Universitat Autònoma de Barcelona

Neurobiological factors associated with treatment response and resistance in Major Depression

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Doctoral thesis of Metodi Draganov

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Doctoral thesis

Presented by

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To my mother

“Cowardice is the most terrible of vices”

The Master and Margarita,

Mikhail Bulgakov

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Abbreviations

5 hydroxytryptamine (5HT)

American Psychiatric Association (APA)

Anterior cingulate cortex (ACC)

Artificial intelligence (AI)

Brain-derived neurotrophic factor (BDNF)

Cerebrospinal fluid (CSF)

Choline (Cho)

Cognitive Behaviour Therapy (CBT)

Corticotropin-releasing hormone (CRH)

C-reactive protein (CRP)

Deep learning (DL)

Default mode network (DMN)

Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5)

Diffusion tensor imaging (DTI)

Dopamine (DA)

Electroconvulsive therapy (ECT)

Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA)

European Staging Method (ESM)

Excitatory amino acid transporters (EAAT)

False discovery rate (FDR)

Fractional anisotropy (FA)

Generalized linear models (GzLM)

Genome-wide association studies (GWASs)

Glutamate (Glu)

Glutamate-Glutamine (Glx)

Grey matter (GM)

Hamilton Depression Rating Scale (HDRS)

Hypothalamic-pituitary-adrenal axis (HPA)

Indoleamine-2,3-dioxygenase (IDO)

Inflammatory response system (IRS)

Interferon gama (IFNγ)

Interferon gamma (IFN-γ)

Interleukin (IL)

Interpersonal Psychotherapy (IPT)

Magnetic resonance spectroscopy (MRS)

Major Depression Episode (MDE)

Major Depressive Disorder (MDD)

Massachusetts General Hospital Staging Method (MGH-s)

Maudsley Staging Method (MSM)

Medial prefrontal cortex (mPFC)

Metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG)

Mindfulness-based cognitive therapy (MBCT)

Minor allele frequency (MAF)

Monoamine oxidase inhibitors (MAOIs)

Multiple-therapy-resistant major depressive disorder (MTR-MDD)

N-acetyl-aspartate (NAA)

National Institute for Health and Clinical Excellence (NICE)

N-methyl-D-aspartate (NMDA)

Orbitofrontal cortex (OFC)

Point Resolved Spectroscopy (PRESS)

Post-traumatic stress disorder (PTSD)

Prefrontal cortex (PFC)

Repetitive transcranial magnetic stimulation (rTMS)

Selective serotonin reuptake inhibitors (SSRIs)

Sequenced Treatment Alternatives to Relieve Depression (STAR*D)

Serotonin reuptake inhibitors (SSRIs)

Serotonin transporter (SERT)

Single nucleotide polymorphism (SNP)

Standard Deviation (SD)

Stimulated Echo Acquisition Method (STEAM)

Subgenual anterior cingulate cortex (sgACC)

Thase and Rush Staging Method (TRSM)

Treatment resistant depression (TRD)

Tricyclic antidepressants (TCA)

Tumor necrosis factor α (TNFα)

Vagus nerve stimulation (VNS)

Voxel of interest (VOI)

White matter (WM)

World Health Organization (WHO)

γ-aminobutyric acid (GABA)

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Summary in Catalan

La depressió és una malaltia heterogènia que afecta una part substancial de la població mundial en algun moment determinat de la seva vida. En els casos lleus, la gent pot sentir tristesa i apatia durant diversos dies per les coses que li agradava fer a la vida quotidiana, mentre que en els casos greus la persona pot necessitar una combinació d'antidepressius i psicoteràpia durant diversos anys. En aquests darrers casos, iniciar el tractament tard, per diversos factors com ara un diagnòstic poc clar, pot dificultar les possibilitats futures d'èxit. Fins el moment, el diagnòstic es basa en un examen clínic dels símptomes exposats. El principal problema ve del fet esmentat que, tot i que la depressió té subtipus altament heterogenis, els metges encara no tenen el coneixement precís dels factors neurobiològics associats, i especialment dels de la depressió resistent al tractament. En aquests casos, el tractament estàndard d'assaig i error amb antidepressius pot tenir resultats perjudicials per a la salut mental del pacient, així com per a la societat si se'n mesuren les pèrdues econòmiques per absència a la feina i el cost d'un tractament més llarg. Per tant, es fa necessari esbrinar els factors neurobiològics associats a la resposta i resistència al tractament per proporcionar pistes per a un tractament dirigit amb més precisió i augmentar la taxa de resposta. Aquesta tesi se centra a crear i ampliar el coneixement existent proporcionant proves de nous marcadors genètics, epigenètics i de neuroimatge per a la resistència al tractament, especialment els relacionats amb la inflamació.

El primer estudi va explorar 153 pacients deprimits que es van puntuar en funció del seu nivell de resistència i, en funció d'això, es van dividir en dos grups: resistents i no resistents. Les dues mostres es van comparar en diverses anàlisis genètiques (al·lel, genotip, haplotip) i epigenètiques (estat de metilació). Els resultats van suggerir que les variants dels gens IL-1β, IL-6 i IL-6R es podrien associar a la resistència.

El segon estudi va investigar possibles alteracions neuroquímiques glutamatèrgiques associades a una pitjor resposta mesurada amb espectroscòpia de ressonància magnètica. Cinquanta pacients van proporcionar mostres per genotipar i es van sotmetre a un protocol estandarditzat per adquirir nivells metabòlics a la zona ventromedial de l'escorça prefrontal. Els resultats van mostrar que una de les mutacions genètiques examinades situada a la zona promotora del gen IL-1β podria estar associada amb un augment dels nivells glutamatèrgics i una major resistència al tractament.

En conclusió, els resultats d'ambdós estudis suggereixen que la depressió resistent al tractament podria representar un subtipus separat caracteritzat per factors neurobiològics específics. El gen IL-1β, juntament amb el complex de gens IL-6 / IL-6R semblen tenir un paper específic en la resposta als antidepressius, de manera que proporcionen una gran oportunitat per a més investigacions i objectius provisionals per desenvolupar nous tractaments personalitzats en un futur proper.

Summary

in English

Depression is a heterogeneous disease affecting a substantial part of the world population at a given point in their life. In the mild cases people might feel sad and loose passion for several days for the things they enjoy doing in everyday life, while in the severe cases the person might need a combination of antidepressants and psychotherapy for several years. In the latter cases, starting the treatment late due to various factors such as unclear diagnose can hinder the future chances of success. At the moment the diagnosis is based on a clinical examination of the exhibited symptoms. The major problem comes from the aforementioned fact that while depression has highly heterogeneous subtypes, clinicians still lack the precise knowledge of the neurobiological factors associated with them, especially evident in Treatment Resistant Depression. In these cases, the standard trial and error treatment with antidepressants can have detrimental results for the patient' mental health, as well as for the society measured in economic losses due to absence at work and cost of longer treatment. Therefore, neurobiological factors associated with treatment response and resistance are highly desirable in order to provide clues for more precisely targeted treatment and increase the response rate. This thesis is focused on building on and expanding the existing knowledge by providing evidence for novel genetic, epigenetic and neuroimaging markers for treatment resistance, especially those related to inflammation.

The first study explored 153 depressed patients that were scored on their level of resistance and based on this they were divided into resistant and non-resistant group. The two samples were compared in several genetic (allele, genotype, haplotype) and epigenetic (methylation status) analyses. The results suggested that variants in the IL-1β, IL-6 and IL-6R genes might be associated with resistance.

The second study investigated potential neurochemical glutamatergic alterations associated with worse response measured by MRI Spectroscopy. 50 patients provided samples for genotyping and underwent a standardized protocol to acquire metabolic levels at the vmPFC area. Results showed that one of the examined genetic mutation located in the promoter area of the IL-1β gene might be associated with increased glutamatergic levels and increased resistance.

In conclusion, the results from both studies suggest that Treatment Resistant Depression might represent a separate subtype characterized by specific neurobiological factors. The IL-1β gene, together with the IL-6/IL-6R genes complex seem to play a specific role in the response to antidepressants, hence providing an exciting opportunity for further investigation and tentative targets for developing novel personalized treatments in the near future.

Chapter 1

Introduction

1.1. The nature of Major Depression – symptoms and diagnosis

Major Depressive Disorder (MDD) is one of the leading causes of disability worldwide affecting over 300 million people (WHO, 2017). According to the most recent Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), in order to be diagnosed with a Major Depression Episode (MDE), a person should display 5 or more symptoms out of 9, with at least one of them being depressed mood or anhedonia (American Psychiatric Association, APA, 2013). These should be exhibited in a period of at least 2 weeks. The secondary clusters of symptoms consist of 2 major groups –somatic and non-somatic–, and include appetite or weight changes, sleep disturbances (insomnia or hypersomnia), psychomotor agitation or retardation, fatigue or loss of energy, problems with concentration and thinking, feelings of worthlessness or overwhelming guilt, and suicidal thinking. The presence of these symptoms is rated with 1 (yes) and 0 (no) and the result is summed to determine the diagnosis of an MDE. There are also 4 additional requirements. First, the symptoms should cause clinically significant distress or impairment in social, occupational, or other important areas of functioning. Second, the episode should not be attributable to physiological effects of a substance or another medical condition. Third, the episode should not be better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders. Finally, the patient should not have previous manic or hypomanic episodes (APA, 2013).

1.2. **Pathophysiology of MDD**

Depression is a highly heterogeneous disease and its pathophysiology is yet to be completely understood. Several theories have been proposed in the last 50 years, but the main obstacle remains the fact that no single hypothesis can explain all manifestations and the broad range of symptomatology of the disorder.

1.2.1. **The Classical Monoaminergic Model**

The most influential classical monoamine hypothesis postulates a dysregulation of the monoaminergic system, concerning mainly the neurotransmitter serotonin (5 hydroxytryptamine: 5HT) in 3 main directions – production and availability, impeded transportation and functional abnormalities of the receptor (for a review see Jesulola et al., 2018). This model is part of the research line investigating the alterations in the neurotransmitters' levels and availability as a prime factor in the pathophysiology of depression. Previous studies have shown elevated enzymes degrading serotonin (Nemeroff, 2008), reduced serotonin transporter (SERT) availability (Joensuu et al., 2007), decreased expression in vivo of serotonin receptors such as 5-HT1A, (Hirvonen et al., 2008) and decreased auto-receptor binding post-mortem in depressed suicide victims (Boldrini et al., 2008). The strongest support for the

monoamine deficiency theory of MDD comes from the fact that most of the antidepressants, mainly monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCA) and selective serotonin reuptake inhibitors (SSRIs) are all aimed at increasing the availability of serotonin at the synaptic cleft by either inhibiting its synaptic reuptake or by inhibiting its metabolism (Kaufman et al., 2016). Although no single gene has been linked to pathophysiology of MDD based on results from genome-wide association studies (GWASs) (Ripke et al., 2013), several genes related to the serotonergic system have been implied. Previous studies have shown a relationship between depression and the genes coding for serotonin 5-HT-1A and 5-HT-2A receptors, as well as serotonin transporter SLC6A4 (5-HTTLPR variant), although the findings remain controversial (Fabbri et al., 2017).

1.2.2. Other models

Several alternative models have been proposed along the leading serotonin hypothesis. There is no doubt that depression carries a high percentage of heritability and therefore **genetic factors** play an important role in the etiology and pathophysiology (Fig.1) of the disease. Support for the genetic models has come from family, twin, and adoption studies, with increased risk of depression for family members. As depression is a heterogeneous disorder, it is expected that various genes of modest effect interact with each other, as well as with environmental factors to increase familial susceptibility (Craddock et al., 1995). Nevertheless, to date no single major gene locus has been determined as accounting for the increased intrafamilial risk.

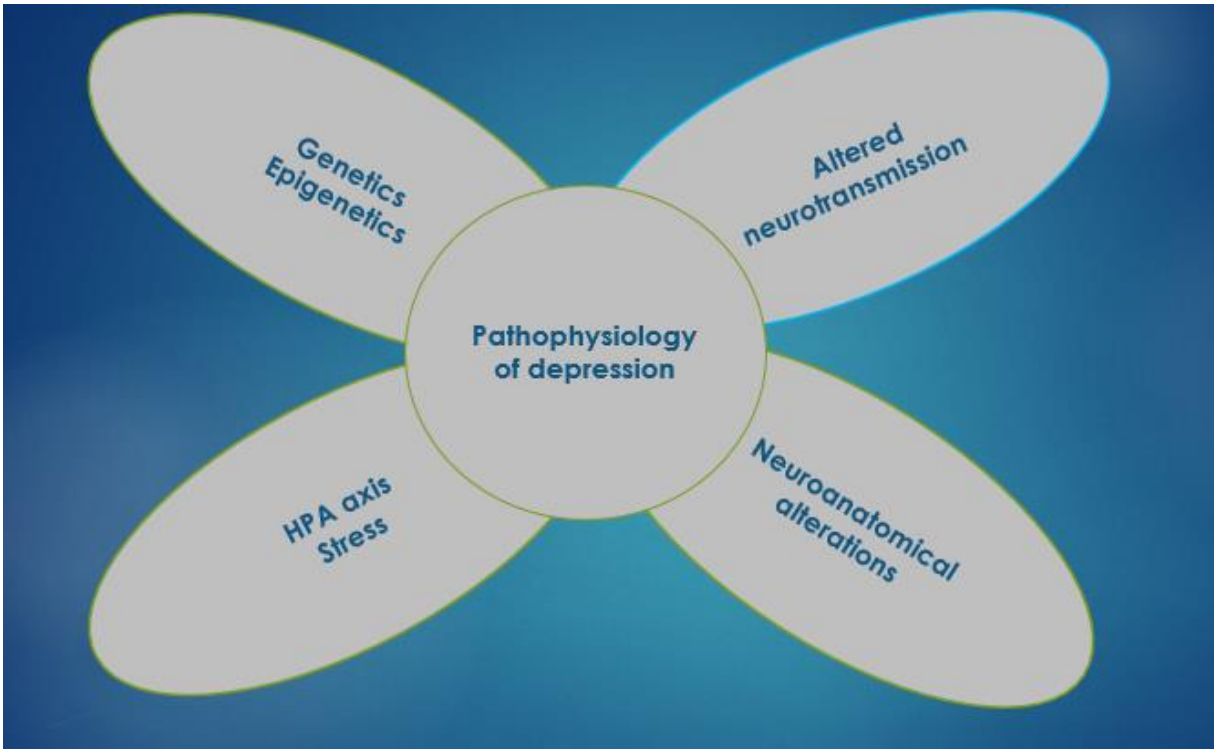
Another aspect of the maladaptive changes related to depression is the alteration of various neuroanatomical structures, subsequently affecting their functionality. With the development of new neuroimaging methods and algorithms for processing of the data, it has become clear that anatomical alterations are not only associated with, but in some cases might even precede the initial phases and predict future development. The mostly affected structures in MDD comprise decreased volume of the **hippocampus** (Chan et al., 2016); increased volume (Hamilton et al., 2008), reduced resting-state functional connectivity (Connolly et al., 2017) and hyperactivity (Godlewska et al., 2012) of the **amygdala**; hyperactivity of the **ventromedial** and hypoactivity of the **dorsolateral** sectors of the **prefrontal cortex** (PFC) (Koenigs & Grafman, 2009).

Another line of research investigates the alterations of the main neurotransmitters in the cortex. Although the serotonin deficiency hypothesis is the leading one, others have investigated reduced levels of **dopamine**. As this neurotransmitter affecting motivation, arousal, reinforcement, and reward, it represents a valid target for investigation with regards to depression. The physiological alterations underlying reduced dopamine (DA) signaling might result from either diminished DA release from presynaptic neurons or impaired signal transduction. This, on the other hand, can be caused by changes in receptor number or function and/or altered intracellular signal processing

(Dunlop & Nemeroff, 2007). **Glutamate** (Glu) has been also studied extensively. A large number of clinical studies suggest that pathophysiology is linked to a dysfunction in the glutamatergic system, probably due to a malfunction in the mechanisms regulating clearance and metabolism of the neurotransmitter (Sanacora et al., 2012). Importantly, in high quantities glutamate can lead to excitotoxicity, neuronal degeneration and death (Hardingham & Bading, 2010). Studies employing magnetic resonance imaging (MRI) Spectroscopy have also reported altered *in-vivo* glutamatergic levels in different brain areas linked to depression (for review and meta-analysis see Moriguchi et al., 2019).

Finally, **stress** has been pointed as a risk factor for developing depression, considering both a continuous exposure (chronic stress) and acute stressful experiences (difficult relationship breakdown, passing away of a family member etc.). A meta-analysis compared depression in mothers of children with (mild chronic stress condition) and without developmental disabilities and reported the former to be at elevated risk (Singer, 2006). A more recent meta-analysis by LeMoult and colleagues (2019) examined 62 studies with a total of 44,066 unique participants and reported that people who experienced early life stress were 2.5 times more likely to develop MDD before reaching the age of 18 years old. Exposure to stress can lead to hyperactivity of the **hypothalamic-pituitary-adrenal** (**HPA**) **axis**, increased production of the hormone cortisol, thus triggering depression (Nemeroff and Vale, 2005). The HPA axis is a complex system interacting with psychosocial, contextual, genetic, and developmental factors (Mayer et al., 2018) helping to deal with acute stress, but undergoing marked changes when presented with chronic stress. In such scenario, the adaptive response of the HPA axis becomes maladaptive causing disruption in the normal functioning. Importantly, increased levels of stress and activation of the HPA axis can initiate an inflammatory immune reaction. This led to the development the **inflammatory hypothesis of depression** discussed in greater details in the next section.

Fig. 1 The four main pathophysiology factors contributing to the development of depression



1.2.3. The inflammatory model

The low rates of responsiveness and the delayed onset of therapeutic effect have pushed an exploration of alternative pathophysiological pathways, with the inflammatory hypothesis gaining grounds in recent years. The monoamine-depletion theory alone cannot completely explain the pathogenesis of depression and several lines of research in the past 20 years have linked inflammatory processes to the onset and maintenance of MDD (Liu et al., 2019). Maes (1999) proposed the inflammatory response system (IRS) theory of depression, which suggested that the occurrence of depression depends on activation of the IRS. The inflammatory hypothesis of MDD is based on the notion that chronic inflammation manifesting through cytokines might alter serotonergic, noradrenergic, and dopaminergic neurotransmission, eventually manifesting in depression symptoms (Miller and Raison, 2016). Cytokines represent a heterogeneous group of mediator molecules produced as regulators of the immune response by immunocompetent cells such as lymphocytes and microphages and can be divided into pro and anti-inflammatory. The proinflammatory classes are either immediately or indirectly involved in the inflammatory process and include interleukin 1, 2, 6, 12, 18; interferon γ (IFNγ) and tumor necrosis factor α (TNFα). On the opposite, the anti-inflammatory group (interleukin 4, 10, 13) suppresses the immune reaction and thus prevents both cell activation and the synthesis of proinflammatory molecules. Finally, some cytokines, such as IL-8, can have both

pro and anti-inflammatory functions depending on their concentration (Shadrina et al., 2018). Three major lines of evidence support the cytokines theory: i) clinical similarities between inflammatory diseases and MDD; ii) elevation of pro-inflammatory peripheral markers and decrements after treatment; and iii) the involvement of inflammatory genes as risk factors.

1.2.3.1. Clinical similarities between inflammatory diseases and MDD

Several early studies have noticed neat similarities in clinical symptomatology between MDD patients and sufferers from cancer and other diseases who have received cytokine therapy. Treatment with proinflammatory cytokines can induce broad spectrum of symptoms strikingly resembling those of MDD and broadly called ''sickness behavior'', which has been characterized by fatigue, psychomotor slowing, decreased appetite and elevated sensitivity to pain in both human studies and animal models (Capuron and Miller, 2011). Administration not only of inflammatory cytokines, but also of their inducers such as endotoxin or typhoid vaccination to otherwise non-depressed individuals tend to facilitate symptoms of depression (Harrison et al., 2009). Alternatively, patients treated with anti-inflammatory agents display clear reduction of depressive behavior (Dantzer et al., 2008). Other clinical similarities between inflammatory diseases and MDD include anhedonia, cognitive impairment and sleep disturbances (Shariq et al., 2018). Epidemiological evidence has also shown a noticeable depressive comorbidity in patients suffering from chronic medical diseases associated with inflammation such as cardiovascular disease, diabetes type 2, autoimmune conditions such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease (Pryce and Fontana, 2017).

1.2.3.2. Elevated peripheral inflammation in MDD

A second line of support for the inflammation theory in MDD comes from the repeatedly confirmed elevated peripheral inflammatory markers. The increased availability of pro-inflammatory cytokines is an important marker, because they can access the central nervous system and interact with a cytokine network in the brain to alter many aspects of brain function relevant to behavior such as neurotransmitter metabolism, functions of the neuroendocrine system and neurocircuits that can affect mood, but also range of other depression-associated features like motor activity, motivation and anxiety (Capuron and Miller, 2011). Studies have consistently shown increased levels of peripheral pro-inflammatory cytokines such as the interleukins IL-1β (Mota et al., 2013), IL-6, TNF-α (Dowlati et al., 2010), IL-8 (Wang and Miller, 2018) and C-reactive protein (CRP) (Haapakoski et al., 2015). Więdłocha et al. (2018) on the other hand conducted a meta-analysis and reported peripheral levels of the pro-inflammatory IL-1β to be decreased following treatment with antidepressants. There is also evidence that electroconvulsive therapy (ECT) can reduce the pro-inflammatory markers (Freire et al., 2017). It has also been shown that elevated levels of pro-inflammatory cytokines can intensify the activity of

corticotropin-releasing hormone (CRH) and lead to hyperactivity of the HPA axis seen over the disease progression (Kopschina et al., 2017). Based on these findings, several research groups have investigated medications that can either block pro-inflammatory cytokine production and/or action, or agents that stimulate anti-inflammatory cytokines in order to achieve therapeutic effect in depressive symptoms (Kappelman et al., 2014). A study by Weinberger and colleagues (2015) used the anti-inflammatory agent infliximab to reduce depressive symptoms, showing promising results.

1.2.3.3. Inflammatory genes and genetic variants in MDD

Depression is a disease caring high genetic risk. Research has shown the heritability to be up to 37% in families and up to 32% among unrelated individuals with genomic similarity (Fernandez-Pujals et al., 2015). Polymorphisms located in the promotor region of the gene coding for the specific cytokine can increase or decrease its production, thus functionally affecting the availability of the molecule and subsequentially promote or reduce inflammation. Common genetic variants of serotonin have been already discussed in section 1.4.1. when reviewing the Monoaminergic theory of depression. Similarly, several genes and genetic variants with regards to inflammation have been investigated in relation to MDD (Barnes et al., 2017).

Probably the gene that has been mostly investigated is IL-1β with its polymorphism rs16944 located in the promoter region (position-511) and comprising of alleles C/T. Younger et al. (2003) first demonstrated that patients homozygous for the 'low-producing' C allele have increased symptoms severity. Later, Fertuzinhos et al., (2004) suggested this variant to confer greater susceptibility for dysthymia and Hwang et al. (2009) reported significantly earlier age of onset for patients with the C/C genotype. More recently Tartter et al. (2015) found the C allele to be linked with increased depressive symptoms following interpersonal stress. Kovacs et al. (2016) reported association of the rs16944 and childhood trauma to lead to elevated anxiety and depression symptoms, while the A allele of IL-1β rs1143643 displayed a protective effect against depressive symptoms after recent life stress. McQuaid et al. (2019) also investigated the relationship between IL-1β rs16944, early-life adversity and depressive symptoms, but found this to be significant only for males. Another genetic variant, rs1143633 has been also associated with increased symptoms when affected by contextual stressors, such as loss, instability, or poverty (Ridout et al., 2014). Most recently, Bialek et al. (2020) reported that patients with the C/C genotypes of rs1143623 demonstrated more severe levels of symptomatology compared to G/C.

With regards to IL-6, the rs1800795 located in the promoter region has been associated with increased depressive symptoms if homozygous for the G allele (Bull et al., 2009) and Udina et al. (2013) replicated these findings. Frydecka et al. (2016) proposed that only patients with the high producing G allele in rs1800795 would develop depressive symptoms in the course of antiviral therapy. Two studies

investigating depression in cancer patients reported interesting results. Doong et al (2015) found an association between patients caring for the high-producing GG genotype of IL-6 of rs2069845, while Saad et al, (2014) suggested that being homozygous for the G allele of rs2069840 was linked with subsyndromal depression. Zhang et al. (2016) investigated rs1800797 and found the frequency of the A allele to be significantly higher among MDD patients compared to controls. Haastrup et al (2012) investigated 2 SNPs of the proinflammatory IL-18 - rs187238 and rs1946518 with regards to onset of depression. They concluded that the major C allele of the rs187238 and the major G allele of rs1946518 were related to depression only when a previous stressful event was experienced. Other pro-inflammatory molecules have also been linked to MDD. Bosker et al, (2011) reported the TNF-α rs769178 genetic variant to be associated with depression. In another study, Myint et al., (2013) investigated the rs3138557 IFN-γ polymorphism. Results suggested that carriers of the interferon-γ CA repeat allele 2 (homo or heterozygous) showed significant association with increase breakdown of the serotonin precursor tryptophan.

The anti-inflammatory genes have also been investigated. Clerici et al, (2009) reported that the 'low-IL-10 producing' A/A genotype of the rs1800896 SNP was significantly related to MDD. Kim and colleagues (2012) reported association between IL-4 and IL-10 genes and post-stroke major depression. They found that carriers of the 'low-producing' C/C genotype of the IL-4 rs2070874 polymorphism or the A/A genotype of the IL-10 rs1800896 were more likely to experience depressive episode after suffering stroke. Holtzman et al., (2012) also reported similar finding with respect to IL-10 rs1800896 in depressed patients with end-stage renal disease. Being homozygous for the A allele of another single nucleotide polymorphism (SNP) of IL-10, namely rs1518111, have also been linked with subsyndromal depression in both oncology patients and their caretakers (Dunn et al., 2013).

Despite the progress in recent years, there are still many unknown aspects with regards to how the different pathophysiological alterations interact and affect one another (eg. how genetic mutations alter neurotransmitters' levels) at the different stages of the illness and treatment course.

1.3. Treatment of MDD

Due to its significant prevalence rates and debilitating nature, a substantial amount of effort has been made in order to develop, study and implement more effective antidepressant treatments for depression. It is beyond the scope of this dissertation to assess in larger detail the process of selecting treatment in clinical settings, especially with the variation in the guidelines in different countries. In short, according to the British Association for Psychopharmacology guidelines (Anderson et. al., 2008), the standard first line of treatment consists of antidepressants in cases of moderate and severe major depression in adults irrespective of environmental factors and depression type; subthreshold depression that has persisted for 2 years or more; in case of a short duration mild major depression where there is a prior history or the depression persists for more than 2–3 months. If the initial treatment is not effective, it can be changed either by augmenting the dose, changing the antidepressant type, or adding another pharmacological agent. Psychological and behavioral therapies are the second line treatments where antidepressants have been used without success, or first line where pharmacological treatments have not yet been administered (eg. in children). Some of the widely used therapies include Cognitive Behaviour Therapy (CBT), Interpersonal Psychotherapy (IPT) and many more. The 3rd line of treatment consists of the so-called physical treatments and includes procedures such as ECT, repetitive transcranial magnetic stimulation (rTMS) and vagus nerve stimulation (VNS), Light therapy for seasonal depression etc.

The National Institute for Health and Clinical Excellence (NICE, 2004) in UK has also been publishing a guidelines model since 2004 with frequent updates and consist of five steps. The first one is Detection – physicians should routinely use screening questions to detect depressive symptoms. The second is Recognized Depression (mild, 4 symptoms), where the most favorable options should include guided self-help, for physical exercise, for computerized CBT etc. Next is the Moderate and severe depression in primary care (5-6 symptoms) or severe (>6 symptoms), where active drug treatment is recommended in all cases. The fourth is to refer patients to Mental Health Services after they have failed to respond to a standard antidepressant. They are divided into four different groups, each with different treatment strategies - acute phase non-responders (augmentation by another antidepressant), treatment resistant cases (CBT, augmentation, lithium), relapse prevention (continued treatment with antidepressants, or if not tolerated psychological therapy) and atypical cases (MAOI if not responding to SSRIs; augmentation with antipsychotic in psychotic depression). The last level is the In-patient care, when there is a risk to life or a serious risk of self-harm, with treatment being combination of medications and possibly ECT (Goldberg, 2006).

The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) was a multisite, prospective, randomized, multistep clinical trial of outpatients with nonpsychotic major depressive disorder conducted in 2004 various treatment options (Rush et al., 2004). The consortium developed a four-stages model for treatment in resistant cases. All steps lasted 14 days and if the respondent did not improve, he moved to the next one. The first consisted of treatment with Citalopram (Celexa). At the next step the patient could choose either to Switch (stop citalopram, randomly receive Bupropion sustained-release; Venlafaxine extended-release; Sertaline or Cognitive therapy) or to Augment (keep citalopram and add one of the following: Bupropion sustained-release; Buspirone or Cognitive therapy). In the third level the patient could choose to Switch (stop current therapy, start Mirtazapine or Nortriptyline) or Augment (keep current therapy and add Lithium or T3 thyroid hormone). The last fourth step was to Switch – stop the current therapy and start either Tranylcypromine or Mirtazapine plus venlafaxine extended-release. The consortium reported the following remission rates - Level 1—33%; Level 2—57%; Level 3—63% and Level 4—67% (Gaynes et al., 2008).

The previous two decades have seen a growing in people reporting depressive symptoms together with a heavy increase of antidepressants use. Data has shown a 95% increase from 2000 to 2011 in Australia (Stephenson et al. 2013); up to 300% in Canada between 1994 and 2002 (Patten et al., 2014), up to 300% in UK between 1993 and 2000 (Spiers et al., 2016); and up to 100% in USA for the 1996 to 2005 (Olfson et al., 2009). Jorm et al., (2017) report that despite this massive surge in the use of antidepressants, most of the available reports suggest no reduction of prevalence of depression, arguing that this lack of reduction might be attributed to the quality of treatment being too poor to affect prevalence or alternatively too poorly targeted.

1.3.1. Profiling response to treatment

In the different countries health government authorities recommend different protocols for starting treatment (staring dose, whether to use medications for mild symptoms etc), when to change the class of the antidepressants, when to use ECT etc. Nevertheless, the standard 1st line consists of SSRIs such as citalopram, the 2nd line involves change of the class of the antidepressants eg. from SSRI to Tricyclic, and the 3rd line can be ECT or experimental procedures such as Deep Brain Stimulation, Transcranial magnetic stimulation etc. After the first episode of depression, around 50% recover and the other 50% relapse and from the relapsing patients, around 15-20% develop chronicity (Richards, 2011).

1.3.2. Socio-demographics

Sociodemographic factors are easy to collect and they have been extensively scanned for possible association with treatment response/resistance. Several factors such as older age has been shown to be related with worse response to antidepressants (De Carlo et al., 2016). Ethnicity has been shown to interact with some genetic factors eg. Caucasian carriers of the homozygous S genotype in the SLC6A4 gene are less responsive to antidepressants that Asians (Duval et al. 2006). Although there is no definite consensus if there are sex differences, several studies have shown that males might respond better to TCAs (LeGates et al., 2019), while females respond better to selective serotonin reuptake inhibitors (SSRIs) (Berlanga et al., 2006). Not living alone, availability of social support and high level of income have also been associated with better response (Van et al., 2008), probably via impact on antidepressants adherence (Gerlach et al., 2017). Finally, a history of childhood traumatic event has been repeatedly linked to bad response (Nikkheslat et al., 2019).

1.3.3.Clinical comorbidities

Patients with comorbid both non-psychiatric and psychiatric medical illness display lower rates of depression improvement and response to antidepressants. Perlman et al., (2019) recently reported some of the common medical comorbidities to be concurrent endocrine disorders, neurological disorders, pancreatic carcinoma, connective tissue disorder, vitamin deficiency, viral infections, obesity, and coronary heart disease. With regards to psychiatric comorbidities, decreased response has been linked to post-traumatic stress disorder (PTSD) and “double depression” (depression and dysthymia) (Kraus et al., 2019) and clinically diagnosed personality disorder (Angstman et al., 2017). Probably the most well replicated comorbidity is the presence of increased anxiety and concurrent anxiety disorders (Schmidt et al., 2011), with comorbid panic disorder and social phobia increasing the risk for resistance 4.2-fold (Bennabi et al., 2015).

1.3.3.1.Symptom profile

Predictors based on symptoms’ profile are the most frequently and pragmatically employed in clinical practice, as they are collected during clinical interviews for diagnostic, prognostic, and treatment preference purposes (Perlman et al., 2019). Moreover, it has been suggested that different symptoms profiles are affected to different extend by various classed of antidepressants, with dopaminergic antidepressants proposed as very effective for anhedonia and loss of motivation, SSRIs more appropriate for comorbid obsessions and compulsions, and SNRIs with more satisfactory effect in cases of excessive fatigue (Kennedy et al., 2016). The profile includes nature and history of current and past symptoms, as well as possible depression subtype. Both early (De Carlo et al., 2016) and late (Kemp et al., 2008)) age at onset have been linked to bad response, as well as increased baseline

severity of the symptoms (Schmidt et al., 2016). Several symptom profiles have also been reported as predictors of worsen outcome, such as anhedonia (Downar et al., 2014) and heightened distress (Khazanov et al., 2020). Groves and colleagues (2018) reviewed cognitive impairments as predictors and reported poor executive function to predicts non-response to SSRIs specifically in older samples. A recent work has also related cognitive profiles with treatment resistance (López-Solà et al., 2020). Suicide ideation (von Brachel et al., 2019), history of a suicide attempt (Kim et al., 2011) and non-suicidal self-injury (Abbott et al., 2019) are also characterized by poorer treatment outcome. Patients with specific personality traits such as neuroticism and stress vulnerability have also been reported as more prone to treatment resistance in late life (Steffens et al., 2018). There is also a positive association between early response to both psychological (Beard et al., 2019) and pharmacotherapy (Wagner et al., 2017), and posttreatment outcomes.

1.4. Treatment resistant depression (TRD)

1.4.1. Prevalence of TRD

However, determining the actual rates of TRD is complicated due to the lack of universal consensus regarding its definition and recently proposed alternative terms, such as multiple-therapy-resistant major depressive disorder (MTR-MDD) (McAllister-Williams et al., 2018). Nevertheless, most studies estimate that around 30% of patients suffering from MDD do not respond to antidepressant treatment and are diagnosed with TRD after 2 unsuccessful courses of treatment with the appropriate dosage (Rush et al., 2006). Prevalence rates for TRD are often assessed in two ways, either as the percentage of patients who fail to respond to antidepressant medication, or the percentage of patients still suffering residual depression related symptoms after an antidepressant treatment. Based on that, several studies have reported different prevalence ranges. Malhi et al (2005) suggested that two years after the onset of depression approximately 20% remain unwell and, even after 5 years, 10% have failed to recover. These protracted episodes of depression with duration larger than two years represent ‘chronic depression’ and are arguably the result of delayed diagnosis or inappropriate treatment. Berlim and Turecki (2007) report similar findings at 15% continuing to experience depressive symptoms after several cycles of antidepressants treatment. It is still a point of debate whether patients with residual depressive symptoms after responding to an adequate short-term treatment such as antidepressant medication or psychotherapy should or should not be regarded as treatment resistant, with the so called ‘’partial response” reported for up to 70% of patients (Nierenberg et al., 2010).

1.4.2. Burden of TRD

Treatment failure has a negative effect on patients' quality of life, is associated with increased risk for suicidal behaviour and has higher economic costs for society (Mrazek et al., 2014). Several attempts have been made in recent years to explore and quantify the burden of TRD compared to depression managed well by first line treatments, with two main lines of investigation – economic costs and patient' health-related quality of life (Johnston et al, 2019).

There is a strong association between increasing the number of treatment steps due to TRD/non-response within an MDD episode and increasing costs. A study in Sweden by von Knorring and colleagues (2006) reported that treatment non-responders had 39% more total costs per patient compared to responders, while in Brazil Lepine et al. (2012) estimated this to 82%. In USA Feldman et al. (2013) reported that medical costs per patient with TRD for 1 year to be increased by 57% compared to patients who received vagus nerve implantation 57% and by 52% compared to general Medicare beneficiaries with managed depression. Pilon et al., (2019) estimated that resistant patients had significantly higher per-patient-per year healthcare resource utilization and total healthcare costs ($25,517 for TRD vs. $14,542 for non-TRD cohort). Lin et al (2019) matched and analysed 45,066 patients in each of the 2 groups and concluded that TRD patients had higher mean hospital cost together with increased combined hospital cost (index hospitalization + all cause readmissions). Sussman et al (2019) assessed 1600 patients in total (800 in each group) and reported that TRD patients had significantly higher mean number of all-cause emergency department visits, outpatient visits and prescriptions over a 12-month follow-up period.

With regards to quality of life, Jaffe et al (2019) examined a total of 52,060 survey respondents with 622 TRD, 2686 non-TRD depressed patients and the remain sample consisting of the general population. From the 3 groups, TRD patients had the lowest health-related quality of life, adjusted mental and physical wellbeing, as well as increased adjusted relative risk for work and impairment (all $p < 0.001$). In a prospective study, Cao et al. (2013) reported significantly improved general health scores for responders than non-responders ($p < 0.001$). Dennehy and colleagues (2014) analysed the public STAR*D database dividing patients to 3 groups – remitters (33%), non-responders (30%) and partial responders (37%). Their results suggested remitters to have the best improvement in quality of life, mental and physical functioning, and social and work-related measurements. Therefore, it can be concluded unequivocally that resistance to treatment leads both to increased economic costs and decreased quality of life of non-responders, presenting a great burden for individual sufferers and society.

1.4.3. Misdiagnosis

There is an ongoing debate over what should be the exact definition of TRD and in what cases we should administer novel or even experimental treatment. After decades of research, the most commonly used definition remains ‘’failure to respond to two antidepressants each given at an adequate dose for an adequate time’’ (Berlim and Turecki, 2007). Nevertheless, there are at least several problems with the whole current approach of TRD (Malhi et al., 2019). They start at the first step, namely misdiagnosis. First, despite the wide use of various forms of psychotherapy and neurostimulation modalities, non-psychopharmacological antidepressant treatments play only a minimal role in diagnosing treatment resistance (Berlim and Turecki, 2007), with electroconvulsive therapy often being the only non-pharmacological treatment assessed as a measure of TRD (Fekadu et al., 2009). Another major problem with affective disorders is that they are longitudinal, but the diagnosis is most often evaluated cross-sectionally, even though it can change after a given period of time. The most obvious example would be the bipolar disorder where the patient is treated for unipolar depression before manifestation of the manic episode. This would eventually lead to months of ineffective treatment with antidepressants, sometimes with more than one trial and the patient might be misdiagnosed with TRD. Another issue lies in the self-reporting symptoms that can sometimes overlap across various mood disorders, with a prominent example of bipolar II disorder that can be mistreated even for borderline personality disorder (Bassett et al., 2017). Instead of receiving proper psychotherapy as most suitable treatment, these patients will again be pharmacologically treated for bipolar disorder, and after few unsatisfactory trials will be diagnosed as treatment resistant. An additional problem comes from the evolving psychiatric taxonomy that in some cases have merged separate diagnoses into one. Malhi et al. (2017) argue that for example DSM-5 (APA, 2013) criteria for the ‘mixed features specifier’ excludes symptoms of distractibility, irritability and psychomotor agitation, although they have been shown to be key symptoms of those experiencing mixed states. This again leads to treatment of mixed depression incorrectly and inadequately, resulting in putative treatment resistance. Finally, there are often cases that do not fit exactly in any of the available diagnosis categories, but in order to be treated they are diagnosed with the one fitting best, which is far from ideal. Of course, clinically speaking this is done so that the patient can be treated and acknowledging the fact that separate diagnosis cannot be created for every single patient with atypical symptoms. Depression is diagnosed based on categorical diagnostic manuals, where a certain number of symptoms are necessary to be present for a given time period. This concept however fails to account for the underlying pathophysiology of the disease and has led to heterogeneous patient population. Moreover, clinicians still lack a reliable objective marker for depression. Both DSM-5 and ICD-10 (WHO, 1992) rely on checklist of dichotomous symptoms suggesting an extended combination of present

symptoms that might lead to the same diagnosis (depression). A person might have decreased appetite and sleeping problems, while another increased appetite with over-sleeping and psychomotor retardation and both might be diagnosed with depression.

1.4.4. Mistreatment

One of the major problems with the current path of developing novel antidepressants and therapies in general comes from the over-restrictive inclusion criteria for patients who do not accurately reflect the heterogeneity of real-world population (Ghaemi, 2008). Clinical trials for novel antidepressants consist mainly of patients with as clear symptoms as possible and often exclude those with comorbidities such as anxiety or psychotic features. In a recent systematic review Gaynes et al. (2020) reported that inclusion criteria as specified by the TRD trials or observational studies they examined generally did not closely align with TRD definitions, with only 20% of defining TRD as two prior treatment failures. Moreover, they suggest that up to 61% and 70% respectively of studies failed to confirm systematically an adequate dose and duration of previous treatments. The results from these trials are then generalized to all of the depressed population, but that is not necessarily a valid conclusion (Sachs et al, 2007). It is no wonder therefore, that as much as 30% of depressed patients do not improve after initial treatment with antidepressants (Conway et al, 2017). They are labeled treatment-resistant and switched to another antidepressant or receive an augmentation, regardless of the fact that the next in line antidepressant has also been tested only on a specific homogenous profile of depressed patients. Often the end result can be deterioration of symptoms after several cycles of antidepressant treatments and increased burden for the patient.

1.5. Staging models for TRD

1.5.1. From binary to staging

For several decades after the introduction of the TRD concept (Heimann, 1974; Lehmann, 1974), resistance was a binary module – the patient either responded well or did not respond. However, as discussed earlier in this chapter, there are large inconsistencies in definitions of what should be considered as good response, partial response and non-response to therapy. Additional problem comes from the fact that there is no universal consensus whether the term resistance should include only antidepressants without ECT and psychological therapies. This ambiguous classification based on vague definitions into binary groups (responders – non responders) can impair the proper

selection of the most efficacious steps over the treatment course. In order to overcome these innate methodological flaws of the binary model, several concepts have been proposed that envision treatment resistance as a continuous scale. Moreover, the optimal staging model for TRD should be able to classify patients with regards to their level of resistance to treatment for MDD, predict chances of future remission and guide clinical treatment selection (Ruhé et al., 2012). Four of the most commonly used and validated models are discussed chronologically.

1.5.2. Thase and Rush Staging Method (TRSM)

The staging model of Thase and Rush (1997) was developed to improve clinical guidance for psychiatrists. In essence, patients are classified in accordance to the number of classes of antidepressants that have proved to be ineffective in their therapy. It has five stages starting with failure to one adequate trial of one major class of antidepressants and reaches stage five with resistance to all classes of antidepressants + resistance to a course of bilateral ECT. Nevertheless, Berlim and Turecki (2007a) pointed out several limitations. One of the main is that the intensity of each course in terms of dosing and duration is not defined. Another issue comes with the assumption that lack of response to two agents of different classes is more difficult to overcome than nonresponse to two agents of the same class. The model also assumes implicit hierarchy of antidepressants (MAOI > TCAs > SSRIs). Regardless of these weaknesses, the model provides easy to use tool that can guide the clinical approach of psychiatrists when treating resistant patients.

1.5.3. European Staging Method (ESM)

The European Staging Method was developed in 1999 (Souery et al., 1999) and it classifies hierarchically respondents into three groups: non-responders; treatment-resistant and chronic treatment-resistant. At the first level are patients that have not improved after their initial trial with antidepressants, while after failing a second trial they move to the next level (treatment-resistant). The TRD stage is additionally staged from one to five depending on the time period of ineffective treatment. Finally, if there is still lack of improvement after more than two antidepressant trials, including augmentation strategy, the patient is classified as chronically-resistant. The ESM advantages come from the inclusion of clear time period of the different stages (6-8 weeks; 12-52 weeks; >52 weeks) and from the quantification of non-response as <50% reduction in scores on Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) or Montgomery Asberg Depression Rating Scale (Montgomery and Asberg, 1979). ESM however makes the assumptions that non-response to two antidepressants of different classes is more resistant to treatment compared to lack of response after two trials of the same class (Ruhé et al., 2012). Some other criticism on the ESM is related to the

arbitrary distinction between treatment resistance and chronic resistance and whether chronic is simply not the 6th stage of the TRD.

1.5.4. Massachusetts General Hospital Staging Method (MGH-s)

The Massachusetts General Hospital Staging model (Fava, 2003) adopts a different strategy and has a total of 3 modules. The first one represents every trial of a single marketed antidepressant for which the patient receives one point. The second module consists of optimization of the dose or the duration, or adoption of augmentation strategies, each of them scored with 0.5 points. The third part of the MGH-s is whether the patient has received electroconvulsive therapy for which 3 points are scored. The main virtues of the model are the inclusion of augmentation and combination options, as well as the lack of assumption regarding the efficacy of different antidepressant classes or a preference for between-class switching. Probably the most important advantage is that the final continuous score might better represent the stages of TRD. In spite of these advances, the MGH-s scores dosage or duration increment equally to augmentation or combination options, with the latter two reported as more efficacious (Ruhé et al., 2009). Nevertheless, Hazari et al. (2013) compared several staging methods and concluded that MGH-s is the preferable choice for routine clinical practice.

1.5.5. Maudsley Staging Method (MSM)

The Maudsley Staging Method is the most recently developed paradigm by Fekadu and colleagues (2009). It again provides a continuous score ranging from 3 to 15 points, which can then be classified in 3 categories mild (scores = 3 - 6), moderate (scores = 7 - 10) and severe (scores = 11 - 15). MSM also introduces 2 novel characteristics of the episode, namely severity and duration. It consists of five modules in total: duration of the episode; severity of symptoms; level of antidepressants failure; augmentation usage and ECT used. MSM is considered as easy to use in clinical practice and the maximum score prohibits extreme outliers, with the possibility of using it as categorical scale adding additional merit. Ruhé et al. (2012) pointed out however that MSM lacks number of augmentation strategies and that dividing the duration into three categories is not interdependent with treatment resistance. As it represents a continuous score, MSM also lacks clear hierarchy of the treatment options, eg. failing one antidepressant is scored with 1 point, while ECT (despite being a more severe treatment) is also scored with 1 point. Nonetheless, MSM has shown very good predictive validity for both short-term (Icick et al., 2014) and longer-term outcomes (Fekadu et al., 2012; Van Belkum et al., 2018). The MSM is considered the most comprehensive staging model, building on and upgrading the

previously available, and therefore it was used for the purposes of the two studies presented in this Thesis.

1.6. Neurobiological markers for TRD

Predictors of response have been warranted at least since it was concluded that not all depressed patients improve after first line of treatment. Knowing that a patient would not respond to standard antidepressants treatment has several benefits such as reduced pharmacotherapy induced health system economic costs, as well as reduced time of individual suffering being on ineffective pharmacological therapy with all the associated side effects. An effective predictor of response would facilitate the use of alternative therapeutic methods that might be more beneficial such as cognitive behavior therapy, Interpersonal therapy or Mindfulness-based cognitive therapy (MBCT) to name few.

To date there is no single reliable marker that can be used to guide treatment decisions (Brand et al., 2015). Nevertheless, a combination of several predictors can be used to construct an algorithm via deep learning (DL) and artificial intelligence (AI) that can achieve much better predictive value. Possible predictors of response to be included in such model can be broadly classified into 2 major groups: non-biological and biological.

1.6.1. Peripheral markers

Peripheral markers are of special interest as they are considerably cheap and easy to collect through urine, blood, saliva and cerebrospinal fluid (CSF) samples. Previous research has investigated levels of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α and It has been repeatedly shown that increased levels are associated with worsen response (Fabbri et al., 2017). C-Reactive Protein levels have shown to be a usable guideline for treatment selection, with decreased serum levels linked to improved treatment response with escitalopram compared to nortriptyline, while nortriptyline was the more effective AD when CRP levels were increased (Hashimoto, 2015). Elevated peripheral cortisol (Strawbridge et al., 2017) and cholesterol levels (Jani et al., 2015), as well as lack of increase in BDNF levels early in treatment (Young et al., 2016) have been also suggested as a predictor of bad response. Decreased levels of metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), a peripheral measure of norepinephrine, have been linked on the other hand with favorable outcome to SSRIs (Strawbridge et al., 2017).

1.6.2. Genetic markers for TRD

Response to antidepressants therapy has been shown to possess marked heritable traits with genetic variants explaining up to 42% of individual differences in antidepressant response (Tansey et al., 2013). As this makes genetic variants a prime candidate for optimal prognostic biomarker for successful treatment in MDD, several genes have been linked to TRD in recent years.

As most of the currently used antidepressants target the serotonergic system, unsurprisingly researchers have searched for polymorphisms in the serotonin related genes. TRD have been associated with variant 5-HTTLPR (rs25531) in the serotonin transporter SLC6A4 (Bonvicini et al., 2010), SNPs in the serotonin receptors HTR1A (rs6295, Malaguti et al., 2011) and HTR2A (rs6313, Kautzky et al., 2015). Another direction of research has focused on genes related to neurogenesis and neuroplasticity, such as BDNF. The most well studied polymorphism of the BDNF gene related to treatment response is rs6265. In this genetic variant (also known as 196G/A or Val66Met), the Met allele decreases the release and availability of Brain-derived neurotrophic factor (BDNF) and has been suggested several times as potential candidate for treatment outcome predictor (Choi et al., 2006; El-Hage et al., 2015). A meta-analysis by Niitsu and colleagues (2013) concluded that heterozygosity (Val/Met) is associated with improved SSRIs response compared to the homozygous genotypes, especially in Asians. Nevertheless, these findings remain controversial and lacking replication with larger cohorts and Lett et al (2016) have suggested that this is due to the significant gene-environment interaction of the polymorphism.

A different line of research has investigated possible involvement of genes responsible for drug metabolism e.g. CYP2D6 and CYP2C19 (Kawanishi et al., 2004), but findings have been hard to replicate. Nevertheless, as treatment resistance might be predisposed due to genetic variation in CYP2D6 altering drug clearance or in CYP2C19 altering the ratio of parent drug to metabolites. Recent clinical guidance for selecting antidepressants have become available (Hicks et al., 2017) suggesting that TCA might be ineffective for patients with specific variants of these genes.

Polymorphisms in genes related to glutamatergic neurotransmission have also been reported as possible candidates. Zhang et al., (2014) reported G allele carriers of rs1805502 in the Glutamate ionotropic receptor NMDA type subunit 2B (GRIN2B) to be more susceptible to resistance, while Milanesi et al., (2015) (rs11218030 and rs1954787) and Minelli et al., (2016) (rs11218030 and rs1954787) proposed variants in the Glutamate ionotropic receptor kainate type subunit 4 (GRIK4) to be associated with bad response.

Several studies have found polymorphisms on inflammatory genes to be associated with treatment response. Previous findings suggest genetic variants of the pro-inflammatory IL-1β to increase the risk for resistance. A study by Younger et al. (2003) reported rs16944 located in the

promoter region to be linked with poorer outcome after 4 weeks of treatment with fluoxetine and further investigation suggested that patients homozygous for the T allele have a significantly faster and more pronounced response to paroxetine treatment (Tadic et al, 2008). Baune et al., (2010) reported GG genotypes of rs16944 and rs1143643 to be related to worse outcome after 6 weeks, while Borkowska et al., (2011) reported rs16944 and rs1143627 to be associated with recurrent episodes. Carvalho et al. (2017) investigated IL-6 and found that patients carrying the IL6-174 rs1800795 "G/C" genotype had a 75% of reduced risk to develop TRD, compared to those with rs1800795 "G/G" genotypes. This is particularly intriguing, because the G allele of this genetic variant has been linked to increased availability of IL-6 in plasma (Zakharyan, 2012). Uher and colleagues (2010) reported a possible association between rs7801617 and escitalopram treatment at genome-wide level, but results have not been replicated since. In a study by Maciukiewicz et al (2015), rs2066992 and rs10242595 were nominally associated with response to duloxetine, but the results did not survive correction for multiple testing. A recent study by Bialek et al (2020) showed homozygosity for the T genotype of the TNF-α variant (rs1799964) to be associated with low effectiveness of pharmacotherapy.

Unfortunately, although providing tentative hints, most of these candidate genes findings remain controversial and lack replication in larger samples. Several GWAS studies and meta-analyses of GWAS studies have also failed to identify and replicate a SNP associated with treatment response at variant level (Gendep Investigators, Mars Investigators, & Star*D Investigators. 2013; Fabbri et al., 2019). Recent investigations using polygenic risk scores (García-González et al., 2017) and exome-wide sequencing (Fabbri et al., 2020) also did not show damaging genetic variants in TRD patients.

1.6.3. Epigenetic markers for TRD

Epigenetics is broadly described as the process of regulating DNA transcription without changing the original sequence and is managed by DNA methylation, histone modifications and non-coding RNAs and dysfunction in this process might hinder the proper regulation of a gene' activity or even transcriptional silencing. Probably the most well studied modification, the DNA methylation manifests by adding a methyl or hydroxymethyl group to the cytosines in cytosine-guanine (CpG) dinucleotides (Herman et al., 2003). Interestingly, these changes can be transmitted through generations and are potentially reversible and accessible for drug treatment, providing a new pathway for the development of novel classes of antidepressant drugs (Menke et al., 2012). Although epigenetics has attended more attention in depression only in the recent decade, several clinical and preclinical studies have already proposed that these mechanisms might affect treatment of major depression. Early studies have showed that tricyclic antidepressants such as amitriptyline reduce methylation levels in animal models (Perisic et al., 2010). Study by Kang and colleagues (2013b) found hypermethylation at the SLC6A4

promoter in patients with a history of childhood adversities and exhibiting more depressive psychopathology, while Domschke (2014) reported hypomethylation in one CpG island in the same region to be associated with non-response to escitalopram after six weeks of treatment. In another study, Kang et al. (2013a) reported hypermethylation of the BDNF gene promoter in suicide victims. Moreover, a pharmacogenetic study found an exome-wide significant variant (exm-rs1321744) located in a brain methylated DNA immunoprecipitation sequencing site to be related to treatment outcome, thus suggesting a highly accurate cross-validated predictive model for treatment remission of major depression (Wong et al., 2014). More recently, Ju and colleagues (2019) assessed the genome-wide DNA methylation before and after treatment with escitalopram. Their results revealed that responders and non-responders had different methylation at 2 positions at the CHN2 gene, and at 1 position at the JAK2 gene. With regards to inflammation, Uddin et al. (2011) reported inversed correlation between methylation of IL-6 CpGs and circulating IL-6 levels in patients with lifetime depression. Powell (2013) investigated IL-11 and suggested that hypermethylation of CpG site 5 was related to good response to escitalopram, while hypomethylation of CpG site 4 was related to good response to nortriptyline.

1.6.4. Neuroimaging markers for TRD

Biomarkers derived from neuroimaging data have the potential to be important contributors to the ultimate aim of guiding early treatment decision making by clinicians. Several neuroimaging techniques have been used to search for reliable predictor for treatment response, with the greater part of the research focused mainly on brain structural and functional changes.

With regards to volumetric alterations, alterations in gray matter of the hippocampus (Sheline et al., 2012), amigdala (ten Doesschate et al., 2014), dorsal and rostral ACC volume (Gunning et al., 2009), precentral gyrus and left thalamus (Li et al, 2010), subgenual anterior cingulate cortex (sgACC) (Sambataro et al., 2018) and left superior lateral orbitofrontal cortex (OFC) (Ribeiz et al., 2013) have been associated with response rates. A meta-analysis of 1728 MDD patients and 7199 controls from 15 research samples worldwide analyzed by the ENIGMA (Enhancing Neuro Imaging Genetics through Meta-Analysis) consortium found smaller hippocampal volumes hippocampal volumes in patients versus healthy controls. There was no difference between healthy controls and first episode patients, as well as first episode and recurrent, suggesting a moderating effect of the illness stage (Schmaal et al., 2016). Cortical thickness in the left rostral ACC region has also been recently proposed as predictor of response to Repetitive transcranial magnetic stimulation (Boes et al., 2018). Moreover, in another recent meta-analysis by the ENIGMA consortium, depressed patients exhibited thinner cortical gray

matter than controls in the OFC, anterior and posterior cingulate, insula and temporal lobes, with the strongest effect in the recurrent adolescent patients (Schmaal et al., 2017).

Several studies using Diffusion tensor imaging (DTI) and fractional anisotropy (FA) to measure the degree of myelin integrity have also shown white matter abnormalities to affect remission. Breitenstein et al. (2014) suggested that decreased FA in cortico-striato-limbic white matter regions might be linked to bad response, while in a recent review Chi et al. (2015) concluded that lower FA in the stria terminalis and higher FA in the cingulum bundle is related to increased response. Recently, van Velzen and colleagues (2020) from the ENIGMA group also reported fractional anisotropy abnormalities when they compared 1305 MDD patients and 1602 healthy controls. Findings suggested subtle, but widespread, decreased FA in the 16 out of 25 WM tracts investigated, with strongest effects in the corpus callosum and corona radiata. The largest differences again were observed between the recurrent patients and controls.

Investigations have been carried out in order to determine functional disturbances in patients with bad response. Much of the research focused on ACC and several studies have shown increased activation to be related to better response in fMRI (Victor et al., 2013; Godlewska et al., 2018) studies. Increased superior temporal sulcus activity (McGrath et al., 2014), resting-state functional connectivity of the left anterior ventrolateral prefrontal cortex/insula, the dorsal midbrain, and the left ventromedial prefrontal cortex (Dunlop et al., 2017), as well as subgenual functional connectivity (Cash et al., 2019) have all been reported as predictors of response. Studies examining predictors for psychotherapy have suggested that increased baseline activity in the ventromedial PFC (Ritchey et al., 2011), as well as increased functional connectivity of the amygdala to the left DLPFC and left anterior insula (Straub et al., 2017) were linked to better outcome. A meta-analysis by Fu et al (2013) examined 20 studies and found increased baseline activity in the anterior cingulate to be associated with good response, while increased baseline activation in the insula and striatum to be linked with poor outcome. A recent meta-review by Perlman and colleagues (2019) found 4 major abnormal activations in several cortico-limbic structures. These include ACC hyperactivity, increased functional connectivity between frontal and limbic areas, decreased connectivity within the default mode network (DMN) and decreased pretreatment amygdala activity as predictors of non-response.

Studies employing in-vivo methodologies such as Magnetic resonance spectroscopy (MRS) have previously shown mixed results, partly due to technical difficulties such as low field strength of 1.5T making it difficult to differentiate between overlapping peaks of metabolites such as glutamate and γ-aminobutyric acid (GABA). Studies have reported decreased myo-inositol in the left PFC to be restored after treatment (Zheng et al., 2010) and higher amygdala choline (Cho) to convey a significantly lower risk for relapse (Henigsberg et al., 2019), but probably the most replicable finding is decreased N-acetyl-aspartate (NAA) levels. NAA is a distinct marker of neuronal integrity and

reductions have been found in left ACC (Gonul et al., 2006), in medial PFC (Taylor et al., 2012), and in the left hippocampus (Lefebvre et al., 2017) normalized after treatment. With the rapid development of novel algorithms and increase of field strength up to 7T, the focus has shifted on glutamatergic metabolites. The recent spike in studies reporting excellent antidepressant efficacy of the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine in both preclinical and clinical investigations (Lener et al., 2017) has also fueled the interest in glutamatergic neurotransmission with new strength. Results suggest patients with lower glutamate quantities in the left dorsolateral PFC at baseline to respond better, as well as normalization in glutamate levels after treatment with rTMS in good responders (Luborzewski et al., 2007; Yang et al., 2014). In addition, increased glutamate + glutamine in the right hippocampus was associated with treatment resistance (Lefebvre et al., 2017). Low baseline glutamate levels in the right ACC have also been implicated as predictors for the success of subcallosal deep brain stimulation therapy (Clark et al., 2020). Other studies have reported high levels of GABA at baseline in the medial PFC (Dubin et al., 2016) to be predictors of good outcome after rTMS.

1.6.5. From Imaging-genetics in MDD to imaging–pharmacogenetics in TRD

As demonstrated from the above concise review of predictors of response to therapy both genetic and neuroimaging studies have failed in most part to produce reliable and reproduceable findings. Therefore, the newly flourished imaging genetics field has tried to provide answers where the two methodologies have separately provided tentative hints. It aims to gain mechanistic insights on the impact of genetic variations on brain neurochemistry, structure and function in depression and shed more light on the gene-environment interaction (Pereira et al., 2018). Most of the studies to date have focused on genes previously associated with the pathophysiology of depression. Frodl et al., (2014) found patients with the BDNF Val66Met 'Met' risk allele to have decreased hippocampal size than non-carriers. Phillips et al., (2015) investigated TRD patients and healthy controls and reported the 5-HTTLPR genetic variant to show a significant diagnosis by genotype interaction effect on hippocampal volume, while Jaworska and colleagues (2016) found patients homozygous for the same variant to have increased volume at the left thalamus and left putamen. Watanabe et al (2015) found patients carriers of the Val/Met genotype of the COMT polymorphism (rs4680) to display volume reduction in the bilateral caudate, together with genotype-diagnosis interaction effects on brain morphology in the right caudate. In addition, Seok et al. (2013) used DTI and found patients with Val/Val genotype of the COMT SNP to have significant reduction in fractional anisotropy in the left inferior longitudinal fasciculus, bilateral middle temporal gyrus, right frontal gyrus, and right cingulum bundle area. In a recent review and exploratory voxel-wise meta-analyses of imaging genetics studies, Pereira et al., (2018) concluded that carrying the 5-HTTLPR short 'S' allele is associated with white

matter microstructural abnormalities mainly in the corpus callosum, while the BDNF Val66Met 'Met' allele was linked to increased gray matter volumes and hyperactivation. Genetic imaging analyses are still scarce with regards to inflammatory genes, but an early study by Baune et al., (2010) has shown the G-allele in two polymorphisms (rs16944 and rs1143643) in the IL-1β gene to be associated with reduced responsiveness of the amygdala and ACC to emotional stimulation. Variants in the pro–inflammatory TNF-α gene have also been related to volumetric abnormalities in MDD. Zhou et al (2018) found high-risk genotype (T-carriers) of rs1799724 to be associated with reductions at the right superior occipital gyrus and Savitz et al., (2013) suggested that TNF mRNA gene expression is correlated with gray matter volume of the caudate in MDD.

With regards to treatment response, to date, there is a limited number of studies combining both pharmacogenetics and imaging techniques and the 5-HTTLPR variant is the mostly studied. A study has associated citalopram treatment with decreased amygdala activity in response to negative faces, with 5-HTTLPR L-allele homozygotes having decreased activation at week 1 and increased activation at week 8 (Ramasubbu et al, 2016). Another study using paroxetine treatment revealed that positive correlation between SERT occupancy and depressive symptoms reduction for the patients with two L-alleles (Ruhe et al., 2009).

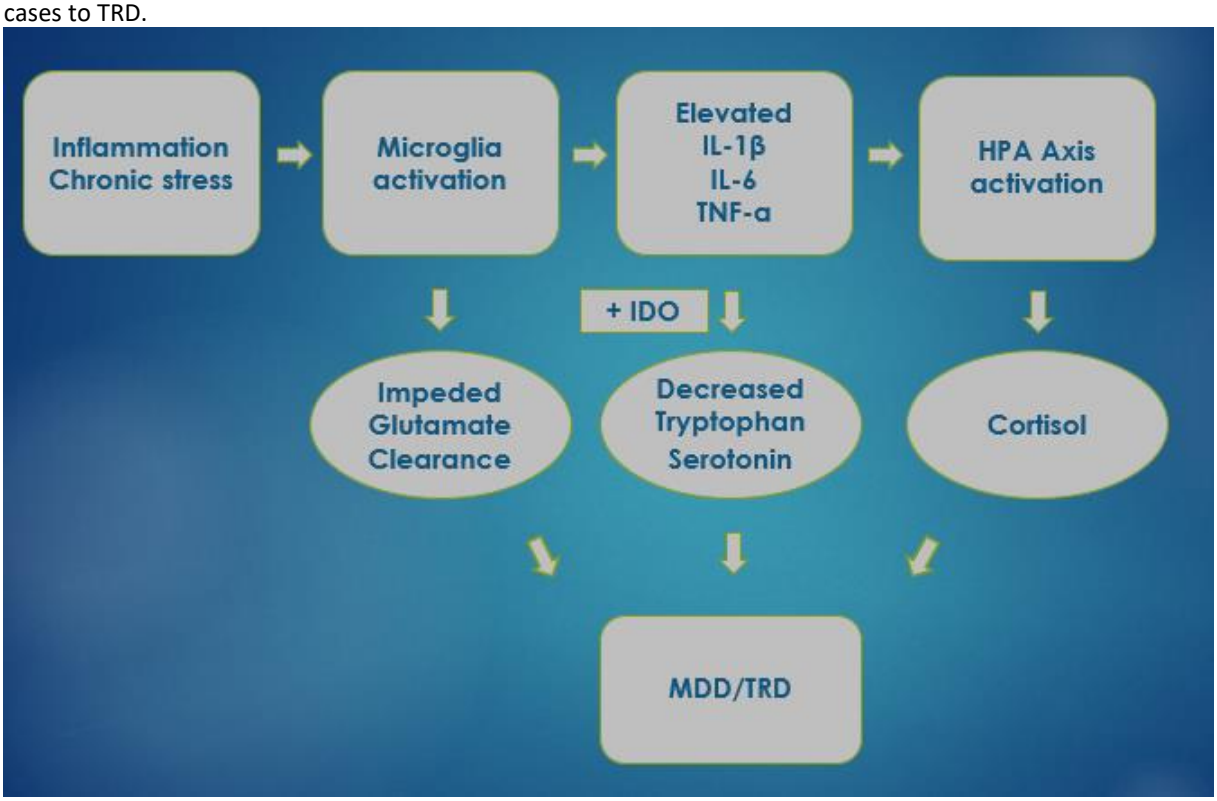
1.7. The role of Inflammation in antidepressants action TRD

Cytokines are a group of signaling agents with extensive immune modulation function (Janssen et al., 2010). Importantly, the major part of the currently used first line antidepressants have effect not only on the neurotransmission, but they also possess immune modulating capacities (Gałecki et al., 2018). Indeed, it has been suggested that inflammation involvement concerns not only the MDD pathophysiology, but also treatment response. The molecular basis of antidepressants action is surprisingly still not yet well understood but is beyond the scope of this review. In brief, as antidepressants have immunosuppressive effect, the increased availability and secretion of pro-inflammatory molecules impedes their action. Inflammatory cytokines can also regulate the expression and activity of monoamine transporters, the main targets of SSRI antidepressants (Miler et al., 2009). Two main lines of research have provided support for such hypothesis. The first comes from research comparing good and bad antidepressants responders. Several studies and reviews have associated bad response with increased levels of pro-inflammatory (such as IL-6, Kiraly et al., 2017) and decreased level of anti-inflammatory cytokines (Arteaga-Henríquez et al., 2019). Strawbridge and colleagues (2019) recently reported TRD patients to display higher levels of various inflammatory molecules such as interleukins 6 and 8, tumor necrosis factor, c-reactive protein and macrophage inflammatory

protein-1. Some have even suggested which antidepressant will be more effective, eg patients with low CRP level (<1 mg/L) respond better to SSRI monotherapy compared to those with increased levels who respond better to combination of bupropion and SSRI (Jha et al., 2017). The second line comes from studies using nonsteroidal anti-inflammatory drugs as adds on to first line antidepressants (or even as monotherapy, Iyengar et al., 2013) and reporting improvement in treatment trajectory. Several studies, clinical trials (Abbasi et al., 2012) and reviews (Kohler et al., 2016) have reported promising results, such as improved effectiveness and shorten onset of antidepressants action (Mendlewicz et al., 2006). Moreover, Strawbridge et al. (2019) found anti-inflammatory agents attenuated at baseline to increase after treatment.

Although imaging genetics studies present an instrument to delineate neural functional architecture associated with genetically driven alterations of a neuropsychiatric phenotype (Raab et al. 2016), such studies investigating a possible inflammation related TRD phenotype are highly warranted but lacking. It is clear that structural, functional and neurochemical alterations are present in both gray and white matter in TRD patients. The alterations in the white matter indicate glial pathology, and this abnormal astrocytic functioning can distort metabolism and subsequently affect glutamate clearance, which in turn, impacts synaptic communication. Pathological oligodendrocyte functioning on the other hand can disrupt the connectivity of neuronal networks, while microglial activation indicates neuroinflammatory activity (Czéh et al., 2018). This combined with activation of the HPA Axis can contribute to development of MDD and subsequently TRD (Fig. 2). Based on this, it was decided that the vmPFC Voxel of interest (VOI) investigated in Study 2 will encompass both white and gray matter.

Fig. 2. Inflammatory and stressful challenges can trigger the activation of the microglia, which in turn overproduce pro-inflammatory cytokines that can contribute to hyper-activation of the HPA axis and the increase in indoleamine-2,3-dioxygenase (IDO) enzyme activity. Alterations in the glial activity can also decrease the clearance of Glutamate. This cumulative process can eventually lead to MDD and in extreme cases to TRD.



Chapter 2

Objectives and Hypotheses

2.1. General aims

The current thesis focuses on clarifying possible factors that could be associated with resistance to treatment in depression. The aim of this research was to investigate how genetic components related to inflammation might delineate a tentative depression phenotype and how these permanent genetic variants are associated with the more dynamic neuro-metabolites levels in brain areas previously linked to the pathophysiology of major depression.

2.2. Specific aims and hypotheses

2.2.1. Study 1

- Aims

- To investigate a pool of 41 polymorphisms in 8 inflammatory genes and detect the ones with strongest association with treatment resistance in major depression adopting a candidate gene approach.
- To examine a possible additional epigenetic predictor for treatment response, namely methylation status, in the genes showing the strongest association in the initial genetic analysis.

- Specific hypotheses

- Based on previous research, we hypothesized that patients with a specific genotype/allele of in the investigated inflammatory genes will be more prone to treatment resistance
- Good and bad responders will have different methylation status in the IL-1β, IL-6 and IL-6R genes

- Novelty of the study

- No previous study has explored predictors of holistic response (as measured by MSM) to treatment in major depression by combining genetic and epigenetic means in inflammatory genes

2.2.2. Study 2

- Aims

To assess the interplay between variants in the IL-1β pro-inflammatory gene, glutamatergic metabolite levels in vmPFC and treatment outcome by means of imaging genetics employing 1^{H}-MR Spectroscopy.

- Specific hypotheses

- Based on the results of Study 1, we selected the polymorphisms in IL-1β gene for further investigation and hypothesized that the allelic variation will be associated with altered levels of Glutamate and Glx in the in vmPFC area.
- Additionally, we hypothesized that increased glutamatergic levels might be evident only when there is an interaction between allelic variation and resistance score.

- Novelty of the study

This is the first study to evaluate the interaction between pro-inflammatory genetic risk variants, glutamatergic levels in the vmPFC area and treatment resistance by means of imaging genetics in depressed patients.

Chapter 3

Methods

The proposed thesis is composed of two articles, one of them published in international peer reviewed journal, and the other submitted for publication. The methods applied are described in great details in the article copies in the Appendix 1 and 2. Therefore, only a concise summary is provided in this section.

3.1. Study 1

3.1.1. Participants

Participants for both studies presented in this dissertation were recruited as a part of a larger project conducted by Dr. Maria J. Portella (supervisor of the current PhD thesis). Study 1 consisted of 153 patients suffering from major depression. They were all recruited from the outpatient psychiatric service at Hospital de la Santa Creu I Sant Pau, Barcelona, Spain. They were all Caucasians and were diagnosed by an experienced clinician according to the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV-TR, 2002). Participants signed an informed consent and received no financial compensation for study participation. Inclusion and exclusion criteria are presented in Table 1.

Table 1. Inclusion and exclusion criteria for participation in Study 1 and 2

Inclusion criteria
Meeting the DSM-IV-TR criteria for MDD
18 years or older
Native Catalan and/or Spanish speaker
Exclusion criteria
Brain trauma with loss of consciousness
Neurological disease
Mental retardation with IQ score <70

Footnote: IQ measured by Vocabulary subtest of the Wechsler Adult Intelligence Scale-IV, Spanish validated version

3.1.2. Measures, tools and methodologies

- Hamilton Depression Rating Scale

The main tool used to score depressive symptoms was the Hamilton Depression Rating Scale (HDRS). It consists of 17 items to be evaluated organized in several areas, such as mood, insomnia, somatic symptoms, everyday activity, suicide ideas etc. with the score range 0 –54. The final score can then be classified in 3 categories: 10 - 13 mild; 14-17 mild to moderate; >17 moderate to severe (see Appendix 3 for the full HDRS). It is widely used both in clinical practice and in psychiatric research to evaluate the level of depression of a given patient and is standardly used in the Psychiatry department of Hospital de Santa Creu I Sant Pau, Barcelona.

- Maudsley Staging Method

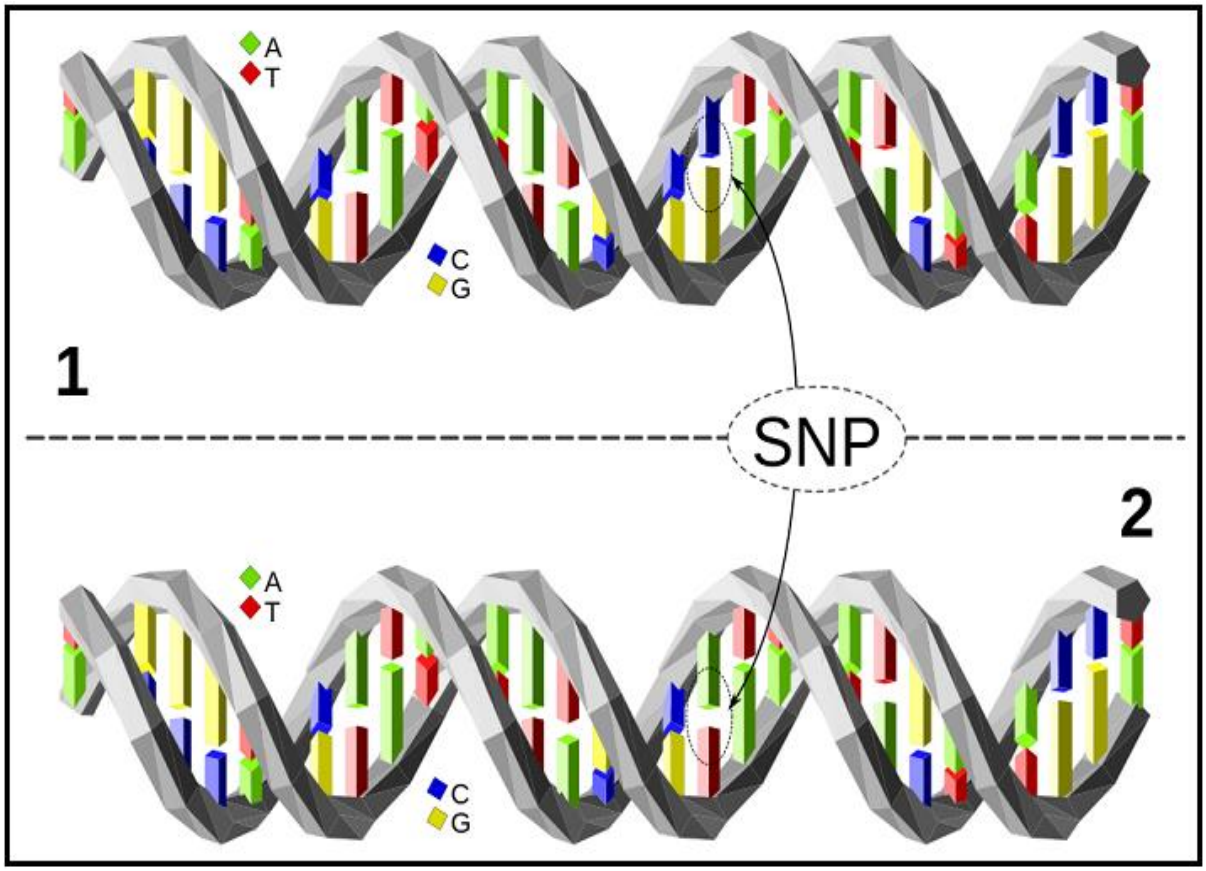
The Maudsley Staging Method developed by Fekadu (2009) and it is a comprehensive tool to stage the level of treatment resistance. It consists of 5 dimensions: Duration of episode; Symptoms severity; Number of failed courses of antidepressants; Augmentation used and ECT used. It has a range 3- 15 and the full version can be found in Appendix 4. MSM was chosen as preferred tool to measure treatment-resistance after careful consideration of the several other available staging tools such as TRSM, ESM, and MGH-s (described in detail in the Introduction). There were several factors for that decision. This staging model accommodates two clinical factors related to treatment resistance, namely severity at baseline and duration, in addition to the treatment factors. This is important because it gives a more holistic picture of the illness progression. As Fekadu argued, staging the treatment resistance based only on the number of treatment failures doesn't say much about the specific nature of the depression itself. For example, mild major depression (2 points at the MSM) resistant to treatment is different from a severe psychotic depression (5 points at the MSM) that also does not respond to treatment. Additionally, including the duration period improves the model, as having an acute episode (eg. 5 months) is distinct to chronicity (eg. 36 months). Moreover, MSM has been recently shown to correlate with the clinical stages of the disease (Reneses et al., 2020). Therefore, it can be argued that MSM is a step further in the development of treatment resistance staging models, because it considers the complex and multidimensional nature of TRD. Finally, it fitted best the research aims of the 2 studies presented in this thesis.

- DNA isolation and genotyping

Participants provided blood samples upon admittance and genomic DNA was extracted from peripheral whole-blood samples (Autopure, Qiagen, Hilden, Germany). 41 SNPs in 8 genes related to

inflammation (IL1-β, IL2, IL6, IL6R, IL10, IL18, TNF-α and IFN-g) were genotyped using the HapMap programme (www.hapmap.org). The basic structure of a polymorphism is explained in Fig. 3. The SNP were selected on the following basis: minor allele frequency (MAF) over 0.05 and r2 threshold of 0.8 in Caucasians. The SNPs were analyzed by real-time PCR using OpenArray1 technology on the QuantStudioTM 12 K Flex Real-Time (Applied Biosystems, Foster City, CA, USA). Quality controls checks (>95% genotyping success per individual and SNPs, Hardy-Weinberg equilibrium) were applied to the genotyping results. Dr. Maria Jesus Arranz, an experienced geneticist and Head of Research Laboratory Unit of Hospital Universitari Mútua Terrassa, Spain, led this process.

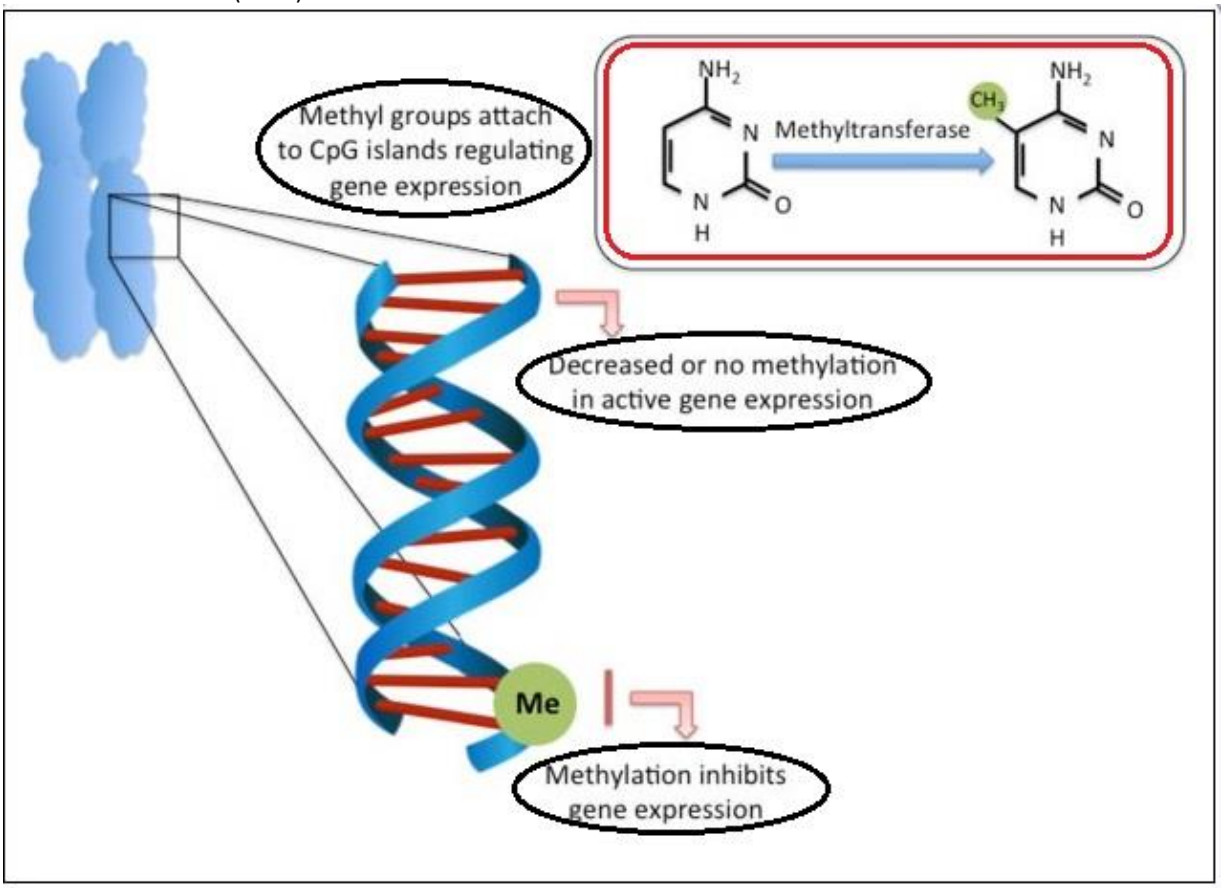
Fig. 3. The image represents a particular change in the DNA producing a single-nucleotide polymorphism (SNP). In order to be polymorphic, there should be at least two possible alleles at a specific locus due to substitution of one or more of the DNA building blocks adenine (A), guanine (G), cytosine (C), and thymine (T). In this example the cytosine and guanine (1) are replaced by adenine and thymine (2). Image adapted from Wikipedia.



- Methylation status

The process of DNA methylation is briefly explained in Fig. 4. In Study 1. 13 CpG islands were selected for analysis and their methylation status in the 5' regulatory region was assessed by bisulfite-pyrosequencing. Primers for PCR amplification and sequencing were designed in PyroMark Assay Design Software v.2.0 (QIAGEN, Hilden, Germany). DNA bisulfite treatment and PCR amplification were performed by means of EpiTech Bisulfite kit and the PyroMark PCR kit (QIAGEN, Hilden, Germany) respectively, following the manufacturer recommendations. Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q24 (QIAGEN, Hilden, Germany). The methylation analysis for this study benefited from the technical support of Dr Juliana Salazar (geneticist at Hospital de Santa Creu I Sant Pau) and Dr. Cristina Gallego-Fabrega (part of the Research Laboratory Unit of Hospital Universitari Mútua Terrassa).

Fig. 4. Schematic representation of the process of DNA methylation and how it impedes gene expression. Methylation changes can be transmitted through generations and are potentially reversible and accessible for drug treatment. Although genetic factors play an important role in MDD and resistance, they cannot explain alone the heterogeneity of the disease. The epigenetic alterations such as DNA methylation provide an additional valuable information about how the life experiences of a patient can affect his genetic machinery. Image adapted from Nevin & Carroll (2015).



3.1.3. Statistical analysis

For this study, all analyses were first run with the MSM as a continuous score, and once again after the sample was divided into responders (MSM < = 7) and non-responders (MSM > 7). The 2 groups were compared on their demographics using Chi-square test for gender and with independent t-tests for age, age at onset, number of depressive episodes and HDRS 17. For the genetic part of the study, linear and logistic regression analyses were employed considering MSM as the dependent variable. (as continuous and binary factors respectively). For the epigenetic part, a logistic regression analysis was performed to examine the methylation status of responders vs. non-responders. Age and gender were used as covariates in all analyses and the results were corrected for multiple comparisons for the allelic and genotype associations employing false discovery rate (FDR). The demographic data were analyzed in SPSS (version 20.0) and the genetic analyses were conducted in Plink (version 1.07).

3.2. Study 2

3.2.1. Participants

50 patients with a diagnosis of MDD recruited at Hospital de Santa Creu I Sant Pau participated in Study 2. Part of the sample coincides with the one investigated longitudinally and published elsewhere during competition of the PhD program (Draganov et al., 2020). All of these 50 patients had available both genetic and MRS data. All patients were on standard antidepressant treatment at the time of blood sampling and image acquisition, following the clinical guidelines of the national health system. Participation was voluntary and no financial retribution was offered. Inclusion criteria were to be native speaker and 18-65 years old, with IQ score > 70 (assessed by Vocabulary subtest of the Wechsler Adult Intelligence Scale-IV, Spanish validated version) and no existing or previous neurological disease. All participants were Caucasians.

3.2.2. Measures, tools and methodologies

- Hamilton Depression Rating Scale

Depression score was measured again by the Hamilton Depression Rating Scale, see a detailed explanation in Study 1 description of used methodologies.

- Maudsley Staging Method

Treatment resistance score was measured again by Maudsley Staging Method, see a detailed explanation in Study 1 description of used methodologies.

- DNA isolation and genotyping

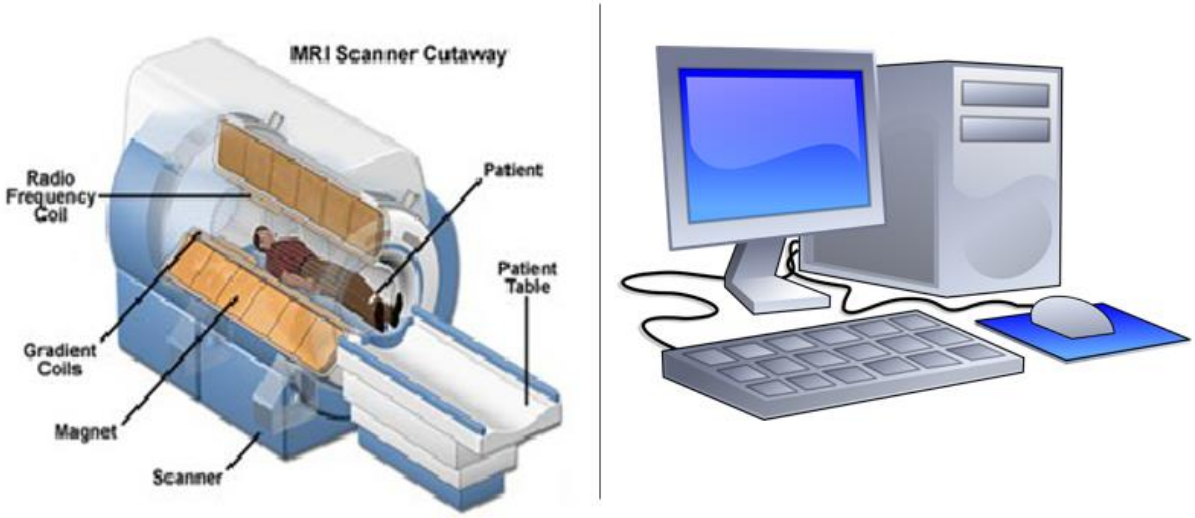
Genetic information was already available as it was extracted for previous analyses, see a detailed explanation for the procedure in Study 1 description of used methodologies.

- ^{1}H MR Spectroscopy

In Study 2, ^{1}H MR Spectroscopy was employed to assess in vivo the glutamatergic metabolites levels of patients at the vmPFC area. The knowledge of the methodology was acquired during earlier analysis resulting in a publication (Draganov et al., 2020, Appendix 5). The MRI (Fig. 5) exploits the fact that hydrogen atoms possess the quantum property of "spin" and aligns along the axis of an applied magnetic field. After excitation and during relaxation, radiofrequency signals are generated and then can be converted into a frequency spectrum. As the nuclei of at least one of the isotopes of most elements possess a magnetic moment, spin, and a charge, when a constant magnetic field is present, the nuclei get excited and their magnetic moments become oriented according to the direction of the applied field in a number of ways, depending on the nuclear spin quantum number, *I*. For protons *I* = 2 (parallel and anti-parallel to the applied field direction; Rouessac & Rouessac, 2013). As electromagnetic radiation in the radio-frequency range causes shifts between the two energy levels, the resonant absorption by nuclear spin occurs only when electromagnetic radiation of the correct frequency (Larmor precession rate) is applied to match the energy difference between the two levels. It is important to note that the absorption depends on the environment of the atomic nuclei (compounds, molecules). This dependence of the transition energy on the position of an atom or molecule allows to obtain a spectrum of the molecular structure. Thus, each signal (or peak) in a spectrum represents the RF energy absorbed by one or different H atoms. The energy released is descending in two relaxation times T1 and T2. The T1 (spin lattice or longitudinal relaxation time) measures the time required for the atomic nucleus to return to its low-energy basal state, while the T2 (spin-spin or transverse relaxation time) is the time when the core is completely out of phase. Therefore, the analysis of these two times can give structural, chemical or functional information for the surrounding tissues of the atomic nuclei. MRS provides a technique to optimize the signal of an ROI (represented by voxels) both in terms of quantity and quality in relation to the noise of background. Two different pulses can be used to obtain the data: STEAM (Stimulated Echo Acquisition Method) or PRESS (Point Resolved Spectroscopy). In STEAM there are 1 RF pulse at 90 degrees and 2 at 180 degrees, while in the PRESS there are 3 pulses at 90 degrees. It is generally accepted that STEAM is

better for metabolites with short T2 and PRESS is used for those with long T2. As each proton differs in the degree to which it is surrounded by other molecules, protons in different compounds resonate at slightly different frequencies. These small alterations in frequency are referred to as chemical shift and are conventionally represented on the right-to-left horizontal axis of an MRI spectrum in ppm units (Fig. 6). The frequency range for H is relatively small and therefore the brain tissue spectra consist of different overlapping signals of the different constituent compounds. Nevertheless, the unique position or chemical shift along the frequency axis of a spectrum allows each metabolite to be accurately identified, and its tissue concentration can be easily determined (for a comprehensive review of the MRI and MRS techniques see Tognarelli et al., 2015). The images for Study 2 were obtained using a 3 T Philips Achieva scanner (software version 2.1.3.2) and a SENSE 8-channel head coil following a standard protocol. The selected VOI included cerebrospinal fluid, white and gray matter. The raw images were processed by a collaborator, namely Dr. Yolanda Vives-Gilabert based at ITACA, Universitat Politècnica de València, Spain.

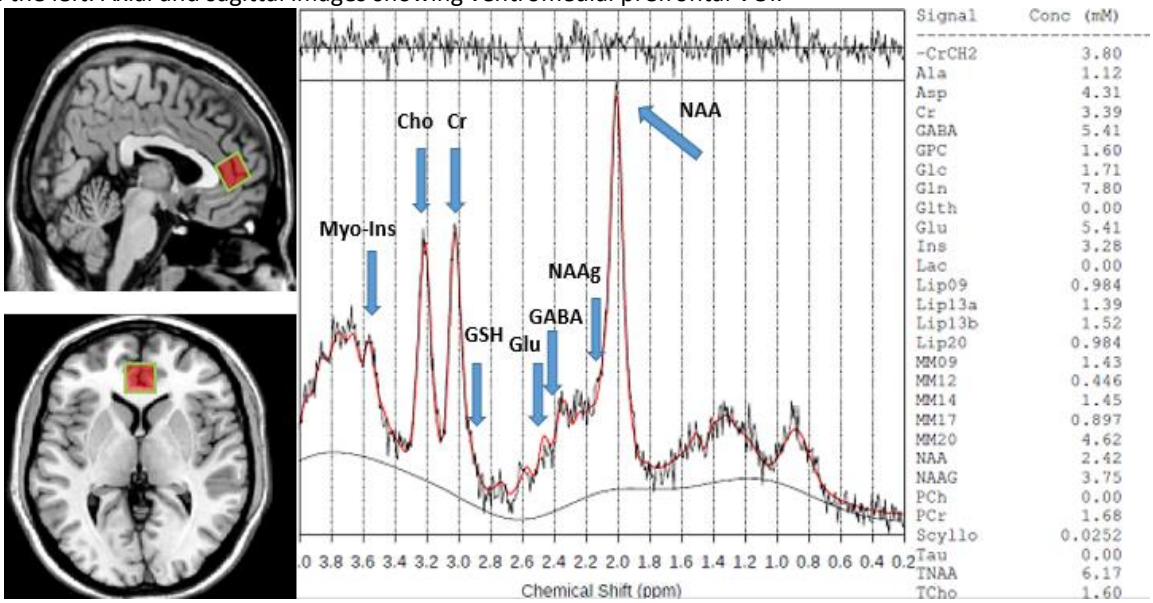
Fig. 5. Acquisition of MRI images for spectroscopic analysis. The MRI consists of: a) A magnet to generate a stable magnetic field, which can be of variable intensity, defining the resonant frequency of each core. Depending on the magnetic field, the atomic nuclei resonate at different MHz.; b) Probe, located inside the magnet, in which the sample is introduced and which consists of the coils responsible for emitting and receiving radio frequencies. The number of coils and their arrangement determine the type and applications of each probe; c) Console, in which the RF pulses are generated and the rest of the electronic part of the spectrometer is controlled; d) Computer, serving as an interface with the spectrometer and with which all the information obtained is analyzed.



After raw data extraction, images were post-processed using TARQUIN software, version 4.3.10. Quality control of the data was performed at 2 stages. The first included visual inspection of images by 2 researchers blindly (Metodi Draganov and Dr. Maria J. Portella), while the second stage included

disqualification of spectra with standard deviations above 30% for quantifications of the main metabolites.

Fig. 6 On the right: A typical post-processed spectroscopy image of MRS data using TARQUIN software, version 4.3.10, PRESS acquisition at 3T, with TR= 2000 ms and TE= 38 ms. The peaks represent the specific metabolite of interest. Quantity of the metabolites is measured in absolute values in millimoles. The initial quality of the spectra is determined by the signal-to-noise ratio, measured by the Cramer-Rao bound (the wave line at the top) and should not exceed Standard Deviation (SD) 20%.
On the left: Axial and sagittal images showing ventromedial prefrontal VOI.



3.2.3. Statistical analysis

For this study, a risk allele carrier models were employed for 3 of the polymorphisms of IL-1β gene that showed strongest signal in Study 1. For the 3 investigated SNPs, the patients were grouped as "A" carriers vs non-carriers (rs1143634); "T" carriers vs non-carriers (rs1143643); "A" carriers vs non-carriers (rs16944). To test whether there is a risk allele and if MSM score affects glutamatergic metabolite levels (Glutamate, Glx), separate generalized linear models (GzLM) were fitted. These were done separately for the 3 SNPs and the 2 metabolites (Glutamate and Glx) totaling 6 models. This method provided the possibility to investigate not only the main effects of risk allele and MSM, but also their interaction. Age, GM, WM and CSF were used as covariates in all analyses. No correction for multiple comparisons was used in Study 2 as the hypothesis was narrowly defined and it was expected that only 6 models should not result in false discoveries.

3.3. Ethical considerations

The sample for the 2 studies was collected as part of a bigger project funded by the Spanish Ministry of Health (PI10/00372) and by the Intramural Call for Basic Research of Institut de Recerca of the Hospital de la Santa Creu i Sant Pau (IIB15/Intramural) that was approved by the Research Ethics Board of Hospital de la Santa Creu i Sant Pau and was carried out in accordance with the Declaration of Helsinki. All participants were debriefed for the aims of their data collection and signed an informed consent that they agree their data to be used for research purposes. They were also informed that they may request their data to be deleted from the databases at any time. The respondents were included in all data sets by an ID number and not real name in order to achieve anonymity. The data collection itself is considered harmless and consisted of blood sample (for Study 1 and 2) and MRI scanning (for Study 2) following standard approved procedures by qualified personal at Hospital de Santa Creu I Sant Pau, Barcelona.

Chapter 4

Results and publications

The original aim was to present this Thesis as work resulted in two articles published in indexed scientific journals. Study 1 has resulted in the article '' Association study of polymorphisms within inflammatory genes and methylation status in treatment response in major depression'' published in European Psychiatry (Draganov et al., 2019). Study 2 has resulted in the article ''Polymorphisms in the IL1-β gene are associated with increased Glu and Glx levels in treatment-resistant depressed patients'' submitted for publication in the journal Psychiatry Research: Neuroimaging on 06/08/2020. It has been under review for several months now but unfortunately the global pandemic has caused severe disturbances and delays in the publishing process as explained by the journal editor. Therefore, copies of the two articles – the first published and the second still unpublished can be found in the Appendix 1 and 2. A concise summary of the Results of the two studies is presented in the following section.

4.1. Study 1: Results

4.1.1. Genetic results

All the explored SNPs were in the Hardy–Weinberg equilibrium and the genotyping success was >95%. Single marker analysis showed uncorrected significant associations with the total MSM result. The allelic distribution of the IL6R rs57569414 variant was associated with MSM total scores (OR=-1.62; p = 0.002). Uncorrected significant associations were also exhibited for the MSM total scores and IL18 rs543810, IL1-β rs1143643, IL6 rs2069824 and IFN-γ rs2069718. The analyses of genotype frequencies suggested nominal associations between the IL6 rs2069824, IL6R rs4075015, IL2 rs1479923 and IL10 rs3021094 genetic variants and MSM scores (p values<0.05 in all comparisons). Finally, uncorrected significant associations between haplotype combinations of the IL1-β, IL6 and IL10 mutations were also detected (p values = 0.02, 0.03 and 0.03, respectively). Logistic regression models (i.e., responders vs non-responders) revealed uncorrected associations between the allelic and genotype distribution of the IL1-β rs1143643 variants and response to treatment of the patient (OR = 2.49, p = 0.0009; and OR = 2.79, p = 0.002, respectively). There was an association of the allele and genotype frequency distribution of the IL6R rs4075015 with the MSM as a binary variable (p = 0.04 and p = 0.03, respectively).

The allele analysis of IL6R rs57569414 (p = 0.009), IL18 rs543810 (p = 0.03) and IFN-g rs1861493 (p = 0.046) revealed significant association with treatment response. Additionally, the genotype distribution of IL2 rs10027390 was nominally linked to treatment response (p = 0.05). Haplotype analyses suggested associations between allelic combinations of the genes IL1-β, IL10, IFN-g and IL6R and treatment outcome (uncorrected p = 0.0006, p = 0.003, p = 0.03 and p = 0.05, respectively). Most of the associations disappeared after FDR correction. Only the allelic association of IL1-β rs1143643 with MSM total scores remained significant and the genotype association of IL1-β displayed a trend towards significance. Haplotype analyses for allelic combinations of IL1-β and IL10 with binary MSM scores also remained significant after FDR correction (p = 0.004 and p = 0.01, respectively).

4.1.2. Epigenetic results

None of the thirteen analyzed CpG sites in the IL6, IL6R and IL1-β genes displayed statistically significant differences in the methylation status when MSM total score was used (p > 0.05 in all comparisons). Nevertheless, when comparing the methylation percentage of the two groups (responders and non-responders), one of the CpG sites in the IL-6R gene exhibited a trend towards significance (1.7 vs. 1.5, respectively; p = 0.05, uncorrected for multiple testing).

4.2. Study 2: Results

4.2.1. Effects of rs1143634

For the Glu results, the omnibus test showed non-significant results (p = 0.1). However, both MSM (Wald $\chi2$ = 27.24, p = 0.004) and SNP (Wald $\chi2$ = 4.94, p = 0.03) were significant, but the interaction of MSM×SNP persisted as non-significant (p = 0.46). The omnibus test was significant for the Glx (Likelihood Ratio $\chi2$ =32.78, p = 0.04), with both MSM (Wald $\chi2$ = 32.73, p = 0.0006) and SNP (Wald $\chi2$ = 5.61, p = 0.018) again reaching significance. The interaction, however, was non-significant MSM×SNP (p = 0.17).

4.2.2. Effects of rs1143643

When examining rs1143643, the omnibus test for the Glu was non-significant (p = 0.17). Neither the factors, nor the interaction showed significance (p > 0.05). For Glx, only MSM reached significance (Wald $\chi2$ = 22.21, p = 0.02).

4.2.3. Effects of rs16944

For the third polymorphism, the omnibus test was significant for the Glu model (Likelihood ratio $\chi2$ = 44.70, p = 0.002). The model also showed a significant main effect of MSM (Wald $\chi2$ = 42.76, p = 0.00001) and a significant MSM×SNP interaction (Wald $\chi2$ = 33.92, p = 0.00009). For the Glx, the omnibus test was significant (Likelihood Ratio $\chi2$ = 33.45, p = 0.041), with the MSM (Wald $\chi2$ = 34.89, p = 0.0003) highly significant. The MSM×SNP interaction was only marginally significant (Wald $\chi2$ = 16.93, p = 0.049). The direction of these findings suggested that non-resistant and resistant ''A''-carriers were indistinguishable for Glu and Glx levels, whereas non-carriers with bad response showed increased levels of Glu and Glx compared to good responders.

Chapter 5

Discussion

5.1. General discussion

5.1.1. Study 1

The main interest of Study 1 was to assess whether treatment response might be associated with specific genetic variants related to inflammation or their methylation status. Results suggest that from the 8 selected genes, IL1-β rs1143643, IL6R rs57569414 and IL6 rs2069824 show the most robust relationship with treatment outcome, with rs1143643 surviving FDR correcting for multiple comparisons. This is supportive of previous research linking rs1143643 with resistance (Baune et al., 2010), but the current Study 1 is the first to show that a specific polymorphism in the gene coding for the receptor of IL-6 might be also related to non-response. With regards to the epigenetic findings, one of the CpG islands at the IL-6R gene was marginally significant.

There are various ways to investigate genetic factors in relation to psychiatric disease, but association studies between a case and a control samples have a main advantage, namely increased power to detect even small gene effects compared to linkage studies across same family, eg. twin studies (Nöthen et al., 1993). As Menke (2012) points out, major depression has a very complex inheritance with involvement of numerous susceptibility genes and association studies might represent the optimal study design to explore, identify and test candidate genes for this disorder. The results from Study 1 point towards the major role of 3 genes and their association with resistance to treatment: IL-1β, IL-6 and IL-6R.

Interleukin-1 beta cytokine is a member of the interleukin-1 super-family and has a potent pro-inflammatory function. It is primarily synthesized and released in the brain mainly by the microglia and astrocytes and (Tsai, 2017). The gene is located on chromosome 2q14 and consists of seven exons and six introns. From the 3 investigated polymorphisms, rs1143643 (located in intron 6) seems to exhibit the most significant association with treatment response in both the allelic and genotype analyses, with patients caring the CC genotype 3 times less likely to be resistant. This result was significant both when considering MSM as score and when comparing responders with non-responders (surviving FDR correcting for multiple comparisons). This is supportive of earlier findings of Baune and colleagues (2010) who also reported that the GG genotype is highly related to worse outcome after 6 weeks of treatment. The other 2 investigated SNPs, namely rs1143634 and rs16944 did not reach significance on their own although they have been previously implied as predictors for treatment resistance. Several studies reported rs16944 as marker for non-response, such as Younger et al. (2003), Tadic et al, (2008) and Borkowska et al., (2011). However, the first 2 investigated specific pharmacological

agents (fluoxetine and paroxetine respectively), and the latter compared healthy controls directly with patients suffering recurrent depression. Study 1 in contrast investigated patients in naturalistic settings on variety of treatments and did not have a control group of healthy subjects, factors that might account for the inconsistency of the results with regards to rs1143634 and rs16944. Interestingly, in Study 1 the haplotype combination of the 3 SNPs was significant (uncorrected), suggesting a possible cumulative effect of the IL-1β polymorphisms. Therefore, even though only rs16944 is in the promotor region of the IL-1β gene, previous findings suggest that haplotypes including rs16944 might be associated with increased IL-1β protein secretion (Hall et al., 2004).

Although only the IL-1β results survived correction for multiple comparisons, some interesting uncorrected results emerged from Study 1, specifically with regards to some of the variants of the IL-6 (rs2069824) and IL-6R (rs57569414) genes (the first coding for the IL-6 cytokine and the latter for the receptor of the same cytokine). There are 2 ways in which the IL-6 cytokine can signal –the so called "classical" or ''trans-signaling" and depending on it, the cytokine IL-6 can have either anti (classical) or pro (trans-signaling) inflammatory function (Rose-John, 2012). The classical comprises of IL-6 binding to the 80 kDa IL-6R and then associating with a second protein, gp130, which thereupon dimerizes and commences intracellular signaling. Interestingly, although gp130 is expressed on all cells, IL-6R is barely present on few cells including hepatocytes and some leukocytes, therefore the cytokine does not have effect where IL-6R is not expressed. Nevertheless, in the trans-signaling the IL-6 bind to a soluble blood-circulating form of the receptor sIL-6R (representing the extracellular part). Therefore, when discussing IL-6 in depression, most of the studies investigate the pro-inflammatory signaling. This differentiation is important to note because a clear knowledge of IL-6 biology is necessary for developing future therapeutic strategies aimed at the impediment of the cytokine. Indeed, studies have suggested that a more promising pharmacological strategy might be to increase the soluble glycoprotein 130 (sgp130) inhibition of IL-6 trans-signaling instead of targeting the classic membrane (Maes et al., 2014).

Previous research has suggested that genetic variants of the IL-6 might be related to treatment resistance in depression. Carvalho et al. (2017) investigated IL-6 and reported that rs1800795 might be associated with a 75% reduced risk to develop TRD. However, we could not replicate this result for rs1800795, possible due to methodological differences such as study design, patients' selection and sample size differences. From the genetic variants of IL-6 investigated in Study 1, the rs2069824 showed the strongest signal, and to the best of our knowledge this is the first study reporting association between this variant and treatment resistance. In addition, a haplotype combination of 9 SNPs at the IL-6 gene showed significant results before FDR correction. This specific polymorphism (rs2069824) is located at the promoter region of the IL-6 gene (Haralambieva et al., 2011) and it might influence the secretion and circulating levels of the IL-6 pro-inflammatory cytokine through the gene expression. Interestingly, in Study 1 a polymorphism at the IL-6R, namely rs57569414 (intron variant;

FDR corrected p = 0.07 in the allele analysis when MSM used as score), and a haplotype combination of 10 SNPs (FDR uncorrected p = 0.05) also emerged as candidates for future investigation. Recently, Sowa-Kućma and colleagues (2018) explored serum levels of the soluble form of the IL-6R and reported an increase in TRD patients compared to the non-resistant ones. As discussed earlier, the availability of the soluble form of the IL-6R enables the robust pro-inflammatory effect of the IL-6 cytokine and despite the fact that rs57569414 is not at a promotor region, a further investigation of this variant with simultaneous measuring of peripheral inflammation and expression of the receptor is highly warranted. Until then, the association of the 2 polymorphisms IL-6 rs2069824 and IL-6R rs57569414 with amplified pro-inflammatory processes remains speculative.

Study 1 is the first to link a polymorphism in the IL-18 pro-inflammatory gene, namely rs543810, to treatment resistant depression. This variant showed a significant signal both at the allele and genotype analyses, although it did not survive correction for multiple comparisons. The available information about possible functional effect of this SNP is scarce. A previous study by Frayling and colleagues (2007) investigated this variant with regards to physical functioning in older age but did not find it to be associated with serum levels. Therefore, further investigation is necessary. Another variant investigated in Study 1, rs1946518 has been linked to onset of depression in patients previously exposed to stressful-life events (Haastrup et al., 2012). A more recent imaging genetics study (Swartz et al., 2017) investigated a haplotype combination including two SNPs (rs187238 and rs1946518) and reported a significant indirect effect of IL-18 risk haplotype on symptoms of depression and anxiety through increased threat-related amygdala reactivity in women. Surprisingly, in Study 1 rs1946518 did not show any association with treatment resistance, but this might be due to a possible effect of this variant only in haplotype combinations or in the presence of previous trauma.

From the anti-inflammatory genes investigated in Study 1, only a haplotype combination of 12 SNPs of the IL-10 gene showed significant signal. The haplotype was significant with both MSM as score (p = 0.03) and bimodal (p = 0.003) but vanished after correcting for multiple comparisons. This lack of results with regards to anti-inflammatory genes only reinforces previous research (Fabbri et al., 2017) that have reported almost exclusively polymorphisms in the pro-inflammatory genes and indirectly supporting the notion that non-response is related to increased inflammation. It could be expected that functional SNPs linked to increased levels of anti-inflammatory cytokines such as IL-10 might be associated with improved treatment, but these remain to be found, probably by significantly increasing the investigated samples.

The genetic results from Study 1 have provided some tentative hints about the role of IL-1β, IL-6 and IL-6R genes and some of their polymorphisms in relation to treatment response. The results are in accordance with previously reported findings suggesting that some patients might be genetically predisposed to be treatment-resistant. It is clear by now that although there is a substantial genetic

component, non-response can be also influenced by environmental factors such as stressful life events. Nevertheless, there is a striking individual variability in vulnerability to environmental factors, with most of the people showing resilience and not developing psychiatric disorders or resistance to treatment despite exposure to stressful events (Dudley et al, 2011). As discussed in the introduction, it has been proposed that epigenetic mechanisms such as DNA methylation can shed more light on the interplay between genetic and environmental factors, manifesting in susceptibility or resilience to stressful events, which thereafter might mediate developing specific depressive phenotypes such as TRD. Therefore, in the second analysis of Study 1, the aim was to explore possible epigenetic alterations in the genes that showed the strongest signal in the genetic analysis. Surprisingly, none of the investigated CpG islands in the IL-1β (2 CpGs), IL-6 (5 CpGs) and IL-6R (6 CpGs) genes showed altered methylation status after correcting for multiple comparisons. The uncorrected results however showed 1 of the CpG island in the IL-6R gene to be marginally hypomethylated in non-responders. Although speculative, this could suggest an alteration in expression of the IL-6 receptor gene that could be associated with fluctuations in IL6 peripheral levels. As discussed earlier, the availability and subsequent effect of the IL-6 cytokine can be easily hindered or augmented by the accessibility of the receptor. Indeed, the methylation results from Study 1 suggest that the IL-6/IL-6R genes complex might be a prime candidate for further epigenetic investigations, with longitudinal studies measuring epigenetic changes before and after treatment especially warranted.

Study 1 comes with several limitations. Probably the most important ones are those related to the "candidate gene" approach – conclusions are based on moderate sample size and should be confirmed in larger independent sample. This is important as several previous reports have suggested polymorphisms to be associated with treatment resistance, but some have failed to be reproduced implying a large number of false-positive reports. This issue was addressed in Study 1 by applying correction for multiple comparisons, after which the results for IL-1β rs1143643 variant remained significant. Given the exploratory nature of Study 1, although the other SNPs did not survive FDR correction, they remain intriguing targets for future research. Another uncertainty comes from the lack of knowledge regarding the functionality of some of the investigated variants and if they can influence the levels of peripheral inflammation. This could be addressed in future by measuring the plasma levels at several longitudinal points. With regards to the epigenetic results, probably the most important limitation comes from the fact that patients were not assessed for previous traumatic events - a factor repeatedly linked to aberrant methylation. Despite these constraints, Study 1 still provide novel and useful information about the genetic and epigenetic alterations linked to worsen treatment prognosis. As response to treatment has a marked genetic component, investigating genetic variants as biomarkers is still theoretically optimal for furthering the personalized antidepressant treatments and will remain at the forefront of research efforts in the foreseeable future.

5.1.2. Study 2

Study 2 focused on investigating further the genetic variants of the IL1-β gene explored in Study 1, their interaction with treatment resistance and the association with glutamatergic levels in the vmPFC area. Interestingly, IL-1β rs16944 showed the most robust results, suggesting that non-carriers of the "A" allele had elevated Glu and Glx levels with the increase of their resistance (genotype*MSM interaction). This indicates that increased glutamatergic levels might be a distinct neurobiological marker for a specific subsample of resistant MDD patients.

The recent positive reports of the rapid anti-depressive effect of the NMDA glutamate receptor antagonist ketamine has provided new neurobiological insights on the mechanisms underlying antidepressant efficacy and is '' poised to transform the treatment of depression'' (Krystal et al., 2019). It has been implicated for a long time that alterations in glutamatergic metabolism play a major role not only in the pathophysiology of depression, but also in developing treatment resistance. After glutamate is released to the synaptic cleft, it is taken up by adjacent cells through excitatory amino acid transporters (EAAT), while the extracellular glutamate is cleared by the astrocytes preventing excitotoxicity (Schousboe, 2003). Postmortem studies have found loss of glial elements such as astrocytes and oligodendrocytes, together with the transporters for excitatory amino acids, responsible for the reuptake and proper recycling of glutamate (Miller, 2013). Moreover, a plethora of studies have reported altered glutamatergic levels measured in-vivo by 1^{H} MR spectroscopy. This method enables quantification of glutamate-related metabolites such as glutamate and glutamine separately, or as a fusion of glutamate, glutamine, GABA, and other metabolites (referred to as Glx), depending on the field strength and signal-to-noise ratio (Yüksel and Öngür, 2010). Nevertheless, results from the available studies and meta-analyses are contradictory with reports for both increased and decreased levels, depending on the differences in regions of interest (ROIs), MRS methodologies, progression of illness, or the anti-depressive therapies used. A meta-analysis by Luykx and colleagues (2012) reported decreased glutamate levels in the ACC of depressed patients compared to controls. Another meta-analysis (Arnone et al, 2015) found reduction of the Glx (but not in Glutamate alone) in the prefrontal cortex in depression, correlating in meta-regression analyses with treatment severity and the number of failed antidepressant treatment trials. In the most recent meta-analysis, Moriguchi and colleagues (2019) explored forty-nine studies with 1180 depressed patients and 1066 healthy controls and reported significant decrease in Glx in the medial frontal cortex in medicated patients, but not in unmedicated patients, suggesting normalization of glutamatergic levels due to treatment. These notable inconsistencies might be accounted for by additional modulatory factors such as elevated inflammation through increased availability of cytokines.

The results from Study 2 that glutamatergic metabolites increase with the progression of treatment resistance support the notion that inflammation might interact and modulate glutamatergic neurotransmission. It can be speculated that carriers of specific polymorphisms and genotypes of the IL-1β gene are more susceptible to developing treatment-resistant depression through elevation of glutamatergic levels and subsequent excitotoxicity. The interplay between inflammation and glutamatergic neurotransmission has been previously investigated and it has been suggested that inflammatory mediators can increase the release of glutamate and inhibit excitatory amino acid removal by astroglia (McNally et al., 2008). Moreover, pro-inflammatory cytokines can alter glutamate metabolism and their levels in peripheral blood can predict response to ketamine the response to ketamine has been predicted by levels of their levels in the peripheral blood in a previous study (Walker et al., 2015), suggesting a synergic effect of inflammation and glutamate (Haroon et al., 2017) on treatment outcome. The findings of Study 2 showed a highly significant main effect of MSM on the metabolites' levels, together with a significant interaction between the IL-1β rs16944 polymorphism and MSM for both Glu and Glx levels. It seems that non-carriers of the "A" allele are more prone to increased glutamatergic levels and resistance, suggesting that the "A" allele might have a protective function. As discussed in more details earlier, the rs16944 is located in the promoter region of the gene and can influence the availability and levels of the IL-1β cytokine. This polymorphism has been repeatedly linked to treatment resistance in previous research, but this is the first time to be examined in relation to glutamatergic levels. An early report by Younger et al. (2003) suggested rs16944 to be associated with poorer outcome after 4 weeks of treatment with fluoxetine and further investigation suggested that patients homozygous for the T allele have a significantly better response to paroxetine treatment (Tadic et al, 2008). Baune et al., (2010) reported GG genotypes of rs16944 and rs1143643 to be related to poorer outcome after 6 weeks, while Borkowska et al., (2011) found rs16944 to be linked with recurrent episodes. As Study 2 is the first imaging-genetics investigation to report association between rs16944 and glutamatergic neurotransmission, the results should be verified in an independent sample. In contrast, it seems that the resistance score and genotype of rs1143634 have distinct effects on the glutamatergic levels, but no interaction. Patients with the ''A'' allele had both increased Glutamate and Glx, and again, increase of the resistance score was linked to rise in the metabolites' levels. The rs1143634 variant has been less studied in treatment-resistance, but it has been repeatedly linked to increased chronic inflammatory diseases by meta-analyses (Yin et al., 2016; da Silva et al., 2018) making it interesting candidate for further exploration. Interestingly, the SNP with the strongest signal in Study 1, rs1143643, failed to reach significance in Study 2 (except for a marginal main effect of MSM on Glx). Nevertheless, this is not abnormal as in Study 1 the measured outcome was the resistance score and not the metabolites' levels. The reduced sample size might also have an effect.

Interestingly, resistance score was significant in all models, except in the one with rs1143643 for Glu (although there was at trend level, p = 0.06). This suggests a strong association between treatment-resistance stage and glutamatergic levels in the vmPFC. In preclinical models, this area of the brain has been implicated as key target for antidepressants, conveying the effects of the pharmacological agents to the rest of the limbic system (Chang et al, 2015) and a recent study by Hare and colleagues (2020) reported ketamine infusion to be associated with increased activity of vmPFC pyramidal neurons in rodents. In human studies with treatment resistant patients, the responders displayed decreased right vmPFC metabolism compared to non-responders measured by positron emission tomography (Pardo et al., 2020). In addition, previous MRS investigations of the vmPFC have reported altered concentrations of Glutamate/Glutamine cortex in depression (Hasler et al., 2007), and in different stages of the illness (Portella et al., 2011). This latter study showed lower levels of glutamate in chronic patients, which would contradict our findings. However, our sample was not divided in terms of illness stage but treatment resistance stage. This controversy may reflect the lack of clarity regarding the relationship between illness manifestations and neurobiological systems. Therefore, Study 2 was designed to combine different technical approaches (neuroimaging and genetics, together with clinical scales) in order to provide a more comprehensive perspective of the pathophysiology and course of MDD and a direct comparison of the results might be irrelevant. The findings from the current study suggest that treatment resistance might be associated with excitotoxicity in the vmPFC area in some of the patients with specific genetic mutations. This excess of the glutamatergic quantities might be related to a dysfunction in the clearance done by astroglia, a process that can be inhibited by inflammatory mediators (McNally et al., 2008).

The results from Study 2 corroborate those of Study 1 that specific genotype and allele variations in these SNPs might be related to treatment-resistance in MDD. Whether this is due to increased levels of inflammation remains speculative as these were not measured, but the available previous literature indirectly suggests so. The IL-1β gene is coding for the highly pro-inflammatory IL-1β cytokine that has been previously related to treatment resistance by several lines of evidence, such as increased baseline levels in non-responders and normalization of after administration of antidepressants (discussed in more details in the Introduction). It has become evident in recent years that resistant patients display elevated pro -inflammatory markers (such as peripheral IL-1β levels) and therefore, plenty of research has focused on the genes coding for the cytokines and their mutations. The polymorphisms of the IL-1β gene investigated thoroughly in Study 1 have been previously linked to increased treatment resistance, but Study 2 is the first to link two of them (rs16944 in the promoter region and rs1143634 in exon 5) to changes in the glutamatergic levels in the vmPFC area.

Study 2 has several limitations. The limited sample size might have decreased the power to detect significant interactions and the findings need to be replicated in larger independent samples. A

power calculation was not available as there were no previous imaging-genetics studies with sufficient data combining MSM, genotype and MRS variables. As patients were not classified based on the antidepressants they used, the effect of the medication at the time of scanning could not be controlled for. Although they were scored in terms of pharmacological treatment failures and clinical characteristics, some of the antidepressants used at the time of scanning might have had an effect on glutamatergic levels. Some methodological issues should be also noted, such as the use of PRESS and 3T, but at the time of study set up MEGA PRESS and 7T were not widely available. A final limitation comes from the lack of a healthy control group, making it difficult to assess if the glutamate and Glx levels were altered or fall within normal cellular neurochemistry. Nevertheless, Study 2 explored a clinically well classified sample with clear *a priori* defined hypothesis (hence the lack of correction for multiple comparisons). The results provide novel findings regarding altered neurochemistry in the vmPFC area that might be related to pro-inflammatory genetic variants. In any case, the inflammatory – glutamatergic pathway emerges as not only important pathophysiological factor, but also provides novel targets for treatment of TRD patients.

5.2. Implications for future research

As stated by Macaluso and Preskorn (2012) "The development and use of a biomarker to identify 'at risk' individuals and to diagnose and/or quantify mental illness is the 'holy grail' of psychiatry". With all the benefits of early accurate prediction of response, this is especially valid for depression. The results from the hereby presented thesis suggest that mutations in genes coding for pro-inflammatory cytokines such as IL-1β and IL-6 might be a reliable predictor for treatment success or failure. As we are still far away from determining the molecular causes responsible for treatment resistance, genetics/epigenetics and imaging genetics present at least a possible avenue for exploring predicting factors. Future research should explore how specific polymorphisms are associated functionally with the levels of peripheral inflammation and whether the increased inflammation is related to structural/functional changes in brain areas previously associated with worsen outcome such as ACC and PFC. Moreover, there is an increasing evidence of epigenetic changes such as aberrations in methylation status in depressed patients due to increased stress exposure levels or prior childhood traumatic events. In this thesis, the results from the exploratory epigenetic analysis point towards methylation changes at 1 of the CpG islands of the IL-6/IL-6R complex. As the hypo/hyper methylation of the receptor might affect its availability and thus the proper function of the cytokine, epigenetic changes in these 2 genes remain to be further explored. Interestingly, the imaging-genetics

results from Study 2 suggest that treatment resistance is characterized with increased glutamatergic levels only in carriers of specific genetic variants. The genotype*MSM interaction of the IL-1β rs16944 associated with increased Glu levels should be confirmed in future larger samples, and in that case future studies might evaluate if these patients are responding better to agents normalizing glutamatergic levels compared to standard therapy. The rs16944 variant is specifically interesting as it is located in the promoter region thus affecting the pro-inflammatory IL-1β cytokine levels and Study 2 for first time suggest that it might be also related to glutamatergic levels in treatment -resistant patients. It has become clear by now that agents that can affect both Glu and inflammation levels such as ketamine have rapid anti-depressive effect even in TRD patients. Therefore, future investigations of ketamine efficacy considering rs16944 genotypes are highly warranted.

With regards to study design, there is one major flaw to be improved. Most studies of potential biomarkers and predictive factors are typically carried out in patients that have already been diagnosed with TRD. Therefore, it is not clear whether findings from such studies can be seen as predictive, causal, risk factors for the response, and not just as consequences of having developed TRD. The aim of this thesis was not to discover predictive causal biomarkers, but nevertheless an effort to account for this defect was made by measuring resistance on a continuous scale. Thus, using MSM score in addition to the binary division responders/non responders should be seen as one of the strengths of the currently presented work. Future studies should however employ longitudinal designs when possible in order to better differentiate between factors associated with TRD when diagnosed for first time in contrast to factors that might be consequences of years of treatment.

5.3. Implication for clinical practice

There are several benefits related to clinical practice that can be acquired with the discovery of novel and reliable neurobiological factors associated with treatment response and resistance. The first one is the better understanding that TRD patients might need a specifically targeted therapy with emphasis on reducing inflammation and normalizing glutamatergic levels. Surprisingly, in 2020 the exact molecular mechanisms involved in the action of antidepressants is still not clear (especially the delayed effect), but as discussed earlier inflammatory cytokines can influence the expression and activity of monoamine transporters (the main targets of SSRIs antidepressants) and further reduce the levels of tetrahydrobiopterin, a cofactor vital for the synthesis of serotonin (Miller et al., 2009). Moreover, several possible pharmacologic targets have been suggested, including the inflammatory

signaling pathways cyclooxygenase, p38 mitogen-activated protein kinase, the nuclear factor-κB, and the indoleamine-2,3-dioxygenase (a metabolic enzyme that breaks down tryptophan into kynurenine) (Haroon et al., 2012). As editing the genetic mutations of the pro-inflammatory genes is still in very early stages, Study 1 and Study 2 provide cautious clues that resistant patients with certain genotypes of the IL-1β gene variants might benefit from therapy targeted at reducing both inflammation and glutamatergic metabolites. Indeed, several reports have suggested that ketamine has anti-inflammatory properties and is able to reduce inflammation (De Kock et al., 2013) and the profound antidepressant effect might be due to the fact that it has effect on both glutamatergic and inflammatory systems. Another clinical benefit from reliable predictors of response is that the patient can be switched early to psychological interventions (eg. CBT), somatic therapies in highly resistant cases (Cusin and Dougherty, 2012), or even more alternative therapies such as psilocybin (Mertens et al., 2020). In any case, the shorter the patient is on ineffective therapy the better, especially keeping in mind the adverse effects of some antidepressants. Therefore, providing personalized treatment tailored to the specific biomarkers and genetic mutations of the patient should be envisioned in near future when treatment algorithms based on machine learning and artificial intelligence become widely available.

Chapter 6

Conclusions

The current thesis investigated the neurobiological factors associated with treatment response and resistance in major depression. Several preliminary conclusions can be drawn from the presented Studies 1 and 2.

- From the investigated 8 inflammatory genes, pro-inflammatory genes appear to have more predictive value compared to anti-inflammatory. This is in line with previous research suggesting increased peripheral levels of inflammatory markers.
- Polymorphisms in the IL-1β (rs1143643), IL-6 (rs2069824) and IL-6R (rs57569414) pro-inflammatory genes show the strongest association with treatment response success or failure.
- It can be speculated that responders and non-responders have different methylation at a CpG island on the IL-6R gene. As this gene is a vital part for the normal secretion of the IL-6 cytokine, epigenetic changes might hinder the proper physiological function of the IL-6/IL-6R complex.
- There are distinct neurochemical alterations taking place in the vmPFC brain area of treatment-resistant patients.
- Polymorphisms in the IL-1β gene might be associated with increased glutamatergic levels in treatment-resistant patients.
- The rs16944 variant located at the promoter region of the IL-1β gene appears to be associated with glutamatergic levels. Bad responders not carrying the A genotype display increased Glu and Glx levels compared to good responders non-carriers.
- The rs1143643 IL-1β variant is not related to glutamatergic changes even though it is linked to treatment resistance.
- Despite the moderate samples and the post hoc approach, results reported in this thesis provide novel clinically translatable insights.

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Appendix 1

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Original article

Association study of polymorphisms within inflammatory genes and methylation status in treatment response in major depression

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ABSTRACT

Background: Although pharmacogenetics for major depressive disorder (MDD) is gaining momentum, the role of genetics in differences in response to antidepressant treatment is controversial, as they depend on multifactorial and polygenic phenotypes. Previous studies focused on the genes of the serotonergic system, leaving apart other pathological factors such as the inflammatory pathway. The main objective of the study was to assess whether treatment response might be associated with specific inflammation-related genetic variants or their methylation status.

Methods: 41 SNPs in 8 inflammatory genes: interleukin (*IL*) *1-β*, *IL2*, *IL6*, *IL6R*, *IL10*, *IL18*, tumor necrosis factor (*TNF*)-*α* and interferon (*IFN*)-*γ* were genotyped in 153 patients with MDD, who were evaluated with the Mausdley Staging Method to determine treatment response profiles. Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q24 in 5 selected CpG islands of *IL1-β*, *IL6* and *IL6R*. Linear and logistic regression analyses were conducted, including age and gender as covariates using PLINK 1.07.

Results: Allelic distribution of *IL1-β rs1143643* was significantly associated with MSM scores (FDR corrected $p = 0.04$). Allelic distribution of *IL6R rs57569414* showed a trend towards significance with MSM scores ($p = 0.002$; FDR corrected $p = 0.07$). Haplotype analyses showed associations between allelic combinations of *IL1-β* and *IL10* with treatment response (FDR corrected $p < 0.01$). Methylation percentage of treatment responders was only higher in an *IL6R* CpG island ($p < 0.05$).

Conclusions: These exploratory findings suggest that *IL1-β* and, marginally, *IL6R* polymorphisms may affect treatment response in major depression. If confirmed, these results may account for the heterogeneous phenotypes of major depression that underlie differences in treatment response.

1. Introduction

Major Depressive Disorder (MDD) is one of the leading causes of disability worldwide affecting over 300 million people. [1] Around 30% of patients suffering from MDD do not respond to antidepressant treatment and are diagnosed with treatment resistant depression (TRD) after 2 unsuccessful courses of treatment with the appropriate dosage [2,3]. Treatment failure has a negative effect on patients´quality of life, is associated with increased risk for suicidal behavior and has higher economic costs for society [4]. In order to improve the efficacy of antidepressants, efforts have been made to identify biomarkers to predict treatment response, particularly within drug targets, i.e. serotonin pathways [5,6]. However, the findings to date have not fulfilled expected impact possibly because an individual's response to pharmacotherapy is multifactorial and involves a complex interplay of both genetic and environmental factors. [7]

Staging TRD has several benefits such as better predicting chances of future remission and guiding clinical treatment selection, minimizing the time that non-responders are on ineffective treatment. Various staging models of treatment resistance have been proposed. The Maudsley Staging Method

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and treatment response was also available. Severity of depression was assessed by the Hamilton Depression Rating Scale, 17 items (HDRS-17). [30] Illness stage was evaluated with the Maudsley Staging Method (MSM) [31], which included duration of index episode, symptom severity and treatment failures. This scale delivers a total score ranging from 3 to 15. For the purpose of the present study, and to better interpret the associations and to obtain odds ratios, MSM scores were also dichotomized between treatment non-response (MSM > 7; n = 62) and response (MSM< = 7; n = 91). After dividing the participants into responder and non-responder groups, the first consisted of 33 males (mean age: 44.03) and 58 females (mean age: 51.04), while in the second there were 15 males (mean age: 51.71) and 47 females (mean age: 54.73) respectively. Differences between the two groups were tested with Chi-square test for gender; and with independent t-tests for age, age at onset, number of depressive episodes, HDRS-17 and MSM scores. Expectedly, there were significant differences only in number of episodes, HDRS-17 and MSM scores ($p < 0.05$).

2.2. DNA isolation

Blood samples were systematically collected from the subjects upon admittance to the study. Genomic DNA was automatically extracted from peripheral whole-blood samples (Autopure, Qiagen, Hilden, Germany).

2.3. Genetic studies

A total of 41 Tag single nucleotide polymorphisms (SNPs) in 8 inflammatory genes (*IL1-β*, *IL2*, *IL6*, *IL6R*, *IL10*, *IL18*, *TNF-α* and *IFN-γ*) were selected for genotyping using the HapMap programme (www.hapmap.org). The SNP selection criteria were: minor allele frequency (MAF) over 0.05 and r2 threshold of 0.8 in Caucasians. The SNPs were analyzed by real-time PCR using OpenArray® technology on the QuantStudio™ 12 K Flex Real-Time (Applied Biosystems, Foster City, CA, USA). Standard quality controls (>95% genotyping success per individual and SNPs, Hardy-Weinberg equilibrium) were applied to the genotyping results.

2.4. Methylation analyses

Specific CpG sites in the 5′ regulatory region of selected genes (see Table 2 for details) were assessed by bisulfite-pyrosequencing. PyroMark Assay Design Software v.2.0 (QIAGEN, Hilden, Germany) was used to design the set of primers for PCR amplification and sequencing. Information on CpG sites locations and primers used is shown in Table 2. DNA bisulfite treatment and PCR amplification were performed by means of EpiTech Bisulfite kit and the PyroMark PCR kit (QIAGEN, Hilden, Germany) respectively, following the manufacturer recommendations. Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q24 (QIAGEN, Hilden, Germany).

2.5. Statistical analyses

Linear and logistic regression analyses for genotype results were conducted considering MSM as the dependent variable (as continuous and binary factors respectively). A logistic regression analysis was performed for methylation results, using binary MSM distribution. Age and gender of patients were included as covariates in all analyses. Statistical analyses were performed using PLINK (version 1.07, Purcel et al., 2007). Significance was set at $p < 0.05$. Correction for multiple comparisons in allelic and genotype associations was carried out with false discovery rate (FDR).

3. Results

3.1. Genetic results

All investigated polymorphisms were in the Hardy–Weinberg equilibrium and showed a genotyping success >95%. Single marker analysis revealed uncorrected significant associations with the total MSM scores. The allelic distribution of an *IL6R rs57569414* polymorphism was associated with MSM total scores (OR=–1.62; p = 0.002). Uncorrected significant associations were also found between MSM total scores and *IL18 rs543810*, *IL1-β rs1143643*, *IL6 rs2069824* and *IFN-γ rs2069718*. Results from the allele association analysis are presented in Table 3. Analyses of genotype frequencies revealed nominal associations between the *IL6 rs2069824*, *IL6R rs4075015*, *IL2 rs1479923* and *IL10 rs3021094* polymorphisms and MSM scores (p values<0.05 in all comparisons). Results from the genotype analysis are displayed in Table 4. Finally, uncorrected significant associations between haplotype combinations of the *IL1-β*, *IL6* and *IL10* polymorphisms investigated were also observed (p values = 0.02, 0.03 and 0.03, respectively).

Logistic regression models (i.e., responders vs non-responders) revealed uncorrected associations between the allelic and genotype distribution of the *IL1-β rs1143643* polymorphisms and response to treatment (OR = 2.49, p = 0.0009; and OR = 2.79, p = 0.002, respectively). The allele and genotype frequency distribution of the *IL6R rs4075015* polymorphism were also associated with MSM as a binary variable (p = 0.04 and p = 0.03, respectively). The allele distribution of *IL6R rs57569414* (p = 0.009), *IL18 rs543810* (p = 0.03) and *IFN-γ rs1861493* (p = 0.046) were also significantly associated with treatment resistance. Additionally, the genotype distribution of *IL2 rs10027390* was nominally associated with treatment response (p = 0.05). Results from the genotype analysis are displayed in Table 4. Haplotype analyses revealed associations between allelic combinations of the genes *IL1-β*, *IL10*, *IFN-γ* and *IL6R* with treatment response (uncorrected p = 0.0006, p = 0.003, p = 0.03 and p = 0.05, respectively).

Most of the associations vanished after FDR correction. Only the allelic association of *IL1-β rs1143643* with MSM scores remained significant and the genotype association of *IL1-β* showed a trend

Table 2
PCR amplification and pyrosequencing's design.

Gene	Number of CpG sites	Primer ID	Primer sequence (5' to 3')	Localization (start-end)
IL6	5	F	AGTGTAGGAAATTTTTAGTTTTGGAATTGT	5139-5258
		R	ACACAACTAAAAACCTACCTCTACTACTAA	
		S	AATTTTTAGTTTTGGAATTGTT	
IL6R	6	F	GGAGGGTTGGGGTAGTTAG	5544-5887
		R	AACAATCTCCCCTTAAAATAACCT	
		S	ACAATCCTATACACAAACC	
IL1-β	2	F	ATGGAAGGGTAAGGAGTAGTAA	9195-9366
		R	ATATCTTCCACTTTATCCCACATAT	
		S	ATGTAAATATGTATTGTTTTTTTGA	

ID: identifier; F: forward; R: reverse; S: sequencing; Localization according to the accession numbers. IL: interleukin. NG_011640 (*IL6*), NG_012087.1 (*IL6R*), NG_008851 (*IL1-β*).

Table 3
Allele associations with the Maudsley Staging Method (MSM) scores used as a continuous or binary (responders vs. non-responders) variable. SNP: single nucleotide polymorphism. FDR: false discovery rate. Significant associations are presented in bold. IFN: interferon; IL: interleukin; TNF: tumor necrosis factor.

		MSM Continuous				MSM Binary			
Gene	SNP	Beta	95%CI	p-value	(FDR)	OR	95%CI	p-value	(FDR)
IFN-γ	rs1861493	−0.68		0.09		0.56	0.32–0.99	**0.05**	(0.37)
IFN-γ	rs2069718	−0.69	−1.35– −0.03	**0.04**	(0.33)	0.74		0.20	
IFN-γ	rs2069727	0.56		0.11		1.12		0.63	
IL1-β	rs1143634	−0.16		0.70		0.70		0.22	
IL1-β	rs1143643	0.86	0.17–1.58	**0.02**	(0.29)	2.49	1.45–4.29	**0.0009**	(**0.04**)
IL1-β	rs16944	−0.34		0.34		0.69		0.14	
IL2	rs10027390	−0.58		0.11		0.66		0.11	
IL2	rs1479923	0.70		0.06		1.39		0.19	
IL2	rs2069772	−0.04		0.93		1.14		0.65	
IL2	rs2069778	−0.16		0.77		0.84		0.63	
IL2	rs3136534	−0.05		0.89		0.99		0.98	
IL6	rs10242595	0.57		0.14		1.21		0.47	
IL6	rs12700386	−0.16		0.74		1.04		0.91	
IL6	rs1800795	−0.15		0.70		1.05		0.86	
IL6	rs1800797	−0.17		0.66		1.00		1.00	
IL6	rs2069824	1.04	0.04–2.04	**0.04**	(0.33)	1.55		0.21	
IL6	rs2069835	1.13		0.07		1.88		0.13	
IL6	rs2069837	0.52		0.41		1.05		0.91	
IL6	rs2069840	−0.39		0.32		0.89		0.66	
IL6R	rs12047973	0.30		0.53		0.99		0.99	
IL6R	rs12083537	−0.21		0.66		0.77		0.43	
IL6R	rs3887104	0.44		0.33		1.23		0.49	
IL6R	rs4075015	0.68		0.05		1.64	1.02–2.65	**0.04**	(0.37)
IL6R	rs4133213	0.06		0.87		0.93		0.78	
IL6R	rs4556347	−0.32		0.42		0.78		0.35	
IL6R	rs57569414	−1.62	−2.61– −0.63	**0.002**	(0.07)	0.35	0.16–0.77	**0.009**	(0.19)
IL6R	rs6690230	0.10		0.80		1.20		0.50	
IL10	rs61815632	0.10		0.83		1.15		0.67	
IL10	rs1518110	−0.52		0.22		1.01		0.97	
IL10	rs1554286	−0.71		0.12		0.83		0.55	
IL10	rs1800890	0.32		0.40		1.59		0.07	
IL10	rs3021094	−0.14		0.79		1.01		0.98	
IL10	rs3024505	0.13		0.81		1.46		0.27	
IL18	rs543810	1.32	0.25–2.04	**0.02**	(0.29)	2.28	1.08–4.79	**0.03**	(0.37)
IL18	rs1946518	0.56		0.12		1.28		0.30	
IL18	rs3882891	0.31		0.36		1.21		0.41	
TNF-α	rs1799724	−0.48		0.43		0.74		0.48	
TNF-α	rs1799964	0.14		0.72		1.10		0.72	
TNF-α	rs1800629	0.21		0.71		1.10		0.81	
TNF-α	rs3093664	0.08		0.91		1.13		0.79	
TNF-α	rs361525	−0.01		0.99		1.12		0.81	

towards significance (see Tables 3 and 4, respectively). Haplotype analyses for allelic combinations of *IL1-β* and *IL10* with binary MSM scores survived FDR correction ($p = 0.004$ and $p = 0.01$, respectively).

3.2. Epigenetic results

None of the thirteen analyzed CpG sites in the *IL6*, *IL6R* and *IL1-β* genes showed statistically significant differences in the methylation status when MSM score was used ($p > 0.05$ in all comparisons). However, when comparing the methylation percentage of treatment responders and non-responders, one of the CpG sites in the IL6R gene showed a trend towards significance (1.7 vs. 1.5, respectively; $p = 0.05$, uncorrected for multiple testing). Results are summarized in Table 5.

4. Discussion

The current study investigated the influence of genetic alterations in inflammatory genes in relationship with treatment response in MDD. Inflammation is thought to play a role in the pathogenesis of depression, but the effect of inflammatory pathways on treatment response is still unclear. Our findings suggest that other factors beyond the serotonergic system may be involved in treatment response in major depression. In our study, *IL1-β* polymorphism showed consistent association with treatment response as measured with the MSM. *IL6* and *IL6R* polymorphisms were marginally associated, although significance did not survive correction for multiple comparisons.

The association of *IL1-β rs1143643* is in agreement with a previous study by Baune and colleagues [21] who reported two *IL1-β* polymorphisms (*rs1143643* and *rs16944*) to be related to SSRI treatment resistance. Another study by Yu and co-workers [22] also reported that the *IL-1β rs16944* polymorphism was associated with poorer outcome after 4 weeks of treatment with fluoxetine. However, we were not able to replicate this finding in our study. This might be due to the fact that in this study patients were on a strict 4-week treatment with fluoxetine, while our patients were on a variety of antidepressants and were assessed after a long period of treatment. In addition, the different ethnicity of the participants may also account for the discrepancy. Nevertheless, we observed a significant association between *IL1-β* haplotype combinations and treatment response.

With regards to *IL6*, the current results suggest that the *rs2069824* polymorphism might be associated with treatment response, although these findings should be taken cautiously as were uncorrected for multiple comparisons. In any case, the *rs2069824* allelic and genotype association with MSM suggest that this variant might be a prime candidate for future investigation.

Table 4
Genotype associations with the Maudsley Staging Method (MSM) scores used as a continuous or binary (responders vs. non-responders) variable. SNP: single nucleotide polymorphism. Significant associations are presented in bold. IFN: interferon; IL: interleukin; TNF: tumor necrosis factor.

		MSM Continuous			MSM Binary		
Gene	SNP	Stat	P value	(FDR)	Stat	P value	(FDR)
IFN-γ	rs1861493	3.51	0.17		4.36	0.11	
IFN-γ	rs2069718	4.77	0.09		4.37	0.11	
IFN-γ	rs2069727	2.92	0.23		0.23	0.89	
IL1-β	rs1143634	0.21	0.89		1.64	0.44	
IL1-β	rs1143643	10.58	**0.005**	(0.1)	12.79	**0.002**	(0.08)
IL1-β	rs16944	0.96	0.62		2.36	0.31	
IL2	rs10027390	3.96	0.14		4.20	0.12	
IL2	rs1479923	4.23	0.12		4.84	0.09	
IL2	rs2069772	0.04	0.98		0.37	0.83	
IL2	rs2069778	0.89	0.64		0.27	0.87	
IL2	rs3136534	0.02	0.99		0.29	0.86	
IL6	rs10242595	3.10	0.21		0.75	0.69	
IL6	rs12700386	0.13	0.94		0.41	0.81	
IL6	rs1800795	0.26	0.88		0.03	0.98	
IL6	rs1800797	0.82	0.66		0.13	0.94	
IL6	rs2069824	6.93	**0.03**	(0.41)	0.14	0.93	
IL6	rs2069835	3.69	0.16		1.64	0.44	
IL6	rs2069837	0.41	0.52		0.001	0.98	
IL6	rs2069840	1.04	0.59		0.51	0.77	
IL6R	rs12047973	3.92	0.14		0.18	0.55	
IL6R	rs12083537	0.77	0.68		0.02	0.99	
IL6R	rs3887104	0.97	0.62		0.48	0.79	
IL6R	rs4075015	4.65	0.09		4.71	0.09	
IL6R	rs4133213	0.44	0.80		0.12	0.94	
IL6R	rs4556347	2.15	0.34		1.11	0.57	
IL6R	rs57569414	10.61	**0.005**	(0.1)	5.59	0.06	
IL6R	rs6690230	3.26	0.19		2.15	0.34	
IL10	rs61815632	0.61	0.74		1.07	0.59	
IL10	rs1518110	2.11	0.35		0.84	0.66	
IL10	rs1554286	3.86	0.15		1.25	0.54	
IL10	rs1800890	0.77	0.68		3.21	0.20	
IL10	rs3021094	5.13	0.08		0.11	0.95	
IL10	rs3024505	0.37	0.83		1.82	0.40	
IL18	rs543810	6.43	**0.04**	(0.41)	5.92	**0.05**	(0.61)
IL18	rs1946518	3.68	0.16		1.44	0.49	
IL18	rs3882891	2.75	0.25		1.22	0.54	
TNF-α	rs1799724	1.07	0.59		0.15	0.93	
TNF-α	rs1799964	0.42	0.81		0.52	0.77	
TNF-α	rs1800629	0.17	0.92		0.001	0.99	
TNF-α	rs3093664	2.97	0.23		0.09	0.96	
TNF-α	rs361525	0.003	0.99		0.02	0.88	

We did not find association with any of the other *IL6* polymorphisms investigated. Carvalho and colleagues [23] had previously reported increased risk for resistance for patients having the *rs1800795*-"G/G" genotype. But, methodological differences such as study design and patients' selection could be underneath this discrepancy. In any case, both studies point towards the fact that genetic variations within cytokine genes, such as *IL6* may be involved in antidepressant treatment outcomes.

Interestingly, we also found *IL6R* gene to be related to treatment response-*rs4075015* and *rs57569414* emerged as possible predictors in both the allele and genotype analyses, together with a marginal haplotype combination-. As the pro-inflammatory signaling of IL6 depends on the soluble *IL6R* [32], it can be suggested that these genetic variants might play a role in this process. Our exploratory epigenetic study revealed marginal difference in *IL6R* methylation, with responders showing a higher methylation than non-responders, suggesting that an alteration in expression of the receptor gene may be associated with fluctuations in IL6 peripheral levels (see for example [33] reporting repeatedly increase plasma levels of IL6 in patients with worse treatment prognosis). Previous studies suggested that methylation changes might occur in MDD patients. An Epigenome wide study (EWAS) conducted in post-mortem frontal cortex from MDD patients revealed alterations in the methylation status of 224 regions, with differences >10%. [34]. Recently, several studies have suggested that the methylation status of genes such as *SLC6A4*, *NR3C1*, *BDNF* and *IL6* may constitute peripheral biomarkers for MDD. [35–37,29], Uddin and co-workers [38] reported an inverse correlation between the level of methylation of *IL6* CpGs and circulating IL6 and levels in patients with lifetime depression. We observed additional associations between pro-inflammatory and anti-inflammatory variants and treatment response. Previous research has suggested that pro-inflammatory *IL18* variants might play a role in MDD. Haastrup and colleagues [39] reported association between the *IL18 rs1946518* variant and increased risk of depression in patients with past stressful life events. However, in our study we did not find any significant association with this polymorphism. Nevertheless, we observed the *IL18 rs543810* allelic distribution associated with MSM when treated as total score and as binary variable, with OR > 2, although such associations did not survive FDR correction. To date, no other study has linked this polymorphism with treatment response, thus the relevance of this finding may also be spurious.

Despite the increasing interest in the inflammation theory of MDD, previous genetic research on cytokines has focused mainly on *IL1-β*, *IL6* and *IL18* variants. In light of our findings, the rest of studied cytokines, i.e., *IL2*, *IFN-γ* and *IL10* were not clearly associated with treatment response in our sample of MDD, but larger studies should explore the involvement of these variants in treatment outcomes. *IL10* haplotype combinations were associated with response in our study. A study by Song and colleagues [40] suggested decreased levels of *IL10* in depressed patients after 6 weeks of treatment and our results supports the notion that *IL10* might be associated with worse treatment prognosis. With all the

Table 5
Average of methylation percentage between non-responders (MSM > 7) and responders (MSM< = 7) in specific CpG islands of the *IL6*,*IL6R* and *IL1-β* genes. The analysis included age and gender as covariates. After adjusting for multiple testing (adjusted p-value) none of the differences were significant. MSM = Maudsley staging method; FC = fold change; IL: interleukin.

CpG	Non- Responders (MSM > 7)	Responders (MSM< = 7)	LogFC	t	p-value
IL6_1	3.516833	3.411333	0.04	0.15	0.88
IL6_2	4.726102	4.622889	0.13	0.21	0.83
IL6_3	2.874068	2.765	0.02	0.09	0.92
IL6_4	7.173158	6.930449	−0.08	−0.19	0.85
IL6_5	6.008772	5.750341	−0.11	−0.29	0.82
IL6R_1	1.423559	1.47686	0.08	0.72	0.47
IL6R_2	1.647627	1.757647	0.13	1.34	0.18
IL6R_3	1.890169	2.015	0.12	1.40	0.16
IL6R_4	1.5152	1.693714	0.20	1.94	**0.05**
IL6R_5	1.292881	1.214217	−0.03	−0.22	0.82
IL6R_6	1.812182	1.980125	0.18	1.49	0.14
IL1-β_1	7.120333	7.583933	0.14	0.38	0.70
IL1-β _2	9.466207	9.94236	0.20	0.46	0.64

reserves due to the exploratory nature of the present study, these findings suggest that not only pro, but also anti-inflammatory cytokines may influence treatment response. The contribution of a single polymorphism or a haplotype to the peripheral cytokine levels is not clear, although a previous study reported a correlation between a *IL1-β* haplotype and a 2–3-fold increase in the secretion levels. [41] Our results might be in line with the previously described mechanism linking increased pro- and anti-inflammatory cytokine levels with worse tratment response. Nevertheless, we did not measure the peripheral serum levels of the investigated cytokines and therefore these conclusions remain speculative.

The present study has several limitations that need to be considered when interpreting the results. It possesses the classical limitations of a candidate genes study - the sample size is moderate and results should be confirmed in a larger independent study, as previous research exploring an already published statistically significant finding for a SNP has often failed to reproduce those findings, implying a large number of false-positive reports. Another pitfall is that it is unclear whether the SNPs have functionally significant effects on the gene or they are simply useful markers. Nonetheless, together with genome wide association studies, candidate gene studies still provide important information about disease mechanisms and the ability to predict individuals who are at risk. A second limitation is that we did not control for differences in antidepressants' type and dose taken by participants. In any case, the naturalistic nature of the present study still provides realistic information about patients that become treatment resistant. A third possible limitation is that information about experienced childhood stressful events was not collected –a factor previously linked to aberrant methylatione. [42] Finally, plasma levels of specific cytokines would have added useful information to check the hypothesis of the study. In any case, a replication of these findings is warranted in future studies.

To conclude, the current findings support that treatment response might be associated with specific genetic variants, and partly by the methylation status, of the inflammation-related gene *IL1-β* and, to a lesser extent, *IL6* and *IL6R*. If confirmed, these results can provide information on additional genetic markers of response and constitute putative new targets for future novel therapies.

Conflict of interests

J.D.A received consultancy and/or lecture honoraria from Lundbeck, Pfizer, Neuraxpharm and Janssen in the last year, none of them with direct relation with this work. Thre rest of authors declare no conflict of interests related with this work.

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Appendix 2

Title Page (with Author Details)

Title: Polymorphisms in the IL1-b gene are associated with increased Glu and Glx levels in treatment-resistant depressed patients

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1. Introduction

Alterations of monoamine (particularly serotonin) levels are thought to be the main neurobiological basis of major depressive disorder (MDD) and monoaminergic pathways are primary targets for treatment. Despite decades of steady advances in the effectiveness of antidepressant therapies, nearly one-third to a half of patients do not respond adequately to standard treatment (Gibbons et al., 2012) and develop treatment-resistant depression (TRD).

Previous studies have pointed towards the influence of the glutamate and inflammatory pathways on treatment response to conventional monoaminergic antidepressants (Miller et al., 2013). Excessive glutamatergic neurotransmission and consequent excitotoxicity are considered a major cause of TRD (Kim & Na, 2016). Ketamine, an N-methyl-D-aspartate (NMDA) glutamatergic receptor antagonist, has been repeatedly associated with rapid antidepressant efficacy in both preclinical (Fond et al., 2014) and clinical reports (Newport et al., 2015). Interestingly, pro-inflammatory cytokines may underlie alterations in expression of glutamate transporters and glutamate release, providing a confluence of the inflammation and glutamate pathways, which has been suggested to play a major role on treatment resistance in MDD. Serum concentrations of pro-inflammatory cytokines such as IL-6 and IL-1β have been reported as elevated in non-responders compared to responders, while successful therapy was associated with their decrement after antidepressants administration (Kohler et al., 2014). An additional line of research has suggested that single nucleotide polymorphisms (SNPs) in inflammatory genes might predict treatment response (Uher et al., 2010). Several studies have reported variants of the IL-1β as prime candidates of such association (Baune et al., 2010; Yu et al., 2003). Indeed, stages of treatment resistance have also been associated with pro-inflammatory genetic variants of IL-6 and IL-1β (Draganov et al., 2019). A possible explanation is that inflammatory cytokines can regulate the expression and activity of monoamine transporters, the main targets of SSRI antidepressants (Miler et al., 2009). In addition, inflammatory mediators can increase the release of glutamate and inhibit excitatory amino acid removal by astroglia (McNally et al., 2008). Cytokines can also alter glutamate metabolism, and the response to ketamine was predicted by increased inflammatory cytokines in the peripheral blood in a previous study (Walker et al., 2015), suggesting a synergic contribution of inflammation and glutamate (Haroon et al., 2017).

The relationship between levels of glutamate in the brain and treatment response has been explored with ^{1}H-Magnetic Resonance Spectroscopy (MRS), a method allowing harmless in vivo measurement of brain metabolites. Price et al. (2009) already examined subgroups determined by antidepressant medication resistance, although Glx (glutamate + glutamine) levels in occipital cortex (OCC) and anterior cingulate cortex (ACC) were not associated with clinical outcome. In another

study, Block et al. (2009) investigated metabolite changes after 8 weeks of treatment, but Glx again did not seem to be associated with treatment response. By contrast, Portella and colleagues (2011) found altered Glu levels through different stages of the illness within the ventromedial Prefrontal Cortex (vmPFC), in which treatment resistant patients had the lowest levels. There is evidence that this brain region is altered both in the neuropathological processes of MDD (Salvadore et al., 2011) and in treatment response (Mayberg's studies). To date, it is not clear whether altered glutamatergic levels might be considered reliable markers for a possible TRD phenotype, or treatment resistance is the result of years of pharmacotherapy. One possible way to investigate vulnerability to treatment resistance is to consider the genetics involved in neurobiological mechanisms such as the inflammatory pathways. The relationship between specific pro-inflammatory polymorphisms, glutamatergic metabolism and treatment resistance has not been investigated so far.

Inflammatory cytokines can alter glutamatergic levels, which in turn can affect treatment response via neuron-glia communication. Thus, exploration of genetic variations within pro-inflammatory genes –previously suggested as risk factors for TRD– might provide some new evidence on treatment resistance in MDD. Furthermore, individuals at high risk for treatment resistance could benefit from early emphasis on psychological interventions and use of non-pharmacological brain stimulation therapies (Brunoni et al., 2013). Therefore, the main objective of this study was to investigate a possible interaction between variants in inflammatory genes, glutamatergic levels and treatment outcome. We hypothesize that pro-inflammatory polymorphisms and treatment resistance are associated with greater MRS alterations, specifically in glutamatergic pathways. Based on our previous research (Draganov et al., 2019), genetic variants in IL-1β gene were selected and ^{1}H-MRS was employed to investigate genotype-dependent differences in glutamate levels in the vmPFC in a sample of depressed individuals.

2. Methods

2.1. Participants

The sample consisted of 50 patients from the outpatient service of the Department of Psychiatry, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. Inclusion criteria were: participants had to be 18-65 years old, right-handed and native Catalan and/or Spanish speakers (please note that Catalan inhabitants are mostly bilingual for these two languages). Exclusion criteria were: clinically significant physical or neurological disease (brain trauma with loss of consciousness) and mental retardation (score <70 on the estimated IQ using the Vocabulary subtest of the Wechsler Adult Intelligence Scale-IV, Spanish validated version). Participants were of Caucasians of European descent and all gave informed consents after a full explanation of the study protocol. Patients did

not receive any financial reimbursement for their participation. All patients were on standard antidepressant treatment at the time of blood sampling and image acquisition, following the clinical guidelines of the national health system. Given the naturalistic approach of this study, time on antidepressants was not controlled for. The study followed the guidance of the Declaration of Helsinki and was approved by the Research Ethics Committee of Hospital de la Santa Creu i Sant Pau.

2.2. Clinical evaluation

Semi- structured interviews were conducted for all participants to collect demographic and clinical information, including comorbid Axis I conditions as described in DSM-IV-TR. Current depressive state was assessed by an experienced psychiatrist or psychologist using the Hamilton Depression Rating Scale 17 items (HDRS-17, Hamilton, 1960) and previous episodes were double-checked through clinical records. Treatment resistance was evaluated using the Maudsley Staging Method (MSM) by calculating a total score ranging from 3 to 15 (Fekadu et al., 2009). This scale includes duration of index episode, symptom severity and treatment failures.

2.3. DNA extraction and SNP genotyping

Blood samples were systematically collected (10ml) from the patients upon admittance to the study. Genomic DNA was automatically extracted from peripheral whole-blood samples (Autopure, Qiagen, Hilden, Germany). As in our previous study (Draganov et al., 2019) the IL-1β gene showed the most robust findings, the three SNPs previously investigated were selected for the current study: rs1143634 (intronic), rs1143643 (exonic, synonymous) and rs16944 (promoter region).

2.4. ^{1}H-MR-spectroscopic data acquisition

We obtained MRS images using a 3T Philips Achieva scanner (software version 2.1.3.2) and a SENSE 8-channel head coil with a dedicated acquisition protocol. This included a 3-dimensional magnetization-prepared rapid-acquisition gradient echo (3D-MPRAGE) whole-brain sequence (turbo field echo, repetition time [TR] 6.7 ms, echo time [TE] 3.1 ms, voxel size 1 × 1 × 1.2), on which ^{1}H-MRS (single-voxel spectroscopy with point resolved excitation spin-echo sequence; TR 2000 ms, TE 38 ms, numbers of signals averaging 128, volume of interest –VOI– of 2 × 2 × 2 cm, AutoWS-Prescan) images were obtained from a selected volume of interest (VOI) within the ventromedial prefrontal region embracing both hemispheres. Therefore, selected VOI included white and grey matter, as well as cerebrospinal fluid (CSF). The ^{1}H-MRS raw data were exported and then post-processed using TARQUIN software, version 4.3.10 (www.tarquin.sourceforge.net). This is an external reference method that provides concentrations (in millimoles) of the single metabolite peaks. For the purposes

of the present study, we included the following metabolites: Glu and Glx (corresponding to Glu+Gln peak). All metabolites were measured in absolute levels (SD% based on the Cramer-Rao lower bound). Spectra quality was assessed blindly by 2 researchers and was qualified as unusable when having standard deviations above 30% for quantifications of the metabolites. See Figure 1 with the exact location of the VOI in the vmPFC and a spectrum of a given patient.

2.5. Statistical analysis

The data were analyzed in SPSS 20.0. The three genotypes in the polymorphisms were analyzed with risk allele carrier models, where patients are grouped as A carriers (including AA and GA genotypes) vs non-carriers (GG genotype) for rs1143634; T carriers (TT and CT genotypes) vs non-carriers (CC genotype) for rs1143643; and A carriers (AA and GA genotypes) vs non-carriers (GG genotype) for rs16944. The effect of the IL-1β polymorphisms on metabolite levels was evaluated by fitting separate generalized linear models (GzLM), one for each polymorphism. Associations between the three main variables (genetic variants, treatment resistance stage and glutamatergic metabolism) were explored. Glu and Glx levels were defined as the outcome variable for each model, allele and MSM scores as between-subject fixed factors, and age, GM, WM and CSF as covariates. GzLMs are tools of choice for analyzing correlated and/or non-normal distributed data (i.e. Kolmogorov-Smirnoff for MSM was $D(50)=0.16$, $p=0.003$; Glu and Glx positively correlated $r = .84$, $p <0.001$). In order to further determine the direction of the associations, patients were grouped on the basis of the three categories of the original MSM scores (Fekadu et al. 2009): patients with mild treatment resistance were considered as non-resistant (MSM < 7), and patients in the other two categories (MSM ≥ 7; moderate and severe treatment resistance) were considered resistant.

3. Results

Table 1 summarizes demographic and clinical characteristics of the final sample. A summary of mean medication load is also displayed in the same table.

3.1. Effects of rs1143634

For the Glu results, the omnibus test was non-significant ($p = 0.1$). However, both MSM (Wald $\chi^2 = 27.24$, $p = 0.004$) and SNP (Wald $\chi^2 = 4.94$, $p = 0.03$) were significant, while the interaction MSM×SNP remained non-significant ($p = 0.46$). The omnibus test was significant for the Glx (Likelihood Ratio $\chi^2 = 32.78$, $p = 0.04$), with both MSM (Wald $\chi^2 = 32.73$, $p = 0.0006$) and SNP (Wald $\chi^2 = 5.61$, $p = 0.018$) again reaching significance, but not the interaction MSM×SNP ($p = 0.17$).

3.2. Effects of rs1143643

contradict our findings. However, our sample was not divided in terms of illness stage but treatment resistance stage. This controversy may reflect the paucity of knowledge regarding the relation between illness manifestations and neurobiological systems. Therefore, this study was designed to combine different technical approaches (neuroimaging and genetics, together with clinical scales) in order to provide a more comprehensive perspective of the pathophysiology and course of MDD.

Generalized linear models revealed differential effects depending on the IL-1β genetic variant investigated. With regards to rs1143634, the current findings were partially in agreement with our hypothesis, where A carriers showed increased levels of Glu and Glx compared to non-carriers, although the interaction allele x MSM interaction was non-significant, suggesting a possible impact of this polymorphism on glutamatergic metabolism and on treatment outcome without a clear interaction. Similarly, Baune and colleagues (2010) also failed to find significant associations between rs1143634 and response to treatment or responsiveness in the anterior cingulate cortex. No statistically significant associations were found when investigating the rs1143643 polymorphism. This result suggests that this polymorphism might be not synergistically associated with treatment stage and glutamatergic metabolism, which would be contrary to our hypothesis. However, a larger sample would be required to confirm or refute our findings.

The finding of a significant interaction between MSM and rs16944 genotype suggest that this SNP may play an important role in the progression of treatment resistance and specifically in later stages with the increase of MSM score. Interestingly, the rs16944 variant, located in the promoter region of the gene, is associated with IL-1β secretion (Hall et al., 2004). Our results show that rs16944-A non-carriers with higher scores on MSM –i.e., more treatment resistant– had increased levels of both Glu and Glx, thus suggesting the involvement of rs16944 variants in treatment outcome through or as a consequence of alterations in glutamate release within a key brain area such as the vmPFC. Noteworthy, in a previous work by our group, the authors failed to find a direct genetic association between rs16944 and treatment outcome (Draganov et al., 2019), while IL1- β rs1143643 and treatment response was indeed significantly associated. The smaller sample size in the current study may account for this discrepancy. However, in this previous study glutamate metabolic levels were not considered. Major depression is a complex disorder, and therefore, several neurobiological systems may be involved to explain such complexity. In addition, there is some opacity whether carrying (Hwang et al., 2002) or non-carrying (Iacoviello et al., 2005) the A allele is associated with increased IL-1β levels, with some arguing that transcription depends on the haplotype combination (Chen et al., 2006). Previous research has shown non-carriers of the A allele to have an early age of onset of depression (Hwang et al., 2009) and to interact with early-life adversity (McQuaid et al., 2019). Kovacs et al. (2016) found that the A allele interacted with childhood trauma increasing depressive symptoms, while more recently McQuaid et al. (2019)

suggested that males, but not females non-carriers were prone to elevated depressive symptoms. With regards to treatment, Tadic et al. (2008) and Baune at al. (2010) reported non-carriers to exhibit poorer response to antidepressant treatment. In the present sample, resistant A carriers had the highest Glu and Glx levels. Although there exists controversy whether glutamatergic metabolism is increased or decreased in more severe stages of MDD (Smaga et al. 2014), evidence indicates that an increased amount or activity of glucocorticoids, glutamate, and proinflammatory cytokines is associated with neurotoxicity, and therefore worse outcomes. In any case, we did not measure IL-1β serum levels, but the observed effect of polymorphisms in the glutamatergic system seems to point towards a possible confluence with treatment outcomes of MDD.

The current study has several limitations. The limited sample size may decrease the power to detect significant interactions and our findings need to be replicated in larger independent samples. A power calculation was not possible as there were no previous studies with sufficient data combining MSM, genotype and MRS variables. This leads to consider the present findings as preliminary. Secondly, although we scored patients in terms of their pharmacological treatment failures and clinical characteristics, the real effect of the medication at the time of scanning could not be controlled for. Another weakness could be the use of PRESS and 3T, but at the time of study set up MEGA PRESS and 7T were not widely available. Finally, the lack of a control group makes difficult to assess whether Glu and Glx levels are altered or fall within normal cellular neurochemistry.

In conclusion, our findings suggest that resistant patients who are non-carriers of the Il-1β rs16944-A allele have increased glutamatergic levels. This would add new evidence to current models on the interaction of inflammatory response on glutamate functioning in the fronto-limbic circuits (the vmPFC) that may lead to worse outcomes and poor treatment response in MDD.

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Keywords

Treatment- resistant depression; Spectroscopy; inflammatory genes; glutamate

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Table 1. Demographics and clinical evaluation of patients. HDRS-17: 17-item Hamilton Depression Rating Scale; MSM: Maudsley Staging Method.

	Total sample N=50	Range
Females N (%)	74	
Males N (%)	26	
Age	48.90 (12.66)	22-71
University education %	58	
Age at onset	35.86 (9.91)	19-54
Axis 1 comorbidity %	16	
N of episodes	2.64 (1.46)	1-7
Smokers %	35	
HDRS-17	18.75 (7.65)	4-35
MSM	7.32 (3.30)	3-14
Medication load*	6.73 (4.54)	2.5-22.5
rs1143634		
A carriers %	28	
Non carriers %	72	
rs1143643		
T carriers %	64	
Non carriers %	36	
rs16944		
A carriers %	54	
Non carriers %	46	

† Footnote: Values represent mean (SD) or otherwise specified. A detailed explanation about the algorithm used to obtain medication load can be found elsewhere (de Diego-Adelino et al., 2014)

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Table 2. Generalized linear models.

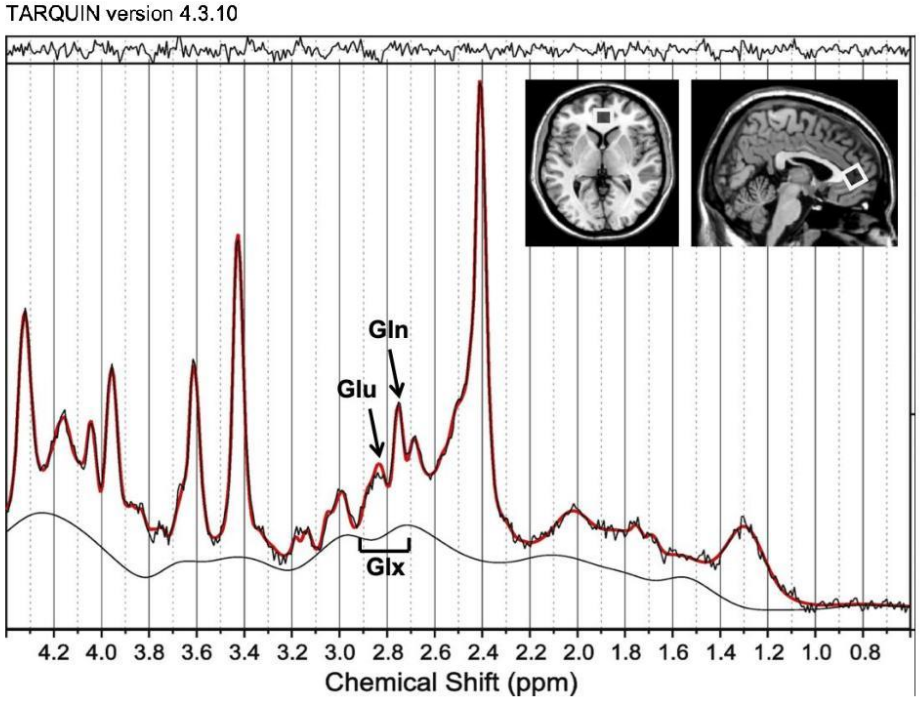
	Absolute Glu levels			Absolute Glx levels		
	LR or Wald $\chi 2$	df	p - value	LR or Wald $\chi 2$	df	p - value
Omnibus Test	28.70	20	0.09	32.78	20	0.04
MSM effect	27.24	11	0.004	32.73	11	<0.001
rs1143634 effect	4.94	1	0.03	5.61	1	0.02
Interaction	7.71	8	0.46	11.62	8	0.17
Omnibus Test	24.83	19	0.17	24.85	19	0.17
MSM effect	19.20	11	0.06	22.21	11	0.02
rs1143643 effect	0.63	1	0.43	0.57	1	0.45
Interaction	4.54	7	0.72	5.21	7	0.64
Omnibus Test	44.70	21	0.002	33.45	21	0.04
MSM effect	42.76	11	<0.001	34.89	11	<0.001
rs16944 effect	0.03	1	0.87	0.13	1	0.72
Interaction	33.92	9	<0.001	16.93	9	0.049

† Footnote: LR= Likelihood Ratio Chi-Square; MSM =Maudsley Staging Method.

Table 3. Estimated means Glu and Glx concentrations (in millimoles) for main effects and interaction for each IL-1β single nucleotide polymorphism. Maudsley Staging Method (MSM) was dichotomized into 2 categories: non-resistant (MSM < 7) and resistant (MSM ≥ 7).

	Absolute **Glu** levels			Absolute **Glx** levels		
MSM Binary	Polymorphism	Mean	S.D.	Polymorphism	Mean	S.D.
	rs1143634			**rs1143634**		
Non-resistant	A carriers	8.67	1.67	A carriers	14.97	4.48
	Non-carriers	8.20	2.11	Non-carriers	13.21	4.55
Resistant	A carriers	10.70	2.19	A carriers	17.58	5.02
	Non-carriers	8.68	1.79	Non-carriers	14.44	3.19
	rs1143643			**rs1143643**		
Non-resistant	T carriers	9.01	2.39	T carriers	14.79	5.19
	Non-carriers	7.87	1.40	Non-carriers	13.11	3.93
Resistant	T carriers	9.22	2.05	T carriers	15.3	3.96
	Non-carriers	8.36	1.86	Non-carriers	13.87	2.08
	rs16944			**rs16944**		
Non-resistant	A carriers	8.51	1.72	A carriers	14.27	3.66
	Non-carriers	8.11	2.38	Non-carriers	13.09	5.91
Resistant	A carriers	8.53	1.93	A carriers	14.48	2.12
	Non-carriers	9.52	2.03	Non-carriers	15.53	4.65

Fig. 1. Representative image after post-processing with TARQUIN software, version 4.3.10, PRESS acquisition at 3T, with TR= 2000ms and TE= 38ms. Dark squares display sagital and axial images showing ventromedial prefrontal Volume of Interest (VOI, white square).



Appendix 3

Patient Name:_____ **Date:**_____

Hamilton Rating Scale for Depression (17-items)

Instructions: For each item select the "cue" which best characterizes the patient during the past week.

1. **Depressed Mood**
   (sadness, hopeless, helpless, worthless)
   0 Absent
   1 These feeling states indicated only on questioning
   2 These feeling states spontaneously reported verbally
   3 Communicates feeling states nonverbally, i.e., through facial expression, posture, voice and tendency to weep
   4 Patient reports VIRTUALLY ONLY these feeling states in his spontaneous verbal and nonverbal communication

2. **Feelings of Guilt**
   0 Absent
   1 Self-reproach, feels he has let people down
   2 Ideas of guilt or rumination over past errors or sinful deeds
   3 Present illness is a punishment. Delusions of guilt
   4 Hears accusatory or denunciatory voices and/or experiences threatening visual hallucinations

3. **Suicide**
   0 Absent
   1 Feels life is not worth living
   2 Wishes he were dead or any thoughts of possible death to self
   3 Suicide ideas or gesture
   4 Attempts at suicide (any serious attempt rates 4)

4. **Insomnia - Early**
   0 No difficulty falling asleep
   1 Complains of occasional difficulty falling asleep i.e., more than ½ hour
   2 Complains of nightly difficulty falling asleep

5. **Insomnia - Middle**
   0 No difficulty
   1 Patient complains of being restless and disturbed during the night
   2 Waking during the night – any getting out of bed rates 2 (except for purposes of voiding)

6. **Insomnia - Late**
   0 No difficulty
   1 Waking in early hours of the morning but goes back to sleep
   2 Unable to fall asleep again if gets out of bed

7. **Work and Activities**
   0 No difficulty
   1 Thoughts and feelings of incapacity, fatigue or weakness related to activities; work or hobbies
   2 Loss of interest in activity; hobbies or work – either directly reported by patient, or indirect in listlessness, indecision and vacillation (feels he has to push self to work or activities)
   3 Decrease in actual time spent in activities or decrease in productivity. In hospital, rate 3 if patient does not spend at least three hours a day in activities (hospital job or hobbies) exclusive of ward chores.
   4 Stopped working because of present illness. In hospital, rate 4 if patient engages in no activities except ward chores, or if patient fails to perform ward chores unassisted.

8. **Retardation**
   (slowness of thought and speech; impaired ability to concentrate; decreased motor activity)
   0 Normal speech and thought
   1 Slight retardation at interview
   2 Obvious retardation at interview
   3 Interview difficult
   4 Complete stupor

9. **Agitation**
   0 None
   1 "Playing with" hand, hair, etc.
   2 Hand-wringing, nail-biting, biting of lips

10. **Anxiety - Psychic**
    0 No difficulty
    1 Subjective tension and irritability
    2 Worrying about minor matters
    3 Apprehensive attitude apparent in face or speech
    4 Fears expressed without questioning

11. **Anxiety - Somatic**
    0 Absent
    1 Mild
    2 Moderate
    3 Severe
    4 Incapacitating
    Physiological concomitants of anxiety such as:
    Gastrointestinal - dry mouth, wind, indigestion, diarrhea, cramps, belching
    Cardiovascular – palpitations, headaches
    Respiratory - hyperventilation, sighing
    Urinary frequency
    Sweating

12. **Somatic Symptoms - Gastrointestinal**
    0 None
    1 Loss of appetite but eating without staff encouragement. Heavy feelings in abdomen.
    2 Difficulty eating without staff urging. Requests or requires laxatives or medications for bowels or medication for G.I. symptoms.

13. **Somatic Symptoms - General**
    0 None
    1 Heaviness in limbs, back or head, backaches, headache, muscle aches, loss of energy and fatigability
    2 Any clear-cut symptom rates 2

14. **Genital Symptoms**
    0 Absent
    1 Mild
    2 Severe
    0 Not ascertained
    Symptoms such as: loss of libido, menstrual disturbances

15. **Hypochondriasis**
    0 Not present
    1 Self-absorption (bodily)
    2 Preoccupation with health
    3 Frequent complaints, requests for help, etc.
    4 Hypochondriacal delusions

16. **Loss of Weight**
    A. When Rating by History:
    0 No weight loss
    1 Probable weight loss associated with present illness
    2 Definite (according to patient) weight loss

    B. On Weekly Ratings by Ward Psychiatrist, When Actual Changes are Measured:
    0 Less than 1 lb. weight loss in week
    1 Greater than 1 lb. weight loss in week
    2 Greater than 2 lb. weight loss in week

17. **Insight**
    0 Acknowledges being depressed and ill
    1 Acknowledges illness but attributes cause to bad food, climate, overwork, virus, need for rest, etc.
    2 Denies being ill at all

Total Score:_____

Appendix 4

The Maudsley Staging Method (MSM)

Parameter/Dimension	Parameter Specification	Score
Duration	Acute (≤12 months)	1
	Subacute (13–24 months)	2
	Chronic (>24 months)	3
Symptom severity (at baseline)	Subsyndromal	1
	Syndromal	
	Mild	2
	Moderate	3
	Severe without psychosis	4
	Severe with psychosis	5
Treatment failures		
Antidepressants	Level 1: 1–2 medications	1
	Level 2: 3–4 medications	2
	Level 3: 5–6 medications	3
	Level 4: 7–10 medications	4
	Level 5: >10 medications	5
Augmentation	Not used	0
	Used	1
Electroconvulsive therapy	Not used	0
	Used	1
Total		(15)

Appendix 5

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Research paper

Glutamatergic and GABA-ergic abnormalities in First-episode depression. A 1-year follow-up 1H-MR spectroscopic study

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ABSTRACT

Background: Previous magnetic resonance spectroscopic (MRS) studies have reported brain metabolic abnormalities in Major Depressive Disorder (MDD). Nevertheless, results have been inconsistent, focusing on fully developed major depression neglecting first episode patients (FED). Longitudinal studies have also been rare and with short follow-up periods. The aim of the current study was to investigate the differences between healthy controls and first episode patients at baseline, together with changes of metabolites after 1 year follow-up in the ventromedial prefrontal cortex.

Methods: ^{1}H-MRS images were obtained from 64 healthy controls and 31 FED patients using a 3T Philips Achieva scanner and processed with TARQUIN software at baseline and after 1 year. Examined metabolites included Glx (corresponding to Glu + Gln-peak), Glu, NAAG, myo-Ins, Cr, GSH and GABA. Clinical improvement was assessed by HDRS-17 scale. Differences in the concentrations of metabolites were evaluated by MANOVA/MANCOVA and GLM repeated measures for longitudinal changes.

Results: FED patients had significantly decreased glutamate levels at baseline ($p < 0.05$) along with significantly elevated GABA ($p < 0.01$) compared to healthy controls. At the follow up, myo- Ins levels were significantly increased compared to baseline ($p < 0.05$)

Limitations: The limited sample size, together with the unexpectedly high response rate after treatment (83%) might suggest decreased representativeness of the sample.

Conclusions: Results indicate glutamatergic and GABAergic changes taking place within the ventromedial prefrontal region even at the early stage of depression prior to any medication treatment.

1. Introduction

Major depressive disorder (MDD) is a potentially devastating disease associated with high mortality risk often characterized by recurrences and chronicity. An early intervention in the initial stages after the debut of the first depressive episode could be key to reduce the time of emotional suffering and its functional impact, improve treatment outcomes (Ghio et al., 2015; de Diego-Adeliño et al. 2010) and perhaps reduce the potential neurotoxic changes associated with recurrences and refractoriness (Carceller-Sindreu et al., 2019; de Diego-Adeliño et al., 2014; Serra-Blasco et al., 2013).

Proton magnetic resonance spectroscopy (^{1}H-MRS) has been successfully applied in the research of MDD focusing on specific brain areas related to the disorder such as prefrontal cortex (PFC; Hasler et al., 2007), anterior cingulate cortex (ACC; Gabbay et al., 2012), occipital cortex and putamen (Godlewska et al., 2018), as well as on whole-brain analysis (for review see Godfrey et al., 2018) employing different field strength (1.5, 3 or 7 Tesla). Studies with field strength above 1.5T have reported alterations in indirect markers of neuronal integrity such as N-acetyl-aspartate or N-acetyl-aspartate-glutamate (NAAG), markers of cell proliferation and degradation such as choline-containing compounds (PCC), markers of glial cell integrity such as myo-inositol, and markers of oxidative stress such as glutathione (GSH) among others (Agarwal and Renshaw, 2012). In addition, novel algorithms have also been able to quantify glutamate-related metabolites separately (glutamate, glutamine (Gln) and GABA) (Lener et al., 2017).

Inconsistency across findings represents a great deal due to discrepancies in methods, but also due to the fact that these results

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considered participants within different stages of illness that included from early to chronic states. Moreover, variation between tissues type (gray matter, white matter, and cerebrospinal fluid) has not always been taken into account, but even small ROIs include signal from different tissue types, and without proper segmentation the quantification of metabolites might be misleading. Although previous research has shown that antidepressants and electroconvulsive therapy (ECT) can increase Glx levels in the PFC of MDD patients (Chen et al., 2014; Njau et al., 2017), most of the available ^{1}H-MRS research compared either medicated depressed vs healthy controls (Grimm et al., 2012), patients in different clinical stages (remitted-recurrent vs. chronic) (Portella et al., 2011), treatment resistant (Baeken et al., 2017) or patients off medications only after a short wash-out period (2 weeks in most cases) before the scan (Abdallah et al., 2017). However, there have been very few studies that have analyzed the presence of alterations in these metabolites in subjects with a first depressive episode (Portella et al., 2011; de Diego-Adeliño et al., 2013) and even less that have explored the changes over time (Machado-Vieira et al., 2015). Therefore, in order to overcome these possible limitations, studies investigating metabolites in drug naïve FED patients are warranted. In one of the few available, Shirayama et al. (2017) found reductions in glutamate levels in the medial prefrontal cortex.

Despite these advances, little is known on the longitudinal effects of depression treatment on metabolic levels and their relationship with clinical outcomes such as reduction of depressive symptoms. Brennan et al. (2017) recently investigated the change in metabolites from baseline to different time points and clinical response and found a tentative association between acute increases in glutamine/glutamate ratios (from baseline to day 7 of citalopram treatment) and clinical response at day 42 in the MDD group. Although the result did not survive correction for multiple comparisons, it corroborates the notion that glutamatergic metabolities might be related to more satisfactory clinical outcomes over the course of treatment of FED patients.

The main objectives of the current study were to compare baseline metabolites in medication free FED patients and healthy controls and examine the longitudinal change in metabolites and clinical response from baseline to the follow-up point (after one year).

2. Methods

2.1. Participants

The sample consisted of 38 patients from the outpatient service of the Department of Psychiatry, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, recruited as part of a bigger project whose main purpose was to establish in vivo neuroimaging markers of clinical illness burden. Seventy-nine healthy controls were recruited in a 2:1 fashion with comparable age and sex distribution to patients among restaurant staff, technical support workers and graduate students at Hospital de la Santa Creu i Sant Pau, receiving monetary compensation for their participation. Exclusion criteria for controls were lifetime psychiatric diagnoses, first-degree relatives with psychiatric diagnoses and clinically important physical or neurologic illness, brain trauma with loss of consciousness, and mental retardation (score <70 on the estimated IQ using the Vocabulary subtest of the Wechsler Adult Intelligence Scale-IV, Spanish validated version). These last two criteria also applied for patients. Semi- structured interviews were conducted for all participants to collect demographic and clinical information, including comorbid Axis I conditions according to DSM-IV-TR criteria. Experienced clinical staff assessed current depressive symptoms using the Hamilton Depression Rating Scale 17 items (HDRS-17, Hamilton, 1960). To be included in the study, patients had to fulfill the criteria for a first episode of depression following DSM-IV-TR, and at least moderate severity on the HDRS-17 (above 17). The study was approved by the Research Ethics Board of Hospital de la Santa Creu i Sant Pau and was carried out in accordance with the Declaration of Helsinki. All participants provided informed consent after a full explanation of the study protocol.

Patients were naïve or minimal exposed (less than two weeks) to antidepressant treatment at the time of the first scanning. A systematic regime of treatment was defined for this sample, in which patients were initially treated with a selective serotonin reuptake inhibitor at therapeutic doses (mostly escitalopram 15 mg/day) and benzodiazepines if required. Subsequent treatment changes were made on an individual basis, following clinical guidelines and avoiding whenever possible changes in antidepressant type.

2.2. MRS scanning procedure

We obtained MRS images using a 3 T Philips Achieva scanner (software version 2.1.3.2) and a SENSE 8-channel head coil with a dedicated acquisition protocol. This included a 3-dimensional magnetization-prepared rapid-acquisition gradient echo (3D-MPRAGE) whole-brain sequence (turbo field echo, repetition time [TR] 6.7 ms, echo time [TE] 3.1 ms, voxel size $1 \times 1 \times 1.2$), on which 1H-MRS (single-voxel spectroscopy with point resolved excitation spin-echo sequence; TR 2000 ms, TE 38 ms, numbers of signals averaging 128, volume of interest –VOI– of $2 \times 2 \times 2$ cm, AutoWS-Prescan) images were obtained from a ventromedial prefrontal region embracing both hemispheres (Fig. 1). Therefore, selected VOI included white and gray matter, as well as cerebrospinal fluid (CSF).

The 1H-MRS raw data were exported and then post-processed using TARQUIN software, version 4.3.10 (www.tarquin.sourceforge.net). This is an external reference method that provides concentrations (in millimoles) of the single metabolite peaks. For the purposes of the present study, we included the following metabolites: Glx (corresponding to Glu + Gln-peak), Glu, NAAG, Ins, Cr, GSH and GABA. All metabolite levels were measured in absolute levels (SD% based on the Cramer-Rao lower bound). Spectra quality was assessed blindly by 2 researchers and the data of 6 patients and 13 healthy controls were disqualified due to low quality of spectra (having standard deviations above 30% for quantifications of the main metabolites). One patient was removed after their diagnosis was changed at follow-up by their psychiatrist. Three healthy controls were removed after visual inspection of distribution (using box plot graphs). The final sample consisted therefore of 31 patients and 63 matched on age and gender controls. For the longitudinal analysis the sample consisted of 18 patients that showed up for the second scan after a follow-up of 12 months on average.

2.3. VOI tissue segmentation

To control for the effect of potential structural differences in the region studied, we assessed tissue composition within the VOIs. The amount of each tissue –gray matter, white matter and CSF– was quantified with SPM12 software (Wellcome Trust Centre for Neuroimaging, www.fil.ion.ucl.ac.uk/spm) running under MATLAB. Images were segmented in native space, which produces a tissue class image (c*) in the same anatomic space of the original non-segmented image. After that, the VOI of 2 cm3 was resituated in the original images using ITK-SNAP software version 3.6.0 (www.itksnap.org) to create a mask of each participant. Then, this was multiplied by his or her own image for the 3 required tissues using MATLAB, and finally we quantified the volume of the resulting multiplied images. Following this procedure, we obtained the amount of gray matter, white matter and CSF.

2.4. Statistical analyses

Statistical analyses were performed in SPSS version 20.0. The two groups were assessed for differences in age, gender and education with t-tests, ANOVA or Chi-square test where appropriate.

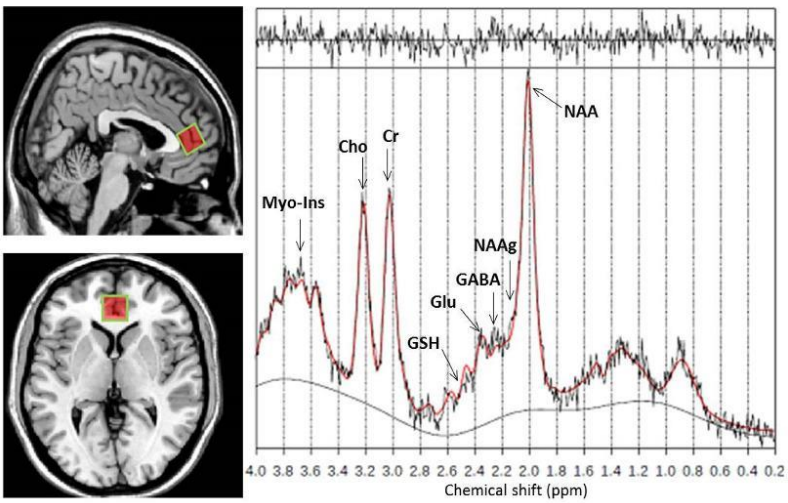

Fig. 1. Axial and sagital images showing ventromedial prefrontal Volume of Interest (VOI); Representative image after post-processing with TARQUIN software, version 4.3.10, PRESS acquisition at 3T, with TR= 2000 ms and TE= 38 ms.

After tissue segmentation, GM, WM and CSF differences between the 2 groups were checked with independent t-tests. Differences in the concentrations of metabolites were evaluated by a multivariate analysis of variance (MANOVA) or covariance (MANCOVA) if differences between groups were to be found in age, GM, WM and CSF. A GLM repeated measures was run to investigate the metabolic changes after 12 months. For those metabolites with missing values <5% linear interpolation and for those with >5% separate one-way ANOVAs (for baseline differences) and paired t-tests (for longitudinal analyses) were performed. To assess the relation of clinical variables and long-term changes in depressive symptoms with the change in metabolites along time, Pearson correlations were carried out.

3. Results

3.1. Demographics, cognitive and clinical results at baseline

The demographic and clinical distribution of the sample is summarized in Table 1. After MRS acquisition all patients started medication (29 patients were prescribed escitalopram, 5 citalopram and 4 fluoxetine at therapeutic doses). The FED and healthy control groups did not differ significantly in terms of age ($F= = 3.80$, $p= = 0.54$), gender ($\chi2= 0.14$, $p= = 0.71$), social status ($\chi2 = 0.70$, $p= = 0.87$) and education ($\chi2 = 5.17$, $p= = 0.08$). There was a difference at baseline in WM ($F= = 26.98$, $p < 0.001$) and GM ($F= = 43.29$, $p < 0.001$).

3.2. Metabolite differences between groups at baseline

All metabolites concentrations in the vmPFC of patients and healthy controls are presented in Table 2. WM and GM were included as covariates to control for the effect of potential structural differences in the region studied. The results from the one way MANCOVA showed a significant difference on glutamate levels ($F= = 5.97$; df =1, 90; $p= = 0.02$), with FED patients having lower values than healthy controls. No significant differences were found for Ins, Cr, Glx and NAAG in the MANCOVA. As GSH and GABA concentrations showed missing values > 5%, separate ANCOVAs (WM and GM as covariates) were carried out. GSH levels (FED $n= = 29$, Controls $n= = 58$) showed to be similar between groups ($F= = 0.02$, $p= = 0.88$), while,

Table 1
Demographics and clinical evaluation of participants.

	Total sample (n = 94)	Controls (n = 63)	Patients (n = 31)	t or $\chi2$	P value
Gender (% of females)	54.74	53.12	58.06	0.14	0.71
Age	40.30(10.6)	41.77(10.1)	37.29(10.8)	1.95	0.54
HDRS	9.16(10.2)	2.44(2.9)	22.81(4.2)	−27.34	0.01
Age at illness onset		NA	37.20(10.3)		
Education				5.17	0.08
Primary	17(18%)	13(21%)	4(13%)		
Secondary	35(37%)	27(43%)	8(26%)		
University	42(45%)	23(36%)	19(61%)		
Social status				0.70	0.87
No information	29(31%)	27(43%)	2(6%)		
Single	30(32%)	16(25%)	14(45%)		
Married/couple	29(31%)	16(25%)	13(41%)		
Divorced/separated	4(4%)	3(5%)	1(3%)		
Widowed	2(2%)	1(2%)	1(3%)		

Footnote: Values represent mean (SD) or otherwise specified.

Table 2
Absolute metabolite concentrations in vmPFC at baseline and follow-up.

Metabolites	Baseline Total sample (n = 94)	Healthy Controls (n = 63)	Follow-up FED (n = 31)	FED (n = 18)
Glx	14.41(2.9)	14.83(2.4)	13.57(3.6)	13.98(1.5)
Glu	8.50(1.8)	8.74(1.7)	8.01(1.8)	8.08(1.6)
GABA[a]	1.29(1)	1.11(0.8)	1.74(1.3)	1.42(0.7)
NAAG	4.46(1.2)	4.41(1.1)	4.55(1.3)	4.37(1)
Cr	5.67(0.9)	5.67(0.9)	5.67(0.8)	5.76(0.7)
GSH[a]	1.10(0.4)	1.09 (0.4)	1.12(0.4)	1.25(0.4)
Ins	4.60(0.9)	4.44(0.7)	4.92(1.3)	5.36(0.8)

Footnote: Values represent mean (SD) or otherwise specified.
Glutamate + Glutamine (Glx); Glutamate (Glu); gamma-Aminobutyric acid (GABA);.
N-acetylaspartate + *N*-acetylaspartylglutamate (total NAA); Creatine (Cr); Glutathione (GSH); Myo-inositol (Ins).
[a] Sample size for GABA was 77 in total; 54 HC and 23 FED patients; and for GSH, was 87 in total; 58 HC and 29 FED patients.

FED patients (n= = 23) had significantly elevated GABA concentrations (F= = 7.65; df=1, 73; p= = 0.007) compared to healthy controls (n= = 54).

3.3. Longitudinal effects

After one year of follow-up, 83% of patients responded to treatment, with 78% in clinical remission (HDRS-17 score ≤ 7). Medication was well tolerated by the majority of patients, and only 6 patients were shifted to another antidepressant –i.e. 1 patient to venlafaxine, 1 to bupropion and 2 to duloxetine. Two were offered combined treatment with trazodone (1 patient) or psychotherapy (1 patient). There were no differences between the patients that finished the follow-up and those who dropped out (HDRS- 17 basal p= = 0.85; age p= = 0.16; age at onset p= = 0.32; gender p= = 0.74). Table 3 displays HDRS-17 and metabolite concentrations at baseline and follow-up, together with percentage change. As there was a significant difference between first and second acquisition in GM (t= = 14.06, p < 0.001) and WM (t= = 8.64, p <0.001) we calculated ratios (GM1/GM2; WM1/WM2) and included them as covariates. Levels of Glu, Glx, GABA, Cr, NAAG and GSH showed no significant change over time (p>0.2). Ins levels significantly increased at the end of follow-up (t = −2.16, p= = 0.047).Finally, Pearson correlations did not reveal significant relationships between clinical changes and metabolite levels along time (p > 0.1).

Table 3
HDRS-17 and metabolite concentrations at baseline and follow-up along with percentage change.

	Baseline n = 18	Follow-up n = 18	Mean% change
HDRS	23.44 (4.30)	4.00 (5.68)	−82.94
Glx	12.90 2.84	13.98 (1.55)	8.37
Glu	7.91 (1.62)	8.08 (1.57)	2.15
GABA[a]	2.00 (1.61)	1.42 (0.67)	−29.00
NAAg	4.60 (1.50)	4.37 (0.98)	−5.00
Cr	5.64 (0.70)	5.76 (0.66)	2.13
GSH	1.08 (0.40)	1.25 (0.39)	15.74
Ins	4.68 (0.99)	5.36 (0.82)	14.53

Footnote: Values represent mean (SD) or otherwise specified. HDRS-17: Hamilton Depression Rating Scale, 17 items. Glutamate + Glutamine (Glx); Glutamate (Glu); gamma-Aminobutyric acid (GABA);.
N-acetylaspartate + *N*-acetylaspartylglutamate (total NAA); Creatine (Cr); Glutathione (GSH); Myo-inositol (Ins).
[a] Sample size for GABA at follow-up was 12.

4. Discussion

The main findings of the study were abnormally lower levels of Glu- and higher levels of GABA in the vmPFC of patients with a first episode of depression compared to healthy controls. After 12 months of follow-up, depressive symptom improvement was not associated with normalization of Glu-and GABA. Interestingly, patients showed significantly increased myo-inositol levels. No other differences were found neither at baseline, nor at follow-up. Clinical changes were not associated to metabolic changes in the follow-up.

These results are suggesting that the reduced levels of glutamate reported in the meta-analysis by Arnone et al. (2015) can be observed at illness onset. A more recent study (Shirayama et al., 2017) also reported abnormal decreased Glu-levels in the medial prefrontal cortex of medication-naïve FED patients. In a previous work with a smaller sample, Portella et al. (2011) pointed towards decreased Glu-levels in first stages of MDD, although the finding did not reach significance, which become more evident in more advanced stages with a consistent negative association with longer duration of illness. These observations altogether suggest the involvement of glutamatergic pathways in the pathophysiology and progressive glutamatergic abnormalities associated to recurrences and refractoriness. Interest in the glutamatergic neurotransmission in MDD is increasing, viewed as an alternative mechanism by which to explain the pathophysiology of the disorder as well as for novel treatment strategies such as NMDA receptor antagonists (Kishimoto et al., 2016).

Strikingly, Glu-levels did not change alongside clinical improvement after follow-up. This fact would partially be in agreement with the above mentioned study (Portella et al., 2011) which reported low levels of Glu-in patients with a recurrent depression in remission, suggesting that Glu-alterations might be more related to the illness neurobiological basis rather than to mood state. Indeed response and remission rates were very high in this sample without a clear normalization of Glu-levels. Similarly, the meta-analysis of Arnone et al. (2015) also concluded that Glu-abnormalities were not affected by antidepressant treatment or by clinical improvement. The results, however, should be interpreted cautiously as glutamate (and glutamine) are present both intracellularly (with metabolic function) in neurons and glial cells and extracellularly (with neurotransmitter function) making it difficult to attribute the disturbances to specifically one of these pools. The fact that only Glu, but not Gln-was diminished may indicate that either the aberration happens not at the precursor glutamine as previously suggested or that Gln-is affected in later stages of depression after treatment with antidepressants.

With regards to GABA, findings suggested elevated levels in FED patients compared to controls at baseline as expected, so as to account for the inhibitory-excitatory imbalance in the pathophysiology of MDD. Although previous reports showed decrements in GABA levels (see Godfrey et al., 2018 for a meta-analysis), other recent evidence has suggested that these results would be counterintuitive in terms of the altered prefrontal metabolic activity. The overall consensus states that GABA concentrations are decreased in patients with depression, at least in some brain regions and in treatment-resistant or severely depressed patients (Pehrson and Sanchez, 2015). However, there are not enough data available to develop a firm consensus on whether these altered GABA concentrations represent consistent biomarkers for depression states, and in fact there is some evidence both for and against this notion (Pfleiderer et al., 2003; McGirr et al., 2015). Indeed, preclinical studies have shown that ketamine blocks NMDA-R on GABA-releasing interneurons in the mPFC, thus decreasing GABA release (Pham and Gardier, 2019). These findings may suggest initial increments of GABA that may interfere with glutamate release at the early commencement of MDD. Other clinical studies, such as Bradley et al. (2018) found increased subcortical striatal GABA levels in a sample of depressed adolescents while Gabbay et al. (2017) found that anhedonic MDD patients had decreased levels in ACC but not non-anhedonic patients. In light of

these findings it is possible that GABA concentrations might depend not only on the VOI, but also on the clinical subtype of depression. Most of the available literature of PFC GABA levels has focused on ACC and long-lasting depression, while vmPFC and FED still need to be further explored.

At follow-up the only difference in the pre and post treatment conditions was the increased level of myo-inositol, which plays an important role in the phosphatidyl inositol second messenger system (PI-cycle) and is a marker for astroglial activation (Kim et al., 2005). Results from the current study suggested an increase in inositol levels in FED patients after treatment, which is in line with previous findings of elevation due to different antidepressants strategies such as medications (Chen et al., 2014), transcranial magnetic stimulation (Zheng et al., 2010) and electro-convulsive therapy (Njau et al., 2016). As concentration of inositol is higher in astrocytes than in neurons (Brand et al., 1993), this result might be interpreted as an improvement of astroglial functioning after treatment, although there seemed not to be altered astrocyte activity in FED patients as no differences between patients and healthy controls were found in inositol at baseline. In any case, it is possible that such a difference came up given the high response and remission rates of the present sample, i.e., 78% of the patients achieved remission (HDRS <7) at follow up.

The main strength of the current study is that it examined FED patients with none or very short-exposure to any antidepressants treatment. As discussed earlier, both antidepressants and ECT can affect metabolites levels and induce long lasting morphological and functional changes on MDD related brain regions, thus the current study eludes these confounding impacts. In addition, Glu~~and glutamine levels were~~ was measured separately instead of the previously combined Glx, which benefits the better understanding of glutamatergic neurotransmission early in the disease. The current study is also one of the few that measured the longitudinal differences in FED patients before and after administration of first antidepressant treatment. Despite these virtues, several important limitations should be taken into account. First, the sample size was moderate and achieved high rate of response to treatment suggesting decreased general representativeness. A second limitation is the lack of measurement of specific clinical symptoms such as anhedonia, as previous research has suggested it might affect the levels of metabolites. Lastly, TARQUIN uses a linear combination of basic functions to fit spectra in the time domain. These simulated sets are usually based on quantum calculations automatically generated to match common acquisition protocols, however, for edited MEGA-PRESS, TARQUIN uses a predefined basis set. This basis set models the GABA peak as two single Gaussian peaks. Full simulation MEGA-PRESS sequence is under development.

To conclude, results provide support for the hypothesis that there are glutamatergic and GABAergic changes taking place within the ventromedial prefrontal region of the brain even at the early stage of depression and prior to any medication treatment. Contrary to our expectations, altered metabolic levels were not normalized after one year of successful antidepressant treatment (83% of patients responded and 78% were in remission) with the exception of increased myo-inositol. These findings highlight the importance of detecting dysfunction in excitatory and/or inhibitory neurotransmitter signaling mechanisms even in the first stages of the illness, so as to develop specific antidepressant treatments with possible modulating agents.

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CRediT authorship contribution statement

Metodi Draganov: Data curation, Formal analysis, Writing - original draft. **Yolanda Vives-Gilabert:** Formal analysis, Methodology, Software, Writing - review & editing. **Javier de Diego-Adeliño:** Conceptualization, Investigation, Supervision, Writing - review & editing. **Muriel Vicent-Gil:** Data curation, Project administration, Writing - review & editing. **Dolors Puigdemont:** Conceptualization, Investigation, Supervision, Writing - review & editing. **Maria J Portella:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Resources, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

All authors declare no conflict of interest.

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