104)}$=1.82, P=0.148; HDRS3, $F_{(3,104)}$=7.67, P=0.002

	HDRS0 Mean (SD)	HDRS1 Mean (SD)	HDRS2 Mean (SD)	HDRS3 Mean (SD)
TT Men (N=10)	25.71 (1.43)	11.73 (1.63)	8.74 (1.56)	5.87 (1.55)
C Men (N=14)	30.18 (1.18)	12.04 (1.35)	6.69 (1.29)	2.83 (1.29)
TT Women (N=53)	24.7 (0.61)	10.77 (0.69)	7.22 (0.66)	7.11 (0.66)
C Women (N=31)	23.26 (0.79)	10.13 (0.91)	6.2 (0.87)	4.40 (0.86)

HDRS0, $F_{(5,103)}$=6.25, P<0.01; HDRS1, $F_{(5,103)}$=1.63, P=0.158; HDRS2, $F_{(5,102)}$=1.26, P=0.286; HDRS3, $F_{(5,102)}$=4.92, P<0.01

Fig. 2 Genotype distribution of the CNR1-rs806368 polymorphism according to the different follow-ups based on CIT treatment. **a** rs806368-C allele carriers presented a better response to antidepressant treatment compared to rs806368-TT homozygous ($F_{(2.76,\ 284.98)}$= 4.987, p=0.003). **b** This effect is originated by the subgroup of male patients that showed a better outcome in response to CIT treatment ($F_{(2.76,\ 284.98)}$=3.233, p=0.026). Tables show the Hamilton scores along the follow-up in relation to genotypes

Moreover, we grouped major depressive patients according to clinical features of severity such as the presence of melancholia, psychotic symptoms or suicide attempts and compared them to the control sample. Our results showed that patients with presence of melancholia or psychotic symptoms presented a higher frequency of rs806371 G carriers than healthy controls. Furthermore, the haplotype analysis showed an association between the haplotype Block 1 and melancholia and psychotic symptoms.

These results highlight the clinical and biological heterogeneity underlying the categorical diagnoses of MD which can easily overcome the power of genetic association studies (Winokur 1997). Categorical diagnostic tools are based on clusters of symptoms and characteristics of clinical course that maybe are not defining the different pathophysiological processes occurring in the disease. The definition of genetically relevant phenotypes in MD could help to increase the success of genetic studies (Hasler et al. 2004). Our results are in line with those defending that a stricter phenotype redefinition could increase power to detect more robust genetic effects (van der Sluis et al. 2010).

MD with melancholia has been identified as a valid subtype of MD that identifies a subset of more severe depressive patients with a particularly high genetic background (Kendler 1997). It has been shown that the genetic or pharmacological blockade of endocannabinoid system in animal models provoked similar symptomatology than melancholic depression (Hill and Gorzalka 2005b). One of the most reliable biological markers of melancholic depression is alterations in the HPA axis. Recent evidences show the role of the endocannabinoid system in regulation of the HPA axis activity (Di et al. 2003; Barna et al. 2004; Patel et al. 2004). Recent studies have shown that CB1 knockout mice present hypersecretion of corticotropin-releasing hormone (CRH) in the PVN (Cota et al. 2003), as well as elevated basal adrenocorticotropin (ACTH) and corticosterone (Barna et al. 2004). Consistent with the findings, glucocorticoid receptors (GR) antagonists have been found to be effective in very severe forms of depression, such as psychotic or endogenous forms of depression (Belanoff et al. 2001; Reus and Wolkowitz 2001). Our findings are in line with these evidences suggesting that severe forms of depression may have specific biological processes.

When we analyzed genetic variability in relation to clinical response or remission in the pharmacogenetic subsample, we did not observe any effect of the single different polymorphisms analyzed in response at fourth week to CIT treatment. However, we found significant effects of rs806368 and rs806371 polymorphisms on remission at 12th week. The TT homozygous of the rs806368 presented more risk of no Remission than the C carriers and TT homozygous of the rs806371 also presented more risk of no Remission than the G carriers. Although previous studies have shown the involvement of the rs806368 in substance use disorder or cannabis dependence (Zuo et al. 2007; Agrawal et al. 2009), no association study considering its role in clinical response has been published.

The results of our longitudinal study showed an influence of the rs806368 polymorphism on the response to treatment. G carrier men presented better response along the follow-up than TT homozygous men or the women group. Specifically,

G carrier men had presented the highest HDRS scores at baseline and the lowest scores at the end of the study being the group with the greatest reduction in HDRS scores. According to that, haplotype analysis showed linkage disequilibrium between rs806368 and rs1049353 polymorphisms in our samples. It has recently reported that rs1049353 has an effect in antidepressant treatment response in MD (Domschke et al. 2008; Mitjans et al. 2012). Domschke and colleagues (2008) reported that the G allele of the rs1049353 confers an increased risk of resistance to antidepressant treatment, particularly in female patients with MD and high comorbid anxiety. In contrast, in a recent work, we described that men carrying the GG genotype presented better response along the follow-up than A carrier men or the women group (Mitjans et al. 2012). Although both studies show the involvement of this polymorphism in clinical response to antidepressant treatment, the results according to sex showed opposite directions.

Differential response mediated by gender remains still controversial (Serretti et al. 2008; Vermeiden et al. 2010; Carter et al. 2012). It might be hypothesized that gender differences in the response could be also reflecting the differences that are found in the aetiology of MD as physiological and epidemiological studies have shown (Biver et al. 1996; Weissman et al. 1996; Nishizawa et al. 1997; Kendler et al. 2001; Legato 2010; Lai 2011). Studies suggesting a role of estradiol in expression regulation of CB1 receptor mRNA (Gonzalez et al. 2000; Hill et al. 2007) could explain the differential response by gender found in this study. However, more research is still needed to better understand the gender specific contribution in antidepressant response. Pharmacogenetics could help to elucidate the role of CNS neurotransmission systems, such as the endocannabinoid system, in response to antidepressants. However, genetics will provide information for just a part of the complex puzzle of clinical response to psychodrugs. Other factors such environmental or clinical will be also necessary in order to understand the total phenotype. Nowadays, a test with widespread clinical use and adoption is still missing (Arranz and Kapur 2008).

All the analyzed polymorphisms are synonymous then not altering amino acid residues. Although synonymous SNPs have often been called silent or unable to affect functional changes, recent reports indicate that there are several mechanisms by which synonymous mutations could bring about such changes (Komar 2007; Sauna et al. 2007). These may have important implications in biology and in the diagnosis and treatment of human diseases. Alternatively, these polymorphisms might not constitute the actual causative variant, but rather reflect association of other polymorphisms in linkage disequilibrium with this locus.

Our study has several limitations. The relatively small size of our pharmacogenetic sample limits the power to detect small differences. However, this study has enough power to detect small–medium effect sizes. Moreover, the possible functional effects of the analyzed markers are still under investigation. We consider that multiple testing corrections are likely to be excessively exclusive in the context of the present study since the selection of the genetic polymorphisms, the sample size and the analyses performed had a directional hypothesis based on previous findings (Cardon and Bell 2001). However, as multiple testing based on Bonferroni's procedures were taking into account; part of our results referred to the single SNP analyses (rs806368 and rs806371) did not survive the correction. Subsequent statistical analyses such as the genotype carrier's analyses or the haplotype analyses, demonstrate the involvement of these polymorphisms in the risk for MD or response to antidepressant treatment.

In summary, *CNR1* gene variants seem to be associated with the etiology of MD and specifically with the severity of MD showing that maybe a redefinition of the phenotype could help to a better understanding of the disease. Additionally, CB1 receptor gene seems to have an indirect effect on clinical response to CIT (SSRIs) basically in remission at the 12th week and along the follow-up.

Further studies focusing on other genes involved in the endocannabinoid system or other systems related to endocannabinoid system could help to elucidate the complex mechanism of aetiology of MD and clinical response to antidepressants.

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Dr. Bárbara Arias
Anthropology Unit, Department of Animal Biology
Faculty of Biology

Supervisor's report on the contribution of the PhD applicant to the article.

Dr. Bárbara Arias Sampériz, assistant lecturer at the Department of Animal Biology of the Faculty of Biology, University of Barcelona and supervisor of the present doctoral thesis by Marina Mitjans, hereby certifies that the participation of the PhD applicant in the article "Screening genetic variability at the CNR1 gene in both major depression etiology and clinical response to citalopram treatment." included the following tasks:

- Participation in the conception and design of the study
- Laboratory tasks
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Dr. Bárbara Arias

Barcelona, November 10th 2014

Exploring genetic variability at PI, GSK3, HPA and glutamatergic pathways in lithium response: association with *IMPA2*, *INPP1* and *GSK3β* genes.

Mitjans M*, Arias B*, Jimenez E, Goikolea JM, Saiz PA, García-Portilla MP, Burón P, Bobes J, Vieta E, Benabarre A. *Co-First Authors

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Exploring genetic variability at PI, GSK3, HPA and glutamatergic pathways in lithium response: association with *IMPA2*, *INPP1* and *GSK3B* genes

Mitjans M*[1], Arias B*[1], Jimenez E[2], Goikolea JM[2], Saiz PA[3], García-Portilla MP[3], Burón P[3], Bobes J[3], Vieta E[2], Benabarre A[2].

[1]Department of Animal Biology, Anthropology Section, Faculty of Biology, University of Barcelona, IBUB, CIBERSAM, Barcelona, Catalonia, Spain [2]Bipolar Disorder Program, Hospital Clinic, University of Barcelona, IDIBAPS, CIBERSAM, Barcelona, Catalonia, Spain [3] Department of Psychiatry, School of Medicine, University of Oviedo, CIBERSAM, INEUROPA. Oviedo, Spain * Co-First Authors

Abstract

Lithium is considered the first-line treatment in bipolar disorder, although response could range from an excellent response to a complete lack of response. Response to lithium is a complex phenotype in which different factors, part of them genetics, are involved. In this sense, the aim of this study was to investigate the potential association of genetic variability at genes related to phosphoinositide (PI), glycogen synthetase kinase-3 (GSK3), hypothalamic-pituitary-adrenal (HPA) and glutamatergic pathways with lithium response. A sample of 131 bipolar patients were grouped and compared according to their level of response: excellent responders (ER), partial responders (PR) and non-responders (NR). Genotype and allele distributions of the rs669838 (*IMPA2*), rs909270 (*INNP1*), rs11921360 (*GSK3B*) and rs28522620 (*GRIK2*) polymorphisms significantly differed between ER, PR, and NR. When we compared the ER *versus* PR+NR, the logistic regression showed significant association for rs669838-C (*IMPA2*) (p=0.021), rs909270-G (*INPP1*) (p=0.009), and rs11921360-A (*GSK3B*) (p=0.004) with lithium non-response. Haplotype analysis showed significant association for the haplotypes rs3791809-rs4853694-rs909270 (*INPP1*) and rs1732170-rs11921360-rs334558 (*GSK3B*) and lithium response. Our study is in line with previous studies reporting association between genetic variability at these genes and lithium response, pointing to an effect of *IMPA2*, *INPP1* and *GSK3B* genes to lithium response in BD patients. Further studies with larger samples are warranted to assess the strength of the reported associations.

Key words: bipolar disorder, lithium, pharmacogenetics, genetic association, phosphoinositide system, hypothalamic-pituitary-adrenal axis, glutamatergic system.

Introduction

Lithium (Li) is still considered the first-line treatment in bipolar disorder (BD) due to its proven efficacy in both acute and maintenance phases [1]. However, an adequate response may range from an excellent response in 24-45% to a complete lack of response in 10-30% of patients [2].

BD is a complex disease that involves abnormalities at neuroprotective, neurochemical, neuroendocrinological, neurostructural and genetic levels [3, 4]. Although Li is thought to target these altered

levels, the precise genetic and molecular mechanism for its therapeutic action remains elusive. In addition, Li's responsiveness is also considered a complex phenotype. Thus, apart from genetic factors, others such as sociodemographical or clinical should be considered in order to understand clinical response [5, 6].

Evidence from molecular biology has shown that Li exerts multiple effects on neurotransmitter/receptor-mediated signalling, ion transport, signal transduction cascades, hormonal and circadian regulation. It also profoundly alters gene expression patterns with a final effect stabilizing neuronal activities, supporting neural plasticity and providing neuroprotection [7]. In this regard, intense interest has been focused upon the two major cell-signalling pathways with which Li interacts: phosphoinositide (PI) and glycogen synthetase kinase-3 (GSK3) pathways. However, other systems have been also involved [6, 8, 9]. In this sense, it is known that chronic Li administration up-regulates glutamate reuptake decreasing glutamate availability in synapse which could contribute to neuroprotective effect attributed to this drug [10]. Moreover, it has been demonstrated that Li leads to a significant activation of the hypothalamic-pituitary-adrenal (HPA) system in patients with major depression [11].

Identifying such a Li-responsive gene network in brain would allow us to distinguish between subsets of genes underlying therapeutic and nontherapeutic actions of Li. The aim of this study was to investigate the potential association of genetic variability at 16 candidate genes related to PI (*INPP1, MARCKS, IMPA1, IMPA2, ITPKC PLCG1*), GSK3 (*GSK3B*, *GSK3A, CREB1*), HPA (*FKBP5, CRHR2, CRHR1*) and glutamatergic (*GRIA2, GABRB2, GRIK2, GRIK5*) systems with Li response in BP.

Materials and Method

Sample:

131 unrelated Caucasian bipolar type I or II outpatients (69 males and 62 females) were recruited from the Bipolar Disorder Program (BDP) at the Hospital Clinic of Barcelona (n=104) and from primary care settings in Oviedo (n=27). The BDP has conducted a prospective data collection on course of illness of all patients in the program since 1992 as previously described [12, 13]. This cross-sectional analysis includes some variables from both prospective and retrospective assessments.

Inclusion criteria were as follows: (a) bipolar I or II DSM-IV-TR diagnosis, (b) age > 18 years, (c) fulfilling criteria for euthymia defined as a score ≤ 8 on the Hamilton Depression Rating Scale (HDRS) [14] and a score ≤ 6 on the Young Mania Rating Scale (YMRS) [15], (d) all patients must receive or have received for at least one year Li as maintenance treatment therapy with doses adjusted to obtain plasma levels within the standard therapeutic range and (e) written informed consent. Exclusion criteria were the presence of (a) mental retardation (defined as IQ<70), (b) severe organic disease and (c) no tolerability to Li. All procedures were approved by each institution's ethics committees. All patients provided written informed consent for the collection of their data

with research purposes, always preserving confidentiality.

Assessment

Clinical and sociodemographic data was collected using a semi-structured interview based on the Structured Clinical Interview for DSM Disorders (SCID) and from available data in medical records. Suicidality was defined as the presence of any suicide ideation or previous suicide attempt.

Definition of Li response

The efficacy of Li treatment was assessed according to the following criteria: (a) excellent responders (ER): patients presenting a 50% reduction of the episodes after the introduction of Li in monotherapy, (b) partial responders (PR): patients presenting a 50% reduction of the episodes after the introduction of Li but on polytherapy (other mood stabilizer, antidepressant or antipsychotics), (c) non-responders (NR): patients who did not reduce at least a 50% of the episodes and patients who required electroconvulsive therapy (adapted from [16]).

Genetic analysis

Candidate genes were selected, either on the basis of the mechanism of action of Li and/or the neurobiology of BD. Several single nucleotide polymorphisms (SNPs) at 16 common genes were selected according to previous literature and/or a tagSNP strategy allowed by the SYSNPS program (www.sysnps.org) (Table 1). Genomic DNA was extracted from blood samples from each participant according to standard protocols. Genotyping, blind to clinical assessment, was performed by competitive quantitative PCR using allele specific probes with FRET signal detection. A randomized 10% of individuals were re-genotyped in order to confirm the pattern reproducibility.

Statistical analysis

Differences in sociodemographic and clinical variables between the groups of Li responders were evaluated with t-test or chi-square (χ^2) test using SPSS v.18. EpiInfo v.3.5.1 was used to calculate Hardy-Weinberg equilibrium for genotype frequencies using χ^2 test.

Genotype and allele association analyses between the groups of patients with different Li response (ER, PR and NR) were performed using χ^2 test. As a second analysis, PR and NR were pooled together and compared to ER. As suicidality significantly differed between the groups of responders (Table 2), the genotypic association analysis was tested using logistic regression under the additive model with suicidality as a covariate. Empirical p-values were generated using the max(T) permutation approach (10000 permutations) for pointwise estimates (EMP1) as well as corrected for all comparisons (EMP2). Odds ratios (OR) with 95% confidence intervals (CI) were estimated for the effects of high-risk genotypes. The study had an 80% power (95% CI) to detect OR in a range of 3.99 to 5.75 for Li no-response (PR and NR pooled together) according to the range of obtained MAFs (MAF: 0.1 to 0.5, respectively). All the analyses were carried out with PLINK, version 2.07 [17].

The study had an 80% power (95% CI) to detect OR in a range of 3.99 to 5.75 for Li no-response (PR and NR pooled together) according to the range of obtained MAFs (MAF: 0.1 to 0.5, respectively).

Haploview 3.2 was used to generate a linkage disequilibrium map. Haplotype analyses were conducted using the 'R' software (v.2.2.1) by the "haplo.stat" package. Suicidality was included in the haplotype analyses as a covariate. Rare haplotypes, those which were less frequent than 1%, were excluded from the analyses. The global significance of the results for haplotype analyses was estimated using permutation (50000 permutations) to confirm the asymptotic p-values.

Results

26 patients (19.8%) were classified as ER, 62 patients (47.3%) as PR and 43 patients (32.8%) as NR to Li treatment.

Sociodemographic and clinical features of the sample are shown in Table 2. No differences for mean age, age at onset or sex were found when comparing patients according to their level of Li response. Significantly differences in suicidality were found between the groups of responders. NR presented higher rates of suicidality than PR or ER ($p<0.001$). Suicidality was included as a covariate in the logistic regression and haplotype analyses.

Genotype distributions of the SNPs were all in Hardy-Weinberg equilibrium (data not shown). Genotypic and allelic frequencies are presented in Table 3.

Significant differences were found in genotypic and allelic distributions between different groups of Li responders for the rs669838 (*IMPA2*) (genotype: χ^2=10.338, df=4, p=0.035; allele: χ^2=8.51, df=2, p=0.015), rs909270 (*INNP1*) (genotype: χ^2=10.132, df=4, p=0.038; allele: χ^2=6.51, df=2, p=0.038), rs11921360 (*GSK3β*) (genotype: χ^2=9.713, df=4, p=0.046; allele: χ^2=8.51, df=2, p=0.0057) and rs28522620 (*GRIK2*) (genotype: χ^2=9.597, df=4, p=0.048; allele: χ^2=8.25, df=2, p=0.016) polymorphisms (Table 3). P-values were not significant after permutation testing. No other associations were found regarding the other SNPs analyzed.

When we pooled together PR+NR *versus* ER and compared the SNPs associated in our previous association analyses, logistic regression showed significant association for rs669838 (*IMPA2*) [β=2.31; p=0.021; OR=2.03; 95% CI (1.11-3.72); EMP1=0.018; EMP2=0.07], rs909270 (*INPP1*) [β=2.58; p=0.009; OR=2.45; 95% CI (1.24-4.82); EMP1=0.008; EMP2=0.028] and rs11921360 (*GSK3B)* [β=2.84; p=0.004; OR=2.52; 95% CI (1.33-4.78); EMP1=0.002; EMP2=0.011] with Li response, being C, G and A the risk alleles, respectively.

Haplotype analysis showed an association of rs3791809-rs4853694-rs909270 haplotype in *INPP1* (D'=0.94, r^2=0.43) and Li response. Frequencies of the T-A-G haploblock were more frequent in PR+NR (0.488) than in ER group (0.306) (p=0.012; sim-p=0.012). On the contrary, T-A-A haploblock was more frequent in ER than in PR+NR group (0.241 vs. 0.12) (p=0.018; sim-p=0.019). The rs1732170-rs11921360-rs334558 haplotype in *GSK3B*

(D'=0.979, r^2=0.742) was also associated with Li response (global p=0.002, global sim-p=0.002). The C-C-A haploblock was significantly less frequent in the group of PR+NR (0.299) than in ER (0.552) (p=0.001; sim-p=0.001). No other significant associations were found regarding the remaining analyzed haplotypes and Li response.

Discussion

This study analyzed the potential association of genetic variability at PI, GSK3, HPA and glutamatergic pathways with Li response in BD.

A large number of studies tried to identify genetic variants within genes of PI system which could predict response to Li. However, the results are still controversial making unclear the role of this system in Li response [18]. Our results are in line with these studies reporting an effect of genetic variability at this system and Li response. Particularly, we found the effect of rs669838 (*IMPA2*) and rs909270 (*INNP1*). The *IMPA2* gene is located in a region thought to be a BD susceptibility locus (18p11.2) [19]. Two trends for association have been previously found between two polymorphisms (rs3786282 and 599+97G>A) of this gene and good response to Li in BD patients [20]. The C937A variant of *INPP1* was associated with response to Li in a Norwegian sample but not in an independent Israeli sample [21]. This finding was not supported in another study [2].

Regarding *GSK3B*, we found association with rs11921360 and Li response. Previously, a functional polymorphism of the *GSK3B* gene and Li response was reported [22], but this was not confirmed in two other studies [2, 23].

Relatively few association studies have been conducted between glutamatergic system and Li response. Genetic variability at *GRIN2B* gene was examined and failed to predict Li outcome [24]. A recent genome-wide association study (GWAs) showed that a SNP in the GluR2 gene was associated with the risk for recurrence among patients treated with Li [25]. In our study, the findings suggest that Li response seems to be associated with genetic variability at *GRIK2* gene. The *GRIK2* gene (6q.21), which encodes for a kainate receptor (GluR6) implicated in synaptic plasticity [26], had been previously reported as candidate gene conferring a predisposition to BD [27] [26].

We did not found any association between response to Li and the genetic variants of the HPA system. To our knowledge, no other studies investigating the role of the genes analyzed in this study related to the HPA system and Li response were available.

Our study has some limitations. Due to the tertiary nature of the BDP, some of the subjects included in this study could be categorized as difficult-to-treat patients, thus generalization of our results should be done with caution. The relative small size of our sample limits the power to detect small differences. Moreover, all the variants associated in the present study are intron variants. It has been suggested that silent SNPs can affect in vivo splicing events or protein folding and, consequently, the final protein function [28]. Recent data from the ENCODE *(Encyclopedia of DNA Elements)* project has revealed the importance of intronic and intergenic variants as regulatory elements of gene expression acting as microRNAs and/or

epigenetic targets [29]. Alternatively, these polymorphisms might not constitute the actual causative variant, but rather reflect association of other polymorphisms in linkage disequilibrium with this locus.

In conclusion, and despite potential limitations, our results indicate a possible role of genes related to PI (*INPP1*, *IMPA2*), GSK3 (*GSK3B*) and glutamatergic system (*GRIK2*) in Li response. As Li response is a complex trait, further studies with larger samples are warranted to assess the strength of the reported associations.

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Conflict of Interest Statement

JM Goikolea has been a speaker or advisory board for Astra-Zeneca, Bristol Myers-Squibb, Eli Lilly, Glaxo-Smith-Kline, Janssen-Cilag, Merck Sharpe and Dohme, Otsuka, Pfizer, Sanofi-Aventis. PA Saiz has been a consultant to or has received honoraria or grants from Adamed, Brainpharma, Bristol-Myers Squibb, CIBERSAM, Ferrer inCode, Lilly, Rovi, Servier, Instituto de Salud Carlos III, Plan Nacional de Drogas. MP Garcia-Portilla has been a consultant to or has received honoraria or grants from Eli Lilly, Janssen, Lundbeck, Pfizer, Otsuka, Roche Farma, Rovi, Servier, the Spanish Ministry of Economy and Competitiveness, Instituto de Salud Carlos III. J Bobes has been a consultant to or has received honoraria or grants from Adamed, Astra Zeneca, Bristol-Myers Squibb, CIBERSAM, D&A Pharma, Elan, European Commission – FP6 and FP7-, Forest, Instituto de Salud Carlos III, Janssen Cilag, Lilly, Lundbeck, Ministerio de Sanidad, Plan Nacional sobre Drogas, Pfizer, Otsuka, Pfizer, Roche, Servier, Shire. E Vieta has received research grants and served as consultant, advisor and speaker for the following companies. Grants: Almirall, Astra-Zeneca, Bristol-Myers-Squibb, Eli Lilly, Generalitat de Catalunya, Gedeon Richter, Glaxo-Smith-Kline, Janssen-Cilag, Johnson & Johnson, Novartis, Pfizer, Sanofi-Aventis, Servier, Seventh European Framework Programme, Spanish Ministry of Science and Innovation. Consultant: Astra-Zeneca, Bristol-Myers-Squibb, Eli Lilly, Forest Research Institute, Gedeon Richter, Glaxo-Smith-Kline, Janssen-Cilag, Jazz, Lundbeck, Merck Sharpe and Dohme, Novartis, Otsuka, Pfizer, Sanofi, Servier, Shering-Plough, Takeda, and United Biosource Corporation. Advisory Board: Astra-Zeneca, Bristol-Myers-Squibb, Eli Lilly, Forest Research Institute, Gedeon Richter, Glaxo-Smith-Kline, Jansen-Cilag, Jazz, Lundbeck, Merck Sharpe and Dohme, Novartis, Otsuka, Pfizer, Sanofi, Servier, Shering-Plough, Takeda, United Biosource Corporation. A Benabarre has

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Table 1. Description of the polymorphisms analyzed.

Gene	CHR position	SNP ID	Location*	A1	A2	MAF (A1)	Function
Phosphoinositide and GSK3β pathways							
INPP1	2q32	rs3791809	190917963	C	T	0.36	Intron
		rs4853694	190927518	G	A	0.27	Intron
		rs909270	190941420	G	A	0.46	Intron
CREB1	2q34	rs10932201	208134502	A	G	0.37	Intron
		rs11904814	208135043	G	T	0.36	Intron
		rs2551923	208150021	T	C	0.22	Intron
GSK3B	3q13.3	rs1732170	121066366	T	C	0.4	Intron
		rs11921360	121094745	C	A	0.34	Intron
		rs334558	121295972	G	A	0.34	Upstream-gene-variant
MARCKS	6q22.2	rs7769769	114286969	A	G	0.49	Intron
		rs352082	114287339	C	G	0.12	Intron
IMPA1	8q21.13-q21.3	rs915	82732945	T	C	0.31	3'UTR
		rs1058401	82733186	T	C	0.26	3'UTR
		rs2268432	82749332	T	G	0.12	Intron
IMPA2	18p11.2	rs669838	11984598	A	C	0.32	Intron
		rs1020294	12007343	A	G	0.31	Intron
		rs1250171	12017028	T	C	0.29	Intron
		rs630110	12019857	A	G	0.32	Intron
ITPKC	19q13.1	rs2290693	45937582	T	C	0.49	3'UTR
GSK3A	19q13.2	rs3745233	47425705	C	T	0.1	Intron
PLCG1	20q12-q13.1	rs2228246	39225477	G	A	0.17	Missense-variant
HPA system							
FKBP5	6p21.31	rs3777747	35686980	G	A	0.45	Intron
		rs1360780	35715549	T	C	0.33	Intron
		rs17542466	35722722	G	A	0.2	Intron
		rs2766533	35793468	A	G	0.47	Intron
CRHR2	7p14.3	rs4722999	30660300	C	T	0.34	Intron
		rs2284219	30680961	A	G	0.38	Intron
		rs255102	30697689	T	A	0.37	Intron
		rs255115	30705448	G	A	0.43	Intron
CRHR1	17q12-q22	rs110402	41235818	A	G	0.32	Intron
		rs242940	41248380	C	T	0.38	Intron
Glutamatergic system							
GRIA2	4q32.1	rs9784453	158609669	A	G	0.38	Unknown
GABRB2	5q34	rs592403	160652562	C	A	0.34	3'UTR
		rs2910284	160761161	A	G	0.35	Intron
		rs2962406	160842679	C	A	0.27	Intron
		rs4426954	160885609	C	T	0.45	Intron
GRIK2	6q16.3	rs2852525	101982434	G	T	0.48	Intron
		rs2787554	101999105	G	C	0.46	Intron
		rs2518261	102088471	T	C	0.42	Intron
		rs2852620	102621626	T	A	0.49	Intron
GRIK5	19q13.2	rs8099939	47212948	T	G	0.41	Intron
		rs4803523	47241774	T	C	0.16	Intron
		rs10407506	47234839	T	C	0.15	Intron

Gene: NCBI gene symbol. CHR: chromosome. SNP: dcSNP symbol. A1 & A2: minor and major allele nucleotides. MAF: minor allele frequency in the sample.*human genome hg18 assembly.

Table 2. Sociodemographic and clinical description of the sample.

	Total n=131	**ER** n=26	**PR** n=62	**NR** n=43	**Statistics**
Age (years) [mean (SD)]	46.8 (13.1)	44.9 (11.9)	46.3 (14)	48.8 (12.4)	F=0.82 df=2 p=0.442
Gender [M:F]	69:62	15:11	31:31	23:20	χ^2=0.45 df=2 p=0.798
Age at onset (years) [mean (SD)]	28.7 (12.3)	26.5 (10.3)	30.13 (13.4)	27.9 (11.4)	F=0.925 df=2 p=0.399
Family history of psychiatric illness [N (%)]	72 (55.4)	10 (40)	34 (54.8)	28 (65.1)	χ^2=4.05 df=2 p=0.132
Suicidality [N (%)]	91 (70.5)	14 (56)	36 (59)	41 (95.3)	χ^2=19.18 df=2 p<0.001

ER: excellent responders; PR: partial responders; NR: no responders

Table 3. Genotype and allele distributions of the polymorphisms analyzed in bipolar patients according to Li response.

SNP	***Sample***	***n***	***Genotype distribution n (f)***			χ^2	***df***	***p***	***Allele distribution n (f)***		χ^2	***df***	***p***
INPP1–rs3791809			**TT**	**TC**	**CC**				**T**	**C**			
	NR	41	20 (0.49)	12 (0.29)	9 (0.22)	7.51	4	0.112	52 (0.63)	30 (0.37)	0.11	2	0.947
	PR	60	25 (0.42)	27 (0.45)	8 (0.13)				77 (0.64)	43 (0.36)			
	ER	26	8 (0.31)	16 (0.61)	2 (0.08)				32 (0.61)	20 (0.39)			
INPP1-rs4853694			**AA**	**AG**	**GG**				**A**	**G**			
	NR	41	24 (0.59)	12 (0.29)	5 (0.12)	6.36	4	0.174	60 (0.68)	22 (0.32)	0.99	2	0.609
	PR	61	34 (0.55)	23 (0.38)	4 (0.07)				91 (0.75)	31 (0.25)			
	ER	26	10 (0.38)	15 (0.58)	1 (0.04)				35 (0.67)	17 (0.33)			
INPP1-rs909270			**AA**	**AG**	**GG**				**A**	**G**			
	NR	41	13 (0.32)	14 (0.34)	14 (0.34)	10.13	4	**0.038***	40 (0.49)	42 (0.51)	6.51	2	**0.038***
	PR	61	14 (0.23)	33 (0.54)	14 (0.23)				61 (0.5)	61 (0.5)			
	ER	26	12 (0.46)	12 (0.46)	2 (0.08)				36 (0.69)	16 (0.31)			

CREB1-rs10932201			**GG**	**GA**	**AA**				**C**	**T**			
	NR	41	14 (0.34)	22 (0.54)	5 (0.12)	1.88	4	0.757	50 (0.61)	32 (0.39)	0.27	2	0.27
	PR	62	22 (0.36)	33 (0.53)	7 (0.11)				77 (0.62)	47 (0.38)			
	ER	26	12 (0.46)	10 (0.39)	4 (0.15)				34 (0.65)	18 (0.35)			
CREB1-rs11904814			**TT**	**TG**	**GG**				**T**	**G**			
	NR	40	14 (0.35)	20 (0.5)	6 (0.15)	4.18	4	0.382	48 (0.6)	32 (0.4)	3.6	2	0.165
	PR	62	30 (0.48)	26 (0.42)	6 (0.1)				86 (0.69)	38 (0.31)			
	ER	26	9 (0.35)	11 (0.42)	6 (0.23)				29 (0.56)	23 (0.44)			
CREB1-rs334558			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	26 (0.63)	15 (0.37)	0 (0)	0.87	4	0.068	67 (0.82)	15 (0.18)	2.38	2	0.304
	PR	59	28 (0.47)	31 (0.53)	0 (0)				87 (0.74)	31 (0.26)			
	ER	25	17 (0.68)	7 (0.28)	1 (0.04)				41 (0.82)	9 (0.18)			
GSK3B-rs1732170			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	16 (0.39)	17 (0.42)	8 (0.19)	5.72	4	0.221	49 (0.6)	33 (0.4)	5.52	2	0.063
	PR	62	20 (0.32)	27 (0.44)	15 (0.24)				67 (0.54)	57 (0.46)			
	ER	26	13 (0.5)	12 (0.46)	1 (0.04)				38 (0.73)	14 (0.27)			
GSK3B-rs11921360			**AA**	**AC**	**CC**				**A**	**C**			
	NR	41	20 (0.49)	17 (0.41)	4 (0.1)	9.71	4	**0.046***	57 (0.69)	25 (0.31)	10.32	2	**0.0057***
	PR	61	32 (0.53)	22 (0.36)	7 (0.11)				86 (0.71)	36 (0.29)			
	ER	25	6 (0.24)	11 (0.44)	8 (0.32)				23 (0.46)	27 (0.54)			
GSK3B-rs334558			**AA**	**AG**	**GG**				**A**	**G**			
	NR	38	18 (0.47)	16 (0.42)	4 (0.11)	5.19	4	0.268	52 (0.68)	24 (0.32)	4.59	2	0.101
	PR	62	25 (0.4)	24 (0.39)	13 (0.21)				74 (0.6)	50 (0.4)			
	ER	25	14 (0.56)	10 (0.4)	1 (0.04)				38 (0.76)	12 (0.24)			
MARCKS-rs7769769			**GG**	**GA**	**AA**				**G**	**A**			
	NR	39	11 (0.28)	18 (0.46)	10 (0.26)	1.51	4	0.825	40 (0.51)	38 (0.49)	1.32	2	0.517
	PR	62	15 (0.24)	30 (0.48)	17 (0.28)				60 (0.48)	64 (0.52)			
	ER	25	8 (0.32)	13 (0.52)	4 (0.16)				29 (0.58)	21 (0.42)			
MARCKS-rs352082			**GG**	**GC**	**CC**				**G**	**C**			
	NR	39	32 (0.82)	6 (0.15)	1 (0.03)	3.37	4	0.498	70 (0.9)	8 (0.1)	0.2	2	0.903
	PR	61	46 (0.75)	15 (0.25)	0 (0.00)				107 (0.88)	15 (0.12)			
	ER	25	19 (0.76)	6 (0.24)	0 (0.00)				44 (0.88)	6 (0.12)			

IMPA1-rs915			**CC**	**CT**	**TT**				**C**	**T**			
	NR	40	22 (0.55)	16 (0.4)	2 (0.05)	2.21	4	0.698	60 (0.75)	20 (0.25)	2.13	2	0.345
	PR	62	27 (0.44)	28 (0.45)	7 (0.11)				82 (0.66)	42 (0.34)			
	ER	26	11 (0.42)	12 (0.46)	3 (0.12)				34 (0.65)	18 (0.35)			
IMPA1-rs1058401			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	26 (0.64)	14 (0.34)	1 (0.02)	4.36	4	0.360	66 (0.81)	16 (0.19)	2.64	2	0.267
	PR	61	30 (0.49)	28 (0.46)	3 (0.05)				88 (0.72)	34 (0.28)			
	ER	26	13 (0.5)	10 (0.38)	3 (0.12)				36 (0.69)	16 (0.31)			
IMPA1-rs2268432			**GG**	**GT**	**TT**				**G**	**T**			
	NR	41	32 (0.78)	9 (0.22)	0 (0)	1.32	4	0.857	73 (0.89)	9 (0.11)	0.24	2	0.888
	PR	62	47 (0.76)	14 (0.23)	1 (0.01)				108 (0.87)	16 (0.13)			
	ER	26	19 (0.73)	7 (0.27)	0 (0)				45 (0.86)	7 (0.14)			
IMPA2-rs669838			**CC**	**CA**	**AA**				**C**	**A**			
	NR	41	25 (0.61)	12 (0.29)	4 (0.1)	10.33	4	**0.035***	62 (0.76)	20 (0.24)	8.51	2	**0.015***
	PR	61	33 (0.54)	19 (0.31)	9 (0.15)				85 (0.7)	37 (0.3)			
	ER	26	6 (0.23)	15 (0.58)	5 (0.19)				27 (0.52)	25 (0.48)			
IMPA2-rs1020294			**GG**	**GA**	**AA**				**G**	**A**			
	NR	41	16 (0.39)	19 (0.46)	6 (0.15)	7.69	4	0.103	51 (0.62)	31 (0.38)	4.18	2	0.123
	PR	62	34 (0.55)	25 (0.4)	3 (0.05)				93 (0.75)	31 (0.25)			
	ER	26	9 (0. 35)	16 (0.61)	1 (0.04)				34 (0.65)	18 (0.35)			
IMPA2-rs1250171			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	21 (0.51)	17 (0.42)	3 (0.07)	1.51	4	0.825	59 (0.72)	23 (0.28)	0.37	2	0.829
	PR	61	31 (0.51)	25 (0.41)	5 (0.08)				87 (0.71)	35 (0.29)			
	ER	26	13 (0.5)	9 (0.35)	4 (0.15)				35 (0.67)	17 (0.33)			
IMPA2-rs630110			**GG**	**GA**	**AA**				**G**	**A**			
	NR	40	22 (0.55)	16 (0.4)	2 (0.05)	3.79	4	0.434	60 (0.75)	20 (0.25)	3.16	2	0.205
	PR	62	25 (0.4)	31 (0.5)	6 (0.1)				81 (0.65)	43 (0.35)			
	ER	26	10 (0.39)	12 (0.46)	4 (0.15)				32 (0.62)	20 (0.38)			
ITPKC-rs2290693			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	14 (0.34)	16 (0.39)	11(0.27)	5.94	4	0.204	44 (0.54)	38 (0.46)	4.86	2	0.087
	PR	61	12 (0.2)	30 (0.49)	19 (0.31)				54 (0.44)	68 (0.56)			
	ER	25	9 (0.36)	13 (0.52)	3 (0.12)				31 (0.62)	19 (0.38)			

GSK3A-rs3745233			**TT**	**TC**	**CC**				**T**	**C**			
	NR	41	35 (0.86)	5 (0.12)	1 (0.02)	4.58	4	0.333	75 (0.92)	7 (0.08)	1.96	2	0.374
	PR	62	47 (0.758)	15 (0.242)	0 (0.00)				94 (0.86)	15 (0.14)			
	ER	26	22 (0.846)	4 (0.154)	0 (0.00)				48 (0.92)	4 (0.08)			
PLCG1–rs2228246			**AA**	**AG**	**GG**				**A**	**G**			
	NR	41	30 (0.73)	9 (0.22)	2 (0.05)	6.07	4	0.194	69 (0.84)	13 (0.16)	0.20	2	0.903
	PR	62	40 (0.65)	22 (0.35)	0 (0)				102 (0.82)	22 (0.18)			
	ER	26	18 (0.69)	8 (0.31)	0 (0)				44 (0.85)	8 (0.15)			
FKBP5-rs3777747			**AA**	**AG**	**GG**				**A**	**G**			
	NR	41	12 (0.29)	21 (0.51)	8 (0.2)	1.13	4	0.889	45 (0.55)	37 (0.45)	0.61	2	0.738
	PR	62	19 (0.31)	28 (0.45)	15 (0.24)				66 (0.53)	58 (0.47)			
	ER	26	10 (0.39)	11 (0.42)	5 (0.19)				31 (0.6)	21 (0.4)			
FKBP5-rs1360780			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	23 (0.56)	16 (0.39)	2 (0.05)	3.84	4	0.428	62 (0.76)	20 (0.24)	3.84	2	0.146
	PR	61	26 (0.43)	26 (0.42)	9 (0.15)				78 (0.64)	44 (0.36)			
	ER	25	10 (0.4)	11 (0.44)	4 (0.16)				31 (0.62)	19 (0.38)			
FKBP5-rs17542466			**AA**	**AG**	**GG**				**A**	**G**			
	NR	41	22 (0.54)	17 (0.41)	2 (0.05)	4.34	4	0.362	61 (0.74)	21 (0.26)	3.36	2	0.186
	PR	61	44 (0.72)	150 (0.25)	2 (0.03)				103 (0.84)	19 (0.16)			
	ER	26	16 (0.62)	8 (0.31)	2 (0.07)				40 (0.77)	12 (0.23)			
FKBP5-rs2766533			**GG**	**GA**	**AA**				**G**	**A**			
	NR	41	11 (0.27)	21 (0.51)	9 (0.22)	1.02	4	0.906	43 (0.52)	39 (0.48)	0.03	2	0.984
	PR	62	18 (0.29)	30 (0.48)	14 (0.23)				66 (0.53)	58 (0.47)			
	ER	25	6 (0.24)	15 (0.6)	4 (0.16)				27 (0.54)	23 (0.46)			
CRHR2-rs4722999			**TT**	**TC**	**CC**				**T**	**C**			
	NR	40	18 (0.45)	19 (0.48)	3 (0.07)	1.73	4	0.785	55 (0.69)	25 (0.31)	3.95	2	0.138
	PR	62	28 (0.45)	25 (0.4)	9 (0.15)				81 (0.65)	43 (0.35)			
	ER	26	10 (0.38)	13 (0.5)	3 (0.12)				33 (0.63)	29 (0.37)			
CRHR2-rs2284219			**GG**	**GA**	**AA**				**G**	**A**			
	NR	41	15 (0.37)	18 (0.44)	8 (0.19)	3.69	4	0.449	48 (0.59)	34 (0.41)	0.61	2	0.738
	PR	61	27 (0.44)	24 (0.39)	10 (0.17)				78 (0.64)	44 (0.36)			
	ER	26	13 (0.5)	6 (0.23)	7 (0.27)				32 (0.62)	20 (0.38)			

CRHR2-rs255102			**AA**	**AT**	**TT**				**A**	**T**			
	NR	41	18 (0.44)	15 (0.37)	8 (0.19)	5.11	4	0.277	51 (0.62)	31 (0.38)	0.02	2	0.989
	PR	61	23 (0.38)	43 (0.54)	5 (0.08)				89 (0.65)	53 (0.35)			
	ER	26	11 (0.42)	10 (0.39)	5 (0.19)				32 (0.62)	20 (0.38)			
CRHR2-rs255115			**AA**	**AG**	**GG**				**A**	**G**			
	NR	39	14 (0.36)	16 (0.41)	9 (0.23)	2.41	4	0.660	44 (0.56)	34 (0.44)	0.03	2	0.987
	PR	61	18 (0.29)	34 (0.56)	9 (0.15)				70 (0.57)	52 (0.43)			
	ER	26	8 (0.31)	14 (0.54)	4 (0.15)				30 (0.58)	22 (0.42)			
CRHR1-rs110402			**GG**	**GA**	**AA**				**G**	**A**			
	NR	41	19 (0.46)	19 (0.46)	3 (0.08)	4.66	4	0.323	57 (0.7)	25 (0.3)	1.89	2	0.388
	PR	61	30 (0.49)	25 (0.41)	6 (0.1)				85 (0.7)	37 (3)			
	ER	26	7 (0.27)	17 (0.65)	2 (0.08)				31 (0.6)	21 (0.4)			
CRHR1-rs242940			**TT**	**TC**	**CC**				**T**	**C**			
	NR	40	15 (0.37)	21 (0.53)	4 (0.1)	2.35	4	0.671	51 (0.64)	29 (0.36)	1.96	2	0.374
	PR	61	25 (0.41)	29 (0.48)	7 (0.11)				79 (0.65)	43 (0.35)			
	ER	26	7 (0.27)	14 (0.54)	5 (0.19)				28 (0.54)	24 (0.46)			
GRIA2-rs9784453			**GG**	**GA**	**AA**				**G**	**A**			
	NR	41	20 (0.49)	17 (0.41)	4 (0.1)	4.33	4	0.363	57 (0.69)	25 (0.31)	3.07	2	0.216
	PR	61	25 (0.41)	23 (0.38)	13 (0.21)				73 (0.6)	49 (0.4)			
	ER	26	8 (0.31)	13 (0.5)	5 (0.19)				29 (0.56)	23 (0.44)			
GABRB2-rs592403			**AA**	**AC**	**CC**				**A**	**C**			
	NR	40	20 (0.5)	15 (0.38)	5 (0.12)	4.56	4	0.335	55 (0.69)	25 (0.31)	4.42	2	0.109
	PR	60	22 (0.36)	28 (0.47)	10 (0.17)				72 (0.6)	48 (0.4)			
	ER	25	14 (0.56)	10 (0.4)	1 (0.04)				38 (0.76)	12 (0.24)			
GABRB2-rs2910284			**GG**	**GA**	**AA**				**G**	**A**			
	NR	41	20 (0.49)	14 (0.34)	7 (0.17)	5.13	4	0.274	54 (0.66)	28 (0.34)	1.85	2	0.397
	PR	62	22 (0.35)	32 (0.52)	8 (0.13)				76 (0.61)	48 (0.39)			
	ER	25	12 (0.48)	12 (0.48)	1 (0.04)				36 (0.72)	14 (0.28)			
GABRB2-rs2962406			**AA**	**AC**	**CC**				**A**	**C**			
	NR	41	21 (0.51)	15 (0.37)	5 (0.12)	4.58	4	0.332	57 (0.69)	25 (0.1	4.70	2	0.095
	PR	61	31 (0.51)	23 (0.38)	7 (0.11)				85 (0.7)	37 (0.3)			
	ER	26	18 (0.69)	8 (0.31)	0 (0)				44 (0.85)	8 (0.15)			

GABRB2-rs4426954			**TT**	**TC**	**CC**				**T**	**C**			
	NR	41	11 (0.27)	21 (0.51)	9 (0.22)	3.56	4	0.468	43 (0.52)	39 (0.48)	2.79	2	0.248
	PR	60	19 (0.31)	25 (0.42)	16 (0.27)				63 (0.53)	57 (0.47)			
	ER	26	11 (0.42)	12 (0.46)	3 (0.12)				34 (0.65)	18 (0.35)			
GRIK2–rs2852525			**TT**	**TG**	**GG**				**T**	**G**			
	NR	41	10 (0.24)	22 (0.54)	9 (0.22)	0.92	4	0.921	42 (0.51)	40 (0.49)	0.14	2	0.932
	PR	61	18 (0.3)	29 (0.47)	14 (0.23)				65 (0.53)	57 (0.47)			
	ER	26	6 (0.23)	15 (0.58)	5 (0.19)				27 (0.52)	25 (0.48)			
GRIK2–rs2787554			**CC**	**CG**	**GG**				**C**	**G**			
	NR	41	8 (0.19)	24 (0.59)	9 (0.22)	5.67	4	0.225	40 (0.49)	42 (0.51)	1.49	2	0.475
	PR	62	24 (0.39)	23 (0.37)	15 (0.24)				71 (0.57)	53 (0.43)			
	ER	26	8 (0.31)	11 (0.42)	7 (0.27)				27 (0.52)	25 (0.48)			
GRIK2–rs2518261			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	19 (0.46)	16 (0.39)	6 (0.15)	4.93	4	0.295	54 (0.66)	28 (0.34)	2.91	2	0.233
	PR	62	18 (0.29)	32 (0.52)	12 (0.19)				68 (0.55)	56 (0.45)			
	ER	25	6 (0.24)	15 (0.6)	4 (0.16)				27 (0.54)	23 (0.46)			
GRIK2–rs2852620			**TT**	**TA**	**AA**				**T**	**A**			
	NR	41	15 (0.37)	18 (0.44)	8 (0.19)	9.59	4	**0.048***	48 (0.59)	34 (0.41)	8.25	2	**0.016***
	PR	62	8 (0.13)	34 (0.55)	20 (0.32)				50 (0.4)	74 (0.6)			
	ER	26	9 (0.35)	12 (0.46)	5 (0.19)				30 (0.58)	22 (0.42)			
GRIK5–rs8099939			**GG**	**GT**	**TT**				**G**	**T**			
	NR	41	18 (0.44)	17 (0.41)	6 (0.15)	2.26	4	0.687	53 (0.65)	29 (0.35)	1.80	2	0.406
	PR	62	21 (0.34)	29 (0.47)	12 (0.19)				71 (0.57)	53 (0.43)			
	ER	26	7 (0.27)	14 (0.54)	5 (0.19)				28 (0.54)	24 (0.45)			
GRIK5–rs4803523			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	32 (0.78)	6 (0.15)	3 (0.07)	2.16	4	0.706	70 (0.85)	12 (0.15)	0.54	2	0.763
	PR	62	43 (0.69)	16 (0.26)	3 (0.05)				102 (0.82)	22 (0.18)			
	ER	25	19 (0.76)	5 (0.2)	1 (0.04)				43 (0.86)	7 (0.14)			
GRIK5–rs10407506			**CC**	**CT**	**TT**				**C**	**T**			
	NR	41	35 (0.85)	4 (0.1)	2 (0.05)	8.79	4	0.066	74 (0.9)	42 (0.1)	2.03	2	0.363
	PR	62	42 (0.68)	19 (0.3)	1 (0.02)				103 (0.83)	61 (0.17)			
	ER	26	17 (0.65)	9 (0.35)	0 (0)				43 (0.83)	16 (0.17)			

*No significant after multiple correction.

Dr. Bárbara Arias
Anthropology Unit, Department of Animal Biology
Faculty of Biology

Supervisor's report on the contribution of the PhD applicant to the article.

Dr. Bárbara Arias Sampériz, assistant lecturer at the Department of Animal Biology of the Faculty of Biology, University of Barcelona and supervisor of the present doctoral thesis by Marina Mitjans, hereby certifies that the participation of the PhD applicant in the article "Exploring genetic variability at PI, GSK3, HPA and glutamatergic pathways in lithium response: association with *IMPA2*, *INPP1* and *GSK3B genes*" included the following tasks:

- Participation in the conception and design of the study
- Molecular analysis design
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Dr. Bárbara Arias

Barcelona, November 10th 2014

Hypothalamic-pituitary-adrenal system, neurotrophic factors and clozapine response: association with *FKBP5* and *NTRK2* genes.

M. Mitjans*, R. Catalán*, M. Vázquez, A. González-Rodríguez, R. Penadés, A. Pons, G. Massana, J. Munro, MJ. Arranz, B. Arias. *Co-First authors

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Hypothalamic-pituitary-adrenal system, neurotrophic factors and clozapine response: association with *FKBP5* and *NTRK2* genes.

M. Mitjans[1,2*], R. Catalán[2,3,4,5 *], M. Vázquez[3], A. González-Rodríguez[3], R. Penadés[2,3,4,5], A. Pons[3], G. Massana[2,3], J. Munro[6], MJ. Arranz[2,7], B. Arias[1,2]

*1) Facultat de Biologia, Universitat de Barcelona / Institut de Biomedicina de la Universitat de Barcelona (IBUB), Barcelona, Spain. 2) Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM). 3) Clinical Institute of Neurociences, Hospital Clínic, Barcelona, Spain .4) Departament of Psychiatry and Clinical Psychobiology, University of Barcelona, Barcelona, Spain. 5) Institut d'Investigació Biomedica Agustı Pi i Sunyer (IDIBAPS), Barcelona, Spain. 6) Optimal Medicine Ltd, 24 Cornhill, London EC3V 3ND, UK.7) Fundació Docència i Recerca Mútua Terrassa - Hospital Universitari Mútua Terrassa / Hospital de la Santa Creu i Sant Pau, Barcelona, Spain.*Co-First Authors.*

Abstract

Clozapine is an atypical antipsychotic drug known as being more effective than traditional antipsychotics for patients with poor response or resistance to treatment. It has been demonstrated that clozapine modulates hypothalamic-pituitary-adrenal (HPA) activity and affects central BDNF levels, which could explain part of its therapeutic efficacy. In this study, we investigated the role of genes related to the HPA axis (*FKBP5* and *NR3C1*) and neurotrophic factors (*BDNF* and *NTRK2*) on clinical response to clozapine in 591 schizophrenia patients. We found significant allelic and genotype associations between *FKBP5*-rs1360780, *NTRK2*-rs1778929 and *NTRK2*-rs10465180 polymorphisms and clozapine response. The haplotypes composed by rs3777747-rs1360780-rs17542466-rs2766533 (*FKBP5*) and rs1619120-rs1778929-rs10465180 (*NTRK2*) were also nominally significant. Our results suggest that genetic variability in *FKBP5* and *NTRK2* genes may partially explain clinical response to clozapine. Further studies are needed in order to clarify the involvement of these genes in clinical response to atypical antipsychotics.

Key words: clozapine, pharmacogenetics, *FKBP5*, *NR3C1*, *BDNF*, *NTRK2.*

Although antipsychotic drugs are the best means available for symptomatically treating individuals suffering from schizophrenia, resistance to antipsychotic treatment has been described in about 50% of schizophrenic patients (Miyamoto et al., 2005). Clozapine (CLZ) is an atypical antipsychotic drug known as being more effective than traditional antipsychotics for schizophrenic patients with poor response or resistance to treatment. Approximately 50% of patients who do not respond to other antipsychotics benefit from CLZ (Reynolds, 2012).

While most of the other antipsychotics basically antagonize the dopamine D2 and serotonin 2A receptors, CLZ exerts its action on a wide range of receptors such as serotonergic, dopaminergic, histamine, adrenergic or muscarinic receptors, which could explain differences in its effectiveness compared to other atypical antipsychotics (Arranz et al., 2011).

Since CLZ is a high-affinity antagonist of serotonin and dopamine receptors, a large number of studies have tried to investigate their role in CLZ response (Souza et al., 2010; Arranz et al., 2011). However, studies involving other CLZ targets are less frequent. .

Several studies have demonstrated that atypical antipsychotics may modulate the hypothalamic-pituitary-adrenocortical (HPA) axis (Walker et al., 2008) and neurotrophic factors (Bai et al., 2003), both related to the aetiology of schizophrenia. Elevated baseline cortisol secretion has been detected in schizophrenic patients, especially in drug naive patients. It has also been found that atypical antipsychotics significantly reduce adrenocorticotropic hormone (ACTH) and cortisol secretion in patients with schizophrenia (Zhang et al., 2005). Furthermore, when patients are withdrawn from atypical antipsychotics, cortisol levels rise in correlation with negative symptoms (Zhang et al., 2005). In this sense, atypical antipsychotics have the potential to dampen HPA activity, which may partially explain their therapeutic action (Walker et al., 2008).

On the other hand, epidemiological, genetic and clinical neurobiological reports indicate that the

pathophysiological origins of schizophrenia may arise from abnormalities in brain development (Arnold, 1999). The brain-derived neurotrophic factor (BDNF) is involved in the development, survival and functional maintenance of neurons, and plays a role in the regulation of expression of dopamine-related systems. Moreover, CLZ has been reported to affect central BDNF levels in a preclinical study (Bai et al., 2003).

Based on this evidence, the aim of this study was to analyse genetic variants in genes related to the HPA axis [*FKBP5* (FK506 binding protein 5) and *NR3C1* (Nuclear Receptor Subfamily 3, Group C, Member 1)] and neurotrophic factors [*BDNF* and *NTRK2* (neurotrophic tyrosine receptor kinase family 2)] and test if they explained variability in clinical response to CLZ in schizophrenic patients.

We collected clinical data from 591 unrelated patients (32.2% females) with schizophrenia according to *Diagnostic and Statistical Manual of Mental Disorders-III-R* (DSMIII-R), all treated with CLZ. Patients were British Caucasians recruited in hospitals in London, Cambridge and Burnley (United Kingdom). Clinical response was retrospectively assessed based on medical notes using the *Global Assessment Scale* (GAS) (Endicott et al., 1976). A 20-point improvement in GAS scores after a minimum of 3 months treatment with CLZ was considered as cut-off for response. According to these criteria, the sample was divided into 437 responders (Rp) and 154 non-responders (n-Rp) to CLZ. Clozapine was the only antipsychotic administered. Ethical approval was obtained for these studies.

Genomic DNA was extracted from blood samples from each participant, according to standard protocols. Several polymorphisms at the *FKBP5* (rs3777747, rs1360780, rs17542466, rs2766533), *NR3C1* (rs2963156, rs1837262, rs4634384, rs4912910), *BDNF* (rs11030076, rs11030096, rs6265, rs1552736) and *NTRK2* (rs1619120, rs1778929, rs10465180, rs4388524) genes were genotyped using KASP™ (Kompetitive Allele Specific PCR) technology by Design (LGC Genomics). Polymorphisms were selected based on previous literature and the SYSNPS program for tagSNPs detection (www.sysnps.org).

EpiInfo v.3.5.1 was used to calculate Hardy-Weinberg equilibrium for genotype frequencies using chi-square tests. Plink v1.03 was used to perform association analyses between the groups of patients with different CLZ response. Odds ratios (OR) with 95% confidence intervals (CI) were estimated for the effects of high-risk genotypes. Empirical p-values were generated using the max(T) permutation approach (10000 permutations) for pointwise estimates (EMP1) as well as corrected for all comparisons (EMP2). The study had an 80% power (95% CI) to detect OR in a range of 1.72 to 1.94 for CLZ response according to the range of obtained MAFs (MAF: 0.47 to 0.16, respectively). Haploview 3.2 was used to generate a linkage disequilibrium map. Haplotype analyses were conducted using the 'R' software (v.2.2.1) by the "haplo.stat" package. Sex was included in the haplotype analyses as a covariate. Rare haplotypes less frequent than 1% were excluded from the analyses. The global significance of the results for haplotype analyses was estimated using permutation (10000 permutations) to confirm the asymptotic p-values.

Genotype distributions of all SNPs were found to be in Hardy-Weinberg equilibrium (data available on demand). Significant differences were observed for genotype (χ^2=7.55, *df*=2, *p*=0.022) and allele (χ^2=4.54, *df*=1, *p*=0.033) distributions of the *FKBP5* rs1360780 polymorphism between Rp and n-Rp (Table 1). TT-homozygous presented 2.11 times higher risk of non-response than C-carriers [χ^2=7.46, *df*=1, *p*=0.006; OR= 2.11; 95%CI (1.22-3.64)]. However, these associations did not remain significant after permutation analyses. The *FKBP5* A-T-A-G haplotype composed by rs3777747-rs1360780-rs17542466-rs2766533 was associated with poor response (*p*=0.012; *simulated p*=0.013) (Table 2).

Regarding the *NTRK2* gene, significant differences were found for genotype and allele distributions for both polymorphisms, rs1778929 [genotype: (χ^2=6.87, *df*=2, *p*=0.032); allele (χ^2=5.76, *df*=1, *p*=0.016)] and rs10465180 [genotype: (χ^2=9.52, *df*=2, *p*=0.008); allele (χ^2=6.58, *df*=1, *p*=0.011)] (Table 1). Rs1778929 TT-homozygous presented 1.7 times higher risk of non-response than C-carriers [χ^2=6.62, *df*=1, *p*=0.011; OR=1.7 95%CI (1.13-2.59)], while rs10465180 CC-homozygous presented 2.15 times more risk of non-response than T-carriers [χ^2=9.39, *df*=1, *p*=0.002; OR= 2.15 95%CI (1.3-3.55)]. However, only the last reported association remained significant after permutation (EMP1=0.003; EMP2=0.033). Haplotype analyses showed that the G-C-T haplotype composed by rs1619120-rs1778929-rs10465180 was associated with better response (*p*=0.009; *simulated p*=0.009) while the G-T-C

haplotype was associated with poor response (p=0.011; *simulated* p=0.011) (Table 2). No other significant results were found between any of the other analyzed polymorphisms and CLZ response.

Our study analysed the role of genes related to HPA axis and neurotrophic factors in CLZ response.

It has been shown that antipsychotics, especially atypical ones such as CLZ, may suppress HPA activity by reducing cortisol levels and this may be one component of the drug's therapeutic action (Walker et al., 2008). The FKBP5 protein is of special interest since it modulates HPA axis reactivity via glucocorticoid receptor (NR3C1) sensitivity and signaling (Binder, 2009). Moreover, the T-allele of the *FKBP5*-rs1360780 has been associated with higher FKBP5 induction by glucocorticoids (Binder, 2009). Our study has shown differences in allele, genotype and haplotype distributions of the *FKBP5*-rs1360780 polymorphism and CLZ response. Although the role of *FKBP5* gene in treatment response has been widely demonstrated, especially in reference to antidepressants (Binder, 2009), there are no studies investigating the role of this gene in atypical antipsychotic response.

With regards to neurotrophic factors, we did not find any associations between the BDNF polymorphisms investigated and CLZ response. This result agrees with a previous pharmacogenetic study which also failed to find association between the *BDNF* Val/Met polymorphism and CLZ response (Hong et al., 2003). Interestingly, we found differences in allele, genotype and haplotype distributions of two *NTRK2* polymorphisms (rs1778929 and rs10465180) between patients who responded and patients who did not respond to CLZ treatment. To our knowledge, these are novel findings and no other studies investigating the role of this gene and CLZ response have been reported.

Our study has some limitations. The possible functional effects of some of the analysed markers are still under investigation. Response assessment was conducted retrospectively from medical notes which could produce inaccuracies. However, response assessments were conducted by two experienced researchers in order to minimise experimental background.

Some of the allele and genotype associations did not survive multiple testing in our sample. However, subsequent haplotype analyses, which are considered a more powerful genetic and statistical approach, were in the same direction of these previous associations supporting our findings.

In conclusion, our results suggest that genetic variants in the *FKBP5* and *NTRK2* genes may play a role in CLZ treatment outcome in schizophrenia patients. Our study provides evidence of the involvement of the HPA axis and of neurotrophic factor in modulating CLZ response. Further studies are necessary to confirm the reported associations. The detection of individual genetic differences in the response to CLZ may provide new strategies for the treatment of schizophrenia.

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Conflicts of interest:
A. Pons has provided consulting services to Janssen-Cilag, Johnson&Johnson and he is speaker/advisory board member for Janssen-Cilag. M. Mitjans, R. Catalán, M. Vázquez, A. González-Rodríguez, R. Penadés, G. Massana, J. Munro, MJ. Arranz and B. Arias do not have conflicts of interest.

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Table 1. Allele and genotype distributions of the polymorphisms analysed according to clozapine response.

	Allele		N	Non-Responders (%)		Responders (%)		Allelic	Non-Responders (%)			Responders (%)			Genotype
	1	2		1	2	1	2	*p-values*	11	12	22	11	12	22	*p-values*
FKBP5															
rs3777747	A	G	559	170 (59)	118 (41)	478 (57.6)	352 (42.4)	0.670	50 (34.7)	70 (48.6)	24 (16.7)	136 (32.8)	206 (49.6)	73 (17.6)	0.905
rs1360780	C	T	582	189 (62.6)	113 (37.4)	597 (69.3)	265 (30.7)	**0.03**	63 (41.7)	63 (41.7)	25 (16.6)	203 (47.1)	191 (44.3)	37 (8.6)	**0.022**
rs17542544	A	G	576	236 (79.2)	62 (20.8)	650 (76.1)	204 (23.9)	0.277	89 (59.7)	58 (38.9)	2 (1.3)	248 (58.1)	154 (36.1)	25 (5.8)	0.078
rs2766533	G	A	584	158 (51.7)	148 (48.3)	436 (50.6)	426 (49.4)	0.751	39 (25.5)	80 (52.3)	34 (22.2)	114 (26.4)	208 (48.3)	109 (25.3)	0.655
NR3C1															
rs2963156	C	T	579	236 (78.6)	64 (21.4)	685 (79.8)	173 (20.2)	0.665	94 (62.7)	48 (32)	8 (5.3)	272 (63.4)	141 (32.9)	16 (3.7)	0.696
rs1837262	A	C	569	195 (66.8)	97 (33.2)	588 (69.5)	258 (30.5)	0.386	65 (44.5)	65 (44.5)	16 (11)	199 (47.1)	190 (44.9)	34 (8)	0.546
rs4634384	C	T	583	164 (53.9)	140 (46.1)	440 (51)	422 (49)	0.383	38 (25)	88 (57.9)	26 (17.1)	105 (24.4)	230 (53.3)	96 (22.3)	0.391
rs4912910	G	A	585	204(66.7)	102 (33.3)	580 (67.1)	284 (32.9)	0.882	71 (46.4)	62 (40.5)	20 (13.1)	197 (45.6)	186 (43.1)	49 (11.3)	0.789
BDNF															
rs11030076	G	A	584	153 (50.3)	151 (49.7)	451(52.2)	413 (47.8)	0.574	39 (25.7)	75 (49.3)	38 (25)	113 (26.1)	225 (52.1)	94 (21.8)	0.705
rs11030096	T	C	579	174 (57.6)	128 (42.4)	453 (52.9)	403 (47.1)	0.159	50 (33.1)	74 (49)	27 (17.9)	114 (26.6)	225 (52.6)	89 (20.8)	0.301
rs6265	C	T	586	250 (82.2)	54 (17.8)	696 (80.2)	172 (19.8)	0.435	104 (68.4)	42(27.6)	6 (4)	279 (64.3)	138 (31.8)	17 (3.9)	0.627
rs1552736	G	A	572	177 (59.4)	121 (40.6)	521 (61.6)	325 (38.4)	0.505	57 (38.2)	63(42.3)	29 (19.5)	156 (36.9)	209 (49.4)	58 (13.7)	0.162
NTRK2															
rs1619120	G	A	576	174 (57.6)	128 (42.4)	516 (60.7)	334 (39.3)	0.346	50 (33.1)	74 (49)	27 (17.9)	155 (36.5)	206 (48.5)	64 (15)	0.631
rs1778929	C	T	581	139 (45.7)	165 (54.3)	461 (53.7)	397 (46.3)	**0.016**	35 (23)	69 (45.4)	48 (31.6)	123 (28.7)	215 (50.1)	91 (21.2)	**0.032**
rs10465180	T	C	581	179 (59.3)	123 (40.7)	580 (67.4)	280 (32.6)	**0.011**	59 (39.1)	61 (40.4)	31 (20.5)	196 (45.6)	188 (43.7)	46 (10.7)	**0.008**
rs4388524	T	C	582	225 (74)	79 (26)	639 (74.3)	221 (25.7)	0.921	83 (54.6)	59 (38.8)	10 (6.6)	239 (55.6)	161 (37.4)	30 (7)	0.951

*Empirical p-values (EMP1, EMP2) of the significant p-values are reported in the text.

Table 2. Haplotype analyses according to clozapine response.

Haplotype	Freq	n-Rp : Rp (freq)	*p-values*	*Sim p-value*	*Global Score Statistics*
FKBP5 (rs3777747-rs1360780-rs17542466-rs2766533) - (D'=0.87; r^2=0.52)					
GCAG	0.393	0.368 : 0.403	0.327	0.328	
ATAA	0.252	0.265 : 0.246	0.455	0.454	
ACGA	0.198	0.176 : 0.204	0.209	0.209	*Global-stat*=11.71, *df*=7
ATAG	0.073	0.103 : 0.061	**0.016**	**0.017**	*p-value* =0.111
ACGG	0.035	0.031 : 0.038	0.804	0.803	*Global sim p-value*=0.123
GCAA	0.032	0.037 : 0.029	0.554	0.579	
ACAA	0.011	0.004 : 0.014	0.348	0.356	
NR3C1 (rs2963156- rs1837262- rs4634384) – (D'=1.0; r^2=0.24)					
CAT	0.482	0.462 : 0.490	0.381	0.392	*Global-stat*=3.32, *df*=3
CCC	0.309	0.317 : 0.306	0.731	0.726	*p*=0.343
TAC	0.205	0.214 : 0.202	0.658	0.658	*Global sim p-value*=0.334
BDNF (rs11030076-rs11030096- rs6265) – (D'=0.89; r^2=0.19)					
GCC	0.440	0.415 : 0.448	0.291	0.288	
ATC	0.285	0.315 : 0.275	0.186	0.186	*Global-stat*=6.85, *df*=5
ATT	0.186	0.176 : 0.189	0.662	0.66	*p* =0.231
GTC	0.068	0.084 : 0.063	0.157	0.158	*Global sim p-value*=0.228
ACC	0.013	0.008 : 0.015	0.348	0.373	
NTRK2 (rs1619120-rs1778929-rs10465180) – (D'=0.52; r^2=0.21)					
GCT	0.499	0.434 : 0.522	**0.01**	**0.01**	
ATC	0.247	0.262 : 0.243	0.437	0.44	*Global-stat=12.55, df*=5
ATT	0.149	0.157 : 0.145	0.688	0.69	***p* =0.03**
GTC	0.085	0.121 : 0.073	**0.01**	**0.009**	***Global sim p-value*=0.02**
GCC	0.014	0.009 : 0.028	0.124	0.152	

Abbreviations: Freq: frequencies; n-Rp: non-responder; Rp: Responders; Sim: Simulated

Dr. Bárbara Arias
Anthropology Unit, Department of Animal Biology
Faculty of Biology

Supervisor's report on the contribution of the PhD applicant to the article.

Dr. Bárbara Arias Sampériz, assistant lecturer at the Department of Animal Biology of the Faculty of Biology, University of Barcelona and supervisor of the present doctoral thesis by Marina Mitjans, hereby certifies that the participation of the PhD applicant in the article "Hypothalamic-pituitary-adrenal system, neurotrophic factors and clozapine response: association with *FKBP5* and *NTRK2* genes" included the following tasks:

- Participation in the conception and design of the study
- Molecular analysis design
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Dr. Bárbara Arias

Barcelona, November 10th 2014

5. Global Summary of Results

Hypothesis 1 "*Genetic variability at genes of the endocannabinoid system will be associated to the lack of clinical response and/or remission to citalopram treatment in major depressive patients"* was tested in the studies:

- **Mitjans et al., 2012**. *Genetic variability in the endocannabinoid system and 12-week clinical response to citalopram treatment: the role of the CNR1, CNR2 and FAAH genes*. Journal of Psychopharmacology, Oct;26(10):1391-8.
- **Mitjans et al., 2013**. *Screening genetic variability at the CNR1 gene in both major depression aetiology and clinical response to citalopram treatment*. Psychopharmacology, Jun;227(3):509-19.

Referring to **hypothesis 1** the following results were found:

In our first study (Mitjans et al., 2012), genetic variability at *CNR1 (rs1049353)*, *CNR2* (rs806368) and *FAAH* (rs324420) genes was analyzed in a sample of 154 depressive patients treated with CIT and evaluated for clinical response (4th week) and remission (12th week).

Clinical response was considered when a decrease of at least 50% in the Hamilton Depression Rating Scale (HDRS) baseline score was observed at 4th week. Following this criteria, 101 patients were classified as responders and 53 as non-responders. Remission of the episode was considered when HDRS score was equal or under 7 by the end of 12th week of follow-up. In this sense, 99 patients were classified as remitters and 54 as no-remitters.

Genotype distributions of all SNPs analyzed were in Hardy-Weinberg equilibrium. No significant differences were found when we compared genotype distribution of the 3 SNPs between responders/no-responders (4th week) or remitters/no-remitters (12th week) to CIT.

The longitudinal 12-weeks follow-up of clinical response showed that rs1049353-GG carriers presented a better response to antidepressant treatment compared to the rs1049353-A allele carriers ($F_{(2.78,\ 270.4)}$= 2.914, p=0.038). Stratification for gender revealed that this effect is originated by the subgroup of male patients, being the GG-homozygous men who showed a better response along the 12 week follow-up (F(2.78, 270.4)=5.85, p=0.001). Regarding *CNR2* gene, we observed that

rs806368-AA carriers presented higher scores on the HDRS scale tan G allele carriers along the 12 week follow-up ($F_{(1, 104)}$=11.432, p=0.001).

Based on the previous evidence, showing that rs1049353 polymorphism of the *CNR1* gene seems to play a role in the response to CIT treatment, we proceed to further analyzed genetic variability at *CNR1* gene (rs806368, rs1049353, rs806371, rs806377 and rs1535255) in the same sample of MDD patients (Mitjans et al., 2013).

Genotype distributions of all SNPs analyzed were in Hardy-Weinberg equilibrium. No significant differences were found when we compared genotype distribution of the 5 SNPs between responders/no-responders (4^{th} week) to CIT.

Significant differences in genotype and allele distributions between remitters and non-remitters at week 12 were observed for two SNPs at *CNR1*: rs806371 (genotype: p=0.045; allele: p=0.016) and rs806368 (genotype: p=0.029; allele p=0.021). Indeed, TT-homozygous for rs806371 had nearly 3 times more risk for non-remission that G-carriers [p=0.012; OR=2.8 95% CI (1.14-7.01)], while TT-homozygous for rs806368 had almost 2.7 times more risk of non-remission that C-carriers [p=0.008; OR=2.64 95% CI (1.20-5.89)]. The haplotype analysis showed a significant association between the rs806371-rs1049353-rs806371 haplotype and remission (Global-stat=10.5; df=4; p=0.032; sim p=0.029), being the C-G-G haploblock less frequent in the non-remitters than in remitters (p=0.007; sim p=0.006).

The longitudinal analyses of the clinical response to CIT showed significant effects of the rs806368 polymorphism. We observed that the C carriers presented a better response to antidepressant treatment along the follow-up than TT homozygous. Stratification for gender revealed that this effect is originated by the subgroup of male patients, being the C-carrier men who presented a better response along the 12 week follow-up ($F_{(2.76, 284.98)}$=3.233, p=0.026).

Hypothesis 2 "*Genetic variability at genes related to phosphoinositide (PI), glycogen synthetase kinase-3 (GSK3), hypothalamic-pituitary-adrenal (HPA) and glutamatergic pathways will be associated the lack of clinical response to Lithium in Bipolar Disorder patients"* was tested in the study:

- "**Mitjans et al. Submitted.** *Exploring genetic variability at PI, GSK3, HPA and glutamatergic pathways in lithium response: association with IMPA2, INPP1 and GSK3B genes.* Submitted to Journal of Clinical Psychopharmacology".

Referring to **hypothesis 2** the following results were found:

Genetic variability at genes related to PI (*INPP1, MARCKS, IMPA1, IMPA2, ITPKC PLCG1*), GSK3 (*GSK3B*, *GSK3A, CREB1*), HPA (*FKBP5, CRHR2, CRHR1*) and glutamatergic (*GRIA2, GABRB2, GRIK2, GRIK5*) systems were analyzed.

The efficacy of Li treatment was assessed according to the following criteria: (a) excellent responders (ER): patients presenting a 50% reduction of the episodes after the introduction of Li in monotherapy, (b) partial responders (PR): patients presenting a 50% reduction of the episodes after the introduction of Li but on polytherapy (other mood stabilizer, antidepressant or antipsychotics), (c) non-responders (NR): patients who did not reduce at least a 50% of the episodes and patients who required electroconvulsive therapy. Following these criteria, 26 patients were classified as ER, 62 as PR and 43 as NR to Li treatment.

Genotype distributions of the SNPs were all in Hardy-Weinberg equilibrium. Significant differences were found in genotype and allele distributions when we compared the three different groups of Li responders for the rs669838 (*IMPA2*) (genotype: p=0.035; allele: p=0.015), rs909270 (*INNP1*) (genotype: p=0.038; allele: p=0.038), rs11921360 (*GSK3β*) (genotype: p=0.046; allele: p=0.0057) and rs28522620 (*GRIK2*) (genotype: p=0.048; allele: p=0.016) polymorphisms. P-values were not significant after permutation testing.

Second, we pooled together PR and NR groups (PR+NR) and compared them to ER. The effect of the genetic distribution of previous associated SNPs on PR+NR versus ER was tested using a logistic regression model including suicidal behaviour as covariate. Results showed significant association for rs669838 (*IMPA2*) [β=2.31; p=0.021; OR=2.03; 95% CI (1.11-3.72); EMP1=0.018; EMP2=0.07], rs909270 (*INPP1*) [β=2.58; p=0.009; OR=2.45; 95% CI (1.24-4.82); EMP1=0.008; EMP2=0.028] and rs11921360 (*GSK3B*) [β=2.84; p=0.004; OR=2.52; 95% CI (1.33-4.78); EMP1=0.002; EMP2=0.011] with Li response (risk alleles: rs669838-C, rs909270-G and rs11921360-A).

Haplotype analysis showed an association of rs3791809-rs4853694-rs909270 haplotype in *INPP1* gene and Li response. Frequencies of the T-A-G haploblock were more frequent in the group of PR+NR (0.488) than in ER (0.306) (p=0.012; sim p=0.012). T-A-A haploblock, instead, was more frequent in ER than in PR+NR (0.241 vs. 0.12) (p=0.018; sim p=0.019). The rs1732170-rs11921360-rs334558 haplotype in *GSK3B* was also associated with Li response (global p=0.002, global sim p=0.002). The C-C-A haploblock was significantly less frequent in the group of PR+NR (0.299) than in ER (0.552) (p=0.001; sim p=0.001).

Hypothesis 3 *"Genetic variability at genes related to neurotrophic factors and HPA axis will be associated to the lack of clinical response to clozapine in Schizophrenic patients*" was tested in the study:

- "**Mitjans et al. Submitted.** *Hypothalamic-pituitary-adrenal system, neurotrophic factors and clozapine response: association with FKBP5 and NTRK2 genes*. Submitted to Pharmacogenetics and genomics".

Referring to **hypothesis 1.3** the following results were found:

Genetic variability at genes related to the HPA axis (*FKBP5* and *NR3C1)* and neurotrophic factors (*BDNF* and *NTRK2)* were analyzed.

Clinical response was retrospectively assessed based on medical notes using the *Global Assessment Scale* (GAS) (Endicott et al., 1976). A 20-point improvement in GAS scores after a minimum of 3 months treatment with CLZ was considered as cut-off for response. According to these criteria, the sample was divided into 437 responders (Rp) and 154 non-responders (n-Rp) to CLZ.

Genotype distributions of all SNPs were found to be in Hardy-Weinberg equilibrium. Significant differences were observed for genotype (p=0.022) and allele (p=0.033) distributions of the rs1360780 (*FKBP5*) polymorphism between Rp and n-Rp. TT-homozygous presented 2.11 times higher risk of non-response than C-carriers [χ^2=7.46, *df*=1, *p*=0.006; OR= 2.11; 95%CI (1.22-3.64)]. However, these associations did not remain significant after permutation analyses. The A-T-A-G haploblock composed by rs3777747-rs1360780-rs17542466-rs2766533 (*FKBP5*) was associated with non-response (p=0.012; sim p=0.013).

Regarding the *NTRK2* gene, significant differences were found for genotype and allele distributions for both polymorphisms, rs1778929 (genotype: p=0.032; allele: p=0.016) and rs10465180 (genotype: p=0.008; allele p=0.011). The rs1778929 TT-homozygous presented 1.7 times higher risk of non-response than C-carriers [χ^2=6.62, df=1, p=0.011; OR=1.7 95%CI (1.13-2.59)], while rs10465180 CC-homozygous presented 2.15 times more risk of non-response than T-carriers [χ^2=9.39, df=1, p=0.002; OR= 2.15 95%CI (1.3-3.55)]. Only the last reported association remained significant after permutation (EMP1=0.003; EMP2=0.033). Haplotype analyses showed that the G-C-T haploblock composed by rs1619120-rs1778929-rs10465180 was associated with better response (p=0.009; sim p=0.009), while the G-T-C haploblock was associated with poor response (p=0.011; sim p=0.011).

6. Discussion and Conclusions

Discussion

The present thesis, which can be framed in the field of pharmacogenetics in psychiatry, was aimed to study how genetic variability at genes related directly or indirectly to the mechanism of drug action explain variability in response to treatment. Specifically three hypotheses have been tested with the final result of four articles. Conclusions derived from these studies are discussed below followed by a global discussion.

Citalopram response in Major Depressive Disorder

It has been suggested the participation of the eCB system in the aetiology of MDD based on its participation in physiological processes altered in the disease (motivation, anxiety, cognitive and vegetative functions) (Hill and Gorzalka, 2005; Mangleri and Piomelli, 2007). Moreover, cannabinoid receptors and the enzymes involved in the synthesis and degradation of eCBs are located along neuroanatomical structures and circuits involved in MDD, including the prefrontal cortex, hippocampus, amygdala, hypothalamus and the forebrain monoaminergic circuits (Herkenham, 1991).

The results of our studies in relation to CIT response showed that genetic variability at genes related to the eCB system could play a role in the understanding of clinical response to CIT treatment. As a summary, we found that: i) there was no association between clinical response at 4th week and genetic variability at *CNR1*, *CNR2* or *FAAH* genes, ii) an association between *CNR1* gene and clinical remission at 12th week was detected iii) an effect of *CNR1* gene on longitudinal response (along the 12th week follow-up) was also found, and iv) *CNR2* gene was involved to the severity of the MDD episode.

With respect to *CNR1* gene, a significant effect of rs806368 and rs806371 polymorphisms and the haploblock rs806368-rs1049353-rs806371 on remission was found. Moreover, the longitudinal study showed an influence of both rs806368 and rs1049353 polymorphisms (*CNR1*) on CIT response along the 12 week follow-up. The effect was clearly related to the male sample.

With respect to the rs806368 polymorphism no association studies considering its role in clinical response to antidepressants have previously published; however

it has been involved in substance use disorder or cannabis dependence (Zuo et al., 2007; Agrawal et al., 2009). Interestingly, the rs806368 polymorphism is located in the 3'-UTR region. The 3'-UTR region is considered a target region for miRNAs (short RNA sequences) that can regulate gene expression both at a transcriptional and translational level and mediate posttranscriptional gene silencing by directly binding this 3' untranslated region (UTR) of target mRNA (Fabbri et al., 2008).

In relation to the rs1049353, several studies to date have examined its role in psychiatric illness, particularly MDD. Although some interesting results have been reported, the direction of the effect is still under investigation. Monteleone and colleagues have shown the contribution of the A allele to the probability of having MDD (Monteleone et al., 2010). More recently, one report has demonstrated the opposite effect of A allele in two separate populations: carriers of A allele are protected against the development of anhedonia and MDD in adult women following early life stress or abuse (Agrawal et al., 2012). However, this effect was not entirely replicated by a second group, although they did note that it was a moderate risk reduction in carriers of the A allele (Pearson et al., 2013).

Specifically related to antidepressant response, Domschke and colleagues demonstrated that individuals carrying the rs1049353 G allele were more likely to exhibit antidepressant resistance than those with the A allele (Domschke et al., 2008), suggesting that the A allele may confer greater antidepressant responsiveness. This effect was found primarily in women, and especially those that presented with melancholic depression with high anxiety (Domschke et al., 2008). On the contrary, our results showed that GG homozygous men exhibit better antidepressant response than A allele carriers. Despite the gender and allele divergences, both studies indicate the involvement of *CNR1* gene to antidepressant response (Domschke et al., 2008; Mitjans et al., 2012), suggesting that there may be some sexual divergence in the role of the eCB system in depression and antidepressant treatment.

It might be hypothesized that gender differences in drug response could also reflect the differences that are found in the aetiology of MDD as physiological and epidemiological studies have shown (Biver et al., 1996; Weissman et al., 1996; Nishizawa et al., 1997; Kendler et al., 2001; Legato, 2010). Studies suggesting a role of estradiol in expression regulation of CB1 receptor mRNA (Gonzalez et al.,

2000; Hill et al., 2007) could explain the differential response by gender found in our study.

These opposite results related to the role of rs1049353 polymorphism in CIT response will require replication and have to be interpreted with caution. Some issues, which are globally discussed below in the global discussion, have to be considered since they should account for the contradictory results: the small sample size and the different definition of CIT response in the studies. In the study of Domschke and colleagues, clinical response was measured by the intra-individual changes of HDRS scores over the 6 weeks study period (Baune et al., 2008). Differently, clinical response in our study was transversally considered when a decrease of at least 50% in the baseline score was observed at week 4 (Baumann, 1996). Clinical remission was also considered, when HDRS scores were equal or under seven by the end of week 12 (Frank et al., 1991). Interestingly, we also considered a longitudinal response based on HDRS change scores during the 12 weeks of CIT treatment which inform about the clinical evolution to mid-long term.

In relation to the fact that genetic variation at *CNR1* gene can predict clinical remission but not the short term clinical response in our sample, it suggests that 4 weeks is not sufficient either to determine whether or not patients will respond to treatment, or to predict clinical mid-long term evolution. These results seem to confirm what defends clinical practice: improving depressive symptomatology caused by antidepressants is slow, requiring usually 6-12 weeks to become apparent (Frazer and Benmansour, 2002).

At molecular level, the rs1049353 polymorphism is a synonymous polymorphism that produces no change in the amino acid threonine at position 453 (Thr453Thr). Although synonymous SNPs have often been called silent or unable to affect functional changes, some reports indicate that there are several mechanisms by which they could bring about such changes. It has been suggested that silent SNPs can affect in vivo splicing events or protein folding and, consequently, the final protein function (Komar, 2007; Sauna et al., 2007). These may have important implications in biology and in the diagnosis and treatment of human diseases. Alternatively, these polymorphisms might not constitute the actual causative

variant, but rather reflect association of other polymorphisms in LD with this locus.

Regarding *CNR2* gene, we observed that rs2501432 was related to severity of depression, indicating that *CNR2* gene appears to be more associated with severity of outcome of the disease than with CIT response. Specifically, rs2501432 G-allele carriers presented higher HDRS scores along the 12 week follow-up than AA homozygous. The rs2501432 polymorphism is a non-synonymous SNP leading to the amino acid substitution of glutamine by arginine at position 63 (Gln63Arg). Interestingly, a study has previously found an association between the rs2501432 polymorphism and increased risk for depression in the Japanese population (Onaivi et al., 2008).

Finally, we did not find association between *FAAH* gene (rs324420) and either response or remission to CIT treatment. The rs324420 is a non-synonymous SNP, which converts a proline residue to threonine (Pro129Thr). Expression studies have shown that individuals carrying this polymorphism may have approximately half of the enzymatic activity of FAAH (Chiang et al., 2004). This reduction in the activity of FAAH might increase levels of the endogenous cannabinoids AEA and 2-AG, thereby increasing the activity of the eCB system. Animal models show that the inhibition of the FAAH enzyme has antidepressant effects (Gobbi et al., 2005). However, to the best of our knowledge, no studies have been previously reported relating the *FAAH* gene to antidepressant response. On a case-control study no significant differences in the genotype and allele frequencies of this polymorphism between MDD patients and healthy controls were found (Monteleone et al., 2010).

With respect multiple testing correction two approaches were performed. In relation to our first study (Mitjans et al., 2012), we consider that there were likely to be excessively exclusive since the selection of the genetic polymorphisms, the sample size and the analyses performed had a directional hypothesis based on previous findings (Cardon and Bell, 2001). In the second study (Mitjans et al., 2013), in which five SNPs in the *CNR1* gene were analyzed, Bonferroni's procedures were taking into account. The significant results referred to the single SNP analyses (rs806368 and rs806371) did not survive the correction. However, approaches including genotype carrier's analyses or haplotypes survived multiple

correction, indicating the involvement of these polymorphisms in CIT remission in our sample.

In conclusion, our results indicate that genetic variability at *CNR1* gene play a role in CIT remission and CIT response along 12 week follow-up. However, more studies are needed to replicate our findings and clarify the direction of the effect. Moreover, further studies are needed in order to analyze in depth the molecular variability associated with endocannabinoid genes in larger samples. New data could help to improve knowledge about the treatment response to antidepressants and also the aetiology of MDD.

Lithium response in Bipolar Disorder

The therapeutic action of Li in BD appears not to result from an effect at a single target site, but rather as the culmination of an integrated re-orchestration of a complex concert of events which effectively adjusts neuronal activity at multiple levels (Jope, 1999). The complex effects of Li stabilize neuronal activities, support neural plasticity, and provide neuroprotection. Three main interacting systems appear most critical: i) modulation of neurotransmitters by Li likely readjusts balances between excitatory and inhibitory activities, and decreased glutamatergic activity may contribute to neuroprotection, ii) Li modulates signals impacting on the cytoskeleton, a dynamic system contributing to neural plasticity, at multiple levels, including GSK3β, cyclic AMP dependent kinase, PKC, neurotrophic factors or HPA activity which may be critical for the neural plasticity involved in mood recovery and stabilization, (iii) Li adjusts signalling activities regulating second messengers, transcription factors, and gene expression (Lenox and Wang, 2003).

Taking this evidence into account, our study analyzed the potential association of genetic variability at PI and GSK3 pathways, HPA axis, and glutamatergic system with Li response in BD. Our results showed that genetic variability at *INPP1*, *IMPA2*, *GSK3β* and *GRIK2* genes could play a role in the understanding of Li response.

Since the effect of Li on the PI pathway has long been considered one of the most important mechanisms of therapeutic action of this drug in BD, a large number of studies have tried to identify genetic variants within genes of this pathway which could predict response to Li. Our results are in line with these studies reporting an

effect of genetic variability at this system and Li response. Particularly, we found an effect of rs669838 (*IMPA2*) in Li response. The *IMPA2* gene is located in a region thought to be a BD susceptibility locus (18p11.2) (Stine et al., 1995). A previous study showed two trends for association between two polymorphisms (rs3786282 and 599+97G>A) of this gene and response to Li in BD patients (Dimitrova et al., 2005).

Our results also showed an effect of rs909270 (*INNP1*) in Li response. Another polymorphism in *INPP1* gene, the C937A, has been previously associated with response to Li in a Norwegian sample but not in two independent samples (Steen et al., 1998; Michelon et al., 2006) .

Regarding genetic variability at *GSK3β* gene, which encode for a key element in the mechanism of action of Li, we found association with rs11921360 and Li response. This association is in line with a previous study that found an effect of another polymorphism of the *GSK3β* gene in Li response (Benedetti et al., 2005). However, it was not confirmed in two other studies (Michelon et al., 2006; Szczepankiewicz et al., 2006).

We found association between rs28522620 at *GRIK2* gene and Li response. The *GRIK2* gene had been previously reported as candidate gene conferring a predisposition to BD (Buervenich et al., 2003; Escamilla and Zavala, 2008). Association studies on glutamatergic system and Li response are scarce in the literature. Firstly, a GWAS showed that a SNP in the GluR2 gene was associated with the risk for recurrence among patients treated with Li (Perlis et al., 2009). Secondly, no association was found between *GRIN2B* gene and prediction of Li outcome (Szczepankiewicz et al., 2009a).

In our study, genetic variability at HPA axis was not associated with Li response. To the best of our knowledge, no other studies investigating the role of the same genes analyzed in our study related to the HPA system and Li response were available. The only study investigating genetic variability in genes involved to HPA axis did not find association between *NR3C1* gene and Li response (Szczepankiewicz et al., 2011).

Our study has some limitations. Due to the tertiary nature of the Bipolar Disorder Program, some of the subjects included in this study could be categorized

as difficult-to-treat patients, thus generalization of our results should be done with caution. Moreover, response definition was evaluated retrospectively, with the inherent limitations associated with recall bias, missing information, or the fact that the treatment has not followed a strict research protocol. The relative small size of our sample limits the power to detect small differences.

The polymorphisms associated to Li response in our study are intronic. Recent data from the ENCODE (*Encyclopedia of DNA Elements*) project has revealed the importance of intronic and intergenic variants as regulatory elements of gene expression acting as microRNAs and/or epigenetic targets (ENCODE Project Consortium, 2012). Alternatively, as commented before, they might not constitute the actual causative variant, but rather reflect association of other polymorphisms in LD with this locus.

In conclusion, our results indicate a possible role of genes related to PI (*INPP1, IMPA2),* GSK3ß *(GSK3B)* and glutamatergic (*GRIK2*) pathways in Li response. Unfortunately, results about *GRIK2* gene have not survived multiple correction, so we cannot conclude firmly its role in Li response.

Our results should be interpreted cautiously taken into account limitations mentioned above. As Li response is a complex trait, further studies with larger samples well-characterized are warranted to assess the strength of the reported associations.

Clozapine response in Schizophrenia

It has been shown that antipsychotics, especially atypical ones such as CLZ, may suppress HPA activity by reducing ACTH and cortisol secretion in patients with SCZ (Zhang et al., 2005; Walker et al., 2008).

Our results showed the involvement of the *FKBP5* gene in CLZ response. Significant allele and genotype associations were found between the rs1360780 polymorphism and CLZ response. The haplotype composed by rs3777747-rs1360780-rs17542466-rs2766533 (*FKBP5*) was also significant being the haploblock A-T-A-G associated with non-response to CLZ.

Several lines of evidence suggest that the *FKBP5* gene is an important functional regulator of the GR complex (Binder, 2009). The binding of *FKBP5* to the GR complex decreases the affinity of cortisol binding, followed by a deficient receptor nuclear translocation, and therefore reduces GR sensitivity (Binder, 2009). It has been demonstrate that the rs1360780 polymorphism (*FKBP5)* has functional effects despite being located within intron 2. Interestingly, the rs1360780 T allele has been associated with higher FKBP5 induction by GR activation (Binder et al., 2004). Although the role of *FKBP5* gene in treatment response has been widely studied, especially in reference to antidepressants (Binder, 2009), there are no studies investigating its role in atypical antipsychotic response, so, we cannot compared our results.

The levels of neurotrophic factors in schizophrenic patients have been reported to be altered. Recently, Zhang and colleagues have shown that BDNF levels were significantly lower in drug-free patients with SCZ (Zhang et al., 2012b). Lee and colleagues have also demonstrated that BDNF levels decreased significantly in unmedicated schizophrenic patients and elevated after successful antipsychotic treatment which parallel symptom improvement of the patients (Lee et al., 2011). Moreover, it has been reported that CLZ up-regulates BDNF mRNA expression in a preclinical study (Bai et al., 2003). BDNF functions through its high-affinity receptor NTRK2 (Squinto et al., 1991), which has also been found decreased in post-mortem schizophrenic subjects (Weickert et al., 2005).

Our results did not show the involvement of any of the polymorphisms analyzed at BDNF gene and CLZ response. Our results agree with a previous pharmacogenetic study which also failed to find association between the *BDNF* Val66Met (rs6265) polymorphism and CLZ response (Hong et al., 2003). Contrary, a previous study found association between a *BDNF* microsatellite and antipsychotic response showing that the long alleles (172–176bp) were more prevalent among antipsychotic responders (Krebs et al., 2000). A more recent study also reported that the short allele was associated with poor response to risperidone (Xu et al., 2010a).

Interestingly, we found differences in allele, genotype and haplotype distributions of two *NTRK2* polymorphisms (rs1778929 and rs10465180) between patients who responded and patients who did not respond to CLZ treatment.

Previous studies have shown that *NTRK2* gene polymorphisms rs2769605, rs1387923, and rs1565445 were associated with mood disorders or antidepressant response (Bremer et al., 2007; Smith et al., 2009; Li et al., 2013). As far as we know, no literature have reported the association between genetic variability at *NTRK2* gene and either SCZ or CLZ response.

Some of the allele and genotype associations in our sample did not survive multiple correction based on permutations. However, subsequent haplotype analyses, which are considered a more powerful genetic and statistical approach, survived multiple correction, indicating the involvement of these polymorphisms in CLZ response in our sample.

One of the limitations of this study is the response definition. Response assessment was conducted retrospectively from medical notes which could produce inaccuracies. However, response assessments were conducted by two experienced researchers in order to minimise experimental background.

In conclusion, our results suggest that genetic variants at *FKBP5* and *NTRK2* genes may play a role in CLZ response in schizophrenic patients. To our knowledge, no other studies investigating the role of these genes in CLZ response have been reported. Present results should be regarded as preliminary and might represent a first step of future extensive research aiming to clarify the role of genes related to HPA axis and neurotrophic factors in CLZ response. The detection of individual genetic differences in the response to CLZ may provide new strategies for the treatment of SCZ, as well as, new knowledge about the aetiology of SCZ.

Global Discussion

The purpose of pharmacogenetic studies is to discover genetic predictors of treatment response with the ultimate goal of identifying the most effective and safest treatment for each individual. In the last few years, a large amount of effort has been directed in pharmacogenetics of mental disorders. In this sense, the candidate gene studies carried out to date have yielded a number of associations between the polymorphism of a given gene and clinical response to psychotropic drugs. New approaches such as GWAS have been appeared opening a new door in the investigation of the importance of genetic factors in the variability in psychotropic drug response. Unfortunately, both approaches have still lead to inconclusive results. Moreover, the vast majority of the genetic variants previously reported by candidate gene studies have not been replicated in GWAS. This lack of conclusive findings might depend on several methodological points; some of them will be discussed below.

One very important issue in all genetic studies is the **phenotype definition**. Clinical response to treatment is one of the phenotypes of interest in pharmacogenetic studies. However, response criteria might vary from one study to another. Accordingly, if this criterion is not equivalent, then results should not be comparables between studies. This is an important matter that should be kept in mind when designing studies.

Following the phenotype definition, **clinical heterogeneity** of the sample can both reduce the statistical power to detect true association and lead to the lack of replication between studies. In this sense, for example, antidepressants are used for the treatment of depression, anxiety as well as pain syndromes in a wide spectrum of psychiatric and neurological disorders. Even within a single DSM diagnostic criterion, it is hypothesized that several different pathophysiological disturbances can lead to different groups of patients. Paradoxically, recent studies showed the existence of common genetic pathways across psychiatric diagnoses. This indicates that pharmacogenetic studies as well as classical genetic risk factors studies have to deal not only to heterogeneity within a diagnostic but also with a probable shared etiology among psychiatric illnesses (Cross-Disorder Group of the Psychiatric Genomics, 2013). This biological overlap might partially explain the non-specific use of psychotropic among disorders opening the debate about

measuring response to a drug in a specific diagnostic or just measuring response to a drug in a diagnose-independent way.

Additionally, treatment response to psychotropic medications, as well as mental disorders, is a **complex phenotype**. As a complex trait, drug response is influenced by a large number of genetic variables in conjunction with clinical, demographic and environmental factors. Factors including age, gender, disease severity, lifestyle habits (diet, smoking, alcohol consumption), concomitant treatments and comorbidities, may influence the way how a person reacts to a drug (Arranz and Kapur, 2008).

Regarding the genetic factors, psychotropic medications may act on a large number of different molecular pathways to exert their therapeutic effect, and in turn they may be acted on by a number of different molecular pathways in the process of their absorption, distribution and elimination. Consequently, multiple variants in distinct and converging genetic pathways may independently and/or interactively contribute to a particular drug response (Ising et al., 2009; Horstmann et al., 2010). However, gene variants will only explain a specific part of global pharmacological response.

In this sense, studies of pathway analysis, gene-gene interaction (epistasis) and gene-environment interaction have been recently started carrying out in the field of pharmacogenetics. Although the number of studies is limited and preliminary, these new approaches seem to be able to explain more about the complexity of drug response. Gene-environment interaction studies have been recently started mainly in the study of antidepressant response in MDD (Klengel and Binder, 2013; Uher, 2014).

Another important matter of concern is the relatively **small size of the samples** studied in candidate gene studies and their consequent insufficient statistical power to detect small to moderate genetic effects. The majority of the studies carried out to date have had rather small sample sizes and short periods of follow-up, largely because it is costly and logistically challenging to ascertain and prospectively evaluate patients. To address this issue, efforts have been made to combine data across studies in meta or mega analyses. Although this approach can be a useful strategy, studies frequently differ so considerably in design, patient

populations and outcome measures raising serious questions about the comparability of results across studies.

Limitations previously commented become even more evident when the pharmacogenetic approach is based in GWAS' methodology. This design requires larger samples than candidate gene studies, patients need to have a specific diagnosis and have to be treated with the same drug. In this sense, the best strategy is to collaborate undertaking multicenter patient recruitment to improve sample sizes. In addition, the use of standardized protocols for defining drug response has to be an important part of this effort. In this sense, ConLiGen project, aimed to perform the largest GWAS to date focused on Li response, including a stringent phenotype definition of response (Box 3). All the patients included have been evaluated for their Li response using the ALDA scale (Grof et al., 2002), thus, obtaining a homogenous and comparable phenotype between all the samples included. Moreover, ConLiGen sample includes only patients with BD type I and II, excluding those Li treated patients with another disease phenotype.

Ethnicity is another important issue in pharmacogenetic studies, since they are susceptible to a form of confounding known as population stratification. It refers to allele frequency differences due to ethnic and/or racial differences between responders and non-responders. Differences in genetic ancestry between studies can also lead to difficulties in replication. It has long been recognized that differences among ethnic groups in the functioning of the drug metabolism enzymes lead to variability in drug response to psychotropic agents. Genetic studies of CYP2C19 have found that 15-30% of specific Asian populations are PMs compared to 3-6% of Caucasians and 2-4 % of Africans (Ng et al., 2004). Moreover, pharmacodynamics also applies to the genetic subtypes of drug receptors or targets that can determine response to a particular drug. As example, the L allele of the 5-HTTLPR gene in Caucasians is associated with better response to SSRIs, while in Asians the same allele is associated with poorer SSRI response (Porcelli et al., 2012).

Another confounding variable is the **placebo effect** that refers to a remarkable phenomenon in which a placebo (a fake treatment) can sometimes improve a patient's condition simply because the person has the expectation that it will be helpful. Although researchers agree that this effect will never be completely

eliminated, it could be minimized. For example, clinical trials comparing efficacy of antidepressants *versus* placebo, have shown that 30% of depressive patients respond to placebo medication (Walsh et al., 2002). Moreover, it is known that clinical response could lack more than 6 weeks to become evident (Gelenberg and Chesen, 2000). Thus, long term follow-up designs will be useful to minimize the number of responders to placebo appearing along the first weeks of pharmacological treatment (Arias et al., 2003).

Although some clinical applications from pharmacogenetics have been seen in some specific diseases, most notably cancer (Ventola, 2011), we are far from its application in the field of psychiatry. The biggest obstacle is that we still do not have a clear understanding of which genetic factors are underlying neither the treatment response to psychotropic medications nor the etiology of mental disorders. The studies carried out to date have yielded a number of associations between the polymorphism of a given gene and a clinical response to psychotropic drugs. However, as commented before, only a minority of them has been consistently replicated in subsequent studies. So, they do not point to any definitive associations that can be used with confidence to predict how a patient will respond to a particular treatment.

Only one pharmacogenetic test has been approved by the FDA for clinical use in psychiatry (de LJ. 2006). This is the AmpliChip CYP450 Test marketed by Roche Molecular Systems, which includes software with an algorithm to predict CYP2D6 and CYP2C19 phenotypes (i.e., PM, IM, EM, and UM) based on the identified alleles. However, there is insufficient evidence for clinical applicability of CYP genotyping prior to prescribing either antidepressants (in particular, SSRIs) or antipsychotics for their respective applications (Thakur et al., 2007; Fleeman et al., 2010; Fleeman et al., 2011).

In this regard, three key characteristics of a pharmacogenetics test are needed in order to its use in clinical practice: i) the **analytic validity** (the ability to detect different alleles accurately, ii) **clinical validity** (the ability to predict clinically meaningful outcomes) and iii) **clinical utility** (the ability to provide information that improves the risk/benefit ratio of clinical treatment).

As an example of the insufficient evidence for clinical applicability of CYP genotyping, the Center for Disease Control and Prevention (CDC) commissioned an independent panel to examine these three key characteristics of CYP450 genotyping when prescribing SSRI antidepressants (EGAPP Working Group2007). They found a strong evidence for the analytic validity of CYP450 genotyping, but only marginal evidence for its clinical validity and almost no evidence for its clinical utility concluding there was, "insufficient evidence to support a recommendation for or against use of CYP450 testing in adults beginning SSRI treatment" (Thakur et al., 2007). Thus, until unambiguous evidence proves the clinical use of this and other genetic tests, caution is advised in their interpretation and application in health care management. Other pharmacogenetic tests including pharmacokinetic and pharmacodynamic targets are currently available in the market; however it has not been proven their efficacy in predicting clinical response to psychotropic drugs (Arranz and Gutierrez, 2011).

In summary, part of the phenotype of psychotropic response, as other complex traits, is a product of interacting polymorphisms in multiple genes. However, the individual's genetic makeup is not the only determinant of variable drug responses, which will provide information for just a part of the complex puzzle of clinical response to psychotropic drugs. Other factors, such environmental, sociodemographical and clinical, will be also necessary in order to understand the total phenotype. Inclusion of large cohorts and prospective trials conducted in multicenter collaborations with clear phenotypic characterization and ethnic homogeneity is also essential. Moreover, progress in technology is providing the opportunity to extend the study of biological predictors of psychotropic response from genetics to genomics and more in general to "omics" such as epigenomics, transcriptomics and metabolomics, thus providing additional knowledge on pathways relevant to drug response.

Considering all the above, our hope is that pharmacogenetics may one day help to unravel the complexities of treatment response and also shed light on the mechanism, of mental disorders leading to a reduction in the burden of mental disorders and improving quality of life for patients, relatives and society.

Conclusions

Our results focused in the analyses of genetic variability at genes coding for proteins involved directly or indirectly in the mechanism of action of psychotropic drugs let us to detect some minor and moderate effects of genetic variants that could explain, at least, part of the lack of response to these drugs. The main conclusions suggested by our studies are:

Citalopram response in Major Depressive Disorder.

1. Genetic variability at *CNR1*, *CNR2* and *FAAH* genes are not associated with clinical response to CIT at 4th week.

2. Genetic variability at *CNR1* gene (rs806368, rs806371) is associated with clinical remission to CIT at 12th week. Individuals carrying the TT genotype showed an OR of 2.8 (rs806368) and 2.7 (rs806371) for non-remission of the depressive episode. The involvement of this gene was supported by the haplotype analysis (rs806371-rs1049353-rs806371) being the haplotype combination C-G-G less frequent in non-remitters than in remitters.

3. The longitudinal study showed an influence of both rs806368 and rs1049353 polymorphisms (*CNR1*) on CIT response along the 12th week follow-up. Regarding rs806368, C allele male carriers showed a better improvement of HDRS scores than TT homozygous. Regarding rs1049353, male GG homozygous showed a better improvement of HDRS scores than A allele carriers. Although the direction of the rs1049353 SNP effect differs to a previous study, both studies indicate the involvement of the *CNR1* gene in CIT response. These results suggest some gender divergence in the role of the eCB system in MDD and antidepressant treatment.

4. Our study shows the possible involvement of the *CNR2* gene on the severity of the depressive episode. The AA homozygous for the rs806368 presented higher scores on the HDRS scale than G allele carriers along the 12 week follow-up.

Lithium response in Bipolar Disorder

5. Our study shows the involvement of genetic variability at PI pathway in Li response. Specifically, the rs669838 (*IMPA2*) and rs909270 (*INNP1*) polymorphisms could explain part of the lack of response to Li treatment in BD. Individuals carrying A allele (rs669838) and G allele (rs909270) have an OR of 2.03 and 2.45, respectively, for non-response. The involvement of INNP1 gene was supported by the haplotype analysis (rs3791809-rs4853694-rs909270) being the haplotype combination T-A-G more frequent in non-responders than in responders.

6. We found association of rs11921360 (*GSK3B*) and Li response. Individuals carrying the A allele have an OR of 2.52 for non-response. The involvement of this gene was supported by the haplotype analysis (1732170-rs11921360-rs334558) being the haplotype combination C-C-A more frequent in responders than in non-responders.

7. Our data suggests a possible role of rs2852620 (*GRIK2)* in Li response showing significant differences in genotypic and allelic distributions between the different groups of Li responders. Further studies focused in glutamate system should be necessary to elucidate the involvement of this system in Li response.

Clozapine response in Schizophrenia

8. Genetic variability at *FKBP5* (rs1360780) may partially explain clinical response to CLZ involving the HPA axis in the therapeutic action of CLZ. Carriers of TT-rs1360780 showed an OR of 2.11 for non-response to CLZ. The involvement of this gene was supported by the haplotype analyses (rs3777747-rs1360780-rs17542466-rs2766533) being the haplotype combination A-T-A-G more frequent in non-responders than in responders.

9. Genetic variability at *NTRK2* (rs1778929 and rs10465180) may partially explain clinical response to CLZ involving neurotrophic factors in the therapeutic action of CLZ. Regarding rs1778929, TT-homozygous showed an OR of 1.7 for non-response. Regarding rs10465180, CC-homozygous

showed an OR of 2.15 for non-response. The involvement of this gene was supported by the haplotype analysis (rs1619120-rs1778929-rs10465180) being the haplotype combination G-T-C associated with non-response.

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