Brain Derived Neurotrophic Factor - Genetic and Epigenetic mechanisms with relevance to the Major Depressive Disorder and Suicide Phenotypes

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Medical Science/PhD

Submitted by
Dr.med.univ. Laura Carlberg
Department of Psychiatry and Psychotherapy
Medical University of Vienna (MUV)
Waehringer Guertel 18-20
1090 Vienna, Austria

Assigned Program:
Mental Health and Behavioral Medicine

Supervisor:
Prim. Priv.-Doz. Dr. Alexandra Schosser, PhD. MBA
Department of Psychiatry and Psychotherapy, Medical University of Vienna
Zentren für seelische Gesundheit Wien, BBRZ-Med, Vienna

Vienna, March 2020

DEPARTMENT OF PSYCHIATRY
AND PSYCHOTHERAPY
MEDICAL UNIVERSITY OF VIENNA

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1 Declaration

This doctoral thesis was carried out at the Department of Psychiatry and Psychotherapy (Medical University of Vienna) and the Clinical Institute of Pathology (Medical University of Vienna). The assessment and collection of the blood samples was performed in the context of the following studies at the Department of Psychiatry and Psychotherapy of the Medical University Vienna: “Studies on the psychosocial and biological (genetic) causes of bipolar and unipolar affective disorder (and schizophrenia)” (Oesterreichische Nationalbank (ÖNB) Grant nos. 5777 and 13198 and Austrian Research Foundation (FWF), Grantno. 7639, all to Harald Aschauer) and the “Genetics of Response to Agomelatine Pilot-Study (GENRAS)” (EK-No201/2010 Alexandra Schosser). The bisulfite conversion and adaptation of protocols for quantitative real-time PCR was kindly supported by Janine Scheibelreiter and Melanie Hassler (Clinical Institute of Pathology, Medical University of Vienna). DNA-purification, data cleaning, quality control, statistical analysis, interpretation of results as well as preparation of the original paper was performed by Laura Carlberg (Department of Psychiatry and Psychotherapy, Medical University of Vienna) under supervision of Alexandra Schosser (Department of Psychiatry and Psychotherapy, Medical University Vienna; Zentren für seelische Gesundheit Wien, BBRZ-Med, Vienna), and with support of the mentors Gerda Egger (Department of Pathology, Medical University of Vienna) and Nestor Kapusta (Department of Psychoanalysis and Psychotherapy, Medical University of Vienna).

The costs for consumables were covered by the research budget of the Department of Psychiatry and Psychotherapy (Medical University of Vienna). Laura Pauline Carlberg was employed at the Department of Psychiatry and Psychotherapy as research assistant and was funded from October 2012 - April 2014 by a grant from the Austrian Science Fund (FWF) in the context of a Clinical research Project (KLIF 220) "Vienna Study on Genetics of Suicidal Behavior in Affective Disorders – toward an integrated genetic, epigenetic and gene expression approach" (principal investigator: Alexandra Schosser).

Abstract

Major depressive disorder (MDD) is disabling for affected individuals, and the remission rate for antidepressant (AD) therapy is about 60-70% only. The pathogenesis of the heterogeneous MDD syndrome is understood incompletely. Hence, comprehensive exploration is needed to establish objective biomarkers and discover novel targets for AD therapy. The brain-derived neurotrophic factor (BDNF) is one possible modulator of MDD neurobiology. Recently, the *BDNF* methylation in peripheral mononuclear blood cells (PBMCs) was observed to correlate with depressive symptoms and diagnosis of MDD, proposing a diagnostic biomarker. Further, the *BDNF* gene is activated by the transcription factor cAMP response element-binding protein (CREB), which was related to suicide.

In the first paper, we analyzed the *BDNF* exon I promoter methylation in peripheral blood mononuclear cells (PBMCs). The sample consisted of two hundred-seven MDD subjects, fifty-nine bipolar disorder (BD) subjects and two hundred seventy-eight control subjects. Our aim was to determine differences of *BDNF* exon I promoter methylation among the diagnostic groups. In the second and third paper we analyzed the genetic effects of *CREB1* and *BDNF* variants on suicide phenotypes (suicide attempts (SA), suicide risk (SR)) in a sample of two hundred-fifty MDD subjects.

BDNF exon I promoter hypermethylation was detected in MDD subjects versus BD subjects ($p=0.0089$) and control subjects ($p<0.001$). In drug-free MDD subjects, the *BDNF* exon I promoter was significantly hypomethylated compared to subjects receiving AD therapy ($p=0.0019$). For *CREB1* and *BDNF* we did not find any association with suicide phenotypes. In a further investigation of treatment response phenotypes, the Val66Met and rs10501087 SNPs showed a significant association with suicide risk (SR) in a subgroup of remitters ($n=34$, 13.6%).

Accordingly, our results reveal that both the MDD phenotype as well as AD therapy has significant impact on the methylation status. In the future, randomized prospective studies should investigate the distinct effect of pharmacological treatment and MDD phenotype on *BDNF* exon I promoter methylation. For the investigation of associations with single common genetic variants our sample may be underpowered, thus, further studies in larger well-defined samples are needed.

Zusammenfassung

Die Major Depression (MDD) ist weltweit eine sehr häufige psychische Störung und einer der Hauptgründe für Invalidität. Die Remissionsrate im Rahmen einer antidepressiven (AD) Therapie beträgt lediglich 60-70%. Dementsprechend muss die Pathogenese der MDD umfassender geklärt werden, um neue Behandlungsansätze und objektive Biomarker zu etablieren. Für den vom zentralen Nervensystem (ZNS) stammenden neurotrophen Faktor (BDNF), wurde wiederholt gezeigt, dass er sowohl an der Ätiologie der MDD als auch an den molekularen Regulationsmechanismen der AD Therapie beteiligt ist. Vor kurzem zeigte eine Studie, dass der Methylierungsgrad von *BDNF* in peripheren mononuklearen Blutzellen (PBMCs) mit den klinischen Symptomen und der Diagnose von MDD korrelieren, dieses Ergebnis postuliert den *BDNF* Methylierungsgrad als einen potentiell wertvollen diagnostischen Biomarker für MDD. Ferner wird *BDNF* durch den Transkriptionsfaktor cAMP response element binding protein (CREB) aktiviert, dieser Vorgang scheint bei Menschen die Suizid begingen beeinträchtigt zu sein.

In der ersten Studie untersuchten wir den Methylierungsgrad am *BDNF* Exon I Promotor in einem Sample von 207 MDD Probanden, 59 BD Probanden und 278 Kontrollpersonen. Das Ziel der Studie war es, Unterschiede im Methylierungsgrad bei den verschiedenen diagnostischen Gruppen festzustellen und einen möglichen Effekt der AD Therapie auf den Methylierungsgrad des *BDNF* Exon I Promotors zu finden. In der zweiten und dritten Studie haben wir die genetischen Assoziationen von *CREB1*- und *BDNF*-Varianten auf die Suizid Phänotypen (Suizidversuche, Suizidrisiko) in einer Stichprobe von 250 MDD Probanden analysiert.

Die Resultate zeigten, dass die *BDNF* Exon I Promotor Methylierung bei MDD Probanden im Vergleich zu BD Probanden ($p=0,0089$) und Kontrollpersonen ($p<0,001$) signifikant erhöht war. Darüber hinaus war die höhere Methylierung bei den MDD Probanden signifikant mit der AD Therapie assoziiert ($p=0,0019$). Eine Korrelation zwischen dem Schweregrad der depressiven Symptome und dem Methylierungsgrad konnte nicht gezeigt werden (p=n.s.). Für *CREB1* und *BDNF* wurde keine signifikante Assoziation mit den Suizid Phänotypen gezeigt. In einer weiteren Untersuchung unterteilt nach Phänotypen des Therapieansprechens zeigten die SNPs Val66Met und rs10501087 eine signifikante Assoziation mit erhöhtem Suizidrisiko (SR) in der Subgruppe der Probanden in Remission (n=34, 13,6%).

Unsere Ergebnisse legen nahe, dass der Methylierungsgrad nicht nur durch den Krankheitsphänotyp beeinflusst wird, sondern möglicherweise auch durch die pharmakologische Behandlung. In Zukunft sollten randomisierte prospektive Studien die unterschiedliche Wirkung der pharmakologischen Behandlung auf die Methylierung des *BDNF* Exon I Promotors in Abhängigkeit vom Krankheitsphänotyp untersuchen. Für die

Untersuchung von Assoziationen mit einzelnen genetischen Varianten ist unsere Kohorte möglicherweise zu klein. Weitere Studien in größeren, genau definierten Gruppen zur Untersuchung von Assoziationen mit einzelnen genetischen Varianten sind hierfür erforderlich.

Publications arising from this thesis

1. **Carlberg L**, Scheibelreiter J, Hassler M, Schloeglhofer M, Schmoeger M, Ludwig B, Kasper S, Aschauer H, Egger G, Schosser A. (2014) Brain-Derived Neurotrophic Factor (BDNF) – Epigenetic regulation in unipolar and bipolar affective disorder. J Affect Disord. 2014 Oct;168:399-406. doi: 10.1016/j.jad.2014.07.022. Epub 2014 Jul 19. PMID: 25106037

2. **Carlberg L**, Schosser A, Calati R, Serretti A, Massat I, Papageorgiou K, Linotte S, Mendlewicz J, Souery D, Zohar J, Montgomery S, Kasper S. (2013) Hint for gender-specific association of creb1 and a history of suicide attempts in MDD: Results from a european multicenter study on treatment resistant depression. Int J Neurosci. 2014 Jun 23:1-20. PMID: 24955721.

3. Schosser A, **Carlberg L**, Calati R, Serretti A, Massat I, Papageorgiou K, Linotte S, Mendlewicz J, Souery D, Zohar J, Montgomery S, Kasper S. (2017) The impact of BDNF gene polymorphisms on suicidality in treatment resistant major depressive disorder – A European Multicenter Study. Int J Neuropsychopharmacol. 2017 Oct 1;20(10):782-787. doi: 10.1093/ijnp/pyx028. PMID: 28977521

List of abbreviations

AD	antidepressant
APA	American Psychiatric Association
BD	bipolar affective disorder
BDNF	Brain derived Neurotrophic Factor
BDI	Beck's Depression Inventory
CNS	central nervous system
CREB	cAMP response element binding protein
DALY	disability-adjusted life year
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders 4th edition
EDTA	ethylene-diamine-tetraacetic acid
GAS	Global Assessment Scale
HRSD-17	17-item Hamilton Rating Scale for Depression
ICD-10	International Classification of Disease 10th Revision
MDD	major depressive disorder
MDE	major depressive episodes
MADRS	Montgomery Asberg Depression Rating Scale
mRNA	messenger ribonucleic acid
ms-qPCR	mold specific quantitative polymerase chain reaction
MAOIs	non-selective monoamine oxidase inhibitors
PBMCs	peripheral blood mononuclear cells
q-rtPCR	quantitative real time polymerase chain reaction
QC	quality control
SCAN	Schedules for Clinical Assessment in Neuropsychiatry
SNP	single nucleotide polymorphism
SNRI	serotonin norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
WHO	World Health Organization
WMA	World Medical Association General Assembly

1 Introduction

1.1 Major Depressive Disorder (MDD)

1.1.1 Overview

Major Depressive Disorder (MDD) affects currently more than 300 million people with a growth of more than 18% between 2005 and 2015 (WHO). Health and performance are severely impaired in those affected by MDD. Long-lasting disability and low labor productivity are also possible consequences of MDD. The overall remission rate for MDD, under adequate treatment, is only about 60%–70% Rush et al. (2006). Both the recurrence rate after a MDD episode and initial treatment response is approximately 30% within the following 6 months (Hardeveld et al. 2013). In addition, MDD-related functional impairment and disabilities (e.g. cognitive decline) often last longer than depressive mood symptoms and can be a relapse factor. Being unable to work due to MDD is considered a loss of resources for the society. The resulting absenteeism leads to high costs due to loss of productivity. According to WHO Europe, approximately 50% of long-term sick leaves and disability benefits are due to mental disorders, mainly MDD. The MDD results in enormous costs and burdens for the individual concerned and his social environment, as well as for the society as a whole. A measure of the burden of an illness is the key figure of the “disability-adjusted life years” (DALYs). This measure takes into account the lifetime lost due to previous death and the impairment of lifetime due to an illness. A DALY is calculated by adding up the years of life lost (YLL) due to mortality and years lived with disability/disease (YLD) (Devleesschauwer et al. 2014). In Europe, MDD is responsible for 4,320,400 DALYs per year (as of 2004), making it number one among neuropsychiatric disorders. In terms of all diseases, MDD contributes to 7.2% of the disease burden. In Austria,

MDD accounted for the main proportion of total DALYs (9,8%). In women in particular, every tenth year lost (10.3% of all DALYs) is due to MDD (Wittchen et al. 2011). The burden on the person concerned results from the depressive symptoms, which are accompanied by limitations in everyday performance and psychosocial consequences. Depression increases the risk of early termination of schooling, leads to less work performance and a higher number of sick days in professional life, and is associated with unemployment and lower income (Kessler et al. 2013). MDD subjects are less likely to ever marry and are at greater risk of marriage and divorce violence. There is also a higher probability of having children as early as adolescence and developing deficits in parenting behavior (Kessler et al. 2013). Overall, MDD causes the greatest impairment in terms of household, job, social life and relationships, even before illnesses such as cancer, cardio-vascular diseases or diabetes mellitus (Ormel et al. 2008, Sumiyoshi et al. 2019). The burden on society due to MDD results from the high treatment costs and absenteeism. In Europe, the cost of mood

disorders is number one in the list of neuropsychiatric disorders. MDD causes the largest share of this with almost 92 billion euros annually, which corresponds to around 3,000 euros per patient per year (Olesen et al. 2012). MDD affects about 25% of patients visiting general practitioners, but physicians have trouble recognizing the symptoms. According to WHO Europe, nearly 1:2 affected with MDD is neither treated with AD nor psychotherapy, as a result of not seeking help due to embarrassment and/or denial, inappropriate services or the institutional failure to recognize the disease. Suicide deaths are on the rise, especially in those affected by MDD not adequately treated (Hardeveld et al. 2013, Li et al. 2017). The clinical diagnosis for MDD is based on psychiatric classification systems such as the Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association 2013) and the International Classification of Diseases (ICD) (World Health Organization 1992). With the current nosology, physicians describe the constellations of symptoms and signs that tend to occur together in the MDD syndrome (e.g. mood, cognition, chronobiology, the autonomic nervous system). However, the MDD syndrome is most likely caused by a complex genetic architecture and a variety of biological pathways (e.g. neurotrophic factors, monoamines (serotonin), the hypothalamic–pituitary–adrenal axis (HPA axis) and inflammatory (cytokines). In order to establish targeted therapy choices, we need to identify objective biomarkers and treatment options interacting within the pathways involved in the MDD pathogenesis.

1.1.2 Epidemiology of Major Depressive Disorder (MDD) and Bipolar disorder (BD)

The current prevalence of MDD is 4-12% of the European population, according to the WHO, and lifetime prevalence for MDD is 4-12% for men and 12-26% for women. Incidence analyses show that MDD can occur at any age for the first time. The period between adolescence (from the age of 15) and the age of 30 has the highest density of incidence (new disease) cases. The incidence and prevalence rates of MDD disorders in children and adolescents up to the age of 16 are significantly lower than in all other age groups. However, depressive symptoms in childhood are not uncommon and may be predictive for the onset of MDD during lifetime. For adults, it is consistently shown that the mean age for the first episode is between 25 and 30 years. Bipolar disorder (BD) occurs earlier between 15-25 years and a lifetime prevalence of 1% was observed (Craddock et al. 1999, Jacobi 2005). The relatives of MDD subjects have an increased risk for unipolar depression, whereas the primary relatives of BD subjects have an increased risk for both bipolar (5-10% lifetime risk) and unipolar affective disorder (Craddock et al. 1999, McGuffin et al. 2003). Further risk factors for MDD are early childhood trauma and stressful life events (McGuffin et al. 2003). In terms of sex differences, evidence shows that from adolescence onwards, a clear predominance of MDD is present in females (Cohen et al. 1993, Angold et al. 1998). Though,

the incidence, symptoms and treatment of MDD all point toward major sex differences, the responsible molecular mechanisms for the greater vulnerability of females so far remain largely unexplained. The higher female risk and stress factors include: poverty, discrimination at work and role overload (role as parent/caregiver, employee, partner, etc.)(Pinquart et al. 2003, Lee et al. 2016). These factors can lead to the onset of MDD, and in part, explain the sex differences.

1.1.3 Diagnostics of Major Depressive Disorder (MDD)

The psychiatric disorders are described and classified in the Diagnostic and Statistical Manual of Mental Disorders (DSM) of the American Psychiatric Association (APA) and the International Statistical Classification of Diseases (ICD) of the World Health Organization (WHO). The well-defined and reliable definition of each disorder helps diagnose and care for patients, as it serves as a common language between physicians, therapists, and anyone else involved. Both the DSM and the ICD classification system present MDD in a descriptive way, neither explicitly nor implicitly referring to their pathogenesis. Both systems follow the so-called categorical approach in which the disorders are considered clearly distinguishable. For each diagnosis, there is a catalogue of symptoms that must be present. We used the Statistical Manual of Mental Disorders (DSM-IV); see Table 1 for the details. In the context of diagnostics, it should be ascertained whether the MDD is based on an organic cause such as a structural brain disease (e.g. tumor, infarction) or a beneficial concomitant disease (e.g. coronary heart disease, thyroid dysfunction). Various medications (e.g. beta blockers, glucocorticoids) can also contribute to the development of depression. Additionally, other mental illnesses associated with depressive symptoms should be taken into account. It is also important to differentiate between pathological grief and post-traumatic stress disorder and depressive adjustment disorder (Shear 2015, Barbano et al. 2019). Likewise, a bipolar illness that often begins with a major depressive episode (MDE) or in which no clearly manic phase can yet be detected can initially be misdiagnosed as unipolar MDD (Anmella et al. 2020). Depressive symptoms can also occur in diseases of the schizophrenic type in all phases of the disease course. In particular, schizoaffective psychoses and the negative symptoms in schizophrenia can imitate depression. Since the MDD can be accompanied by symptomatic decrease of concentration and memory, it is difficult to distinguish between the onset of dementia (“pseudo dementia”), especially in older patients, particularly since MDD and dementia are often comorbid (Sekhon et al. 2020). Psychiatric and somatic comorbidities are common among depressive disorders. Approximately 60-70% of patients have another, 30-40% even several mental illnesses, including in particular anxiety disorders and substance abuse disorder, especially of alcohol (Otte 2008, Foulds et al. 2016). Looking at somatic comorbidities, patients with MDD are more likely to suffer from cardiovascular and

cerebrovascular diseases, diabetes mellitus, irritable bowel syndrome and some tumor entities compared to the general population (Wang et al. 2019).

Table 1. Primary DSM-IV depression disorders, criteria for adults

Depressive Diagnoses	Symptoms
Major Depressive Episode: - 5 or more depressive symptoms for ≥ 2 weeks - Must have either depressed mood or loss of interest/pleasure - Symptoms must cause significant distress or impairment - No manic or hypomanic behavior ***Minor Depressive Episode:**** - 2–4 depressive symptoms for ≥2 weeks - Must have either depressed mood or loss of interest or pleasure - Symptoms must cause significant distress or impairment - No manic or hypomanic behavior	1. Depressed Mood 2. Markedly diminished interest or pleasure in most or all activities 3. Significant weight loss (or poor appetite) or weight gain 4. Insomnia or hypersomnia 5. Psychomotor retardation 6. Fatigue or loss of energy 7. Feelings of worthlessness or excessive or inappropriate guilt 8. Diminished ability to think or concentrate, or indecisiveness 9. Recurrent thoughts of death (not just fear of dying), or suicidal ideation, plan, or attempt
Dysthymic Disorder - Depressed mood for most of the time for at least two years - Presence of 2 or more of symptoms of dysthymia - Never without symptoms for 2 months or more over 2 year period - Symptoms must cause clinically significant distress or impairment - No major depressive disorder in first two years, no manic, hypomanic, or mixed episodes.	1. Significant weight loss (or poor appetite) or weight gain 2. Insomnia or hypersomnia 3. Fatigue or loss of energy 4. Low self-esteem 5. Diminished ability to think or concentrate, or indecisiveness 6. Feelings of hopelessness

* not a formal diagnosis but considered a research category requiring further study

From: 1, Introduction

Screening for Depression in Adults and Older Adults in Primary Care: An Updated Systematic Review [Internet].
Evidence Syntheses, No. 75.
O'Connor EA, Whitlock EP, Gaynes B, et al.
Rockville (MD): Agency for Healthcare Research and Quality (US); 2009 Dec.

Source, with permission: (O'Connor et al. 2009)

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1.1.4 Bipolar Disorder (BD)

The bipolar I disorder (BD) is categorized by one or more manic episode(s) according to the DSM-IV or ICD-10 criteria. The ICD-10 recognizes the manic single episode as its own diagnostic category, the DSM-IV pursues the concept that even one manic episode assures the diagnosis of BD, as in more than 95% of patients sooner or later major depressive episodes (MDE) will occur. In bipolar I disorder, both manic episodes and MDE occur. The MDE lasts at least 14 days and the manic episode lasts at least 7 days. Manic episodes are often severe and lead to negative consequences (debts, marital problems). The central features of mania are the acceleration and disinhibition of psychomotor functions, disorganized thinking and behavior, lack of insight/judgment, as well as psychotic symptoms (delusions, hallucinations) and catatonia in the most severe cases. In several patients, depressive symptoms occur simultaneously which in extreme cases lead to a mixed affective episode, i.e. the simultaneous presence of all symptoms of mania and MDD. Bipolar II disorder is characterized by recurrent MDEs and a minimum of one hypomanic episode for 4 days. While the gender ratio is evenly distributed in bipolar I disorder, women are predominant in bipolar II disorders, just as in MDD (Weissman et al. 1996). The symptoms of MDE in BD may equal those in MDD, psychomotor deceleration and reduced mental functions are core symptoms; the affective state is dominated by inhibition of emotions to the point of emotional numbness, a deceleration of thinking, speech and motor skills, in many cases also an undirected psychomotor restlessness, disturbance of the biorhythm with symptoms such as sleep disturbances as well as early waking and a typical morning depression, physical symptoms such as constipation or nausea and an increasing willingness to commit suicide. "Rapid cycling" is when at least four or more episodes of mania, hypomania, or MDE occur within twelve months.

1.1.5 Course and prognosis of Major Depressive Disorder (MDD)

MDD is typically characterized by an episodic course, meaning the disease phases are limited in time (Ustun et al. 2004). The patterns of depressive disorders show great inter-individual variability. A depressive episode can completely remit, leaving the patient symptom-free in the subsequent period. In the case of incomplete remission, residual symptoms are persisting and the risk of a new depressive episode is increased. Dysthymia is characterized by a sub-syndromal depressive symptomatology that has existed for at least two years, from which an additional depressive episode can develop. In the latter case one speaks of the so-called double depression. If a depressive episode persists for more than two years without improvement or remission in the interval, it is called a persistent depressive disorder (American Psychiatric Association 2013).

1.1.6 Mortality in Major Depressive Disorder (MDD)

MDD causes a higher mortality; on the one hand due to committed suicide, on the other with premature death due to accidents, physical causes or a typical unhealthy lifestyle (e.g. malnutrition and lack of exercise). For example, MDD following a myocardial infarction (MI) independently increases the risk for cardiac mortality within the next 6 months (Joukamaa et al. 2001, McGuffin et al. 2003). In stroke patients, it has been shown that co-morbid MDD is associated with an elevated chance for mortality (McGuffin et al. 2003). The exact mechanism of this relationship remains to be solved. However, there are indications of the influence for certain mediating factors, such as physical inactivity, unhealthy diet and other health-related behaviors.

1.1.7 Suicide characteristics and Epidemiology

Since the 1990s, suicide mortality is on the rise and suicide is the second leading cause of death after the accidental death up to the age of 29 years in both sexes (see Figure 1.). According to data published by the WHO (2017) within the last decade, the suicide mortality worldwide has changed. As observed, there is a rise in Eastern Europe and Asia, currently the highest rates of suicide mortality are in China and India (see Figure 2.) (Varnik 2012). MDD is related to suicide and self-harming acts (Wolfersdorf 2008). In addition, chronic illnesses (e.g. therapy refractory MDD) belong to the psychological factors that are also associated with suicides. An estimated 65-90% of all suicides are caused by mental illness, most commonly MDD (Krug et al. 2002). Approximately 3-4% of all patients suffering from MDD die by suicide, three times more men than women (Statistisches Bundesamt , Wolfersdorf 2008). Suicide attempts are carried out ten to twenty times more frequently than suicides, although numerous cases remain unreported and many attempts are not recognized as such (K Jost 2007). For older people, the suicide -risk and -rate are significantly higher, especially for men. Statistically, the amount of suicide attempts decrease with age, whereas the amount of completed suicides are on the rise after age 75 (Wilk K 2007). In the elderly, MDD can lead to unintended suicide due to loss of appetite, or inadequate fluid intake, these conditions can quickly lead to life-threatening conditions. Historically in 1642, the term suicide was established to distinguish between the murder of another person and suicide (Minois 1999). The term suicide is composed of the Latin words sui (himself) and caedere (kill). The broader term suicidal behavior, by contrast, encompasses various behaviors, ranging from suicidal thoughts, the development of suicide plans, suicide attempts, to completed suicide. Suicidal thoughts can be wide-ranging in nature and consist of thinking about one's own death or death wishes; but they can also include the concrete ideas of an action, as the person could actively take his own life. The widespread definition of suicide attempt comes from a working group of the World Health

Organization (WHO) and was first published in English and referred to as parasuicide (Platt et al. 1992). A suicide attempt is a non-fatal outcome that would cause self-harm without third-party intervention. In this definition suicide attempts with an appellative or manipulative character are also included, which mainly express the desire for a break or a change in the situation (Antretter et al. 2002). Suicide is the pre-considered self-harm with fatal consequences (Kidd 2003). Overall, however, there is no universally valid and accepted nomenclature in this field of psychiatric research. One current suggestion is based on the combination of three key components of suicidal behavior: the idea of being death, the act, and death as a result of the act (Marusic 2004). In a completed suicide, in contrast to accidents or suicide attempts, all 3 components are present. This model of a nomenclature is particularly useful because it offers a clear distinction between completed suicide, suicide attempts, suicidal thoughts and other non-death related behaviors. Suicide methods distinguish between violent or hard methods such as shooting, hanging or jumping from high altitude and nonviolent or soft methods such as intoxication with drugs and other substances (Asberg et al. 1976). The choice of the suicide method is influenced by various factors, such as the gender of the person, a possible psychiatric illness, but also the availability of the method. In the United States of America, where a large proportion of the population has access to firearms, suicide by shooting is the most common method (Shenassa et al. 2003). On the other hand, suicide attempts are clearly dominated by intoxications, which are less lethal than violent methods (Shenassa et al. 2003). In the study by Shenassa et al. (2003) lethality is 96.5% for firearms, 90.4% for hanging or suffocation, 74.0% for blunt violence, and only 6.5% for intoxication. Access to suicide aids is a crucial factor in determining whether self-harming behavior is fatal or not (Barnhorst et al. 2018). Overall, it can be said that men are more likely to resort to violent means (for example firearms or hanging), while women tend to use less violent means (for example, self-poisoning) (Perry et al. 2019). In addition, the availability of different tools has a major impact on the national accumulation of suicide methods. In Austria, hangings account for about 46% of all suicides, followed by shooting at 16% and self-poisoning at 10% (Grabenhofer-Eggerth A 2016). In North-, Central- and South America, most people (46%) use the firearm, with a risk highest where Weapons are present in the household (Allchin et al. 2019). A systematic review shows that, especially in rural areas of developing countries, death from poisoning with pesticides is chosen. The toxicity, the ready availability and the lack of safety precautions during storage of the Pesticides lead to a major part of global suicides (Gunnell et al. 2017). In 2012 Wu et al., found that the environment of a social group influences the accumulation of a specific suicide method. For example, in Hong Kong or Singapore, where most people live in tall building complexes, the plunge is one of the most popular suicide method (Wu et al. 2012). Since 1998, the use of charcoal burning has been practiced as a suicide method in China and Hong Kong to create the lethal gas carbon monoxide. This suicide method extended to

Taiwan and became one of the most common methods in the following 8 years (Chang et al. 2014, Byard 2019). It is clear that almost all people who purposely want to commit suicide show signs of hopelessness, depressive mood and suicidal thoughts, with or without evidence of mental illness (Turecki et al. 2016). In order to prevent these people from committing suicide and offering them the opportunity to think about their intentions and thus survive the crisis, it would be a big step in the direction of suicide prevention to restrict access to suicide aids (Florentine et al. 2010, Martinez-Ales et al. 2019). For instance, the use of pesticides, one of the most common methods of suicide in the world, could be reduced by banning the use of the most harmful pesticides, keeping pesticides safe in rural communities, and ensuring the availability and quality of pesticides supply in case of intoxication would be improved (Pollock 2019). On the other hand, the limitation on the method of hanging is much more difficult to achieve. In order to be able to initiate targeted prevention measures that are meaningful for the region, it is important to understand which methods of suicide are preferred in the respective community and how they can be restricted (for example, making access to firearms difficult, installing barriers at level crossings or bridges) (Pollock 2019). Suicide attempts are also a big burden for communities and of course the person concerned. There is a great demand on health services as a result of self-injuries and their treatment and possible disabilities as well as the psychological and social burden on relatives and friends of the person concerned (WHO 2013). However, there are insufficient data on suicide attempts, as they are often not recognized as suicide attempts or documented as such or do not lead to any contact with the health care system (Grabenhofer-Eggerth A 2016). According to estimates and international studies, at about every 10th to 30th suicide attempt a suicide victim dies (Kolves et al. 2013). Since suicidal thoughts or suicide attempts often go unnoticed, data can only be captured after the death of a person. Limiting the availability of suicide methods provides a universal and effective approach to suicide prevention. Successful examples of this approach include the detoxification of domestic gas, the restricted sale of pesticides, safety barriers at bridges and buildings, stricter weapon laws and a reduction in availability of barbiturates (Lester 1990, Ohberg et al. 1995, Carlsten et al. 1996, Beautrais 2001, Bridges et al. 2004). A sex-difference as far as the choice of suicide method is concerned is well known. In comparison to men, women prefer soft methods such as intoxication (25.7% versus 12.3%), while the use of firearms plays a minor role for women (1.2% versus 7.6%) (Wiesner 2004). The study by Turecki and Brent (2016) found out that 45% of subjects committing suicide visit a doctor during the month before suicide (Turecki et al. 2016). This illustrates that the subjects try to get help and suicide could often be prevented. In order to reduce suicide rates, diagnostic procedures must be optimized and objective biomarkers need to be identified, because to this day, every 40 seconds, in anyplace of the world somebody commits suicide and a higher number of subjects attempt suicide. With more than 800.000 people committing suicide per year,

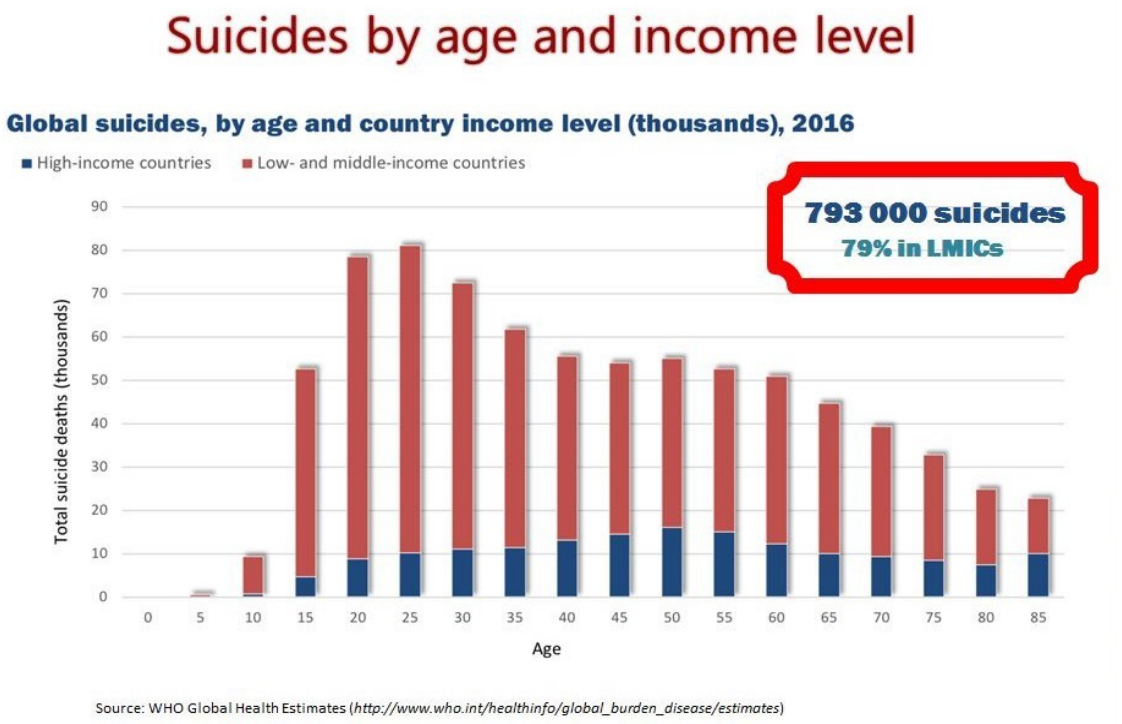
suicide is the number 10 in the world's leading cause of death and, accordingly, a global public health problem (Hawton et al. 2009). Durkheim (1897) recognized in his study in the late 19th century that social integration and marital status had an influence on the suicide rate (Kolodziej-Sarzynska et al. 2019). Socio-economical status has a great impact on quality of life and unemployment increases the risk for suicide. In the United States (US) unemployed women are at higher risk to die by suicide than unemployed men (Kposowa et al. 2019). Today it is known that suicide rates vary, among other things, with socio-economical status, age, gender, region, season, ethnicity, and probably unreliable death registration. The recording of an unexpected death as a suicide varies from country to country and requires different preconditions. In Luxembourg, for example, the existence of a farewell letter is a precondition for assigning the death to suicide (Hawton et al. 2009). Death by drug intoxication may also account for a proportion of unrecognized suicides which could not be accurately measured in magnitude so far (Wicki Zürich, 6. Januar 2017). Kohlbrenner et al. (2016) describe that suicidal behavior plays a major role, especially in socio-economically often underprivileged and often discriminated parts of the world population (King et al. 2008, Haas et al. 2011, Swannell et al. 2016, Kolodziej-Sarzynska et al. 2019). With about 75% of global suicides, the majority of suicides happen in low- and middle-income countries as there is a lack of health services and available resources, and there is insufficient support or treatment for those in need (WHO 2019a). In the 2013-2020 Mental Health Action Plan published by the World Health Organization (WHO), WHO member states agreed to reduce their suicide rate by 10% by 2020 (WHO 2013). To achieve this goal, several risk factors and preventive measures need to be discussed. On the way to identifying risk factors and even more in search of possible protection against these risks, many questions arise. Where and when do most suicides happen? Is there a causal relationship between the suicide rate and the weather/temperatures or the hours of daylight in a region (Hiltunen et al. 2011)? Are mostly jobseekers or drug- and alcohol addicts taking their own lives (Dragisic et al. 2015, Lee et al. 2019)? Do all people who think about suicide have a mental disorder? What role do social and financial resources play (Watzka 2012)? When does a suicide come from an impulse and when is it planned for a long time? Does the rate decrease with difficult access to suicide aids, such as pesticides (Gunnell et al. 2007)? How to report on suicidal behavior in the media and what are the expected implications (Sugg et al. 2019)? The fact that a person takes his own life can usually not be explained by a single factor. For a better understanding, the interactions between genetic risk, social, maladaptive personality, somatic, and environmental factors need to be considered. Connections between mental disorders and suicide have been proven (Hoertel et al. 2015). The experiences of sexual violence and child sexual abuse are also significantly associated with suicidal behavior (O'Brien et al. 2013). Impulsiveness and disinhibition by alcohol and drugs increase the risk of suicidal behavior (Khemiri et al. 2016). Tabooing in society inhibits

suicide prevention. However, increasing the awareness of society about suicide carries the risk of stigmatization, especially in relation to mental illness and suicide, which may avoid people at risk from seeking help. The screening and consequent education, in the sense of psychoeducation, should begin as early as possible. Objective biomarker screenings are not yet available for the risk of suicide (Calati et al. 2019). As described above, in nearly half of the cases in the month before their suicide, people who took their own lives sought out a doctor, which makes it clear that it is also of great importance in the established area to treat a holistic condition in the case of physical ailments to consider the patient's history. The biopsychosocial disease model provides an increasingly recognized construct for this (Wright et al. 2019). Particularly at risk of suicide are people who have already attempted suicide. In their study, Ghanbari et al. (2015) found that 25% of people who were discharged from a psychiatric ward or emergency room following a suicide attempt committed another suicide attempt. In addition to the aftercare of the people who committed suicide, it also requires a follow-up care of bereaved and friends of suicide victims (Ghanbari et al. 2015). In order to develop a holistic strategy and thus to make progress, it is of great scientific and world health interest to consider both risk factors and preventive measures and to formulate a guide that can serve as a source of information for the health sector. The WHO Global Health Assessment (2015) largely refers to the WHO mortality database, which receives data from WHO member states. This allows a closer look at different regions and groups. In 2015, an estimated amount of 788.000 people committed suicide. This corresponds to a global age-standardized suicide rate of 10.7 per 100.000 inhabitants. About twice as many men as women commit suicide (13.6 to 7.8 per 100.000 inhabitants) (WHO 2019b). The exception is still China, where most suicides are committed by women (WHO 2018a). In 2012 the age-standardized suicide rate of 12.7 per 100.000 inhabitants was slightly higher in high-income countries than in low- and middle-income countries (LMICs) with 11.2 suicides per 100.000 inhabitants (WHO 2019b). In the LMICs which are divided into six regions by the WHO (Africa, Central and South America, Eastern Mediterranean, Eastern Europe, South-East Asia, Western Pacific), age-adjusted suicide rates vary enormously: in Southeast Asia the rate is almost 17.7 per 100.000 three times as many committed suicides as in the WHO region of Central and South America, where there are 6.1 per 100.000 inhabitants (WHO 2019b). If one compares the age-standardized rates of the suicides of the individual countries, the differences emerge even more clearly. In 2015 the age-standardized suicide rates of the included 172 WHO member states (with a population of 300.000 or more) ranged from 0.3 (Barbados) to 34.6 (Sri Lanka) per 100.000 inhabitants. This is about a 115 times higher suicide rate of the state with the highest suicide rate compared to the country with the lowest suicide rate. Since the year 2000, the rate has remained relatively stable. In 2000 Barbados (1.9 per 100.000 inhabitants) and Sri Lanka (38.3 per 100.000 inhabitants) were already close to the maximum and minimum values as of 2015 (WHO 2019). In its report

published in 2014, the WHO described that mortality records should be considered cautiously, as only 60 of the 172 WHO Member States had good quality data (WHO 2014). This means that only the data from 60 countries could be used directly and that of the other 112 Member States provided data based on modeling methods. Clearly recognizable is the positive association between good quality of data in civil registers and high-income countries. To make matters worse, countries with mortality data of "poor quality" often pursue inadequate registration or misclassification of suicides due to illegality of suicidal behavior (WHO 2018b). Although efforts have been made for decades to improve the procedures for collecting country-specific mortality data, and thus to increase accuracy, regional differences still persist. That these discovered deviations are real discrepancies could not be ruled out (WHO 2018b). Although Stack (2000) did not yet know exactly why, he also found that significantly more men die from suicide than women (Stack 2000). It has long been claimed that men are even three times more likely to be affected by suicide. It has now been shown that this large inequality in suicide frequency is likely to be a problem for high-income countries (WHO 2019b). The National Institute of Mental Health has published data showing that men in the United States die nearly four times as often as women by suicide (NIMH 2019). Globally speaking, in high-income countries, the male-to-female ratio of the age-adjusted suicide rate is 3.5:1, whereas in the LMICs it is only 1.6:1. This corresponds to a 57% higher male suicide rate worldwide (WHO 2019b). In most regions of the world, the suicide rate is lowest for those under the age of 15 and highest for those over 70 years old. In the other age groups, i.e. 15 to 70 years, the distribution differs according to gender and region (WHO 2019b). In some regions, the ratio of men to women remains relatively constant across age groups, while it varies widely in other areas. For example, in Chile the age-specific suicide rates range from 16.2 to 16.5 (difference of 0.3) per 100.000 inhabitants for those aged 15-70 years, while in Japan between 18.4 and 31.8 (difference of 13.4) per 100.000 inhabitants (WHO 2018b). In high-income countries, suicide rates of adolescent and older women are significantly lower than those of LMICs, whereas suicidal rates in middle-aged men are significantly lower in LMICs than in high-income countries (WHO 2019b). In Austria, suicide frequency differs in age. After the age of 75, the risk of death from suicide against the average population (age median of 42.3 years, 2015 increases to double and at the age of 85, even threefold (Statista 2017). In absolute terms, however, most suicides occur in middle age. Except for the 15 to 19-year-olds, who had a slight increase in the suicide rate in 2013 and 2014, the rate of suicides between 1970 and 2014 was decreasing in all age groups (Grabenhofer-Eggerth A 2016). Among all mortalities, the percentage for age related deaths from suicide even reach their highest number (suicide ratio) in the age group 20-29 years (Watzka 2012). Worldwide, from 2000 to 2012, the number of suicides decreased from 883.000 to 804.000 by about 9% as the world's population grew. In Austria, since the peak in 1986, with 2139 suicides, there has been a steady decline in suicide rates

(Sonneck G 2012). Since the global economic crisis in 2008, the decline has stagnated somewhat and almost reached a plateau. In 2014, about 3.1 times as many men (n=989) as women (n=324) committed suicide among the total of 1313 suicides. The suicide rates therefore correspond to 23.7 per 100.000 inhabitants for men and 7.4 per 100.000 for women. Again, especially men over the age of 70 have a high risk of suicide (Grabenhofer-Eggerth A 2016). Often, an impulsive reaction to a situation that feels hopeless for the suicidal person leads to suicidal behavior (Stanley et al. 2019).

Figure 1. WHO: Suicides by Age and income level



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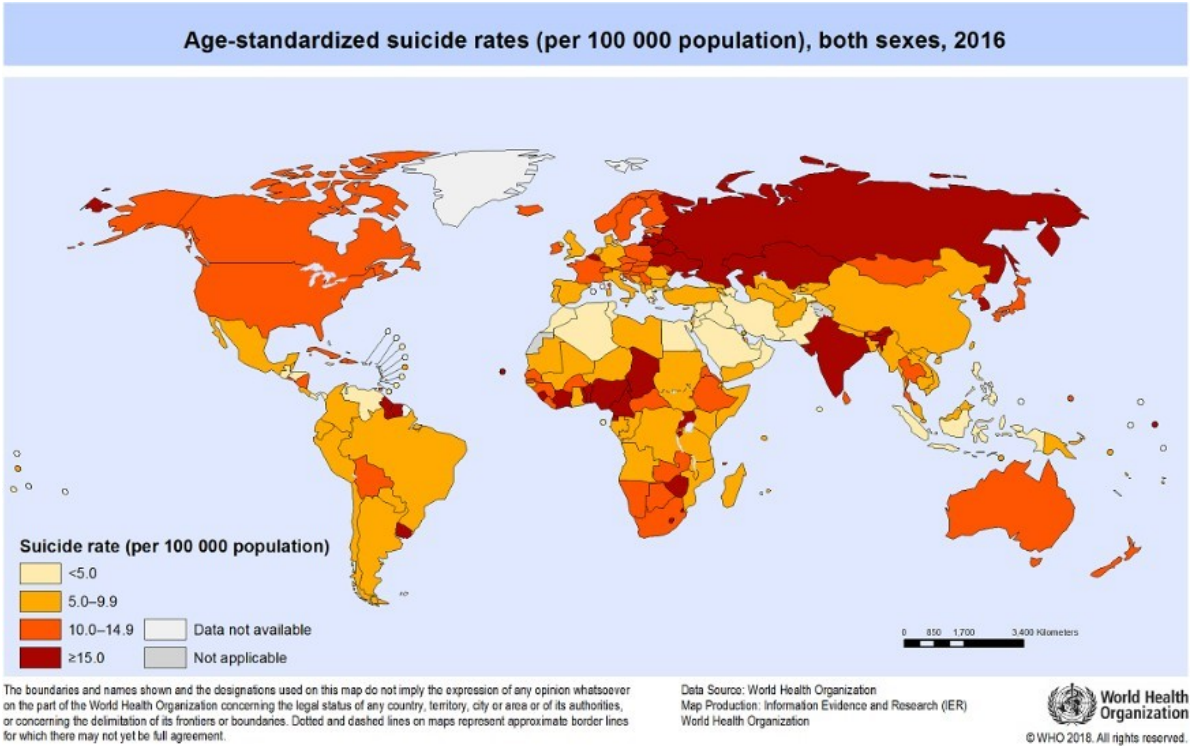
Available online: https://www.who.int/mental_health/suicideprevention/age_income_level_2016.JPG?ua=1

Figure 2. WHO: Age-Standardized Suicide Rates (Per 100,000)



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Available online: http://www.who.int/gho/mental_health/suicide_rates_male_female/en/

1.1.8 Cognitive theories of Major Depressive Disorder (MDD)

The first discovery of the model of learned helplessness was accidental; the study was originally performed on negative reinforcement in dogs (Seligman et al. 1967). In a common study design, two groups of animals (“group 1” and “group 2”) were exposed to electric shocks. The group 1 animals were able to terminate the shock by their behavior. The group 2 animals were exposed to the same kind of electric shocks but had no influence on their termination. Consequently, in contrast to the group 1 animals, the group 2 animals did not try to escape from the electric shocks and developed general inactivity. In humans, the "learned helplessness" can result in MDD. Through the repeated experience of lack of control over situations and unpleasant consequences, passivity and the feeling of not being able to act or to be unable to control one's life (helplessness) is learned and solidified (McGuffin et al. 2003). One limitation of this model is that no explanation was given why certain individuals develop MDD when exposed to uncontrollable stressors whereas others did not (Abramson et al. 1978). The "learned helplessness theory" was further developed into the "reformulated learned helplessness theory" and in the succeeding "hopelessness theory of depression"

(Abramson et al. 1978, Abramson 1989). The "hopelessness theory of depression" integrated the idea that subjects have a higher risk to develop MDD if the cause of a negative life event is attributed as global ("affects my whole life"), stable ("will always be this way"), and internal ("it is my fault") (Seligman et al. 1979).

1.1.8 Treatment of Major Depressive Disorder (MDD)

MDD is usually treated in settings with specialized outpatient care units and primary care units. The treatment goals are: cure of depressive symptoms, prevention of mortality/suicide, restoration of professional and psychosocial performance, recovery to mental equilibrium, and reduction of the probability of recurrence (Lam et al. 2016). In addition to acute treatment, the therapy concept includes maintenance therapy after response/remission and the prophylaxis of future episodes (Lam et al. 2016). Psychopharmacological treatment and psychotherapy are first line treatment options for MDD. The latter treatment modalities include sleep deprivation therapy (also called "wake therapy"), electroconvulsive therapy (ECT), light therapy and physical activity. ECT is mainly used in patients with severe MDD who have not responded to treatment trials with antidepressants and psychotherapy (therapy-resistant depression) especially with psychotic symptoms (Milev et al. 2016). The bright light therapy is mainly used in seasonal affective disorder (SAD). In terms of treatment escalation, several steps are available, for treatment with antidepressants, four weeks are assumed to be one stage, for psychotherapeutic therapy one stage lasts between four and twelve weeks (van Straten et al. 2015). At the end of the stage duration, the efficacy of the selected treatment method must be evaluated. If response has occurred, treatment is continued until remission. If there is no response, treatment should be adapted or transferred to the next stage of therapy. Pharmacotherapy is based on the drug class of antidepressants (AD). The most common antidepressants are: Selective serotonin reuptake inhibitors (SSRIs), Selective serotonin/norepinephrine reuptake inhibitors (SNRI, SSNRI), Noradrenergic and specifically serotonergic antidepressant (NaSSA), Tricyclic antidepressants. The average rate of patients responding to the ADs (response rate) is 50-75% in all ADs groups. In comparison, placebo provides an average response rate of 30-40%. In addition to a mood enhancing effect, antidepressants can also develop sleep-inducing, calming and anxiolytic effects. The full effect unfolds after several days to 4 weeks of continuous intake. To detect any side effects and to be able to make timely adjustments, the use of antidepressants should be closely monitored. At least weekly care in the acute phase is recommended (Archer et al. 2012). After four weeks, the success of the chosen treatment is examined. The duration of treatment is usually at least six months and will continue on the full remission to avoid relapse.

1.1.10 Treatment of Bipolar Disorder (BD)

With increasing knowledge about the treatment of BD and the associated recognition of the complexity of the disease, it has become clear how important it is even within the acute treatment period to already take into account any necessary long-term therapy (mood stabilizer). Acute therapy starts as soon as an acute episode occurs. The acute therapy is continued until the acute symptoms have improved significantly. According to the S3 guidelines of bipolar disorders, in acute therapy mood-stabilizers are indicated (DGPPN e.V. 2012). In case of severe restlessness, other sedative medications like benzodiazepines may also be indicated. For acute treatment of a depressive episode, antidepressants are essential. However, patients are at risk to switch into a manic episode. Depending on the severity and type of symptoms, various medications, psychotherapy and non-drug treatments are used. Maintenance therapy follows the acute therapy and is intended to stabilize the condition of the affected person so far that there is no direct relapse. The further purpose is to maintain a stable euthymic affective state. Relapse prevention begins as soon as the affected person's mood has returned to the habitual condition and intends to prevent long-term recurrence of a new episode. How long the relapse prophylaxis is needed depends on the number of episodes. For three or more episodes within 5 years, continuous pharmacological treatment to prevent new episodes is indicated (DGPPN e.V. 2012). In BD, every affected person has her/his own spectrum of symptoms, which can vary in severity. Therefore, it is important to always choose the medication individually. Today, three main groups of drugs are used, the mood stabilizers, antidepressants (AD) and atypical antipsychotics. Electroconvulsive therapy (ECT) is an efficient treatment for severe depressive and manic episodes (remission rate: 80%). ECT is recommended when the pharmacological and psychotherapeutic treatment is not effective or pharmacological treatment is not possible. However, ECT can only be performed in patients with good physical condition.

1.1.11 The neurotrophic hypothesis of Major Depressive Disorder (MDD)

The pathogenesis of MDD is tied to a general genetic predisposition, resulting in an increased vulnerability when additional stressful life events occur (e.g. job-loss, partnership issues). The individual vulnerability derives from biological factors (e.g. genetic predispositions, genomic imprinting), early childhood trauma, and involves personality traits such as neuroticism (Abramson et al. 1978, Kessler 1997, Sullivan et al. 2000, Peters 2014). There are different theories to explain the neurobiology of MDD. The monoamine (serotonin (5-HT), noradrenaline (NE) and dopamine (DA)) hypothesis claims that MDD is derived from deficiency of 5-HT and/or NE in the synaptic cleft. Antidepressant (AD) drugs act on

monoamine transporters or receptors increasing 5-HT and/or NE in the synaptic cleft. However, antidepressant response occurs with a delay of 2-4 weeks after initial treatment with fast increase of 5-HT by AD therapy (e.g. SSRIs) in the synaptic cleft and therefore the monoamine hypothesis is not sufficiently explaining MDD pathogenesis. Interestingly, monoamine depletion studies failed to induce MDD in control subjects. However, relapse was induced in subjects who had prior MDE but where currently in remission without AD treatment (Ruhe et al. 2007). Recent studies about the neurobiology in MDD, stress and AD treatment have introduced theories beyond "the monoamine hypothesis of MDD". The brain-derived neurotrophic factor (BDNF) is a regulatory protein that mediates neuronal growth and maturation (Abramson et al. 1978). It was shown that exposure to stress decreased BDNF in limbic brain regions, which are important in MDD and cognition (Kaplan et al. 2010). The treatment with ADs increases BDNF in the same brain structures (Kaplan et al. 2010). The "neurotrophic hypothesis" was underlined by observations in MDD subjects showing reduced BDNF protein/mRNA in the hippocampus and prefrontal cortex including structural changes with reduced volume in these regions (Kaplan et al. 2010). The reduction of BDNF in the hippocampus seems to be induced by stress-mediated glucocorticoids and by intensification in serotonergic transmission (Duman et al. 2006, Kaplan et al. 2010). In studies of the anterior hippocampus from brain tissue in MDD subjects, BDNF levels are lower in these regions, compared to control subjects, but increased in MDD subjects with current AD treatment (Chen et al. 2001b). Antidepressants (AD) might reverse hippocampal atrophy and neuronal cell loss by inducing BDNF expression (Kaplan et al. 2010). However, BDNF itself might not be enough to understand the complex etiology of MDD but given that BDNF expression was reduced in MDD subjects and increased by AD, BDNF is a potential biomarker to monitor AD treatment response and might also be a new target for treatment (Chen et al. 2001b, Castren et al. 2010).

1.1.12 Genetics of Major Depressive Disorder (MDD)

There is a scientific consensus that the pathogenesis of MDD is partially due to genetic factors, first of all as a familial accumulation of MDD is well documented (Lieb et al. 2002). The heterogeneity of MDD is also evident in its complex genetic background. It is assumed that MDD is based on polygenic causes with small effects. Both common variants with low penetrance and rare variants with a stronger effect could contribute to the development of MDD (Domschke et al. 2012, Flint et al. 2014). Research into the genetic causes of MDD has therefore been challenging so far. Family studies provided first indications about the heritability of MDD. Subjects with a first-degree relative who is suffering from MDD have an increased risk to develop MDD as well. The meta-analysis of several family studies showed an odds ratio of 2.84 (Sullivan et al. 2000). However, family studies cannot differentiate

between genetic influences and shared environmental factors. For this purpose, twin and adoption studies are performed. Twin studies revealed that the concordance rate (concordance is the match of important characteristics in twins) for MDD is 44% for identical twins and 20% for dizygotic twins. The estimated heritability ranges from 17-75% in MDD, with a mean of 37% (McGuffin et al. 2003, Kendler et al. 2006b, McGuffin et al. 2015). The influence of various environmental factors on an individual basis is given as 60-65%, and the influence of the shared environment is estimated at less than 5% (McGuffin et al. 2015). In contrast, the few existing adoption studies, found especially parental MDD as an environmental risk factor for the child to develop MDD (Tully et al. 2008, McAdams et al. 2015, McGuffin et al. 2015, Hannigan et al. 2018, Kendler et al. 2018). Several twin studies found a difference in the heritability of MDD between women and men, but overall the results are conflicting (Kendler et al. 2006b, Flint et al. 2014). Furthermore, a considerable overlap among the genetic and environmental factors impacts the differences in susceptibility for MDD (Rappaport et al. 2018). In certain subtypes of severe or recurrent MDD, a higher heritability of up to 70% is discussed (McGuffin et al. 2015). In order to localize the genetic causes of a disease at the molecular level, linkage- or association studies are performed that cover the entire genome, as well as candidate gene studies in which individual genes are examined, which due to their function could play a role in the pathogenesis of MDD. Despite the significant heritability of MDD, these methods have so far provided only a few promising and sometimes conflicting or non-replicable results (Cohen-Woods et al. 2013, Mullins et al. 2016). Linkage studies investigate the heritability of genetic markers in relatives with a positive family history of MDD or with traits associated with increased vulnerability to MDD. If such a marker occurs accidentally in the family frequently linked to the disease, chromosomal regions that influence the disease can be determined from the location of this marker on the genome. Like this, a large number of chromosomal “risk loci” for MDD have already been identified (Camp et al. 2005, Middeldorp et al. 2009, Breen et al. 2011). The most promising results include regions on chromosomes 2, 3, 8, 11, 12, 15 and 18 (Camp et al. 2005, Flint et al. 2014, McClain et al. 2019). For some of the markers, however, linkage is only assumed because the level of significance has not been reached (Camp et al. 2005, Middeldorp et al. 2009, Knowles et al. 2016). In addition, the results were only partially reproducible. Furthermore, some of the loci appeared to be gender specific (Camp et al. 2005). One study identified a possible link to chromosome 17 in a region that contains the serotonin transporter gene (Middeldorp et al. 2009). These findings thus result in so-called “positional” candidate genes, which can be examined in association studies. Association studies observe the relationship between genetic markers and diseases by comparing patients and healthy controls. Here too, susceptibility genes can be inferred from the joint appearance of marker and disease. Technological progress in the field of DNA sequencing and chip technology enables genome-wide analyzes of several million markers in several

thousand individuals. However, the genome-wide association studies (GWAS) published so far have not been able to achieve any clear, genome-wide significant results (Schwabe et al. 2019). Kohli et al. (2011) found a genome-wide significant association on chromosome 12q in a meta-analysis. However, there is no coding gene sequence in this region. The closest annotated gene is *SLC6A15*, encoding for an amino acid transporter which transports neutral amino acids and is a member of the solute carrier family 6 protein family (Kohli et al. 2011). A recently published bivariate genome wide association analyses of the wider MDD phenotype found 8 new genetic loci for depression (Amare et al. 2019). In contrast to genome-wide approaches, candidate gene studies analyze variants of individual a priori selected genes and their association with a certain disease phenotypes. Most of the candidate genes are selected based on hypotheses about the pathomechanism of a disease. In the case of MDD, the monoaminergic neurotransmitter system seems to play a role. Therefore, a large number of studies already exist on the relationship between MDD and the genes of serotonin, dopamine and norepinephrine transporters and receptors as well as tryptophan hydroxylase and monoamine oxidase (Cohen-Woods et al. 2013). Furthermore, genes of the hypothalamic-pituitary-adrenal axis were examined due to the relationship between MDD and stress, as well as the brain-derived neurotrophic factor (BDNF) and other potential susceptibility genes (Cohen-Woods et al. 2013). Nearly 200 candidate genes with relation to: neurotransmission (e.g. a functional polymorphism (SNP) near the serotonin transporter (*5-HTTLPR*), neuroplasticity (e.g. *BDNF val66met*) or to the hypothalamic–pituitary–adrenal (HPA) axis have been investigated. With this candidate gene approach, most results remain conflicting and only a few findings have been replicated. Also, further genome-wide association studies (GWAS) of subjects with MDD have brought limited success. In 2013, a GWAS mega-analysis of 9240 MDD subjects and 9519 control subjects did not detect powerful and replicable findings (Major Depressive Disorder Working Group of the Psychiatric et al. 2013). The observed incongruity concerning the evident heritability of MDD in addition with the lack of significant genetic variants associated with MDD, suggests that gene environment interactions (GxE) are adding a major part to the development of MDD (Kendler et al. 2002, Kendler et al. 2006a). The gene expression of relevant genes can be altered by environmental influences through epigenetic mechanisms (e.g. *DNA* methylation) (Lolak et al. 2014). Recent evidence suggests that neuroplasticity in the mature brain is partly regulated by epigenetic mechanisms (Mateus-Pinheiro et al. 2011). The discovery of epigenetic mechanisms elaborating in the pathophysiology of MDD might help to understand the neurogenic processes behind MDD symptoms.

1.1.13 Genetics of Bipolar Disorder (BD)

Affective disorders cumulate within families. As already mentioned, ancestors of BD subjects have a higher risk of both BD and MDD, while in ancestors of MDD there is only a greater number of MDD subjects, compared to the subjects without familiar risk of affective disorders (McGuffin et al. 1989). The evidence of increased heritability in BD and MDD as well as several shared candidate genes point to a genetic overlap between MDD and BD (Craddock et al. 2005, Craddock et al. 2006, Kato 2007). However, according to McGuffin et al. (2003), about 71% of the genetic variance for the manic episode syndrome is not in common with MDD (McGuffin et al. 2003).

1.1.14 Epigenetics of Major Depressive Disorder (MDD)

Overall, the genetic studies have not generated the anticipated results elucidating the heritability of MDD known from twin studies. Instead, there was a discrepancy between the presumed influence of genetics in the development of MDD and the susceptibility genes actually found. Since the genetic studies carried out to date cannot entirely explain the heritability of MDD, this is referred to as the “missing heritability” phenomenon (Maher 2008, Manolio et al. 2009, Genin 2020). The lack of clarification about MDD heredity suggests the involvement of epigenetic mechanisms. In addition, there are other reasons for the influence of epigenetics on the development of MDD: On the one hand, the altered incidence in monozygotic twins can be partly explained by epigenetic differences or distinctive environmental influences. On the other hand, environmental factors can themselves cause changes in epigenetics (Mill et al. 2007). The term epigenetics is used to sum up various mechanisms that influence the activity of genes. These are modifications of the genetic material that regulate gene expression without changing the actual DNA sequence (Schroeder et al. 2012). These modifications are possibly stable over time and can be influenced by environmental factors and presumably be passed on to the next generation (Roth et al. 2009, Nestler 2014). The epigenetic mechanisms known to date include DNA methylation, the modification of histones and the positioning of nucleosomes (Portela et al. 2010). Nucleosomes form the smallest packaging unit of DNA and consist of a piece of the DNA strand that is wound around a histone octamer. The DNA-histone package can be loosened or strengthened by histone modifications including methylation, acetylation, phosphorylation and ubiquitination. Euchromatin enables expression of the genes by binding transcription factors and heterochromatin prevents transcription (Schroeder et al. 2012). The precise positioning of a nucleosome in relation to the DNA strand also plays an important role in transcription control as the nucleosome occludes underlying DNA sequences (Portela et al. 2010). However, this thesis focuses on the study of DNA methylation, as it is the most studied epigenetic mechanism on the subject of MDD. The methylation of DNA takes place

on CpG dinucleotides (cytosine/guanine). DNA methyltransferases mediate the covalent binding of a methyl group to the cytosine base of the CpG dinucleotide (Schroeder et al. 2012). In general, DNA methylation of promoter regions in genes usually leads to a reduction in gene activity (silencing) in various ways. This is done both directly by preventing the binding of transcription factors and indirectly via methyl-CpG-binding proteins (Reamon-Buettner et al. 2007, Portela et al. 2010). Areas with increased occurrence of the CpG dinucleotides are referred to as CpG islands. They are found primarily in promoter regions where they are involved in gene regulation (Portela et al. 2010). Epigenetic mechanisms can favor the development of MDD in different ways: stress-induced epigenetic modifications, stochastic changes during maturation and hormonal influences on the epigenetic signature can predispose to MDD (Nestler 2014, Guintivano et al. 2016). Stress in the early stages of life in particular seems to cause long-term vulnerability to the development of MDD. Since epigenetic mechanisms are able to map such gene-environment interactions (GxE), they can cause changes in the neuronal function and thus in the risk of disease (Menke et al. 2014). A large number of studies confirm epigenetic changes caused by early life stress in the animal model (Dalton et al. 2014). It is above all the pituitary-adrenal axis (also HPA axis, "stress axis") affected by epigenetic modifications (Paslakis et al. 2011, Raabe et al. 2013). But these changes are also detectable in humans. A post-mortem study shows an increased methylation of the glucocorticoid receptor promoter in suicide victims with anamnestic child abuse (McGowan et al. 2009). However, environmental methylation changes have also been found in other genes associated with MDD, e.g. in the neurotrophic system with genes like *BDNF* (Menke et al. 2012). In addition, even prenatal stressors such as maternal smoking, MDD, partner violence or war can influence the methylation pattern of different genes in the child (Dalton et al. 2014). To identify previously unknown candidate genes with changed methylation in MDD, a genome-wide analysis (epigenome-wide analysis; EWAS) is possible analogous to the procedure in genetics. These methylation analyzes allow the methylation differences of several hundred thousand CpG dinucleotides to be investigated depending on the underlying method (How Kit et al. 2012). One of the first analyses was carried out post-mortem with brain tissue from MDD patients compared to healthy controls. The biggest methylation differences were found in the genes LASS2 (ceramide synthase 2), CPSF3 (cleavage and polyadenylation specific factor 3), ZNF263 (zinc finger protein 263) and PRIMA1 (proline rich membrane anchor 1), each with higher methylation in patients with MDD (Sabunciyan et al. 2012). Additional genome-wide methylation analyzes were carried out using twin studies. There was no difference in the average global methylation between the twins with MDD and their healthy siblings (Byrne et al. 2013, Malki et al. 2016). Overall, however, many genes with deviating methylation were identified that had previously been associated with the MDD (Davies et al. 2014, Dempster et al. 2014, Cordova-Palomera et al. 2015). In particular, hypermethylation of the ZBTB20 gene (zinc finger and BTB domain

containing 20), hypermethylation of the serine/threonine kinase STK32C and hypomethylation of the gene WDR26 (WD repeat domain 26) are associated with MDD (Davies et al. 2014, Dempster et al. 2014, Cordova-Palomera et al. 2015). Furthermore, these twin studies found increased variability in the genome-wide methylation profile of affected twins compared to siblings not affected by MDD (Byrne et al. 2013, Davies et al. 2014, Cordova-Palomera et al. 2015). A large number of methylation studies also exist for candidate genes for MDD that are already known from genetic studies, including genes for neurotropic factors and components of the neurotransmitter system (Dalton et al. 2014). For BDNF, an increased methylation of several CpG dinucleotides and a reduced mRNA level in the brain tissue of suicide victims were demonstrated (Keller et al. 2010). Patients with post-stroke depression also had an increased methylation status in the BDNF promoter (Kim et al. 2013). Another study also showed an altered methylation profile of the BDNF gene in MDD patients and postulated this as a potential marker for the diagnosis of MDD (Fuchikami et al. 2011). Regarding the neurotransmitter system, genes of neurotransmitter receptors, transporters and degrading enzymes are to be mentioned: In the gene promoter a $GABA_A$ receptor subunit hypermethylation was identified post-mortem in suicide victims, which correlated with an increased occurrence of DNA methyltransferase (Poulter et al. 2008). In contrast, a hypomethylation of a CpG dinucleotide was found in the HTR2A serotonin receptor gene, however only in patients with bipolar depression and schizophrenia (Ghadirivasfi et al. 2011). In the area of the first exon-intron transition of the MAOA gene, reduced methylation could be demonstrated in women with MDD, whereas no association was found for the gene of the catechol-O-methyl-transferase (COMT) between methylation level and MDD (Dempster et al. 2006, Melas et al. 2013). With the COMT gene, however, hypomethylation was also found in patients with bipolar depression and schizophrenia (Abdolmaleky et al. 2006).

1.2 Brain Derived Neurotrophic Factor (BDNF)

1.2.1 BDNF Function in Neurons and its Impact on Cognition

The neurotrophins are a group of signaling molecules in the brain and are further related to maturation of synapses during development, synaptic plasticity, cell differentiation, neural growth, and axon targeting. BDNF is the best described neurotrophin in terms of its mode of action in neuropsychiatric disorders e.g. MDD (Lohof et al. 1993, Levine et al. 1995, Levine et al. 1998, Kossel et al. 2001, Duman et al. 2006). BDNF was shown to act as a signal for appropriate axonal growth, synaptic plasticity (Yoshii et al. 2010). In learning processes and memory BDNF is essential as well (Poo 2001, Lu et al. 2014). BDNF operates through the tropomyosin receptor kinase B (TrkB) (Levine et al. 1998, Pillai 2008) and is secreted in an activity-dependent manner. BDNF and TrkB are localized at pre- and postsynaptic positions

(Waterhouse et al. 2009). The presynaptic BDNF signaling stimulates release of neurotransmitters, while postsynaptic BDNF signaling enhances numerous ion channel functions (Rose et al. 2004). BDNF performs at excitatory as well as inhibitory synapses (Kovalchuk et al. 2004). The BDNF effect on synapses appears instantly and induces translation/transcription of more BDNF in dendrites, which might maintain long-term potentiation (LTP) activation through constant TrkB stimulation (Kang et al. 1996, Kovalchuk et al. 2004). The activation of TrkB also leads to binding of the transcription factor cAMP response element-binding protein (CREB1) on the *BDNF* promoter and initiates gene transcription accounting for the positive transcriptional feedback (Lu et al. 2008). LTP is an inter-neuronal long-term potentiation of synaptic transmission, which is important in cognitive processes (Nagappan et al. 2005). In behavioral paradigms the depletion of BDNF led to diminished LTP and loss of memory (Patterson et al. 1996, Monteggia et al. 2004, Lu et al. 2008).

1.2.2 BDNF in Major Depressive Disorder (MDD)

In affective disorders, such as MDD, BDNF is crucial in the molecular mechanisms (Cowansage et al. 2010). BDNF is assumed to be a molecular substrate of the stress-response, as BDNF expression was reduced by stress, a major risk factor for MDD (Martinowich et al. 2007). It was further observed that BDNF concentration in the CNS was impaired by persistent stress and ADs stimulated the increase of BDNF in the CNS (Castren et al. 2010). The hypothalamic-pituitary-adrenal axis (HPA) regulates hormonal stress reactions, cognition and memory and sends signals to the hippocampus (McEwen 2006). MDD subjects exhibited a decline in hippocampal volume (Bremner et al. 2000). In post mortem samples of MDD and/-or suicide subjects, the BDNF serum concentration and hippocampal BDNF levels were significantly low in this subjects compared to controls (Castren et al. 2007, Castren et al. 2010, Thompson Ray et al. 2011). The decreased hippocampal volume in MDD subjects might be caused by a stress-induced reduction of BDNF expression (Yu et al. 2011). Additionally, the prefrontal cortex also shows structural changes e.g. reduced volume, linked to lower BDNF and TrkB levels in MDD subjects (Dwivedi et al. 2003b, Castren 2004, Pandey 2004). On the other hand, BDNF protein was increased in samples of the nucleus accumbens (NAc) in MDD subjects (Krishnan et al. 2007). The amygdala, which is involved in memory, in attentional and emotional processes, was increased in volume in MDD subjects (Tebartz van Elst et al. 2000, Frodl et al. 2004). Animal studies revealed stress induced higher BDNF levels in the amygdala (Yu et al. 2011). However, this was not yet confirmed in human MDD subjects.

1.2.3 CREB1 in Major Depressive Disorder (MDD)

The transcription factor cAMP response element-binding protein (CREB) initiates *BDNF* expression and plays a major role in neuropsychiatric disorders including MDD (Tao et al. 1998, Conti et al. 2002). *CREB1* encodes for the transcription factor cAMP response element-binding protein (CREB). CREB might be linked to completed suicide and response to ADs in MDD subjects (Odagaki et al. 2001, Dwivedi et al. 2003a, Young et al. 2004). Further studies postulate additional evidence for the significant impact of certain *CREB1* SNPs on the suicide phenotype among MDD subjects (Perlis et al. 2007a, Perlis et al. 2007b, Zubenko et al. 2008). A higher frequency of suicides is well known in subjects suffering from treatment resistant MDD (Souery et al. 2007). Interestingly, in male subjects with substance abuse disorders, suicide is increased compared to female subjects with substance abuse disorders (Arsenault-Lapierre et al. 2004). CREB concentration was significantly decreased in suicide completers among MDD subjects (Dwivedi 2003a). Other studies have shown that CREB activity in the prefrontal cortex and hippocampus is decreased in MDD subjects (Tardito et al. 2006, Wallace et al. 2009). This can be reversed as a result of the chronic administration of ADs (Blendy 2006). CREB is a very well analyzed transcription factor regarding its role in neuropsychiatric disorders (Marsden 2013). Postmortem studies postulate decreased function of CREB in the prefrontal cortex and in the hippocampus of MDD subjects (Dwivedi et al. 2003b, Yamada et al. 2003). One study performed by Serretti et al (2011) reveals a significant association for the A allele of rs7569963 within *CREB1* on treatment resistance in MDD subjects (Serretti et al. 2011). A former study detected gender specific associations for certain *CREB1* SNPs and higher susceptibility for MDD in females (Zubenko et al. 2003a, Zubenko et al. 2003b).

1.2.4 BDNF and Antidepressants (AD)

In animal models, BDNF exhibited antidepressant effects after straight infusion in the hippocampus (Shirayama et al. 2002). These antidepressant effects were blocked through BDNF knockout in forebrain regions (Monteggia et al. 2004, Groves 2007). Insertion of BDNF in the midbrain and intracerebroventricularly was observed to enhance the activity of monoaminergic neurons (Siuciak et al. 1996). Of note, serotonergic neurons in rat brains were stimulated by BDNF infusion and noradrenaline concentration was raised in the hippocampus (Siuciak et al. 1996, Altar 1999). The above described BDNF activity on the serotonergic and noradrenergic neurons connect the monoaminergic with the neurotrophic hypothesis of MDD. However, different effects were shown for distinct brain areas – in spite of that, the AD-like effect of BDNF injection into the midbrain and hippocampus has been consistently reported. The well-known, delayed antidepressant effect of ADs indicates that a key point in this activity could be BDNF alteration. In post mortem studies of MDD subjects,

cortical and hippocampal BDNF was increased of subjects after chronic AD therapy, whereas BDNF serum levels where stabilized after chronic AD therapy in MDD subjects (Duman et al. 2006). The transcription factor cAMP response element-binding protein (CREB1) activates the *BDNF* gene (Tao et al. 1998, Conti et al. 2002). Further, for all main classes of ADs the initiation of CREB1 expression in the hippocampus was observed (Blendy 2006). The AD substances: selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (SNRIs), tricyclic antidepressants, the monoamine oxidase inhibitors (MAOIs), and atypical antidepressants, as well as electroconvulsive therapy (ECT) were all shown to increase BDNF (Nibuya et al. 1995, Kuroda et al. 1998, Russo-Neustadt et al. 1999, Fukumoto et al. 2001, Coppell et al. 2003, Jacobsen et al. 2004). Another cellular influence of numerous ADs is the induction of neurogenesis in the hippocampus (Sahay et al. 2007, Pittenger et al. 2008). However, there are conflicting results as several preclinical studies did not find these stress- or ADs induced alterations in BDNF levels, or found contrasting results (Martinowich et al. 2007). These observed discrepancies are possibly an effect of different research models e.g. types of stressors, stress duration, altered choice of endpoint, rodent-/mouse strain, and examined brain region. However, the present interpretation of the BDNF hypothesis may be too simplistic and BDNF alone might not be enough to explain depression models, but BDNF was observed to have impact on the susceptibility to develop MDD. It is not yet clear how the reduced BDNF activity influences the vulnerability to MDD (Advani et al. 2009, Autry et al. 2012). Of note, AD treatment probably activates CREB1 and several other transcription factors and hereby numerous growth factors including BDNF are elevated in hippocampal regions and have an impact on neurogenesis (Nestler et al. 2002, Sairanen et al. 2005, Pittenger et al. 2008). Overall, ADs might stimulate neuroprotective pathways, and enhance neuronal plasticity in brain regions of emotional processing, disturbed in MDD. However, so far it is not fully understood how BDNF is altered by ADs. The mechanisms are complex and might appear on different levels; therefore we need more investigations to elucidate the antidepressant effect of BDNF.

1.2.5 BDNF in Bipolar Disorder (BD)

According to the literature, serum BDNF is decreased both in MDD subjects and bipolar disorder subjects with MDE, and is concomitantly increasing with clinical improvement (Teixeira et al. 2010). However, in further studies, decreased BDNF concentrations in BD subjects were found during both manic episodes and MDE, irrespective of therapy status (with or without antidepressants/mood-stabilizers) (de Oliveira et al. 2009). BDNF serum levels could function as potential state marker for mood episodes in BD patients as several groups observed that the key element for restoring BDNF serum levels is not the treatment

itself, but rather the improvement of clinical symptoms (Machado-Vieira et al. 2007, Tseng et al. 2008).

1.2.6 BDNF polymorphisms in Major Depressive Disorder (MDD)

Researchers have studied the *BDNF* gene for single nucleotide polymorphisms (SNPs) that could be linked to MDD. A common polymorphism in the human *BDNF* gene is SNP rs6265, located at position 196, leading to a switch in guanine to adenine base. The SNP rs6265 further causes an amino acid exchange from valine to methionine and has already been extensively studied in connection with psychiatric disorders like MDD (Bath et al. 2006, Baj et al. 2013). The Val66Met destabilizes the BDNF mRNA and has been related to irregular intracellular protein transport and degradation (Baj et al. 2013). Morphological brain alterations and reduced hippocampal sizes were observed in carriers of the Val66Met mutation compared to Val/Val homozygotes (Szeszko et al. 2005, Bath et al. 2006, Bueller et al. 2006, Montag et al. 2010). Moreover, data support the hypothesis that human subjects carrying the Met allele reveal irregular hippocampal activation, together with inferior episodic memory and verbal recognition memory (Egan et al. 2003, Goldberg et al. 2008). Additional SNP variations were identified in the *BDNF* and *NTRK2* (TrkB) genes that may have influence on molecular mechanisms of AD treatment response (Hennings et al. 2013). It is still under investigation whether the presence of certain BDNF polymorphisms may predispose/protect the individual to develop MDD (Yulug et al. 2010). Several animal studies underline the idea that the Val66Met polymorphism could help as a prognosticator of potential onset of MDD (Chen et al. 2006, Chen et al. 2008b). In humans it was observed that Met66 allele carriers are at higher hazard for MDD under certain circumstances than Val66 homozygote subjects (Hwang et al. 2006). The BDNF Val66Met SNP was observed to be associated with MDD and as risk factor for suicidal behavior (Sarchiapone et al. 2008, Licinio et al. 2009, Schenkel et al. 2010, Wells et al. 2010). Several other studies did not observe any significant association of the Val66Met SNP with MDD itself, but with the Val66Met SNP and BDNF serum levels (Chen et al. 2008a, Verhagen et al. 2010, Xu et al. 2010). Both, MDD and control subjects carrying the Val66Met SNP were observed to have reduced BDNF serum levels compared to Val homozygote subjects, irrespective of sex (Ozan et al. 2010). Genetic factors were observed to influence both the lack of response to ADs and the incidence of adverse effects to psychopharmacological therapy (Schosser et al. 2009). Though the Val66Met polymorphism does seem to affect human cognition, the impact of this mutation to the pathological characteristics of MDD or suicide remains unclear (Dwivedi 2010). To this point, it is uncertain whether BDNF polymorphisms contribute to manifestation of MDD symptoms or ADs efficacy, so further studies will be needed to examine this possibility.

1.2.7 BDNF polymorphisms in Bipolar Disorder (BD)

A recent meta-analysis including more than 90.000 individuals showed that the above-described *BDNF* Val66Met polymorphism is significantly associated with BD in Europeans (Li et al. 2016). Also family-based association studies of Caucasian BD subjects found positive results with the Val66Met SNP polymorphism (Neves-Pereira et al. 2002, Sklar et al. 2002). Overall, Val66Met may affect the neuroplasticity and neurogenesis in BD subjects and therefore add to the pathophysiology of BD and other mood disorders like MDD.

1.2.8 BDNF DNA methylation in Major Depressive Disorder (MDD)

Epigenetic mechanisms modify gene expression beyond changes in DNA sequence; these alterations are possibly transmitted through generations and might still be environmentally modifiable throughout life, and could explain discrepancies between phenotypes with high heritability and lack of sufficient findings in conventional genetics (Wu et al. 2001, Urdinguio et al. 2009). Epigenetic modifications are essential for numerous cellular processes among others the regulation of gene expression. Likewise, epigenetics has impact on patho-physiological processes (Portela et al. 2010). It was observed, that epigenetics modifies the development of several mental disorders, including MDD (Nestler 2014, Nestler et al. 2016). The epigenetic mechanisms can be clustered into three general categories: DNA-methylation, histone modification and nucleosome positioning. Another epigenetic mechanism, non-coding RNA (ncRNA)-mediated regulation, also impacts the pathophysiology of MDD (Cogswell et al. 2008, Li et al. 2013, Roy et al. 2017). In humans, DNA methylation is the best analyzed epigenetic mechanism and was observed to be a potentially stable epigenetic modulation on a given locus (Hochberg et al. 2011). The DNA methylation depends on the activities of DNA methyltransferases (DNMTs). The DNMTs add a methyl group to the C-5' position of cytosines. The cytosine-phosphate-guanine (CpG) dinucleotides often cluster in the genes promoter sections, as the so-called CpG islands (Portela et al. 2010). About 60% of genes contain CpG islands, which are usually kept free of methylation to allow for transcription. The consequence of DNA methylation at CpG sites is the inhibition of transcription factors binding into regulatory promoter regions. Therefore, DNA methylation produces suppression of gene-transcription. It is well observed that environmental signals like stress influence DNA methylation (Weber et al. 2007). Brain tissue is proposed as the best sample for DNA methylation analyses in psychiatric disorders. Nevertheless, its accessibility is limited to postmortem collections. Peripheral blood mononuclear cells (PBMCs) provide alternative non-invasive samples with superior accessibility, which might also reflect the biochemical and molecular changes occurring in

the brain as a surrogate marker. Additionally, it has been described that DNA methylation status acquired from blood samples shows a correlation with the methylation status detected in post mortem brain tissue. Recently, (Stenz et al. 2015) found a correlation between *BDNF* methylation of PBMCs and post mortem brain tissue from MDD subjects. Consequently, several studies using PBMCs as a non-invasive surrogate for DNA methylation status have been performed, permitting the identification of potential circulating biomarkers for MDD diagnosis. BDNF stimulates proliferation, differentiation and survival of neurons and is crucial for neural plasticity and cognitive function (Kaplan et al. 2010). In post mortem analyzes, high *BDNF* methylation and low BDNF mRNA concentration were found in the Wernicke region of the brain in suicide committers (Keller et al. 2010). Further, recent evidence shows that the *BDNF* exon I promoter methylation status, obtained from PBMCs, correlated adversely with gene expression (D'Addario et al. 2012). Fuchikami et al. (2011) established a categorization based on the *BDNF* exon I promoter methylation status, which correlated with the symptom severity and diagnosis of MDD in contrast to healthy control subjects. In numerous animal models, epigenetic modifications affected different pathways leading to depression-like behaviors (Schroeder et al. 2010). In rodents, systemic as well as hippocampal administration of DNA methyltransferase (DNMT) inhibitors induced increased BDNF gene expression and antidepressant-like effects (Sales et al. 2011).

1.2.9 BDNF DNA methylation in Bipolar Disorder (BD)

The *BDNF gene* in association with neural adaptions to stress and antidepressants has recently been studied extensively in order to elucidate BD pathophysiology (Henikoff et al. 1997, Shirayama et al. 2002, Hashimoto et al. 2004, Castren et al. 2010, Grande et al. 2010). D'Addario et al. (2012) observed reduced *BDNF* gene expression paralleled by an increased *BDNF* promoter methylation in BD-II subjects in contrast to BD-I and control subjects. The increased *BDNF* promoter methylation in BD-II subjects compared to BD-I subjects indicates that the difference in the *BDNF* gene regulation among BD subjects could be related to the mood state i.e. MDE or manic episode. Further studies need to evaluate the *BDNF* methylation as potential biomarker in BD subjects.

1.2.10 The Influence of BDNF SNP variations on DNA methylation at BDNF

DNA methylation is a molecular mechanism, which supposedly connects genotypes and phenotypes (e.g. complex disease attributes). It is unclear whether genetic variants play a role in inter-individual differences in DNA methylation levels. The genetic variants might affect the probability of DNA methylation in specific gene regions, in addition to the generally hypothesized mechanisms (e.g. environmental factors). SNPs may impact the probability of

DNA methylation and the location of a SNP may determine how it is linked with the epigenome or phenotype. Several studies found a correlation between genetic variants at specific gene loci and DNA methylation level in human brain and other cell lines (Gibbs et al. 2010, Hellman et al. 2010, Shoemaker et al. 2010, Zhang et al. 2010, Bell et al. 2011, Gertz et al. 2011). A local correlation between genetic variants and DNA methylation level (cis-meQTLs) has been found in previous studies (Gibbs et al. 2010, Shoemaker et al. 2010). There is further evidence that genetic variants at CpG sites (meSNPs) can influence DNA methylation on the affected allele (Zhi et al. 2013). However, DNA methylation modulates the gene expression and may consequently augment or diminish effects driven by individual genetic variants. Recently, three *BDNF* SNPs (rs6265, rs7103411 and rs908867) were shown to modulate the association between MDD and *BDNF* promoter I methylation (Januar et al. 2015). In the MDD sample carriers of rs6265 and rs7103411 minor allele and the carriers of rs908867 major allele had *BDNF* promoter I hypermethylation (Januar et al. 2015). The *BDNF* variant rs6265 is positioned in a protein-coding region and it was observed that the Met allele is associated with elevated BDNF protein concentrations (Lang et al. 2009). SNPs in promoter and intronic regions have both been shown to influence gene regulation, implying that proximity within the gene might not be as important as previously suggested (Cooper 2010, Moyer et al. 2011).

1.3 Aims and research questions

The main aim of this thesis was to determine and test whether the *BDNF* exon I promoter methylation status is associated with the MDD phenotype compared to control subjects. Further, the differences in the methylation status between MDD subjects with and without AD treatment were assessed. The first publication listed in the results section deals with the following scientific questions:

1. Is the hypermethylation of the *BDNF* exon I promoter associated to the major depressive disorder (MDD) phenotype?

2. Does antidepressant (AD) treatment influence *BDNF* exon I promoter methylation levels in MDD subjects?

3. Is there a correlation between the severity of MDD symptoms with *BDNF* exon I promoter methylation levels?

4. Has age a significant impact on the *BDNF* exon I promoter methylation levels?

5. Is there a gender difference in *BDNF* exon I promoter methylation levels within MDD, BD and control subjects?

6. Is hypermethylation of the *BDNF* exon I promoter associated with a specific genotype (SNPs; rs11030096, rs925946, rs10501087, rs6265, rs11030102, rs11030104, rs11030108, rs988748, rs12273363, rs908867, rs1491850 and rs1491851)?

The second and third publications analyzed the effects of certain *CREB1* and *BDNF* variants on the suicide phenotype in MDD patients. The specific aims can be summarized as:

1. To test a set of five SNPs in *CREB1* (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690) for association with suicide risk and/or lifetime history of suicide attempts.

2. To test for association between *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs12273363, rs908867, rs1491850, and rs1491851) and suicide risk and/or lifetime history of suicide attempts.

3. To test for associations between *BDNF* SNPs and treatment response phenotypes.

2 Results

2.1 Brain-derived neurotrophic factor (BDNF) - Epigenetic regulation in unipolar and bipolar affective disorder.

Prologue

The brain-derived neurotrophic factor (BDNF) is one possible modulator of the pathophysiology in affective disorders. In order to establish further treatment targets, a better knowledge of the molecular background in affective disorders such as MDD and BD is needed. In the paper "Brain-derived neurotrophic factor (BDNF) - Epigenetic regulation in unipolar and bipolar affective disorder", we analyzed the *BDNF* exon I promoter methylation status in peripheral mononuclear blood cells (PBMCs) of MDD, BD and control subjects in order to elucidate its role in the pathophysiology and treatment response. MDD subjects had a significantly higher *BDNF* exon I promoter methylation status compared to BD and control subjects. Further, the AD treatment accounted for the significant increased methylation status in MDD subjects.

Journal of Affective Disorders 168 (2014) 399–406

Contents lists available at ScienceDirect

Journal of Affective Disorders

journal homepage: www.elsevier.com/locate/jad

Research report

Brain-derived neurotrophic factor (BDNF)—Epigenetic regulation in unipolar and bipolar affective disorder

Laura Carlberg [a], Janine Scheibelreiter [b], Melanie R. Hassler [b], Monika Schloegelhofer [a], Michaela Schmoeger [a], Birgit Ludwig [a], Siegfried Kasper [a], Harald Aschauer [a], Gerda Egger [b], Alexandra Schosser [a,c,n]

[a] Department of Psychiatry and Psychotherapy, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria
[b] Clinical Institute of Pathology, Medical University of Vienna, Austria
[c] Zentrum für Seelische Gesundheit LEOpoldau, Vienna, Austria

article info

Article history:
Received 29 January 2014
Received in revised form
16 June 2014
Accepted 12 July 2014
Available online 19 July 2014

Keywords:
Affective disorders
Depression
Antidepressant therapy
Epigenetics
BDNF methylation
Genetic association study

abstract

Background: Alterations of brain-derived neurotrophic factor (BDNF) DNA methylation at specific *BDNF* promoters and corresponding gene expressions are associated with pathology and the response to antidepressant (AD) therapy in affective disorders such as major depressive disorder (MDD) and bipolar disorder (BD).

Methods: Genomic DNA was derived from peripheral blood mononuclear cells (PBMCs) and was bisulfite converted. Percentage of methylated reference (PMR) was calculated based on results from quantitative real-time PCR following the MethyLight protocol. For statistical analysis parametric procedures were performed as appropriate.

Results: In this study 544 subjects were included, 207 MDD subjects, 59 BD subjects and 278 control subjects. The *BDNF* exon I promoter methylation resulted to be significantly increased in MDD subjects compared to BD subjects (p ¼ 0.0089) and control subjects (p o0.001). Furthermore, the increase of methylation in MDD subjects was significantly associated with AD therapy (p ¼ 0.0019) but not to the clinical features of depression such as the severity of symptoms (p ¼ n.s.). None of the 12 investigated single nucleotide polymorphisms (SNP) showed significant genotype–methylation interactions.

Limitations: Although based on previous findings, the DNA methylation was evaluated within only one CpG island of the different alternative *BDNF* gene transcripts.

Conclusions: The results suggest that the methylation status might not only be affected by the disease phenotype but might also be further influenced by pharmacological treatment, therefore harbouring the possibility of identifying new insights for treatment options.

1. Introduction

Major depressive disorder (MDD) is a common psychiatric disorder. Worldwide, more than 350 million people suffer from symptoms of depression. MDD is internationally one of the leading causes of invalidity, and is a major contributor to the global burden of disease. At its worst, MDD can result in suicide, which causes an estimated one million deaths every year (WHO, 2012). New biomarkers need to be identified, in order to support a reliable and precise diagnosis and prognosis in psychiatric disorders, better understanding of pathogenesis and pathophysiological mechanisms, and for predicting disease progression and monitoring therapeutic interventions and development of improved therapeutic agents (Kobeissy et al., 2013).

A polygenic multifactorial model best explains the aetiology of MDD, where certain genes, in combination with environmental factors (such as poor nutrition, exposure to toxins, psychological trauma and certain stressors), can result in depressive symptoms of variable severity once the individual threshold is exceeded. Furthermore, epigenetic modifications such as DNA methylation play a major role in the pathogenesis of psychiatric disorders (Bondy, 2011; Peedicayil, 2007). There is evidence that DNA methylation may be influenced by genotype and perhaps presents an interface between the environment and the genome, and therefore provides an interesting target for peripheral biological markers (Mill et al., 2008; Peedicayil, 2007, 2008).

The brain-derived neurotrophic factor (BDNF) belongs to the family of neurotrophins and is known to be an important regulator of neuronal survival, development, function and synaptic plasticity

[n] Corresponding author at: Department of Psychiatry and Psychotherapy, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria.
E-mail address: alexandra.schosser@meduniwien.ac.at (A. Schosser).

http://dx.doi.org/10.1016/j.jad.2014.07.022
0165-0327/& 2014 Published by Elsevier B.V.

in the central nervous system. Several studies revealed that BDNF is broadly expressed in the brain (Timmusk et al., 1993; Angelucci et al., 2005). Accumulating evidence suggests that BDNF may be involved in the pathophysiology of MDD, as well as the molecular mechanisms of response to antidepressant (AD) therapy (Taliaz et al., 2010; Angelucci et al., 2005; Molendijk et al., 2011; Castren, 2013). Recently published studies showed that epigenetic modifications that include DNA methylation of *BDNF* promoters are associated with the pathophysiology of MDD, among other psychiatric disorders (Ikegame et al., 2013; D'Addario et al., 2013; Kang et al., 2013; Fuchikami et al., 2011). The *BDNF* gene expression was shown to negatively correlate with DNA methylation at *BDNF* exon I promoter in peripheral blood mononuclear cells (PBMCs) of subjects with affective disorders (D'Addario et al., 2012). Previously reported data showed increased *BDNF* DNA methylation in Wernicke's area of the brain in subjects, who committed suicide. Depression is associated with high risk of suicide (Keller et al., 2010). Importantly, *BDNF* DNA methylation changes have been shown to be consistent across tissues including brain and peripheral blood, supporting the potential usefulness of the *BDNF* DNA methylation level as a biomarker for MDD (Ikegame et al., 2013). Since *BDNF* gene transcription is very complex due to alternative promoter regions, within this study, we focused on *BDNF* exon I promoter, which has been described as the brain-specific inducible promoter (Falkenberg et al., 1993).

The current study investigates the methylation level of the *BDNF* exon I promoter in combination with 12 *BDNF* single nucleotide polymorphisms (SNPs; rs11030096, rs925946, rs10501087, rs6265, rs11030102, rs11030104, rs11030108, rs988748, rs12273363, rs908867, rs1491850 and rs1491851) in a sample of 207 major depressive disorder (MDD) subjects, 59 bipolar disorder (BD) subjects and 278 control subjects, in order to test the *BDNF* methylation status as a potential biomarker in affective disorders and to elucidate a potential functional impact of genotype–methylation interactions. The second aim of our study was to analyse the effect of antidepressant therapy (AD) on DNA methylation levels in subjects with MDD.

2. Methods

2.1. Subjects

The study consisted of 597 participants recruited in Vienna: 210 subjects with major depression (MDD), 60 subjects with a diagnosis of bipolar disorder (BD) and 327 control subjects. For methylation analysis 544 subjects (*n*¼ 207 MDD; *n*¼ 59¼ BD; *n*¼ 278 controls) were available (*n*¼ 53 were excluded due to insufficient DNA concentration); for genotype analysis all 597 study subjects were available. The assessment was performed in the context of the following studies at the Department of Psychiatry and Psychotherapy of the Medical University Vienna: studies on the psychosocial and biological (genetic) causes of bipolar and unipolar affective disorder (and schizophrenia) (Oesterreichische Nationalbank (ÖNB) Grant nos. 5777 and 13198 and Austrian Research Foundation (FWF), Grant no. 7639, all to H.A.) and the "Genetics of Response to Agomelatine Pilot-Study (GENRAS)" (EK-No 201/2010 A.S.).

2.2. Diagnostic procedures

Unrelated in- and outpatients of white European ethnicity were identified at the Department and interviewed face-to-face using either the Schedule for Affective Disorders and Schizophrenia (SADS), Lifetime version (Endicott and Spitzer, 1987), Structured Clinical Interview for DSM Disorders (SCID) (Wittchen et al., 1997) or the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (Wing et al., 1990). All subjects were diagnosed by experienced psychiatrists according to DSM-IV criteria, based on structured and unstructured psychiatric interview and information from medical records (Gmitrowicz and Kucharska, 1994). Exclusion criteria were primary substance use disorders or primary organic or neurological cause of psychiatric symptoms. MDD subjects were free of a personal and family history (first-degree) of BD or schizophrenia. BD subjects were excluded if they or a first-degree relative were ever diagnosed with schizophrenia, or they had experienced mood incongruent psychotic symptoms. Control subjects were of white European ethnicity, free of any current or past psychiatric illness and without any psychiatric history in first-degree relatives. The Local Ethical Committee approved each study protocol and all participants gave informed written consent. The Beck Depression Inventory (BDI) was administered to a subsample of MDD subjects and control subjects. BDI is a 21-item self-report inventory that assesses the severity of depressive symptoms (scores range from 0 to 63 with higher scores reflecting more severe depression) (Beck, 1961), it has strong internal consistency, test–retest reliability, and validity (Beck et al., 2001).

2.3. Methylation analysis

Genomic DNA was extracted from PBMCs using the PUREGENE DNA Purification Kit (Gentra Systems Inc., Minneapolis, Minnesota, USA) and the Nucleon BACC Genomic DNA Extraction Kit (Amersham Biosciences). Samples not reaching DNA concentrations Z10 ng/ml were excluded from methylation analysis. DNA (10 ng/ml) was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo Research), according to the manufacturer's protocol. Unmethylated cytosines were hereby converted to uracil whereby methylated cytosines remained unchanged. To determine the DNA methylation status at the exon I promoter region of the *BDNF* gene, we used methylation-specific quantitative PCR following the MethyLight protocol using SYBR green instead of fluorescent probes for quantification (Campan et al., 2009). *BDNF* primers amplifying the methylated exon I BDNF promoter were generated according to published sequences, ALU-C4 amplification was used as a methylation-independent quantity control PCR (for detailed information on Primers, see Table 1) (D'Addario et al., 2012). In the primer and amplified region, 138 common SNPs were found in Z1% of samples using SNPer software (http://snpper.chip.org/bio/snpper-enter). M.SssI-treated DNA was used as a universally methylated reference sample and as a basis for the serial dilutions for ALU-C4 standard curves. Universally unmethylated DNA served as a negative control. Calculations were then based on the mean Ct (cycle threshold) value calculated from duplicate reactions. The percentage of methylated reference (PMR) value was calculated by the formula shown by (Campan et al., 2009):

$$100 \times \frac{\text{BDNF mean value sample}}{\text{ALU mean value sample}} = \frac{\text{BDNF mean value M:SssI}}{\text{ALU mean value M:SssI}}$$

2.4. Genotyping

Genotyping was performed using the Sequenom MassARRAY® iPLEX Gold technology at Beckman Coulter Genomics, Morrisville

Table 1
The primers for bisulphite-converted DNA.

BDNF_m	fo 5′-GTAGTTTTCGTAGGATGAGGAAGC-3′ re 5′-AATATAAATTAACAACCCCGATACG-3′
BDNF_u	fo 5′-GTAGTTTTTGTAGGATGAGGAAGTG-3′ re 5′-TATAAATTAACAACCCCAATACACA-3′
ALU-C4	fo 5′-GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA-3′ re 5′-ATTAACTAAACTAATCTTAAACTCCTAACCTCA-3′

(USA), and 12 *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs11030102, rs11030104, rs11030108, rs988748, rs12273363, rs908867, rs1491850 and rs1491851) were genotyped in the context of the current study. The SNP (rs6265) is located in exon 11 and results in an amino acid substitution from valine to methionine at codon 66 (Val[66]Met). The Sequenom MassARRAY® iPLEX Gold assay uses PCR amplification and primer extension, resulting in an allele-specific difference in mass between extension products. The mass difference allows the data analysis software to differentiate between SNP alleles; for detailed information see (Schosser et al., 2004).

2.5. Statistical analysis

The statistical analyses were performed using Prism version 5 (Graph-Pad Software, San Diego, CA); for 3 group analyses we performed analysis of variance (ANOVA) followed by Bartelett's test and post-hoc *t*-test. The *p*-values were two-tailed and $p<0.05$ was considered to be statistically significant; results are declared as mean ± SEM. Pearson's correlation coefficient test was performed to test for correlation between the PMR values and clinical variables (Beck-Depression-Inventory (BDI) scores; age). To test for genotypic association with each SNP, a standard chi-square (χ^2) statistic was calculated using SPSS Statistics version 20 for MAC. The computer program FINETTI (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to calculate the Cochran–Armitage trend statistic to test for allelic association. *p*-values <0.05 were considered to be statistically significant and all reported *p*-values are two-tailed. UNPHASED version 3.0.10 program was applied using three-marker slide windows to analyse for haplotypic association (Dudbridge, 2003). UNPHASED uses the standard Expectation-Maximisation (EM) algorithm in order to estimate haplotypes from genotypes. The rare haplotype frequency threshold was taken as 0.01. UNPHASED uses unconditional logistic regression to perform likelihood ratio tests under a log-linear model of the probability that an allele or haplotype belongs to the case rather than control group. The global null hypothesis is that the odds ratios of all haplotypes are equal between cases and controls. Individual haplotypes were also tested for association by grouping the frequencies of all other haplotypes together. Multiple testing corrections were performed by application of the false discovery rate (FDR) to both single-marker and haplotype analyses (Benjamini et al., 2001). In the case of single-marker analyses, it was assumed that 12 independent tests were performed when testing 12 SNPs.

3. Results

3.1. Demographic and clinical characteristics of subjects

The clinical and demographic characteristics of the subjects collected in the context of the different studies are shown in Table 2.

3.2. Increased BDNF methylation in MDD subjects

To examine the DNA methylation level of the *BDNF* exon I promoter, we applied quantitative methylation specific PCR on bisulfite converted genomic DNA isolated from PBMCs. For this analysis, DNA of 207 MDD subjects, 59 BD subjects and 278 control subjects, was available. The percentage of methylated reference (PMR) values at the *BDNF* exon I promoter resulted to be significantly increased in MDD subjects (3.5870.23%; $n = 207$) compared to BD subjects (2.3670.32%; $n = 59$; $p = 0.0089$; *t*-test) and control subjects (2.0170.13%; $n = 278$; $p < 0.000$; *t*-test). No significant increase of PMR in BD subjects compared to control subjects was observed ($p > 0.05$; *t*-test) (see Fig. 1). No significant correlations were found for PMR values and the total BDI sum score (available from $n = 81$ MDD subjects), as inferred from the Pearson's correlation coefficient ($p = 0.74$). In gender-specific

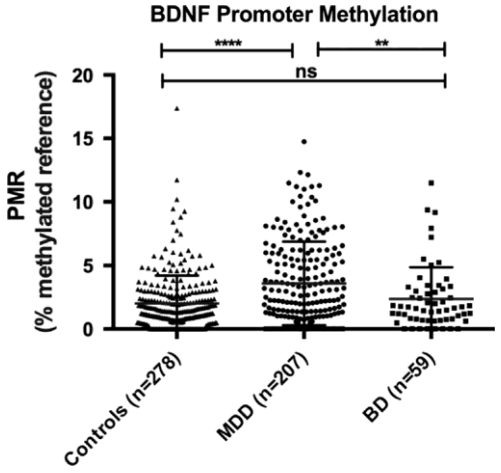

Fig. 1. The extent of methylated DNA at *BDNF* exon I promoter in peripheral blood mononuclear cells from controls, subjects diagnosed with major depressive disorder (MDD) and bipolar disorder (BD). Scatter dot plots with mean values and error bars are shown.

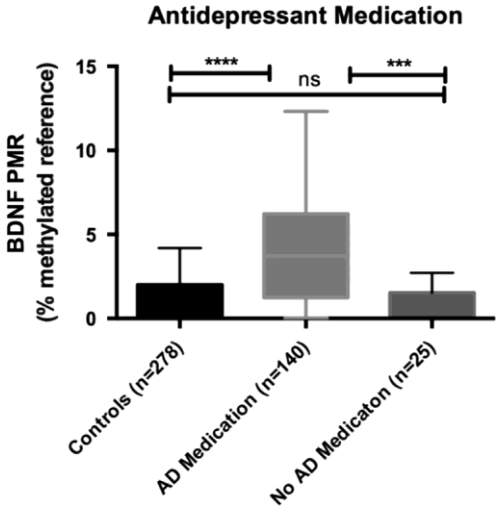

Fig. 2. The extent of methylated DNA at *BDNF* exon I promoter in control subjects, subjects diagnosed with major depressive disorder (MDD) under current antidepressant (AD) and without (No AD) medication. Box plots with whiskers from minimum to maximum are shown.

Table 2
Demographic characteristics of subjects.

Group	Age (Years:mean7SEM)	BDI sum score (Mean7SEM)	Gender (Male/Female) *n*	Gender (Male/Female) %
Control subjects ($n=327$)	31.870.55	1.870.27 ($n=81$)	123/204	37.6/62.4
Bipolar disorder ($n=60$)	4371.90	n.a.	33/27	55/45
Major depressive disorder ($n=210$)	46.0371.07	22.57 1.12 ($n=119$)	89/121	42.4/57.6

Table 3
The effect of antidepressant medication on DNA methylation. One-way ANOVA and unpaired *t*-test: Subjects with major depression (MDD) were grouped by genotype and analysed, based on mean percentage of methylated reference (PMR) values, for treatment status with antidepressant medication (AD) (NoAD: *n* ¼ 25; AD: *n* ¼ 140) and compared to control subjects (*n* ¼278).

SNP	Genotype	ANOVA: CON vs. NoAD vs. AD	*t*-test: CON vs. NoAD	*t*-test: AD vs. NoAD	*t*-test: CON vs. AD
rs1491851	CC NoAD (*n*¼5) CC CON (*n* ¼ 74) CC AD (*n* ¼ 30)	*p*o0.0001	n.s.	*p* ¼ 0.0129	*p*o 0.0001
	CT NoAD (*n*¼14) CT CON (*n* ¼ 213) CT AD (*n* ¼ 69)	*p*o0.0001	n.s.	*p* ¼ 0.0124	*p* ¼ 0.0003
	TT NoAD (*n*¼6) TT CON (*n*¼53) TT AD (*n* ¼ 32)	*p*¼0.0018	n.s.	*p* ¼ 0.0386	*p*o 0.0001
rs11030096	CC NoAD (*n*¼5) CC CON (*n*¼464) CC AD (*n* ¼ 33)	*p*¼0.0007	n.s.	n.s	*p*o 0.0001
	CT NoAD (*n*¼14) CT CON (*n* ¼ 143) CT AD (*n* ¼ 69)	*p*o0.0001	n.s.	*p* ¼ 0.0026	*p*o 0.0001
	TT NoAD (*n*¼6) TT CON (*n* ¼ 65) TT AD (*n* ¼ 28)	*p*¼0.0001	n.s.	*p* ¼ 0.0498	*p*o 0.0001
rs925946	GG NoAD (*n*¼12) GG CON (*n* ¼ 144) GG AD (*n* ¼ 62)	*p*o0.0001	n.s.	*p* ¼ 0.0050	*p*o 0.0001
	GT/TT[a] NoAD (*n*¼13) GT/TT[a] CON (*n*¼127) GT/TT[a] AD (*n* ¼ 68)	*p*o0.0001	n.s.	*p* ¼ 0.0020	*p*o 0.0001
rs10501087	CC/TC[a] NoAD (*n*¼11) CC/TC[a] CON (*n*¼113) CC/TC[a] AD (*n* ¼ 46)	*p*o0.0001	n.s.	*p*¼ 0.0154	*p*o 0.0001
	TT NoAD (*n*¼14) TT CON (*n*¼158) TT AD (*n* ¼ 84)	*p*o0.0001	n.s.	*p*¼ 0.0012	*p*o 0.0001
rs11030102	CC NoAD (*n*¼14) CC CON (*n* ¼157) CC AD (*n* ¼ 80)	*p*o0.0001	n.s.	*p*¼ 0.0103	*p*o 0.0001
	CG/GG[a] NoAD (*n*¼11) CG/GG[a] CON (*n* ¼ 115) CG/GG[a] AD (*n* ¼ 50)	*p*o0.0001	n.s.	*p* ¼ 0.0019	*p*o 0.0001
rs11030104	AA NoAD (*n*¼14) AA CON (*n* ¼ 159) AA AD (*n* ¼ 85)	*p*o0.0001	n.s.	*p* ¼ 0.0015	*p*o 0.0001
	GA/GG[a] NoAD (*n*¼11) GA/GG[a] CON (*n* ¼112) GA/GG[a] AD (46)	*p*o0.0001	n.s.	*p*¼ 0.0154	*p*o 0.0001
rs11030108	AA/GA[a] NoAD (*n*¼13) AA/GA[a] CON (*n* ¼130) AA/GA[a] AD (*n* ¼ 69)	*p*o0.0001	n.s.	*p*¼ 0.0017	*p*o 0.0001
	GG NoAD (*n*¼12) GG CON (*n* ¼142) GG AD (*n* ¼ 62)	*p*o0.0001	n.s.	*p*¼ 0.0142	*p*o 0.0001
rs988748	CC NoAD (*n*¼14) CC CON (*n* ¼157) CC AD (*n* ¼ 84)	*p*o0.0001	n.s.	*p*¼ 0.0017	*p*o 0.0001
	CG/GG[a] NoAD (*n*¼11) CG/GG[a] CON (*n* ¼115) CG/GG[a] AD (*n* ¼ 47)	*p*o0.0001	n.s.	*p*¼ 0.0134	*p*o 0.0001
rs12273363	CC/CT[a] NoAD (*n*¼12) CC//CT[a] CON (*n*¼100) CC/CT[a] AD (*n* ¼ 36)	*p*o0.0001	n.s.	*p* ¼ 0.0031	*p*o 0.0001
	TT NoAD (*n*¼13) TT CON (*n* ¼ 171) TT AD (*n* ¼ 95)	*p*o0.0001	n.s.	*p* ¼ 0.0085	*p*o 0.0001
rs1491850	CC NoAD (*n*¼6) CC CON (*n* ¼ 48) CC AD (*n* ¼ 15)	*p*¼0.0161	n.s.	*p* ¼ 0.0268	*p*o 0.0001
	CT NoAD (*n*¼12) CT CON (*n* ¼ 140) CT AD (*n* ¼ 63)	*p*o0.0001	n.s.	*p* ¼ 0.0031	*p*o 0.0001
	TT NoAD (*n*¼7) TT CON (*n* ¼ 83) TT AD (*n* ¼ 35)	*p*o0.0001	n.s.	n.s.	*p*o 0.0001
rs6265	AA/GA[a] NoAD (*n*¼10) AA/GA[a] CON (*n* ¼ 102) AA/GA[a] AD (*n* ¼ 37)	*p*o0.0001	n.s.	*p* ¼ 0.0328	*p*o 0.0001
	GG NoAD (*n*¼15)	*p*o0.0001	n.s.	*p* ¼ 0.0008	*p*o 0.0001

Table 3 (*continued*)

SNP	Genotype	ANOVA: CON vs. NoAD vs. AD	*t*-test: CON vs. NoAD	*t*-test: AD vs. NoAD	*t*-test: CON vs. AD
rs908867	GG CON (n ¼ 172) GG AD (n ¼ 93) AA/GA[a] NoAD (n¼3) AA/GA[a] CON (n ¼38) AA/GA[a] AD (n ¼ 33) GG NoAD (n¼22) GG CON (n¼236) GG AD (n¼98)	[b] p o 0.0001	n.s. n.s.	n.s. p¼0.0002	p¼0.0031 p o0.0001

[a] The groups o 5 homozygous for one allele were combined with the heterozygous group of the same SNP for analysis.
[b] Bartlett's test (One-way ANOVA) was not performed due to the small sample size.

analysis of the MDD and BD subjects, no significant differences regarding the PMR values (*t*-test: p ¼ 0.16) were evident. In order to detect a potential age-related alteration in DNA methylation we performed Pearson's correlation coefficient for age and PMR in each group (control subjects: r ¼ −0.1, p ¼ 0.79; MDD with AD r ¼ 0.11, p ¼ 0.22; MDD without AD r ¼ 0.23, p ¼ 0.30; BD: r ¼ 0.42, p ¼ 0.002).

3.3. Genetic association of BDNF SNPs with disease phenotype

We genotyped 12 *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs11030102, rs11030104, rs11030108, rs988748, rs12273363, rs908867, rs1491850 and rs1491851) in the sample set we used for methylation analyses, in order to test for genetic association with the MDD and BD phenotype, as well as for potential methylation–genotype interactions (see below). For this analysis, DNA of 210 MDD subjects, 60 BD subjects and 327 control subjects, was available. Our case-control association test was analysed as dichotomous trait applying standard chi-square (χ^2) statistics. In haplotype analyses, the 3-marker haplotype, A-T-T of rs908867-rs1491850-rs1491851, showed significant associations with the combined affective disorder group (BD and MDD; n¼ 270; individual: p¼ 0.007; global: p¼ 0.040) and the group of MDD subjects alone (n¼ 210; individual: p¼ 0.003; global: p¼ 0.053), however not resisting multiple testing correction. In single-marker analyses, we found three significant allelic (p¼ 0.04; p¼0.03; p 0405) associations of the SNPs rs11030102, rs12273363 and rs1491850 with the MDD phenotype, which also did not remain significant after multiple testing correction. No significant genotypic association was obtained with the group of BD phenotype (n¼ 60) for single-marker analyses. The results showed that none of the 12 SNPs was significantly associated with the MDD or BD phenotype after multiple testing correction.

3.4. BDNF methylation–genotype interactions

To examine a putative correlation of the *BDNF* genotype with DNA methylation levels of the *BDNF* exon I promoter, we performed parametric tests for each SNP, with genotype as the independent variable and PMR value as the dependent variable. For tests with 6 of the 12 genotyped SNPs, the smaller homozygous group (n r 20) was combined with the heterozygous group for analyses resulting in two groups (i.e. AA/AG vs. GG), due to the little number of individuals homozygous for one allele, as shown before (Haeffel et al., 2012). For these 6 SNPs (rs6265, rs10501087, rs11030104, rs908867, rs988748, rs12273363) an unpaired *t*-test was performed resulting in a significant difference between the homozygous and the heterozygous/homozygous genotype for one SNP (rs908867; AA/AG: 2.45 7 0.13%, (n¼438) vs. GG: 3.39 7 0.32%, (n¼83)), p ¼0.0038, not resisting multiple correction. For the other 6 of the 12 SNPs (rs11030096, rs925946, rs11030102, rs11030108, rs1491850, rs1491851), allele frequencies of the minor allele were sufficient (nZ20 for each possible genotype) to include each genotype (i.e. AA vs. AG vs. GG) in statistical analyses, in order to perform ordinary one-way ANOVA. However, no significant differences in PMR values between the different genotypes for the tested SNPs were evident after multiple testing correction (data not shown).

3.5. The effect of antidepressant medication on BDNF methylation

A sub-group of MDD subjects was further analysed for the effect of antidepressant medication on DNA methylation levels at the *BDNF* exon I promoter. For 165 subjects the detailed information about treatment status was available; n ¼ 140 subjects were treated with antidepressant medication (AD) whereas n¼ 25 subjects had no antidepressant medication (No AD). Results show significantly increased PMR values in MDD subjects with AD therapy (4.1370.28%; n ¼ 140) compared to MDD subjects without AD therapy (1.7270.28%; n ¼ 25; p ¼ 0.0019; *t*-test) and compared to control subjects (2.0170.13%; n ¼ 278; p o0.0001; *t*-test). No increase of PMR in MDD subjects without AD therapy compared to control subjects was observed (p 40.05; *t*-test) (see Fig. 2). In a further exploratory analysis, the subjects were grouped by genotype and compared regarding their treatment status, based on the PMR value. Results show increased *BDNF* methylation for MDD subjects treated with AD medication compared to MDD subjects without AD medication and healthy controls, within groups of the same genotype for each of the 12 selected SNPs (see Table 3).

4. Discussion

In order to examine BDNF as a potential biomarker and further elucidate its role in the pathophysiology of depression, we determined the methylation level of the *BDNF* exon I promoter and also genotyped a total of 12 *BDNF* SNPs from PBMCs of subjects with affective disorders and control subjects.

Our data showed significantly increased DNA methylation in MDD subjects compared to BD subjects and control subjects, which is in line with a recent study that reported an increased *BDNF* exon I promoter methylation in MDD subjects compared to control subjects in a smaller sample of 85 participants, analysed from PBMCs (D'Addario et al., 2013). Recently, Fuchikami et al. (2011) were able to accurately classify subjects into diagnostic groups of MDD and control subjects based on DNA methylation levels, and this classification was in accordance with the clinical diagnosis. However, they showed mostly hypomethylation of the *BDNF* exon I promoter for MDD subjects compared to control subjects in a smaller Asian sample of 38 participants. In general, for various psychiatric conditions, subjects show increased *BDNF* DNA methylation levels at the *BDNF* promoters in the brain and peripheral blood, often associated with decreased BDNF levels (Rao et al., 2012; Keller et al., 2010; D'Addario et al., 2012, 2013; Perroud et al., 2013; Kim et al., 2013; Toledo-Rodriguez et al.,

2010). In line with a recent study, our data showed that methylation levels did not correlate with BDI sum scores reflecting the severity of depressive symptoms (D'Addario et al., 2013). Previous studies found low levels of BDNF protein in serum of MDD subjects but no direct correlation of severity of depression and BDNF serum levels (Birkenhager et al., 2012; Molendijk et al., 2011), suggesting that *BDNF* methylation status and BDNF protein levels are trait markers and do not have direct effects on the depression characteristics such as its severity. Importantly, the epigenetic status is dynamic and basically can change in response to various factors. It is well established that the genomic landscape of DNA methylation gets altered as a function of age (Teschendorff et al., 2013); hence we also investigated potential age-related alterations in DNA methylation in our sample, although results have to be taken with caution due to mean ages varying between 31.8 and 46.03 years (see Table 2). In order to establish *BDNF* methylation as a biomarker in psychiatric disorders, further analyses are needed in larger well-defined samples, to determine the potential outcomes of the DNA methylation levels on clinical symptoms and precisely define the factors that affect DNA methylation at the *BDNF* gene such as adverse life events and therapeutic interventions.

Considering psychopharmacological treatment as an important regulating agent on DNA methylation, largely in accordance with recent findings, our data showed that DNA methylation in MDD subjects under current antidepressant (AD) therapy was significantly higher than in MDD subjects without AD therapy and control subjects. Interestingly, there was no significant difference between MDD subjects without AD therapy and control subjects. Therefore, the significantly increased DNA methylation levels in MDD subjects compared to control subjects might be due to antidepressant medication as opposed to the disease phenotype, suggesting that antidepressants may increase DNA methylation in PBMCs. However, MDD subjects showed significantly increased DNA methylation levels compared to BD subjects as well, the latter possibly pharmacologically treated (among others with AD) as well. As a consequence, at least based on our results, methylation changes cannot be explained by pharmacological treatment alone. Further randomized case-control studies in MDD and BD patients with and without pharmacological treatment are therefore needed to separate effect of pharmacological treatment and effect of phenotype itself. Strengthening the findings of increased *BDNF* exon I promoter methylation levels in MDD subject treated with AD therapy, we further showed, in rather exploratory sub-analyses of groups with the same genotype, an increased DNA methylation in MDD subjects receiving AD therapy compared to MDD subjects without AD therapy and control subjects, for each of the 12 genotyped *BDNF* SNPs. Strengthening our hypothesis, D'Addario et al. (2012) showed higher methylation levels of the *BDNF* exon I promoter in BD subjects receiving AD therapy compared to AD-free subjects. In a second study, D'Addario et al. (2013) showed higher levels of methylation in MDD subjects receiving AD therapy compared to MDD subjects receiving AD therapy plus mood stabilizers (D'Addario et al., 2013). To our knowledge, no study has so far investigated the effect of AD on methylation status and/or expression of *BDNF* promoter I in brain tissue, although blood cells have proven to be useful material to investigate the methylation status of certain gene regions (Weksberg et al., 2002, Cui et al., 2003). Reinforcing the hypothesis that AD therapy may have regulating effects on epigenetic modifications, there is evidence that *BDNF* H3K27 methylation levels change in response to AD therapy (Lopez et al., 2013). Thus, our results as well as data published by other groups provide support for the impact of AD therapy on epigenetic processes associated with the *BDNF* gene. The evaluation of *BDNF* methylation levels may have relevance for molecular mechanisms of AD therapy. Further studies are needed in larger well-defined cohorts to elucidate the role of *BDNF* methylation in the pathway of AD therapy.

Besides, a leading hypothesis of depression supports a role for neurotrophins such as BDNF in the cellular adaptations that underlie the therapeutic actions of antidepressants (Duman and Monteggia, 2006; Warner-Schmidt and Duman, 2006, 2007; Krishnan et al., 2008). Further, two recent meta-analyses reported positive correlations between serum levels of BDNF and antidepressant responses (Brunoni et al., 2008; Sen et al., 2008).

Analysing genotype–methylation correlations, our results showed relation of one SNP (rs908867) with the DNA methylation levels at *BDNF* exon I promoter with significantly increased methylation levels in subjects carrying the homozygous (GG) genotype. This finding is in line with a previous study, which reported that the *BDNF* methylation status was linked with genotype, and consequently had a differential effect on major psychosis (Mill et al., 2008). In contrast, two other studies on *BDNF* genotype–methylation interactions did not find any significant genotype–methylation interactions in Korean subjects with post-stroke depression (Kim et al., 2012, 2013). It is important to bear in mind that there were no significant associations between the genotyped *BDNF* SNPs and MDD or BD phenotype in our study. Results should be interpreted with caution, as the absence of genetic associations could possibly be due to the lack of statistical power. However, it is still unclear how *BDNF* methylation status influences BDNF secretion, but taking into account that the SNP rs908867 lies within the *BDNF* promoter region, genotype–methylation interactions could be a possible pathway with regulatory impact on *BDNF* gene expression. In order to elucidate the role of genotype in epigenetic changes such as DNA methylation, further studies are needed in larger well-defined samples using a combined genetic, epigenetic and gene expression approach.

The current study has several limitations. First, the sample size (n ¼ 278 for control subjects, n ¼ 207 for MDD subjects and n ¼ 59 for BD subjects) in the current study is relatively small. Further studies using larger samples are necessary for the clinical application in the future. Second, although there is evidence that the levels of gene expression in blood correlate with DNA methylation, it is uncertain whether the methylation profiles of DNA from peripheral blood in humans are representative for the brain (D'Addario et al., 2012). Furthermore, the detected differences in methylation were rather small and it is unclear whether these changes might result in a biological significant change in gene expression. In this context, further studies examining the methylation profiles and gene expression levels of the *BDNF* gene in post-mortem human brain are required. Finally, although carefully selected based on previous findings (D'Addario et al., 2012), we evaluated the methylation profiles within only one CpG island of the *BDNF* gene. However, BDNF has several alternative transcripts and the methylation patterns might be different at these loci (Falkenberg et al., 1993). Regarding the assay we used for measuring DNA methylation patterns, we were only able to amplify DNA molecules with 3 distinct CpG sites methylated as defined by the PCR primers. However, more complex and alternative methylation patterns might exist, which can only be detected by high resolution analyses such as bisulfite sequencing based approaches.

In conclusion, analysing DNA methylation levels at the exon I promoter of the *BDNF* gene in peripheral mononuclear cells (PBMCs), we found significantly increased DNA methylation in subjects diagnosed with MDD. These alterations in methylation levels from peripheral blood might be due to antidepressant (AD) therapy, although we found significantly increased DNA methylation levels in MDD compared to BD subjects as well, the latter possibly pharmacologically treated (among others with AD) as well. We conclude that, at least in our sample, methylation changes cannot be explained by pharmacological treatment alone, but also by disease phenotype (MDD vs. BD). Besides, D'Addario et al. (2012) found significant hypermethylation of the *BDNF*

promoter region in BP-II patients, especially in those pharmacologically treated with AD plus mood stabilizers, compared to those with mood stabilizers only. Moreover, lithium and valproate treatment showed lower methylation levels compared with other drugs. Therefore we hypothesize that this might explain higher methylation levels in MDD than BD found in our study as well. Without doubt, further randomized case-control studies in MDD and BD patients with and without pharmacological treatment are therefore needed to separate effect of pharmacological treatment and effect of phenotype itself, holding the possibility of identifying new insights for potential new therapy targets. Considering the advantage of blood samples in terms of accessibility, the DNA methylation status of the *BDNF* gene could potentially be useful for developing biomarkers in psychiatric disorders.

Author contributions

Laura Carlberg: study design, data collection, data interpretation, manuscript writing, approval of final manuscript version.

Janine Scheibelreiter: study design, data collection, data interpretation, manuscript writing, approval of final manuscript version.

Melanie R. Hassler: data collection, data interpretation, manuscript writing, approval of final manuscript version.

Monika Schloegelhofer: data collection, data interpretation, manuscript writing, approval of final manuscript version.

Michaela Schmoeger: data collection, data interpretation, manuscript writing, approval of final manuscript version.

Birgit Ludwig: data collection, data interpretation, manuscript writing, approval of final manuscript version.

Siegfried Kasper: study design, data collection, data interpretation, manuscript writing, approval of final manuscript version.

Harald Aschauer: study design, data collection, data interpretation, manuscript writing, approval of final manuscript version.

Gerda Egger: study design, data collection, data interpretation, manuscript writing, approval of final manuscript version.

Alexandra Schosser: study design, data collection, data interpretation, manuscript writing, approval of final manuscript version.

Conflict of interest

The authors Carlberg, Schosser, Scheibelreiter, Hassler, Schloeglhofer, Schmoeger, Ludwig, Aschauer, and Egger, declare no conflict of interest. Dr. Kasper has received grant/research support from Bristol Myers-Squibb, Eli Lilly, GlaxoSmithKline, Lundbeck, Organon, Sepracor and Servier; has served as a consultant or on advisory boards for AstraZeneca, Bristol-Myers Squibb, Eli Lilly, GlaxoSmithKline, Janssen, Lundbeck, Merck Sharp and Dome (MSD), Novartis, Organon, Pfizer, Schwabe, Sepracor, and Servier; and has served on speakers' bureaus for Angelini, AstraZeneca, Bristol Myers-Squibb, Eli Lilly, Janssen, Lundbeck, Pfizer, Pierre Fabre, Schwabe, Sepracor, and Servier.

Role of funding source

This study was supported by Oesterreichische Nationalbank (OeNB) Project no 12723 (A.S.); Austrian Research Foundation (FWF) Project no. 7639, and by Oesterreichische Nationalbank (OeNB) Project nos. 5777 and 13198 (H.A.).

Acknowledgements

None.

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2.2 Association study of *CREB1* polymorphisms and suicidality in MDD: Results from a European multicenter study on treatment resistant depression.

Interlude

The transcription factor cAMP response element-binding protein (CREB1) activates the *BDNF* gene (Tao et al. 1998, Conti et al. 2002). Further, for all main classes of ADs the initiation of *CREB1* expression in the hippocampus was observed and expression of *CREB1* was significantly lower in MDD patients who committed suicide compared in non-suicidal subjects (Blendy 2006; Dwivedi 2003a). In the paper "Association study of *CREB1* polymorphisms and suicidality in MDD: Results from a European multicenter study on treatment resistant depression", we analyzed the effects of certain *CREB1* variants on suicide phenotypes in MDD patients. No significant genetic association was found between a set of five SNPs in *CREB1* (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690) and the phenotype: suicide risk and/or lifetime history of suicide attempts.

International Journal of Neuroscience, 2014; 00(00): 1–8

ISSN: 0020-7454 print / 1543-5245 online
DOI: 10.3109/00207454.2014.936554

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ORIGINAL ARTICLE

Association study of *CREB1* polymorphisms and suicidality in MDD: results from a European multicenter study on treatment resistant depression

Laura Carlberg,[1] Alexandra Schosser,[1,2] Raffaella Calati,[3] Alessandro Serretti,[3] Isabelle Massat,[4] Konstantinos Papageorgiou,[1] Neslihan A. Kocabas,[5] Julien Mendlewicz,[6] Joseph Zohar,[7] Stuart A Montgomery,[8] Daniel Souery,[6] and Siegfried Kasper[1]

[1]*Department of Psychiatry and Psychotherapy, Medical University Vienna, Austria;* [2]*Zentrum für Seelische Gesundheit LEOpoldau, Vienna, Austria;* [3]*Department of Biomedical and NeuroMotor Sciences, University of Bologna, Bologna, Italy;* [4]*Laboratory of Experimental Neurology, Universite´ Libre de Bruxelles, ULB, FNRS, Bruxelles, Belgium;* [5]*Department of Toxicology, Faculty of Pharmacy, University of Gazi, Etiler Ankara, Turkey;* [6]*Laboratoire de Psychologie Medicale, Universite´ Libre de Bruxelles and Psy Pluriel, Centre Europe en de Psychologie Medicale, Bruxelles, Belgium;* [7]*Chaim Sheba Medical Center, Tel-Hashomer, Israel;* [8]*Imperial College, School of Medicine University of London, London, UK*

Purpose: Mood disorders are present in more than 90% of suicides, and a genetic vulnerability to suicidality is well established. Numerous lines of evidence relate the transcription factor Cyclic adenosine monophosphate Response Element Binding protein (CREB1) to suicide, and to the aetiology of major depressive disorder (MDD). Our aim was to test for association between *CREB1* single nucleotide polymorphisms (SNPs) and both suicide risk (SR) and a personal history of suicide attempt (SA) in MDD patients. *Materials and Methods*: A sample of 250 MDD patients collected in the context of a European multicenter resistant depression study and treated with antidepressants over a period of at least 4 weeks were genotyped for five *CREB1* SNPs (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690). To assess suicidality, the Mini International Neuropsychiatric Interview (MINI) and the Hamilton Rating Scale for Depression (HAM-D) were applied. *Results*: Neither single-marker nor haplotypic association were found between SR and/or a personal history of SA with any of the investigated SNPs after multiple testing correction. For females, an association between rs2709376 and a personal history of SA was found ($p \leq 0.016$), however not resisting multiple testing correction. *Conclusions*: Although we found significant *CREB1* single marker association with a personal history of SA in female MDD patients, this finding could not be confirmed in haplotypic analyses after multiple testing correction. Larger well-defined cohorts are required to confirm or refute a possible association of *CREB1* and SA in female MDD patients.

KEYWORDS: CREB1, depression, suicide, genetic association

Introduction

Major depressive disorder (MDD) is a common public health problem worldwide, with a mean lifetime prevalence of 16.1% [1]. It has been shown in several clinical studies that 30 to 40% of adequately treated MDD patients fail to respond to antidepressant medication [2, 3]. The concept of treatment-resistant depression (TRD) generally describes MDD patients failing to achieve treatment-response after an adequate treatment with antidepressant drugs, and patients suffering from TRD are at higher risk of suicidal ideation and suicide, respectively [4, 5].

According to the World Health Organisation (WHO), suicide is the fourth leading cause of death and the sixth leading cause of disability and infirmity worldwide among people between the ages of 15 and 44 [6]. More than 90 percent of suicide victims have a diagnosable psychiatric illness [7]. Family studies suggest that suicide attempt (SA) and fulfilled suicide show familial accumulation [8], with heritability estimates of suicidal behaviour between 30% and 55% [9–11]. Among

Received 30 January 2014; revised 16 June 2014; accepted 16 June 2014.

Correspondence: Siegfried Kasper, M.D., Professor and Chair, Department of Psychiatry and Psychotherapy, Medical University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria. Tel: +43 1 404003568. Fax: +43 1 404003099. E-mail: sci-biolpsy@meduniwien.ac.at

others, a recently published genome-wide association study (GWAS) of suicidal thoughts and behaviour in MDD indicates a genetic architecture of multiple genes with small effects [12].

Cyclic adenosine monophosphate Response Element Binding Protein (CREB1) is a transcription factor that controls the transcription of numerous neuronally expressed genes such as brain derived neurotrophic factor (*BDNF*), that has been related to the pathophysiology of affective disorders and the mechanism of action of antidepressants [13–18]. Besides, CREB1 seems to be involved in both aetiology and pharmacotherapy of MDD [19, 20]. A number of studies have analysed the effect of different kinds of antidepressant treatments on the signalling pathways that regulate phosphorylation and transcriptional activity of CREB1, however with conflicting results. Nevertheless, CREB1-regulated gene expression is a major target of antidepressant action [21].

There is evidence for CREB1 playing an important role in the neurobiology of suicidal behaviour. In postmortem brain samples of depressed patients, CREB1 mRNA expression has been found to be decreased, and alterations in protein levels have been reported [22–24].

Although studies investigating a possible genetic association of *CREB1* with MDD and related phenotypes show conflicting results, some possible associations with treatment resistance in MDD patients have been reported [25–28]. Serretti et al. investigated five single nucleotide polymorphisms (SNPs) in *CREB1* in a sample of MDD patients for association with antidepressant response, remission and treatment resistance, and reported on an association of certain *CREB1* SNPs with treatment resistance [28].

Within the current study, we investigated an extension of the sample used by Serretti et al. [29]. Our primary aim was to test a set of five SNPs in *CREB1* (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690) that has been shown to capture relevant genetic variation in *CREB1* [25, 26, 28] for association with both SA and suicide risk (SR). As our secondary aim, based on previous results postulating gender differences in association between *CREB1* and MDD, we performed gender-specific sub-analyses with the SA and the SR phenotypes [25, 26].

Materials and Methods

Subjects and diagnostic interviews

Patients suffering from MDD were recruited in the context of the European multicentre project "Patterns of treatment resistance and switching strategies in unipolar affective disorder" [29]. The following seven centres have contributed to the project: [1] Department of Psychiatry and Psychotherapy, Medical University of Vienna, Austria; [2] Department of Psychiatry, Chaim Sheba Medical Center Tel-Hashomer, Israel; [3] Department of Psychiatry, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; [4] Department of Psychiatry, University Hospital Gasthuisberg, Leuven, Belgium; [5] Hôpital la Salpetriere, INSERM U302, Paris, France; [6] Sint-Truiden, Psychiatric Center, Sint-Truiden, Belgium; [7] Institute of Psychiatry, University of Bologna, Bologna, Italy.

The diagnostic procedure as previously described for this sample [30] was performed by experienced psychiatrists using the Mini International Neuropsychiatric Interview version 5.0.0 (MINI), modified for the Group for the Study of the Resistant Depression (GSRD) [5, 31]. The Hamilton Rating Scale for Depression (HAM-D) 17-item version was administered to all subjects at the end of the last adequate antidepressant treatment [32].

To assess suicidality, we used: (1) the MINI item on suicidality (presence or absence of current suicidal risk), and (2) the HAM-D suicidality item (score 0 to 4) [30]. A present suicidal risk is defined within the MINI by actuality of at least one of the following suicide related items: having in the past month thought that it would be better being dead or wishing to die, wanting to harm oneself, thinking about suicide, having a suicide plan, attempting suicide, and ever attempted suicide at least once in the lifetime. Treatment adequacy was defined as at least 4 weeks of treatment at a dosage at optimal range. Treatment response was defined as HAM-D ≤ 7 and remission as HAM-D ≤ 7. Details on inclusion/exclusion criteria have been previously described [30]. Moreover, detailed information on diagnostic procedures, recruitment methods, and treatment response phenotypes has previously been published by our group [5]. Written informed consent was obtained from each individual and ethics approval was provided by the local ethics committees of all participating centres.

Genotyping

We analysed a minimum set of SNPs (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690) essential to cover all common genetic variations in *CREB1,* based on previous research by [26]. For the detailed protocol of DNA analysis, see [28].

Statistical analysis

A personal history of suicide attempts (SA, yes/no) and suicide risk (SR, yes/no) were analysed as dichotomous traits. To test for genotypic association with each SNP, a standard chi-square (χ^2) statistic was performed using PASWStatistics version

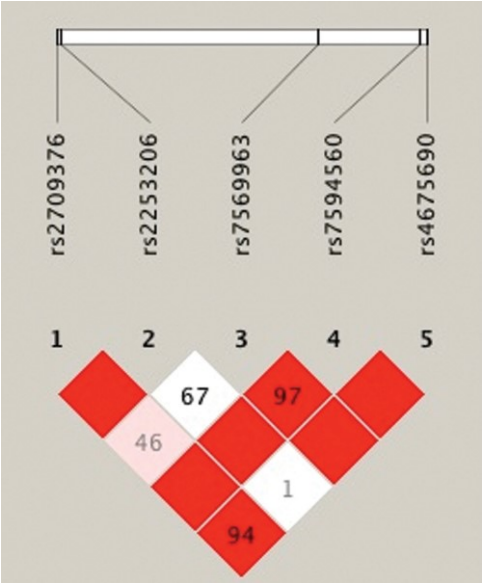

Figure 1 Haploview 4.0 program [34] was used to perform LD analysis of all SNP in our sample. Each diamond represents the correlation (r^2) between each pair of SNPs with darker shades representing stronger LD.

18 for MAC. The computer program FINETTI (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to calculate the Cochran-Armitage trend statistic to test for allelic association. UNPHASED version 3.0.10 program [33] was applied using three-marker slide windows to analyse for haplotypic association [30]. Haploview 4.0 program [34] was used to perform linkage disequilibrium (LD) analysis of all SNPs in our sample (see Figure 1). The measure of LD, denoted as D'and r^2, was calculated from the haplotype frequency using the EM (Expectation Maximisation) algorithm. To test for deviation from the Hardy–Weinberg Equilibrium (HWE), the computer program FINETTI (http://ihg.gsf.de/cgi-bin/hw/ hwa1.pl) was used as previously described [30]. All *p*-values reported in this study were two-tailed. Multiple testing corrections were performed by application of the false discovery rate: Benjamini and Hochberg's FDR-controlling procedure to both single-marker and haplotype analyses [35]. In the case of single-marker analyses, it was assumed that five independent tests were performed when testing 5 SNPs. Haplotype analyses were corrected for the number of sliding windows used. The PS program was used to calculate the power of our case-control sample [36]. Assuming a population frequency of 0.3, which confers risk of disease at an odds ratio of 1.3 of our MDD sample to detect association at $p = 0.05$, we have 15.6 and 15.0% power for SR, respectively, SA.

Results

Patient characteristics are shown in Table 1. We investigated 250 MDD patients (97.2% Caucasian, 0.8% Asian, 1.6% African, 0.4% North-American) including 182 females (72.8%) and 68 males (27.2%), the mean age was 51.23 years (SD 15.5) for females and 50.28 years (SD 14.5) for males. For further exploratory analyses, MDD patients were divided in subgroups according to treatment response 42.8% (= 107) and non-response 57.2% (n=143), as well as remission 13.6% (n=34) and non-remission 86.4% (= 216). The mean HAM-D-17 score was 17.33 (SD 7.81) for the entire sample [30].

CREB1 and suicide attempt (SA) and suicide risk (SR) phenotypes in MDD patients

Analysing the SR phenotype, no single-marker or haplotypic association was found for any of the SNPs tested. As for the SA phenotype, we found significant genotypic and allelic association (p 0.05 and = 0.0499) for rs2709376 SNP before multiple testing correction (see Table 2). Association with the latter phenotype

Table 1. Patient characteristics of the present sample (n = 250) compared to the sample investigated by Serretti et al. (n = 190).

	MDD patients (n = 250)	Serretti et al. (n = 190)
Mean age, mean ± SD	52.0 ± 15.2	57.06 ± 15.19
Males	50.2 ± 14.5	56.84 ± 12.98
Females	51.2 ± 15.5	57.14 ± 15.95
Missing		n = 24
Sex, *n* (%)		
Males	68 (27.2%)	49 (25.79%)
Females	182 (72.8%)	141 (74.21%)
Ethnicity, *n* (%)		
Caucasians	243 (97.2%)	188 (98.94%)
Asians	2 (0.8%)	0 (0.00%)
African	4 (1.6%)	1 (0.53%)
North-American	1 (0.4%)	1 (0.53%)
Mean HAM-D, mean ± SD		
All	17.33 ± 7.8	18.07 ± 7.79
Suicide risk, *n* (%)		
Present	147 (58.8%)	114 (60%)
Absent	103 (41.2%)	76 (40%)
Personal history of SA		
Present	74 (29.6%)	53 (28.19%)
Absent	174 (69.6%)	135 (71.81%)
Missing	2 (0.8%)	2
Response, *n* (%)		
Responders	107 (42.8%)	63 (36.42%)
Non-responders	143 (57.2%)	110 (63.58%)
Missing		*n* = 17
Remission, *n* (%)		
Remitters	34 (13.6%)	20 (10.53%)
Non-remitters	216 (86.4%)	170 (89.47%)

Table 2. *CREB1* Single-Marker (a) and haplotypic (b) association analyses with the SA phenotype, in the entire sample (n = 250) of MDD patients and in female MDD patients only (n = 182). Uncorrected p-values are shown.

CREB1—entire sample	Number of patients		Genotypes		Alleles		HWE	
SNP ID	N°(+)SA	N°(−)SA	chi²	*p*	chi²	*p*	(−)SA	(+)SA
			Table 2a					
rs2709376	73	174	3,846	0,05	3,85	0,0499	n.s.	n.s.
rs2253206	74	172	4,3	0,117	0,68	0,409	n.s.	n.s.
rs7569963	74	174	2,309	0,315	2,19	0,139	n.s.	n.s.
rs7594560	73	172	1,171	0,557	0,97	0,326	n.s.	n.s.
rs4675690	74	172	1,792	0,408	0,17	0,678	n.s.	n.s.
CREB1—females	Number of patients		Genotypes		Alleles		HWE	
SNP ID	*N*°(+)SA	*N*°(−)SA	chi²	*p*	chi²	*p*	(−)SA	(+)SA
rs2709376	51	129	5,704	0,016	5,79	0,016	n.s.	n.s.
rs2253206	52	127	3,843	0,146	0,56	0,456	n.s.	n.s.
rs7569963	52	129	1,71	0,425	1,11	0,291	n.s.	n.s.
rs7594560	51	127	1,015	0,602	1, 00	0,317	n.s.	n.s.
rs4675690	52	127	1,259	0,533	0,11	0,744	n.s.	n.s.
CREB1—SNP	Haplotypes							
			Table 2b					
rs2709376	C							
rs2253206	A	A						
rs7569963	G	G	G					
rs7594560		T	T					
rs4675690			C					
Global *p*	0.035	0.037	0.194					
Individual *p*	0.040	0.003	0.051					
Frequency cases/controls	0.16/0.10	0.25/0.13	0.32/0.24					

[CREB1 = cyclic adenosine monophosphate (cAMP) Response Element Binding Protein (CREB1), SNP = single nucleotide polymorphism, N = number, HWE = Hardy Weinberg Equilibrium].

was also found for two of the three-marker haplotypes: C-A-G of rs2709376-rs2253206-rs7569963 and A-G-T of rs2253206-rs7569963-rs7594560 (individual: $p \leq 0.040$ and $p \leq 0.003$; global: $p \leq 0.035$ and $p \leq 0.037$) (see Table 2). However, none of the significant p-values resisted multiple testing correction.

Gender-specific analysis of *CREB1* and suicide attempt (SA) and suicide risk (SR) phenotypes in MDD patients

Although gender-specific sub-analyses support both genotypic ($p \leq 0.016$) and allelic ($p \leq 0.016$) association of rs2709376 with the SA phenotype in females (see Table 2), and the three-marker haplotype containing this SNP (T-A-G of rs2709376-rs2253206-rs7569963) showed an gender-specific association in females as well (individual: $p \leq 0.022$; global: $p \leq 0.089$), again, none of the significant p-values resisted multiple testing correction. The same holds true for three-marker haploptype A-G-T of rs2253206-rs7569963-rs7594560 (individual: $p = 0.014$; global: $p = 0.126$).

In the males, no single-marker association was found with the SA phenotype (data not shown). Before correction for multiple testing, the three-marker haplotype C-A-G of rs2709376-rs2253206-rs7569963 showed an individual p-value of 0.047 with the SA phenotype in males, however with non-significant global p-value. As for the SR phenotype, no association was found for females. Before correction for multiple testing, significant allelic association was shown for rs4675690 ($p \leq 0.03$) in males (see Table 3). Again, none of the significant p-values resisted multiple testing correction.

CREB1 and suicide attempt (SA) and suicide risk (SR) phenotypes in subgroups of treatment response

The subgroup of treatment responders showed genotypic association of SNP rs2253206 with the SA phenotype (genotypic: $p \leq 0.007$). Within remitters, a significant individual p-value ($p \leq 0.017$) of the three-marker haplotype containing this SNP (C-A-G of rs2709376-rs2253206-rs7569963) with the SR phenotype was

Table 3. *CREB1* Single-Marker association analyses with the SR phenotype in the sub sample of male MDD patients only ($n = 68$). Uncorrected p-values are shown.

CREB1—males SNP ID	Number of patients		Genotypes		Alleles		HWE	
	N°(+)SR	N°(−)SR	chi^2	p	chi^2	p	(−)SR	(+)SR
rs2709376	37	31	1,482	0,223	1,48	0,223	n.s.	n.s.
rs2253206	37	31	2,694	0,26	2,35	0,126	n.s.	n.s.
rs7569963	37	31	0,448	0,799	0,44	0,506	n.s.	n.s.
rs7594560	37	31	2,586	0,274	1,33	0,259	n.s.	n.s.
rs4675690	37	31	4,756	0,093	4,71	0,03	n.s.	n.s.

[CREB1 = cyclic adenosine monophosphate (cAMP) Response Element Binding Protein (CREB1), SNP = single nucleotide polymorphism, N = number, HWE = Hardy Weinberg equilibrium].

found, however with non-significant global p-value. No significant single-marker or haplotypic association of the SR phenotype was found in non-remitters or non-responders. Again, none of the significant p-values survived multiple testing correction.

LD was performed for all *CREB1* SNPs (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690), and strong LD was found between rs7569963, rs7594560, and rs4675690 (see Figure 1).

Discussion

Within the present study, we investigated a possible association of *CREB1* with SA and SR phenotypes in a sample of 250 MDD patients, finding no significant single-marker or haplotypic association for neither SA nor SR phenotypes with any of the SNPs tested after multiple testing correction. Since gender-specific associations with *CREB1* had been reported in a MDD sample [25, 26], we further performed gender-specific sub-analyses, finding significant *CREB1* single-marker association of one SNP (rs2709376) with the SA phenotype in females, although neither being confirmed in haplotypic analyses nor resisting multiple testing correction. As for the SR phenotype, one SNP (rs4675690) showed significant single-marker association in males, again not resisting multiple testing correction.

Analysing suicidal behaviour in a cohort of MDD cases provides an a priori high-risk group for suicidal behaviour that is appropriate for uncovering the genetic contribution to this complex phenotype. Besides, mood disorders are present in 90% of suicides [7], and focusing on suicidal behaviour within a single disorder allows the distinction between genes related to suicide per se from those associated with MDD itself [37, 38]. The transcription factor CREB1 regulates the expression of several genes that are involved in neuronal functioning i.e. the gene encoding for the neurotrophic factor BDNF [39]. Dwivedi et al. (2003) studied the role of CREB1 and BDNF in suicide subjects and found reduced CREB1 and BDNF protein concentrations in the prefrontal cortex (PFC) and hippocampus [23, 40]. There is further evidence showing that antidepressants caused an increase of CREB1 and BDNF in the brain of rats [41]. The activation of CREB1 may be a common action of antidepressant treatment that leads to up-regulation of specific target genes, such as *BDNF*, and hereby modulates neuronal functioning. Heterogeneous, findings in association studies provide contrasting evidence for the role of *CREB1* on MDD or related phenotypes [27, 42–47]. The divergence could possibly be caused by factors such as a different study design, the variation of investigated SNPs or gender differences. Investigating suicidal behaviour using a broad definition of MDD is a potential limitation of some of the reported studies. In contrast, we studied a more homogeneous group of MDD patients, namely predefined treatment resistant patients with a reported higher rate of suicidality [5], which has not been investigated before.

In rather exploratory analyses examining small subgroups of treatment-response, one SNP (rs2253206) showed significant genotypic association with the SA phenotype in the subgroup of treatment responders, however not resisting multiple testing correction. There is evidence for *CREB1* playing an important role in treatment response to antidepressant drugs in patients with MDD [42, 44, 45]. However, in recent association studies several SNPs in *CREB1,* could be associated to treatment resistance in MDD patients [28].

As for the limitations of our study, the definitions of SR and a personal history of SA, defined as SA and SR phenotypes, were obtained from two items of the HAM-D and the MINI interviews. Neither the instruments nor our study on TRD were designed mainly to examine suicidality. Prospective studies primarily addressed to assess suicidality are needed to provide detailed data on suicide phenotypes, in order to elucidate its aetiology. Furthermore, our sample size of MDD cases is a limitation to identify genes of small effect size, that are expected to be involved in suicidal behaviour, due to limited statistical power. Consequently, it is possible that our findings are false positives (especially since they do not resist multiple testing correction), and replication in

larger independent samples is essential to verify or refute their role. Thus, these preliminary findings have to be interpreted with caution. Last but not least, we only investigated five SNPs within the *CREB1* gene, chosen based on previous research in order to cover the main genetic variation in *CREB1,* therefore possibly missing the effects of rare variants or SNPs not in strong LD with the five SNPs investigated in the current study.

Conclusions

In conclusion, we found no association for the SR and SA phenotype with any of the *CREB1* polymorphisms investigated. As for gender-specific analyses, although an uncorrected *CREB1* single-marker association with the SA phenotype in females was found, this finding neither resisted multiple testing correction nor was supported by haplotypic analyses. Therefore, these results cannot be taken as evidence for gender-specific association of CREB1 with a personal history of SA in our MDD sample. Further research in larger, well-defined samples is required to confirm or refute a possible (gender-specific) association of *CREB1* and SA MDD.

Declaration of Interest

Drs. Carlberg, Schosser, Calati, Massat, Kocabas, and Papageorgiou declare no conflict of interest. Dr. Seretti is or has been consultant/speaker for: Abbott, Astra Zeneca, Clinical Data, Boheringer, Bristol Myers Squibb, Eli Lilly, GlaxoSmithKline, Janssen, Lundbeck, Pfizer, Sanofi, Servier. Dr. Mendlewicz is a member of the Board of the Lundbeck International Neuroscience Foundation and of Advisory Board of Servier. Prof. Zohar has received grant/research support from Lundbeck, Servier and Pfizer, has served as a consultant or on advisory boards for Servier, Pfizer, Solvay, and Actelion, and has served on speakers' bureaus for Lundbeck, GSK, Jazz and Solvay. Dr. Montgomery has been a consultant or served on Advisory boards: AstraZeneca, Bionevia, Bristol Myers Squibb, Forest, GlaxoSmithKline, Grunenthal, Intellect Pharma, Johnson & Johnson, Lilly, Lundbeck, Merck, Merz, M's Science, Neurim, Otsuka, Pierre Fabre, Pfizer, Pharmaneuroboost, Richter, Roche, Sanofi, Sepracor, Servier, Shire, Synosis, Takeda, Theracos, Targacept, Transcept, UBC, Xytis and Wyeth. Dr. Kasper has received grant/research support from Bristol Myers-Squibb, Eli Lilly, GlaxoSmithKline, Lundbeck, Organon, Sepracor, and Servier; has served as a consultant or on advisory boards for AstraZeneca, Bristol-Myers Squibb, Eli Lilly, GlaxoSmithKline, Janssen, Lundbeck, Merck Sharp and Dome (MSD), Novartis, Organon, Pfizer, Schwabe, Sepracor, and Servier; and has served on speakers' bureaus for Angelini, AstraZeneca, Bristol Myers-Squibb, Eli Lilly, Janssen, Lundbeck, Pfizer, Pierre Fabre, Schwabe, Sepracor, and Servier. This study was supported by an unrestricted grant from Lundbeck for the GSRD. Lundbeck had no further role in the study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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Supplement 1. *CREB1* Single-Marker association analyses with the SA phenotype in a subsample of MDD patients responding to antidepressive treatment ($n = 107$).

CREB1 treatment responders SNP ID	Gene	Number of patients		Genotypes		Alleles		HWE	
		N° (+)SA	N° (−)SA	chi^2	p	chi^2	p	(−)SA	(+)SA
rs2709376	CREB1	31	73	0.549	0.459	0.55	0.459	n.s.	n.s.
rs2253206	CREB1	32	72	9.926	0.007	1.70	0.192	0.025	n.s.
rs7569963	CREB1	32	73	1.933	0.380	0.17	0.678	n.s.	n.s.
rs7594560	CREB1	32	73	1.275	0.529	0.36	0.546	n.s.	n.s.
rs4675690	CREB1	32	72	4.383	0.112	0.80	0.372	n.s.	n.s.

[CREB1 = cyclic adenosine monophosphate (cAMP) Response Element Binding Protein (CREB1), SNP = single nucleotide polymorphism, N° = number, HWE = Hardy Weinberg equilibrium]. The p-value for HWE of rs2253206 remained not significant after correction with FDR.

Supplement 2. *CREB1* haplotypic association analyses (2- and 3-marker slide windows are shown) with SR phenotypes in a sub sample of patients showing remission in depressive symptoms under antidepressive treatment ($n = 34$).

SNP		Haplotypes			
rs2709376	1	C			
rs2253206	2	A	G		
rs7569963	3		G	G	
rs7594560	4			C	T
rs4675690	5				C
Global p		0.046	0.201	0.449	0.240
Individual p		0.0099	0.069	0.197	0.123
Frequency cases/controls		*0.44/0.15*	*0.55/0.77*	*0.10/0.20*	*0.60/0.40*

SNP		Haplotypes		
rs2709376	1	C		
rs2253206	2	A	A	
rs7569963	3	G	G	G
rs7594560	4		T	C
rs4675690	5			T
Global p		0.081	0.410	0.404
Individual p		0.017	0.141	0.217
Frequency cases/controls		*0.28/0.06*	*0.30/0.15*	*0.10/0.23*

[CREB1 = cyclic adenosine monophosphate (cAMP) Response Element Binding Protein (CREB1), SNP = single nucleotide polymorphism, N° = number, HWE = HardyWeinberg Equilibrium].

2.3 The Impact of *BDNF* Polymorphisms on Suicidality in Treatment-Resistant Major Depressive Disorder: A European Multicenter Study.

Interlude

For the *BDNF* gene and the MDD phenotype as well as the suicidal behavior phenotype several associations have been described. However, the results remain conflicting. In the paper "The Impact of *BDNF* Polymorphisms on Suicidality in Treatment-Resistant Major Depressive Disorder: A European Multicenter Study" the effects of certain *BDNF* variants on suicide phenotypes in MDD patients were analyzed. No significant genetic association was found for the *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs12273363, rs908867, rs1491850, and rs1491851) with the phenotype suicide risk and/or lifetime history of suicide attempts. In an additional exploration of treatment response phenotypes, the Val66Met and rs10501087 SNPs showed a significant association with suicide risk in a subgroup of remitters (n = 34, 13.6%).

OXFORD

International Journal of Neuropsychopharmacology (2017) 20(10): 782–787

doi:10.1093/ijnp/pyx028
Advance Access Publication: June 27, 2017
Brief Report

brief report

The Impact of BDNF Polymorphisms on Suicidality in Treatment-Resistant Major Depressive Disorder: A European Multicenter Study

Alexandra Schosser, MD, PhD; Laura Carlberg, MD; Raffaella Calati, PhD; Alessandro Serretti, MD; Isabel Massat, MD, PhD; Christoph Spindelegger, MD; Sylvie Linotte, MSc, MD, PhD; Julien Mendlewicz, MD, PhD; Daniel Souery, MD, PhD; Joseph Zohar, MD; Stuart Montgomery, MD; Siegfried Kasper, MD

Department of Psychiatry and Psychotherapy, Medical University, Vienna, Austria (Drs Schosser, Carlberg, Spindelegger, and Siegfried); Zentrum für Seelische Gesundheit, Leopoldau, Austria (Dr Schosser); INSERM U1061, University of Montpellier, FondaMental Foundation, Montpellier, France (Dr Calati); Department of Biomedical and NeuroMotor Sciences, University of Bologna, Bologna, Italy (Dr Serretti); Laboratory of Experimental Neurology, National Fund of Scientific Research, Brussels, Belgium (Dr Massat); Université Libre de Bruxelles, Brussels, Belgium (Ms Linotte and Dr Mendlewicz); Laboratoire de Psychologie Medicale, Université Libre de Bruxelles and Psy Pluriel, Centre Europe en de Psychologie Medicale, Brussels, Belgium (Dr Souery); Chaim Sheba Medical Center, Tel-Hashomer, Israel (Dr Zohar); Imperial College, School of Medicine, University of London, United Kingdom (Dr Montgomery).

Correspondence: Siegfried Kasper, MD, Professor and Chair, Department of Psychiatry and Psychotherapy, Medical University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria (sci-biolpsy@meduniwien.ac.at).

Abstract

Background: Numerous studies have reported associations between the brain-derived neurotrophic factor (*BDNF*) gene and psychiatric disorders, including suicidal behavior, although with conflicting results.
Methods: A total of 250 major depressive disorder patients were collected in the context of a European multicenter resistant depression study and treated with antidepressants at adequate doses for at least 4 weeks. Suicidality was assessed using the Mini International Neuropsychiatric Interview and Hamilton Rating Scale for Depression, and treatment response using the HAM-D. Genotyping was performed for the functional Val66Met polymorphism (rs6265) and 7 additional tagging single nucleotide polymorphisms within the *BDNF* gene.
Results: Neither BDNF single markers nor haplotypes were found to be associated with suicide risk and lifetime history of suicide attempts. Gender-specific analyses revealed nonsignificant single marker (rs908867) and haplotypic association with suicide risk in males after multiple testing correction. Analyzing treatment response phenotypes, the functional Val66Met polymorphism as well as rs10501087 showed significant genotypic and haplotypic association with suicide risk in remitters (n = 34, 13.6%).

Received: December 20, 2016; **Revised:** April 14, 2017; **Accepted:** June 20, 2017

Conclusions: Considering the sample size, the present findings need to be replicated in larger samples to confirm or refute a role of BDNF in the investigated suicidal behavior phenotypes.

Keywords: BDNF, suicidality, depression, pharmacogenetic

Introduction

Suicide is a significant public health issue and a major cause of death, with the WHO estimating that suicide accounts for 1.5% of the deaths throughout the world. Suicidal behavior refers to the occurrence of suicide attempts that range from suicide death, to highly lethal but failed suicide attempts, to suicide attempts of low lethality (Mann, 2002). It is strongly linked with psychiatric disorders, in particular mood disorders and substance abuse (Arsenault-Lapierre, 2004). Approximately 90% of suicide attempters are estimated to have a psychiatric disorder. Patients suffering from MDD have an estimated 6% to 15% lifetime risk of suicide (Davies et al., 2001).

Major depressive disorder (MDD) constitutes a major clinical problem with a mean lifetime prevalence of 16% (Wittchen, 2011). Treatment-resistant depression is usually seen as the failure to reach response after an adequate treatment, and different definitions that need to be validated before their application in clinical practice have been proposed (Souery et al., 2007).

Family, twin, and adoption studies all support a genetic contribution to suicidal behavior, with an estimated heritability of suicide death of approximately 43% (McGuffin et al., 2001).

Neurotrophic factors such as brain derived neurotrophic factor (*BDNF*) gene are hypothesized to be markers of suicidality (Sher, 2011), and altered BDNF levels may play a role in the pathogenesis of suicidal behavior by resulting in long-term changes in the brain that can lead to neuropsychological deficits (for review, see Eisen et al., 2015). In a large meta-analysis based on 12 studies, the *BDNF* Val66Met polymorphism (rs6265) was found to have a statistically significant effect on the risk of suicide, with the Met allele and Met-allele carriers being associated with a history of suicide attempt (Zai et al., 2012). In a systematic review and meta-analysis to explore associations between BDNF levels and suicidal behavior, the meta-analysis of studies examining serum BDNF levels and attempted suicide revealed no significant association. Similarly, the qualitative review of the literature revealed that the current evidence does not provide consistent support for an association between BDNF and suicidal behavior phenotypes (completed suicide, suicidal ideation, suicide attempt) (Eisen et al., 2015); however, both cases had significant methodological limitations, thus making it difficult to draw sound conclusions.

We carried out an association study investigating 8 *BDNF* single nucleotide polymorphisms (SNPs) in a sample of 250 MDD patients collected in the context of a European multicenter treatment-resistant depression study. The functional Val66Met SNP (rs6265) as well as 7 additional tagging SNPs, covering the *BDNF* genomic region, were selected for genotyping. This is the first study investigating *BDNF* modulation of suicidality in treatment-resistant depression patients. The primary aim of the study was to test for association between *BDNF* SNPs and suicide risk and/or lifetime history of suicide attempts, and the secondary aim was to investigate possible associations between *BDNF* SNPs with and treatment response phenotypes.

Methods

Sample collection was performed in the context of the European multicenter project "Patterns of Treatment Resistance and Switching Strategies in Unipolar Affective Disorder" (Schosser et al. 2012a). Seven centers took part in this large multicenter study on treatment-resistant depression: (1) Department of Psychiatry and Psychotherapy, Medical University of Vienna, Austria; (2) Department of Psychiatry, Chaim Sheba Medical Center Tel-Hashomer, Israel; (3) Department of Psychiatry, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; (4) Department of Psychiatry, University Hospital Gasthuisberg, Leuven, Belgium; (6) Hôpital la Salpetriere, INSERM U302, Paris, France; and (7) Sint-Truiden, Psychiatric center, Sint-Truiden, Belgium.

Subjects and Diagnostic Interviews

A total of 250 unrelated MDD patients were recruited, diagnosed by experienced psychiatrists using the Mini-International Neuropsychiatric Interview version 5.0.0 (MINI) (Sheehan et al., 1998), and modified for the European Group for the Study of Resistant Depression (Souery et al., 2007). The Hamilton Rating Scale for Depression (HAM-D) (Hamilton, 1967) 17-item version was administered to all patients at the end of the last antidepressant treatment for the current episode.

Inclusion criteria were: (1) male or female inpatients or outpatients ≥18 years of age, (2) patients with primary diagnosed MDD (i.e., mood disorder preexisting to any other psychiatric disorder), and (3) having received at least one adequate antidepressant treatment (that is at least 4 weeks of treatment at a dosage at optimal range of an antidepressant) during the current or last episode of depression.

Treatment resistance was defined as not reaching a HAM-D-17 score ≤17 after at least 2 adequate consecutive antidepressant trials administered during the last episode (Schosser et al. 2012a). Nonresistance was defined as a HAM-D-17 score ≤17 after a single antidepressant treatment or at the second trial after one failure. It was not required that drugs from 2 different classes of medication were used in order to define resistance status.

Exclusion criteria were: (1) patients with a mood disorder secondary to any primary "nonaffective" psychiatric condition, (2) patients not receiving at least one adequate antidepressant treatment during the last depressive episode, (3) patients unwilling to participate in the study, and (4) patients unwilling to give an informed consent. Detailed information on the diagnosis, the recruiting method, and treatment response phenotypes is described elsewhere (Souery et al., 2007; Schosser et al., 2012a).

Suicidality was assessed using the MINI section on suicidality and the HAM-D item 3 on suicidality (score 0–4: 0 = absent, 1 = feels life is not worth living, 2 = wishes he were dead or any thoughts of possible death to self, 3 = suicidal ideas or gestures, 4 = attempt at suicide [any serious attempt rates 4]) as described previously (Schosser et al., 2012b; Höfer et al., 2013, 2016; Carlberg et al., 2015). The MINI defines a current suicidal risk as presence of at least one of the following suicide related items: having in the past month thought that it would be better being dead or wishing to die (item C1), wanting to harm oneself (item C2), thinking about suicide (item C3), having a suicide plan (item C4), attempting suicide (item C5), and ever attempted suicide at least once in the lifetime (item C6a). Assessment of the item "lifetime history of suicide attempt" was performed using the MINI item C6a, and the item "suicide risk" was assessed using the MINI item C6c (current suicide risk).

As described previously (Souery et al., 2007; Schosser et al., 2012a), treatment response was defined as HAM-D ≤ 17 and remission as HAM-D ≤ 7. The study protocol was approved by ethical committees of all participating centers. Written informed consent was obtained from all participants.

Genotyping

Genomic DNA was extracted from the whole blood using standard phenol-chloroform extraction procedure. Genotypes of 8 *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs12273363, rs908867, rs1491850, and rs1491851) were obtained using the Sequenom iPLEX genotyping technology by Cogenics (Morrisville) as previously described (Kocabas et al., 2011).

Statistical Analyses

To test for deviation from Hardy-Weinberg Equilibrium (HWE), the computer program FINETTI (https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) was used to perform exact statistics, and cases and controls were separately considered.

To test for genotypic association with each SNP, a standard chi-square (χ^2) statistic was calculated using PASW Statistics version 18 for MAC. The computer program FINETTI was used to calculate the Cochran-Armitage trend statistic to test for allelic association. UNPHASED version 3.0.10 program (Dudbridge, 2003) was applied using 3-marker slide windows to analyze for haplotypic association. UNPHASED uses the standard Expectation-Maximisation algorithm to estimate haplotypes from genotypes. The rare haplotype frequency threshold was taken as 0.01. UNPHASED uses unconditional logistic regression to perform likelihood ratio tests under a log-linear model of the probability that an allele or haplotype belongs to the case rather than control group. The global null hypothesis is that the odds ratios of all haplotypes are equal between cases and controls. Individual haplotypes were also tested for association by grouping the frequencies of all other haplotypes together. Haploview 4.0 program (Barrett et al., 2005) was used to perform linkage disequilibrium analysis of all SNPs in our sample (data not shown).

To calculate the power of our case-control sample, we used the PS program (Dupont and Plummer, 1990). Assuming a disease allele and/or genotype with a population frequency of 0.3, which confers risk of disease at an odds ratio of 1.3, we have 17.4% or 15.4% power of our MDD sample to detect association at $P = .05$ for suicide risk or lifetime history of suicide attempt, respectively.

All P values reported in this study were 2-tailed, and the statistical significance was set at the 0.05 level. Multiple testing corrections were performed by application of the false discovery rate (FDR; Benjamini et al., 2001) to both single-marker and haplotype analyses. In the case of single-marker analyses, it was assumed that 8 independent tests were performed when testing 8 SNPs. Haplotype analyses were corrected for the number of sliding windows used.

Results

We investigated a total of 250 MDD patients (as previously described, Schosser et al., 2012b, Höfer et al., 2013, 2016, Carlberg et al., 2015) (females: 72.8%, males: 27.2%; mean age: 50.97 ± 15.2; Caucasians: 97.2%, Asians: 0.8%, Africans: 1.6%, North Americans: 0.4%; mean HAM-D-17-score: 17.33 ± 7.81) in the context of a European multicenter resistant depression study. The minor allele frequencies (MAFs) of the investigated polymorphisms did not differ between Caucasian (C) and non-Caucasian (NC) patients (rs11030096: MAF-C = 0.46, MAF-NC = 0.50; rs925946: MAF-C = 0.23, MAF-NC = 0.21; rs10501087: MAF-C = 0.20, MAF-NC = 0.14; rs6265: MAF-C = 0.19, MAF-NC = 0.14; rs12273363: MAF-C = 0.16, MAF-NC = 0.14; rs908867: MAF-C = 0.08, MAF-NC = 0.07; rs1491850: MAF-C = 0.40, MAF-NC = 0.43; rs1491851: MAF-C = 0.44, MAF-NC = 0.43).

The primary aim of the current study was to test for association of 8 *BDNF* SNPs with either suicide risk or lifetime history of suicide attempts. We previously reported (Schosser et al., 2012b) that 21.9% of MDD patients with but only 10.7% without a lifetime history of suicide attempts had a family history (first-degree relatives) of suicide death or suicide attempts ($P = .021$).

Suicide risk (yes/no, 59.1% yes) was analyzed as dichotomous trait applying a standard chi-square (χ^2) statistics, finding neither genotypic nor allelic association with any of the tested SNPs. The same holds true for haplotypic association analysis using 3-marker slide windows (data not shown). Gender-specific analyses revealed nonsignificant genotypic ($P = .046$ [FDR $P = .184$]) and allelic ($P = .046$ [FDR $P = .184$]) association between the rs908867 SNP and suicide risk in male MDD subjects (n = 68) after multiple testing correction (FDR P values in brackets).Three-marker haplotypes containing this SNP showed significant individual P values even after multiple testing correction [FDR $P \leq .036$] but nonsignificant global P values (see Table 1). Two different 3-marker haplotypes, G-C-G of rs925946-rs10501087-rs6265 and C-G-T of rs10501087-rs6265-rs12273363, showed significant individual

Table 1. Suicide Risk Males: Haplotypic Association Analyses

SNP	Haplotypes					
rs11030096	T					
rs925946	T	G				
rs10501087	T	C	C			
rs6265		G	G	G		
rs12273363			T	T	T	
rs908867				A	A	A
rs1491850					T	T
rs1491851						T
Global *P*	.326	**.0068 (.014)**	**.014 (.021)**	.313	.228	.650
Individual *P*	.119	**.003 (.009)**	**.003 (.009)**	**.042 (.050)**	.039	.015
Frequency cases/controls	0.31/0.19	0 / 0.11	0 / 0.11	0.12/0.02	0.12/0.02	0.03/0.0

Abbreviations: BDNF, brain-derived neurotrophic factor; HWE, Hardy Weinberg equilibrium SNP, single nucleotide polymorphism.
3-marker slide windows are shown.
False discovery rate (FDR) P values after multiple testing correction in brackets. Significant P values in bold.

(P = .003 in both cases [FDR P = .009]) and global (P = .0068 and P = .014 [FDR P = .014 and FDR P = .018]) P values.

Investigating the lifetime history of suicide attempts (yes/no, 29.8% yes), we found neither single marker nor haplotypic association with any of the tested SNPs. The same holds true for gender-specific analyses (uncorrected individual P = .for T-G-T haplotype of rs11030096-rs925946-rs10501087 in females) after multiple testing correction (data not shown).

The secondary aim of the study was to further perform subanalyses to test for association of the 2 suicide phenotypes with either treatment response/nonresponse or remission/nonremission, as described previously (Schosser et al., 2012b). Treatment response was defined as an HAM-D score ≤ 17 after 4 weeks of treatment with antidepressants at adequate dose, and 42.8% of our 250 MDD cases were defined responders (n = 107), whereas 57.2% were defined as nonresponders (n = 143). Remission was defined as an HAM-D score ≤7 after 4 weeks of treatment with antidepressants at adequate dose, thus 13.6% (n = 34) of MDD patients remitted, whereas 86.4% (n = 216) were nonremitters. Association analyses of between *BDNF* SNPs with and treatment response in MDD were previously published (Kocabas et al., 2011).

Regarding suicide risk, there was neither single marker nor haplotypic association with any of the SNPs tested in treatment responders (n = 107, data not shown). In nonresponders (n = 143), a P value of 0.036 was found for allelic association with the functional Val66Met polymorphism (rs6265), however, not resisting multiple testing correction and was thus taken as nonsignificant finding. Similarly, neither the genotypic P value of the same SNP (P = .111) nor the haplotypes including this SNP were significant. The same holds true for the other *BDNF* SNPs tested.

Investigating suicide risk in nonremitters (n = 216), neither single marker nor haplotypic association was found (data not shown). However, in remitters (n = 34), the rs10501087 and the functional Val66Met polymorphism (rs6265) showed significant genotypic association with suicide risk (P = .009 [FDR P= .024] for rs10501087 and P=.003 [FDR P=.016] for rs6265, FDR P values in brackets), as well as significant haplotypic association (Table 2). Of note, MDD patients without suicide risk were out of HWE for both SNPs (P=.015 [FDR P=.031] for rs10501087 and P=.0039 [FDR P = .016] for rs6265), but not those with suicide risk.

Discussion

Since mental disorders, especially depression, are present in more of 90% of suicides (Asenault-Lapierre et al., 2004), analyzing suicidal behavior in a cohort of depression cases provides an a priori high-risk group for suicidal behavior that is appropriate for uncovering the genetic contribution to this complex phenotype.

There is evidence that neurotrophins such as BDNF are involved in the neurobiology of suicidal behavior (Deveci et al., 2007; Kim et al., 2007). Associations between the *BDNF* gene and suicidality phenotypes were reported in some (Kim et al., 2008; de Luca et al., 2011; Pregelj et al., 2011; Zai et al., 2012) but not all studies (Zarrilli et al., 2009; Murphy et al., 2011).

In the present study, we further elucidated the impact of *BDNF* gene on suicidal behavior (suicide risk and lifetime history of suicide attempt) in a sample of 250 MDD cases collected in the context of a treatment-resistant depression study and treated with antidepressants at adequate doses for at least 4 weeks. MDD subjects were genotyped for 8 tagging SNPs covering the *BDNF* genomic region, including the functional Val66Met polymorphism. Response, remission, and treatment resistance as well as suicidality information derived from the MINI and the HAM-D were recorded.

Neither *BDNF* single markers nor haplotypes were found to be associated with suicide risk. In gender-specific analyses, a nonsignificant trend towards single-marker association between rs908867 SNP and suicide risk in males was observed after correction for multiple testing. Of note, we also found an association between suicide risk in males and haplotypes containing this SNP, as well as 2 significant adjacent 3-marker haplotypes that resisted multiple testing correction, although the sample size is a limiting factor (68 male subjects). No significant single marker or haplotypic association was identified between *BDNF* SNPs and lifetime history of suicide attempts, neither in the whole sample nor in gender-specific analyses.

Analyzing treatment response phenotypes, no association with suicide risk was found in responders, nonresponders, and nonremitters after multiple testing correction. As for remitters, the functional Val66Met polymorphism (rs6265) as well as rs10501087 SNP showed significant genotypic and haplotypic association with suicide risk. However, although resisting multiple testing correction, this finding should be interpreted with caution, since the sample size in this subgroup is a clear limitation (34 subjects). Therefore, confirmation in larger samples is essential. As for personal lifetime history of suicide attempts and treatment response phenotypes, neither single-marker nor haplotypic association was identified after multiple testing correction.

Table 2. Suicide Risk, Remitters: Haplotypic Association Analyses

SNP	Haplotypes					
rs11030096	T					
rs925946	T	G				
rs10501087	T	C	C			
rs6265		A	A	A		
rs12273363			T	T	C	
rs908867				G	G	A
rs1491850					C	T
rs1491851						T
Global *P*	.341	**.014 (.017)**	**.002 (.004)**	**.035 (.035)**	.254	.840
Individual *P*	.182	**.009 (.014)**	**.002 (.004)**	**.002 (.004)**	.106	.473
Frequency cases/controls	0.13/0.29	0.31/0.06	0.35/0.06	0.35/0.06	0.05/0.21	0.00/0.10

Abbreviations: BDNF, brain-derived neurotrophic factor; HWE, Hardy Weinberg equilibrium SNP, single nucleotide polymorphism.
2- and 3-marker slide windows are shown.
False discovery rate (FDR) P values after multiple testing correction in brackets. Significant P values in bold.

The current study has several limitations. First, suicide risk and lifetime history of suicide attempts were defined from items of the MINI and HAM-D-17 scale. Neither the instruments nor our treatment-resistant depression study were primarily designed to address suicidality. Another limitation of the current study is that our sample of MDD cases had limited power to identify genes of small effect size that are assumed to be involved in suicidal behavior. This issue especially holds true for our association signal with suicide risk in remitters, but also for our association finding with suicide risk in males. Therefore, it is possible that the reported findings are false positives, and replications in independent samples are essential to confirm or refute their role. Our investigation was performed within a single disorder (MDD), which could be seen as a further limitation of this study. However, focusing on suicidal behaviour within a single disorder allows the distinction between genes relating to suicide per se from those associated with the disorder itself (Schosser et al., 2011).

In conclusion, considering the small sample size, the present findings need to be replicated in larger samples to confirm or refute a role of BDNF in the investigated suicidal behaviour phenotypes.

Conflict of Interest

Raffaella Calati received a grant from FondaMental Foundation, France. Alessandro Serretti is or has been consultant/speaker for: Abbott, Astra, Zeneca, Clinical Data, Boheringer, Bristol Myers Squibb, Eli Lilly, GlaxoSmithKline, Janssen, Lundbeck, Pfizer, Sanofi, and Servier. Christoph Spindelegger received a travel grant from Lundbeck and speaker's fees from Bristol Myers Squibb. Julien Mendlewicz is a member of the Board of the Lundbeck International Neuroscience Foundation and of the Advisory Board of Servier. Daniel Souery has received grant/research support from GlaxoSmithKline and Lundbeck; has served as a consultant or on advisory boards for AstraZeneca, Bristol-Myers Squibb, Eli Lilly, Janssen, and Lundbeck. Joseph Zohar has received grant/research support from Lundbeck, Servier, and Pfizer; has served as a consultant or on advisory boards for Servier, Pfizer, Solvay, and Actelion; and has served on speakers' bureaus for Lundbeck, GSK, Jazz, and Solvay. Stuart Montgomery has been a consultant or served on advisory boards for: AstraZeneca, Bionevia, Bristol-Myers Squibb, Forest, GlaxoSmithKline, Grunenthal, Intellect Pharma, Johnson & Johnson, Lilly, Lundbeck, Merck, Merz, M's Science, Neurim, Otsuka, Pierre Fabre, Pfizer, Pharmaneuroboost, Richter, Roche, Sanofi, Sepracor, Servier, Shire, Synosis, Takeda, Theracos, Targacept, Transcept, UBC, Sytis, and Wyeth. Siegfried Kasper received grants/research support, consulting fees, and/or honoraria within the last 3 years from Angelini, AOP Orphan Pharmaceuticals AG, AstraZeneca, Eli Lilly, Janssen, KRKA-Pharma, Lundbeck, Neuraxpharm, Pfizer, Pierre Fabre, Schwabe, and Servier. All other authors, no conflicts of interest.

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3 Discussion

In this thesis, we analyzed the *BDNF* exon I promoter methylation status in peripheral mononuclear blood cells (PBMCs) of MDD, BD and control subjects. We could identify a significantly higher *BDNF* exon I promoter methylation status in MDD subjects compared to BD and control subjects. Further, the AD treatment accounted for the significant increased methylation status in MDD subjects. Our findings add valuable information on the role of epigenetic mechanisms within the *BDNF* gene in the pathophysiology and treatment response of affective disorders. In a sample of treatment resistant MDD subjects, no significant impact of selected *CREB1 and BDNF* SNPs on suicide phenotypes was found.

3.1.1 Disease phenotype and DNA methylation at the BDNF exon I promoter

The main observation of the conducted study was the significantly increased level of methylation at the *BDNF* exon I promoter in MDD subjects in comparison to subjects suffering from bipolar disorder (BD) and healthy controls. Previous findings, suggesting decreased BDNF protein in the serum of MDD subjects and increased BDNF after AD treatment, are in line with the herein presented results (Sen et al. 2008, Satomura et al. 2011, Takebayashi et al. 2012, Brunoni et al. 2014). Hence, DNA methylation in regulatory gene-regions is expected to be of importance in gene silencing (Milutinovic et al. 2004). *BDNF* promoter methylation in PBMCs is commonly analyzed in the field of epigenetics in affective disorders (D'Addario et al. 2012). Several studies observed that the *BDNF* methylation status measured in peripheral tissue (PBMCs, buccal tissue) correlates with MDD diagnosis and represents *BDNF* methylation status in the CNS (Januar et al. 2015, Stenz et al. 2015). A recent study could show that higher *BDNF* promoter methylation and low cortical thickness has inverse correlation in patients with MDD, but not in control subjects (Na et al. 2016). Similar to our study, numerous studies focused on *BDNF* exon I promoter methylation in MDD, however, with inconsistent results (Fuchikami et al. 2011, D'Addario et al. 2013, Kim et al. 2013b, Song et al. 2014). Significantly higher *BDNF* promoter I methylation was likewise seen in post stroke depression, though being a different type of depression than investigated in this study (Kim et al. 2013b). In conformity with our observations, D'Addario et al. (2013) found the *BDNF* exon I promoter in MDD subjects hypermethylated related to controls. Fuchikami et al. (2011) performed a small case-control study in Japanese MDD and control subjects (n=38). The results of Fuchikami et al. (2011) were contrary to our results and showed a significant hypomethylation in the *BDNF* exon I promoter of MDD subjects. Another Japanese study (n=180) found a significantly decreased *BDNF* exon I promoter methylation in buccal tissue of MDD subjects as compared to controls (Song et al. 2014). We can hypothesize, that the obvious differences in ethnicity and tissue specificity might explain the different results. No significant association was found for *BDNF*

methylation and depression in maltreated children (n=190), not equivalent to the sample in this study (Weder et al. 2014). The inconsistent findings of *BDNF* methylation in the above-described studies could be due to different ethnicity (Caucasian, Asian) and sample heterogeneity (MDD, post-stroke depression, depression in maltreated children). Our study is so far one of the largest in the field of psychiatric methylation analyzes, but still relatively small in order to detect slight methylation differences. According to the PCR primers we used in our essay, we were only capable to amplify DNA molecules with three different CpG sites for measuring the DNA methylation. Based on previous findings we selected one CpG island within the *BDNF* exon I promoter for our analyses. However, in the human *BDNF* gene three CpG islands can be detected in the promoters of exons I, II, and VI (Boulle et al. 2012). BDNF has different transcripts and the methylation patterns might be altered at these loci. Regarding the discrepancy between DNA methylation and BDNF expression levels, as several alternative BDNF transcripts have been described, it would be of high interest to correlate BDNF expression of the different transcripts and DNA methylation of all promoters. D'Addario et. al (2012) verified that the levels of *BDNF* exon I promoter methylation correlate negatively with *BDNF* gene expression in PBMCs. Of note, despite the dynamic epigenetic mechanisms and tissue specific differences, the DNA methylation in humans between two individuals was significantly correlated between PBMCs and the cortex and cerebellum (Davies et al. 2012). As studies on brain tissue are not feasible in humans, the advantage in collecting blood samples in order to analyze the *BDNF* methylation status is evident. A potential confounding factor in our study is the recruitment of in- and outpatients at the Department of Psychiatry and Psychotherapy of the Medical University Vienna only. These patients might have a longer history of disease and therefore be more severely affected than patients receiving their AD treatment from general practitioners. To further discover the importance of *BDNF* methylation in MDD, more studies examining the methylation status with the background of individual differences are required. It is necessary to monitor the *BDNF* methylation status in the same subject before and after the occurrence of MDD in future longitudinal studies.

3.1.2 Antidepressants (AD) and DNA methylation at the BDNF exon I promoter

In the present study, we observed that MDD subjects receiving antidepressant (AD) treatment had significantly higher methylation levels at the *BDNF* exon I promoter compared to MDD subjects without AD treatment. Moreover, the subsample of MDD with AD treatment accounted for the significant difference of MDD subjects compared to control subjects. Our data suggest a down-regulated *BDNF* gene expression and therefore reduced BDNF serum concentration in MDD subjects under AD treatment. Moreover, D'Addario et al (2012) discovered a significantly hypermethylated *BDNF* exon I promoter in bipolar disorder II (BD-

II) subjects under treatment with ADs and mood stabilizers, compared to bipolar disorder I (BD-I) subjects treated with mood stabilizers only. In those BD-I subjects treated with the first line mood stabilizing drugs lithium and valproate, the *BDNF* promoter was significantly hypomethylated compared to other treatments (D'Addario et al. 2012). This evidence underlines our results and implies that the differences in the *BDNF* gene regulation among subjects with affective disorders (MDD and BD) could in part be regulated by the psychopharmacological therapy as opposed to the disease phenotype. Contrarily, MDD subjects had significantly higher methylation levels at the *BDNF* exon I promoter in comparison to BD subjects potentially treated with ADs as well. Consequently, based on our data, methylation effects cannot be explained by AD treatment alone. In rodents, hippocampal *BDNF* expression was induced by AD treatment (Nibuya et al. 1995, Russo-Neustadt et al. 1999, Shirayama et al. 2002). Clinical studies in MDD patients mostly found an increase of serum BDNF after AD treatment (Brunoni et al. 2008). One post mortem brain study found low hippocampal BDNF levels in MDD and BD subjects without AD treatment, compared to MDD and BD subjects who received AD treatment (Chen et al. 2001b). Overall, both, BDNF and ADs, were observed to interact with each other. In detail, ADs may reactivate activity-dependent and BDNF-induced cortical plasticity and further adjust neuronal networks to improve adaption to environmental challenges (Castren et al. 2010). In a subset of MDD patients ADs show poor efficacy, this could in part be explained by a lack of *BDNF* gene regulation following treatment. The severely affected and pharmacologically treatment resistant MDD patients are often treated with electroconvulsive therapy (ECT). A recent meta-analysis suggests a potential role for BDNF concentration changes obtained from serum as a biomarker of treatment response after ECT, as serum concentration of BDNF in MDD patients receiving ECT was significantly higher than in those without ECT (Rocha et al. 2016). In this regard, our finding is of particular interest, postulating *BDNF* methylation as a potential marker of efficacy for AD treatment. It also points toward BDNF regulation being a key target for the effects of AD treatment. One limitation of our study is that we did not control for the AD class in MDD patients. However, we observed higher methylation levels at the *BDNF* exon I promoter in MDD subjects with AD treatment but it remains unclear whether higher methylation is an effect caused by ADs or the higher methylation levels cause the more severe MDD course and therefore AD treatment is required. Nevertheless, in order to support the function of BDNF in MDD treatment, investigation of the particular effects of different classes of ADs, as well as ECT and psychotherapy effects on *BDNF* methylation in correlation to the response of symptoms, is required.

3.1.3 Symptom severity in MDD and DNA methylation levels at the BDNF exon I promoter

We observed a significantly elevated *BDNF* exon I promoter methylation in MDD subjects but neither in BD subjects nor in control subjects. However, we did not observe a correlation between the methylation status and severity in MDD symptoms. The applied Beck Depression Inventory (BDI) is a 21-item, self-rating and is widely performed in research and clinical routine in order to measure the severity of MDD symptoms. The patients rated the intensity from 0 to 3 out of 21 items consisting of clinical observations systematically combined into symptoms and intrusive negative cognitions. In our sample, the mean BDI of 22.5 in MDD subjects equates to a moderate MDD episode (BDI score 20-28). The literature is so far inconclusive regarding the correlation between clinical symptoms and DNA methylation status. In the study published by Hee-Ju Kang et al. (2015) higher *BDNF* methylation levels were associated with MDD diagnosis and severity of symptoms, measured by Montgomery Asberg Depression Rating Scale (MADRS), in a sample of breast cancer patients (Kang et al. 2015). In a recent study by D'Addario et al. (2012) no *BDNF* methylation changes in BD-I and BD-II subjects were found according to their clinical mood status (D'Addario et al. 2012). According to the recently published meta-analysis by Molendijk et al. (2014) changed serum BDNF concentration represents a peripheral indicator of MDD (Molendijk et al. 2014). The lack of consistency for correlations between severity of depressive symptoms and *BDNF* methylation status might be due to the heterogeneity of methods and MDD samples. The MDD syndrome consist of a variety of mental and vegetative symptom clusters, which could be addressed separately according to their pathogenesis, if adequate sample sizes could be achieved (Fried et al. 2015).

3.1.4 Age effects on DNA methylation at the BDNF exon I promoter

Regarding age effects, in our sample (Control subjects (n=327) mean age 31.8±0.55 BD (n=60) mean age 43±1.90, MDD (n=210) mean age 46.03±1.07, we did not observe any significant correlation for *BDNF* exon I promoter methylation status and age. There is one study reporting a significant correlation between *BDNF* exon I promoter methylation and age (Ihara et al. 2018). It needs to be mentioned that age has been reported to modify DNA methylation patterns. A recently published review of the literature resumes that overall, in the healthy human aging, two phenomena are present called epigenetic drift and epigenetic clock (Jones et al. 2015). The epigenetic drift was defined by the divergence of the epigenome caused by stochastic changes in methylation as a result of individual environmental influences (Jones et al. 2015). The epigenetic clock defines specific sites that show age-related changes across individuals and some, even across tissues (Jones et al. 2015).

3.1.5 Gender differences in DNA methylation at the BDNF exon I promoter

In this sample (Control subjects (n=327) male/female (123/204), BD (n=60) male/female (33/27), MDD (n=210) male/female (89/121)) participated. According to in the methylation status of the *BDNF* exon I promoter we did not observe any significant gender differences. However, theoretically the sex hormones testosterone and estradiol have important effects on mediating *BDNF* expression and BDNF performance (Carbone et al. 2013). In rodents, a recently performed experiment determined that an extended period of hypoestrogenicity interrupts the estradiol induction of neurotrophins such as BDNF. DNA methylation was postulated as one of the important epigenetic pathways that may explain the estradiol insensitivity of *BDNF* after a long period post ovariectomy (Moreno-Piovano et al. 2014). A stressful life event could therefore result in a different *BDNF* methylation and consequently lead to reduction in BDNF expression in females compared to males, as observed in rodents (Niknazar et al. 2016). This hypothesis, suggesting a pathogenesis for the higher susceptibility to MDD in females, needs more observations to shed light on the variety of gender related behaviors and brain functions in humans.

3.1.6 Interactions between genotype and DNA methylation at the BDNF exon I promoter

In terms of genotype-methylation interactions, we observed one significant association for the homozygous (GG) genotype at SNP (rs908867) with increased *BDNF* exon I promoter methylation. In a recently performed study in a sample of late-life MDD, the major allele carriers of the same SNP (rs908867) as well as minor allele carriers of the SNP rs6265 and rs7103411 showed a significant association with increased *BDNF* methylation (Januar et al. 2015). One study observed significant association in the rs6265 SNP for genotype and methylation status in post mortem brain tissue of the major psychosis disease phenotype (Mill et al. 2008). However, two further studies analyzing genotype-methylation interactions in a post stroke depression phenotype did not observe relevant association in a Korean sample (Kim et al. 2012, Kim et al. 2013b). The SNP rs908867 lies within the *BDNF* promoter region and genotype-methylation interactions could possibly have a regulatory impact on *BDNF* gene expression. To elucidate this potential pathway, further studies operating with a shared methodology including genetics, epigenetics and gene-expression analyzes are needed.

3.1.7 Disease phenotype association with BDNF genotype

No significant associations were observed between the genotype of the 12 selected *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs11030102, rs11030104, rs11030108, rs988748, rs12273363, rs908867, rs1491850 and rs1491851) and MDD or BD disease phenotype. Due to the heterogeneity of the MDD syndrome and its complicated genetic

background, standard methods applied to assess genetic association with SNPs may be underpowered and our data have to be described with cautiousness.

3.2.1 Effect of CREB1 variants on suicide phenotypes MDD subjects

In the second publication, we analyzed the effect of a set of five SNPs in *CREB1* (rs2709376, rs2253206, rs7569963, rs7594560, and rs4675690) on suicide phenotypes in MDD subjects. In our sample of MDD subjects, no significant genetic association with suicide risk and/or lifetime history of suicide attempts resisted multiple testing correction. Generally, within association studies on candidate genes, the allelic and genotypic frequencies of selected variants are compared between distinct phenotypes in order to identify potential biomarkers.

3.2.2 Demographic and clinical characteristics of the sample

3.2.3 Age

The mean age of our sample was 52.0±15.2 years (mean±SD), with 52.2±14.5 years for male subjects and 51.2±15.5 years for female subjects. The average age in our study at 52 years is reasonably representative for MDD patients, considering that the average age at first depressive episode is estimated to be at about the age of 30 years and the first episodes are often minor episodes and untreated (Dold et al. 2018). In a subsample of our sample (n=190) published by Seretti et. al (2011) 35% (n=17) of the male subjects (n=49) and 30% (n=43) of the female subjects (n=141) reported an early onset of MDD, defined as first depressive Episode at the age <26 years according to the literature (Zubenko et al. 2002, Zubenko et al. 2003a). A not inconsiderable proportion of the MDD patients have their onset of disease in childhood or adolescence (Fava et al. 2000). The age of onset of the initial major depressive Episode (MDE) and the course of the disease can vary significantly from person to person, but in recent years there has been a clear shift in the onset of MDD from middle to early adulthood, mainly in women (Bretschneider et al. 2018). The increased occurrence of minor depressive episodes and a younger age of onset are associated with changing living conditions, life events and stress factors of today (i.e. negative interpersonal relations and social-cultural changes) (Bernaras et al. 2019). However, there are also hints that the symptom composition can vary over the lifespan showing a decrease of vegetative symptoms in older age (Mezuk et al. 2012). Consequently, in the literature different sample characteristics are described in some aspects. If this shifted age distribution would be based on various entities within MDD, it could reduce the comparability of the studies.

3.2.4 Gender

The well-known higher prevalence of MDD among women was also reflected in this study, with 72.8% female participants and 27.2% male participants in the present sample. According to relevant studies, women suffer from MDD twice as often as men (Kessler 2003, Bourne et al. 2019). Women show a significantly earlier onset of MDD, a longer episode duration and a higher relapse rate for further MDEs (Winkler et al. 2005, Bretschneider et al. 2018). While there are no uniform gender differences in the occurrence of depressive disorders in childhood, there is evidence of an increasing risk of illness for girls and young women (Winkler et al. 2005, Hyde et al. 2008, Conklin et al. 2018). From the age of 15, the rate of depressed girls is almost as high as that of adult women (Conklin et al. 2018, Vu et al. 2019). Considering the profile of the symptoms, the female image of MDD differs in many ways from the male, especially at the beginning of the disease (Seidler et al. 2016). In addition to the main symptoms that both sexes experience equally often, MDD in women is characterized by lassitude and loss of energy, somatization of anxiety, irritable bowel syndrome (IBS) as well as loss of interest and loss of initiative (Asgeirsdottir et al. 2015). The male pattern of MDD is characterized by irritability, aggressiveness, anti-social behavior, reduced impulse control, reduced stress tolerance and substance abuse, mainly alcohol (Seidler et al. 2016, Price et al. 2018). One possible explanation, the male MDD can be overseen by physicians, is that alcoholism, substance abuse and anti-social behavior mask MDD in male subjects (Egeland et al. 1983, Vu et al. 2019). Essential factors men receive less support than women: are a lack of help-seeking behavior, dysfunctional stress processing patterns and a gender bias in MDD diagnosis (Seidler et al. 2016). The common denominator of these factors is the historical construct of traditional masculinity in the western society, which continues to fulfill normative functions for boys and men despite the change in roles and the progress in individualization (Salk et al. 2017). In general, MDD is considered to be a more typical disease for women, with two to three times higher lifetime prevalence in women compared to men, repeatedly confirmed by numerous epidemiological studies. Even though this difference in prevalence seems to be a stable finding, there are reasons to question it: Is it the true prevalence or are we dealing with a chronically reproduced artifact that is methodological or diagnostic? Externalizing symptoms that are not included in the standardized MDD diagnosis and may be overlooked. Important note, since in some studies, likewise in our study, patients were assessed exclusively in psychiatric inpatient- or outpatient units, there might possibly be a gender bias in the inclusion process, as the primary care doctor might see also male MDD patients with more unspecific symptoms. However, one possible confounding factor of this study could be the distortion in the estimation of individual consumption of alcohol, especially in the question of the consumption of narcotics. Primary substance addiction and -abuse are frequent comorbidities of affective disorders and alcohol is very widespread in our society. The self-

and third-party assessment may differ in terms of alcohol consumption. Similarly, a lack of insight into certain mental illnesses may have led to misstatements. However, whether this has an influence on the study results could only be discussed with corresponding comparative studies. Of note, the suicide rate is also increased in male subjects among substance abuse disorders (Arsenault-Lapierre et al. 2004). Though, physical diseases as a possible consequence of primary addictive substance abuse have been examined in advance and lead to exclusion from our study.

3.2.5 Ethnicity

Ethnicity can influence the distribution of genetic polymorphisms, however, in this study a methodological impairment due to ethnicity is rather small, since the sample consisted almost exclusively of Caucasians. The whole sample n= 250 (100%) consisted of caucasians n= 243 (97.2%), Asians n= 2 (0.8%), African n= 4 (1.6%) and North-American n= 1 (0.4%).

3.2.6 Treatment response

Regarding treatment response, in our sample, 13.6% (n=34) of the n=250 MDD patients were able to achieve remission, whereas 86.4% (n=216) could not achieve remission. Remission was defined as a HAM-D score of ≤7 after 4 weeks of treatment with at least one antidepressant in a sufficient dose. 42.8% of the 250 MDD cases were defined responders (n=107), whereas 57.2% were defined as non-responders (n=143). The response to therapy was defined as a HAM-D score ≤17 after 4 weeks of treatment with antidepressants in a sufficient dose. The inconsistency in reported data may be due to the fact that the response and the remission are defined differently by different authors and are therefore measured differently in many studies. This is based on using different scales to assess the severity of the MDD symptoms. The most common scales are the Hamilton Depression Rating Scale (HAM-D), which was used in our study, and the Montgomery-Asberg Depression Rating Scale (MADRS). Several authors also define remission as an absolute decrease in HAM-D score of ≤7 points (Montoya et al. 2016). However, when normal levels of functioning are taken into account the literature suggests even lower cutoffs with a HAM-D score of ≤5 points (Romera et al. 2011). There is evidence that on the longitudinal course of MDD (up to 12 years naturalistic follow-up), those patients with sub threshold residual symptoms throughout remission are disposed to severely and chronic future MDD courses (Judd et al. 2000). According to Rush et al., in the average population of patients in eight-week efficacy studies of antidepressants, the response rate is about 50% and the remission rate is 30-40% of the intention-to-treat group (Rush et al. 2006). In our sample, 42.8% of the 250 MDD cases were defined responders and 13.6% of the cases were defined as remitters. Indicating a more

severely course of MDD in our sample. The treatment resistance in our sample was defined if a HAM-D score of ≤17 points could not be achieved after at least two antidepressant (AD) treatments in an adequate dose for 4 weeks each (Schosser et al. 2012). In our study, data on the TRD status were available for a subsample (n=122), exhibiting TRD in 58% (n=71), also a hint on a more severely MDD course in our subjects (Serretti et al. 2011). The term “treatment-resistant depression” (TRD) is problematic in the scientific and clinical context, since to date there are various definitions of this term and, in addition, overlaps with the terms “treatment-refractory depression” or “chronic depression” are often used (Ng et al. 2019, Gaynes et al. 2020). However, according to a recent study, 60% of all patients who are suffering from MDD do not respond to the initial antidepressant (AD) medication, or respond inadequately (Dold et al. 2017). Nevertheless, in the case of an inadequate response to therapy, the so-called “pseudo-resistance” should be ruled out as the very first step (Dold et al. 2017). Pseudo-resistance describes a lack of treatment success, which is mostly caused by insufficient dosage or duration of treatment. In addition, non-adherence, insufficient plasma levels, occurrence of undesirable side effects, current psychosocial stress and relevant and possibly not treated psychiatric and/or somatic comorbidities suggest "pseudo-resistance" (Dold et al. 2017). In this context, the important role of accurate psychiatric exploration should be mentioned. For our study, only experienced psychiatrists performed the diagnostic interviews. Furthermore, therapeutic drug monitoring (TDM) can detect possible deviations in the metabolism due to enzyme variants (especially in the cytochrome P450 system of the liver), which can be associated with insufficient AD plasma levels (Hiemke et al. 2018). Taking all this into account, the term “complex depression/MDD” is also used in the literature especially for clinical practice, which includes the various sub-types discussed above (Brown et al. 2019). TRD is a clinical condition that is increasingly relevant in daily practice, but a consensus on the definition of TRD needs to be established (Brown et al. 2019). According to numerous studies the prevalence of TRD is between 15-30% of all MDD cases (Dold et al. 2017). Our sample consisting of predefined treatment resistant MDD subjects provides a high-risk cohort to analyze suicide phenotypes and to reveal a possible genetic impact. A higher frequency of suicides is well known in subjects suffering from treatment resistant MDD (Souery et al. 2007).

3.3.1 Assessment of the suicide phenotypes (suicide risk/history of suicide attempts) within MDD

In our MDD sample, suicide risk was present in 58.8% (n=147) and absent in 41.2% (n=103) of cases. A personal history of suicide attempts was reported in 29.6% (n=74) and absent in 69.6% (n=174) of the sample. Data on suicide phenotypes were missing for 0.8% (n=2). The statistical analyzes were applied with a standard chi-square test for the dichotomous trait

suicide risk (yes/no) and a history of suicide attempts (yes/no). The diagnostics of suicidality comprises three important aspects: the assessment of the strength of the subjective wish to commit suicide/the level of the objective risk for suicide, recording the probability of a (repeated) suicide attempt and the determination of the underlying disorder. We assessed suicidality at inclusion with the Mini International Neuropsychiatric Interview (MINI), section on suicidality. The MINI is a short, structured interview that has been developed for psychiatric diagnostics of axis I disorders, both in clinical areas and in research. The diagnoses are made according to DSM-IV criteria (Sheehan et al. 1998, Pettersson et al. 2018). According to the MINI, the current suicide risk is described when a minimum of one rated item was/or is positive within the last month. "The items are: C1) Think that you would be better off dead or wish you were dead? C2) Want to harm, hurt or injure yourself? C3) Think about suicide? C4) Have a suicide plan? C5) Attempted suicide? 6c) (current suicide risk). In your lifetime: C6a) Did you ever made a suicide attempt?" (Roaldset et al. 2012). The Hamilton Depression Scale (HAM-D) was administered to all subjects at inclusion and after 4 weeks of adequate antidepressant (AD) treatment. The Hamilton Rating Scale for Depression (HAM-D) is an external assessment method with 17 items that is used to record depressive symptoms. Further, item 3 can assess suicide risk with a score (0-4). One possible limitation of our study is that it was primarily designed to assess the response to AD treatment but not suicidality among MDD patients. No self-rating scale for suicide risk was applied in our study; however, according to the literature for clinical usability, no scale has accomplished to predict suicide precisely so far (Runeson et al. 2017, Steeg et al. 2018). Though, the MINI is a commonly performed instrument to evaluate suicidality among MDD subjects (Li et al. 2017). Overall, our data are consistent with the literature reporting suicide risk among MDD patients from 47% to 69%, compared to 58.8% in our sample (Asnis et al. 1993, Sokero et al. 2003). In several clinical studies, evidence was found that attempting suicide is a major risk factor for later suicide (Bostwick et al. 2016, Parra-Uribe et al. 2017, Irigoyen et al. 2019). Earlier suicide attempts, according to numerous studies, are considered to be one of the most important predictors of the occurrence of later completed suicides, further thoughts of suicide and suicide attempts (Bostwick et al. 2016). A personal history of suicide attempts was evident in 29.6% and absent in 69.6% of our sample, in accordance with the literature (Isometsa 2014). The number of people who die of a further suicide attempt after a first suicide attempt differs depending on the country and the sample examined and is described at 5,4% in a recent observational retrospective-prospective cohort study (Bostwick et al. 2016). As also explained in the introduction, there are significant gender differences with a higher ratio of suicide attempts to suicide in women (Moore et al. 2018). An important finding from studies that assessed people after attempting suicide is the high percentage of suicides within the first months up to five years after attempting suicide. Out of 25 patients presenting with suicide attempt or self-harm, one will commit suicide within

the following five years (Carroll et al. 2014). Long-term studies also demonstrated an increased lifetime risk of suicide, peaking in the first one to two years, and an increased mortality risk for natural causes of death (Hawton et al. 2006). There are consistent results in the literature for suicide risk factors, such as the existence of MDD (Asnis et al. 1993). As performed in our study, investigating suicide phenotypes within one disorder allows the distinction between genes related to suicide phenotypes per se from those associated with the MDD phenotype.

3.3.2 The impact of CREB1 variants on CREB function and neuronal processes related to MDD and suicide phenotypes

This association study examined the statistical association between 5 single nucleotide polymorphisms (SNPs) and suicide phenotypes (suicide risk/suicide attempt) in MDD subjects. The SNPs examined in this work can influence gene function: Of the 5 SNPs, 2 are located in the promoter region of the *CREB1* gene. The promoter of a gene is a complex regulatory region to which transcription factors and RNA polymerases bind. Thus, among other things, gene expression can be increased or weakened (Barrett et al. 2012). Within the candidate gene approaches, selections of certain candidate genes are based on previous findings. However, valid replications are very rare. The discrepancies among candidate gene studies may be partially explained by methodological differences concerning assessment, sample sizes, and population group among others (Arranz et al. 2000). Regarding the subject selection for the present study, the genetic descent of the study participants was an important criterion for participation, our sample consisted of 97.2% Caucasians. The genotype and allele frequencies in ethnically diverse populations can vary considerably, and thus the results are influenced by population-related genetic factors (Winterer et al. 2004). Among the investigated SNPs in our study, the minor allele frequencies (MAFs) did not vary between Caucasian (C) and non-Caucasian (NC) subjects. *CREB1* encodes for the transcription factor cAMP response element-binding protein (CREB) that induces *BDNF* expression and plays a key role in neuroplasticity (Tao et al. 1998, Conti et al. 2002). The expression of the transcription factor CREB was significantly lower in MDD subjects who completed suicide than in non-suicidal ones (Dwivedi 2003). Further, for all main classes of ADs the initiation of CREB expression in the hippocampus was observed (Blendy 2006). Several studies indicate that CREB mediated molecular functions are decreased in MDD subjects and that ADs work by reversing this effect (Tardito et al. 2006, Wallace et al. 2009). In the context of MDD, CREB is among the best-studied transcription factors (Marsden 2013). Postmortem studies suggest limited function of CREB in the prefrontal cortex and in the hippocampus in patients with MDD (Dwivedi et al., 2003, Yamada et al. 2003). Functional studies with knockout mice show that *CREB1* is linked to important functions of the nervous

system: Mantamadiotis et al. (2002) found a degeneration of the brain in the area of the hippocampus and striatum in the absence of CREB (Mantamadiotis et al. 2002). Neurodegenerative causality is as well discussed in MDD and suicide subjects (Serafini et al. 2014). Other authors have shown an abnormality in neuronal migration during brain development in the absence of CREB (Diaz-Ruiz et al. 2008). An impairment of brain development as a component of the etiology is also considered in MDD and suicide subjects (Turecki 2014, Eggart et al. 2019). Aguado et al. (2009) found a changed pattern of neuronal connectivity and activity in the absence of CREB (Aguado et al. 2009). Pandaya et al. (2012) also mention a network disorder of the brain with regard to possible causes of MDD (Pandya et al. 2012). From other studies using mouse models, it was concluded that this transcription factor is of no small importance in memory formation (Kim et al. 2013a). *CREB1* is of great importance in the nervous system and therefore also for important accomplishments such as memory formation and learning processes (Ortega-Martinez 2015). With regard to MDD and suicide, functional disorders and structural anomalies of the brain are discussed as possible disease-promoting factors. An impairment of memory is known in MDD as well as in suicide subjects and a recognized accompanying symptom (Darcet et al. 2016). One study, performed by Serretti et al. (2011) within a subsample of our sample, analyzing an effect of genetic variance within *CREB1* on treatment resistance in MDD subjects, they found a significant association for the A allele of rs7569963 (Serretti et al. 2011). A former study detected gender specific associations for certain *CREB1* SNPs and higher susceptibility for MDD in females (Zubenko et al. 2003a, Zubenko et al. 2003b). We detected one *CREB1* association with a personal history of suicide attempts in female MDD subjects, in a further sex-specific analysis, however, not resisting multiple testing correction. The constantly growing amount of findings, support the relation of CREB to suicide and antidepressant response in patients suffering from MDD (Odagaki et al. 2001, Dwivedi et al. 2003a, Young et al. 2004). Further studies postulate additional evidence for the significant impact of certain *CREB1* SNPs on suicide phenotypes among MDD subjects (Perlis et al. 2007a, Perlis et al. 2007b, Zubenko et al. 2008). The investigated polymorphisms of the *CREB1* gene have so far been inconclusively researched. Numerous studies have shown that *CREB1* influences various phenotypes (i.e. cognitive impairment) that are associated with affective disorders. In addition, other polymorphisms of the *CREB1* gene, possibly linked to markers, can influence the memory performance in the context of MDD and suicide phenotypes. Increased or decreased expression of *CREB1*, which is modulated by other genes, is also plausible. Certain transcription factors can also be activated via antidepressants (AD). Long-term treatment with ADs led to overexpression of *CREB1* (Blom et al. 2002, Blendy 2006). Chen et al. (2001) was able to show in the animal model that such an up regulation of *CREB1* led to an antidepressant like effect in certain areas of the brain (Chen et al. 2001a). Knowledge of the influence of *CREB1* on memory performance can be used to develop new drugs that

target the therapy of cognitive and affective symptoms in diseases such as MDD. Studies performed in the animal model already showed a positive effect in this direction. For example, the administration of the PDE-4 inhibitor HT-0712 had a significant effect on long-term memory in aged mice. The expression of CREB-regulated genes in the hippocampus of aged mice supported by HAT-0712 provides an indication that this medication incorporates CREB-dependent mechanisms in vivo (Peters et al. 2014). An improvement in cognitive functions in various neurocognitive diseases was also observed with DI-3-n-butylphthalide (DI-NBP), a successfully synthesized and stable chemical drug that enhances microcirculation and protects mitochondria (Chen et al. 2019). Chronic administration of DI-NBP led to an increased expression of BDNF and phosphorylation of CREB (Wang et al. 2016). Virus-related overexpression of *CREB1* in the CA1 region of the hippocampus led to an improvement in long-term memory in older rats and thus suggests that modulating *CREB1* could be a possible approach in the therapy of age-related cognitive decline (Yu et al. 2017). However, the fact that transferability from animal models to clinical studies in humans is only possible to a limited extent has a limiting effect on these research results. In conclusion, valid negative findings are an important contribution although the negative results of our study could be solely related to the lack of statistical power. Therefore, further studies are needed to identify small effects exerted by single SNPs.

3.3.3 Effect of BDNF variants on suicide phenotypes in MDD subjects

The third publication analyzed the effect of certain *BDNF* SNPs (rs11030096, rs925946, rs10501087, rs6265, rs12273363, rs908867, rs1491850, and rs1491851) on suicide phenotypes as well as on treatment response phenotypes in MDD patients. After correction for multiple testing, no significant genetic association with suicide risk and/or lifetime history of suicide attempts was found. The detailed demographic and clinical characteristics of the sample have been discussed in sections 3.2.2-3.2.6 of this chapter.

3.3.4 Effect of BDNF variants on suicide phenotypes in subgroups of treatment response phenotypes in MDD subjects

We performed a further analysis in subgroups of treatment response phenotypes, in our study, consisting of 250 MDD subjects, treatment response was defined as HAM-D-17 score ≤17, remission as HAM-D-17 score≤7 and treatment resistance as HAM-D-17 score >17 following two trials of at least 4 weeks of AD treatment at optimal dosage range (Schosser et al. 2012). We found two SNPs, the Val66Met and rs10501087, were significantly associated with suicide risk in a subgroup of remitters (n = 34, 13.6%). This finding may be a false positive finding due to the small sample size and lack of statistical power. However, so far,

this is the first study analyzing the effect of *BDNF* variants on suicide phenotypes in defined AD treatment groups among MDD subjects. In general, an increased risk for suicide is well known among clinicians within the first to sixth week of AD treatment. In a recent meta-analysis, data from antidepressant trials were evaluated, which had been reviewed by the US Food and Drug Administration (FDA) upon market approval between 1987 and 2013. Across all studies, 0.8 percent of the subjects who received an antidepressant committed suicide or suicide attempt compared to the control group (placebo) with 0.3 percent (Hengartner et al. 2019). In the literature, heterogeneous definitions of suicidal behavior or the suicide phenotypes are evident, which is problematic. Suicidal behavior occurs in different psychiatric disorders with multiple characteristics and a complex genetic structure. Courtet et al. (2005) therefore plead to categorize suicidal ideation as a separate phenotype and to differentiate from (repeated) suicide attempts, suicide and violent suicide (Courtet et al. 2005). In our study, the assessment for a current suicide risk/or lifetime history of suicide attempt was performed with the Mini Neuropsychiatric Interview (MINI) and the Hamilton Depression Rating Scale (HAM-D-17). However, the instruments are not primarily designed to address suicide phenotypes. A further limitation of our study was the design, as we primarily aimed to investigate treatment resistant MDD. This study was therefore not conceptualized to tackle suicide phenotypes; for ethical reasons, subjects reporting concrete suicide plans were excluded from this study. Suicidal behavior is very complex and is influenced by both genetic and environmental factors (GxE). It is assumed that multiple genes, each having only a small effect size are involved in the etiology of MDD and suicide phenotypes. For the *BDNF* gene and MDD as well as the suicide phenotypes, several genetic associations have been described. In particular the rs6265 SNP has been intensively studied as the Met allele impairs the BDNF function and was associated with decreased volume of the hippocampus in humans (Dwivedi et al. 2003b, Youssef et al. 2018). Our work deals with the rs6265 *BDNF* polymorphism, which presumably influences the treatment response of MDD subjects. The Val66Met polymorphism has an effect on the intracellular transport and secretion of BDNF in nerve cells (Egan et al. 2003). In animal experiments, the Met *BDNF* variant had a disruptive effect on intracellular trafficking and secretion of BDNF in neurons of the hippocampus (Harrisberger et al. 2015). When the brain volume was examined using magnetic resonance imaging (MRI) for various ethnic groups, cortical volume reduction was found on both sides in the hippocampus and dorso-lateral pre-frontal cortex (DLPFC) in carriers of the Met allele compared to carriers of the Val/Val allele (Pezawas et al. 2004). The rs6265 SNP in the *BDNF* gene is an exchange polymorphism between guanine and adenine and leads to valine to methionine exchange in region 5' in codon 66 (Val66Met) on chromosome 11p13. It is positioned in exon 9 in the BDNF gene (Huang et al. 2006). The BDNF protein itself is not affected by the SNP, but its precursor proBDNF, from which the mature BDNF (mBDNF) is formed (Seidah et al. 1996). The variant

Val66Met leads to a low intracellular BDNF level and a reduced activity-dependent BDNF secretion. Due to the defective formation of a transport signal, the secretion of the BDNF is disturbed. Although this does not produce less BDNF, it is released from the cell to a lesser extent (Egan et al. 2003). Chen Z-Y et al. (2006) demonstrated a disrupted interaction of the BDNF molecule with a transport protein, the sortilin. Another study examined the association between the Val66Met polymorphism and the susceptibility to developing MDD. Carriers of the Val66Val allele showed a higher risk of for MDD (Ribeiro et al. 2007). If the carriers of the Val66Val allele actually developed MDD, they showed better executive functions and memory functions than carriers of the Val66Met allele (Ribeiro et al. 2007). Though, the results remain conflicting. Another risk factor for MDD and suicide phenotypes is early life adversity, however, as a limiting factor of our study, we did not assess childhood trauma. To test for associations with single common genetic variants, our sample may be underpowered. Nonetheless, gaining new insights on the MDD and suicide endophenotypes is fundamental. We performed a genetic association study in order to elucidate the complex and polygenic heredity of MDD with the possibility of identifying susceptibility genes with small effects. A disadvantage of our study, however, is that it is based on biological hypotheses and therefore only known candidate genes can be analyzed. In a recently published paper Border et. al (2019) stated that the long studied MDD candidate gene polymorphisms do not have detectable effects on MDD phenotypes (Border et al. 2019). Though, we investigated 8 *BDNF SNPs* within MDD subjects and compared the allelic and genotypic frequencies of selected variants between the phenotypes suicide risk (yes/no) and a personal history of suicide attempts (yes/no). While phenotypes are defined by behavioral characteristics (i.e. suicide risk) or disease diagnoses (i.e. MDD), endophenotypes represent underlying brain functions or their disease-related changes. Certain gene variants with a variety of protein transcripts are understood to shape the endophenotype together with early environmental factors (GxE). This, in turn, is believed to be jointly responsible for the development of the psychopathological phenotype (i.e. MDD), with environmental influences being able to contribute to this both as a risk and as a protective factor. Recently it was shown that subjects with obesity, smoking, and high-risk drinking behavior were at greater risk for developing MDD (Zhang et al. 2018). Whereas protective factors for MDD were a good physical health status and performing physical activity on a regular basis (Zhang et al. 2018). Of note, in a subsample of our sample (n=190) smoking was increased with 61% (n=114), compared to the general population of Austria with about 24% (Comission 2017). In order to better understand the complex phenotypes of psychiatric disorders and further elucidate their endophenotypes, it requires better understanding of not merely the genetic variation, but also environmental effects (GxE) that affect the gene-expression (i.e. epigenetics) and the complex pathways of gene products (i.e. proteomics) (Braff et al. 2017).

3.4.1 Conclusion and outlook

This study observed significantly hypermethylated *BDNF* exon I promoter in PBMCs of MDD subjects in comparison to BD and control subjects. For age, gender, and genotype no relevant effect on *BDNF* exon I promoter methylation was observed. No significant genetic effect was found for *CREB1* and *BDNF* on suicide phenotypes. The increase in methylation levels might be an effect of AD therapy in MDD subjects, although BD subjects were, most likely, as well treated with ADs and mood stabilizers. Overall, we conclude that methylation differences in our sample are not completely explained by psychopharmacological treatment, but as well by MDD disease phenotype. Of note, there was no significant difference observed in methylation levels of BD subjects, most likely receiving psychopharmacological treatment, compared to control subjects. The observed effect sizes are relatively small and it should be considered that it is not yet known how these observations could translate into biological differences. In general, there are several genetic and epigenetic mechanisms, which finally lead to specific gene-expression patterns. However, the phenotypic differences, large enough to be classified as the MDD syndrome, might be a result of such small variations persisting over a long period in the epigenome, or in numerous genes of the same pathway. Eventually, we need longitudinal studies monitoring epigenetic changes within childhood, prior to MDD onset and a follow up throughout the different depressive episodes and upon symptom remission, in order to gain more understanding of the MDD pathology. However, more complex and alternative methylation patterns can only be detected by high-resolution analyses such as bisulfite sequencing based approaches. To separate the effect of pharmacological treatment and disease phenotype, further randomized case-control studies in drug free MDD and BD subjects and those receiving treatment are necessary. Our findings might add to the myriad of neurobiological findings and further elucidate the pathogenesis and potential therapeutic targets of affective disorders.

4 Materials and Methods

This doctoral thesis was carried out at the Department of Psychiatry and Psychotherapy (Medical University of Vienna), within the doctoral program "Mental Health and Behavioral Medicine".

The assessment and collection of the blood samples was performed in the context of the following studies at the Department of Psychiatry and Psychotherapy of the Medical University Vienna: “Studies on the psychosocial and biological (genetic) causes of bipolar and unipolar affective disorder (and schizophrenia)” (Oesterreichische Nationalbank (ÖNB) Grant nos. 5777 and 13198 and Austrian Research Foundation (FWF), Grantno. 7639, all to Harald Aschauer), the “Genetics of Response to Agomelatine Pilot-Study (GENRAS)” (EK-No201/2010 Alexandra Schosser) and in the context of the European multicenter project “Patterns of Treatment Resistance and Switching Strategies in Unipolar Affective Disorder” of "The Group for the Study of the Resistant Depression (GSRD) (PI: Siegfried Kasper)" funded by an unrestricted grant from Lundbeck.

The epigenetic analyses were performed at the Clinical Institute of Pathology (Medical University of Vienna), the bisulfite conversion and adaptation of protocols for quantitative real-time PCR was performed according to the MethyLight protocol using SYBRgreen instead of fluorescent probes for quantification (Campan et al. 2009). The BDNF primers were selected according to prior studies (D'Addario et al. 2012). According to the formula by Campan et. al (2009) the percentage of methylated reference (PMR) value was calculated:

$$100 \times ((\text{BDNF mean value})\text{sample}/(\text{ALU mean value})\text{sample})/((\text{BDNFmean value})\text{M.Sss1}/(\text{ALUmean value})\text{M.Sss1})$$

Genotyping of DNA samples was performed at Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, King’s College London, London, UK and at Laboratory of Experimental Neurology in the Department of Neuroscience at the Mario Negri Institute for Pharmacological Research, Milan, Italy.

The statistical analysis was performed at the Department of Psychiatry and Psychotherapy, Medical University of Vienna. The analysis were performed with the program Prism version 5 (Graph-Pad Software, San Diego, CA) and consisted of the ANOVA models with subsequent post-hoc *t*-tests, and Bartlett's tests to check for equality of variance. To control for possible confounding variables, e.g. age, depressive symptomatology/disease phenotype, Pearson's correlations coefficient test was performed. With the SPSS Statistics version 20 for MAC

genetic association was tested with standard chi-square ($\chi 2$). The Cochran–Armitage trend statistic to test for allelic association was performed with the program FINETTI (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). All results where corrected for multiple testing.

5 List of tables

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7 REFERENCES

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Zubenko, G. S. and Hughes, H. B., 3rd, 2008. Effects of the G(-656)A variant on CREB1 promoter activity in a glial cell line: interactions with gonadal steroids and stress. Am J Med Genet B Neuropsychiatr Genet 147Bl, 579-585.

Zubenko, G. S., Hughes, H. B., 3rd, Stiffler, J. S., et al., 2003a. Sequence variations in CREB1 cosegregate with depressive disorders in women. Mol Psychiatry 8l, 611-618.

Zubenko, G. S., Hughes, H. B., Stiffler, J. S., et al., 2002. Genome survey for susceptibility loci for recurrent, early-onset major depression: results at 10cM resolution. Am J Med Genet 114l, 413-422.

Zubenko, G. S., Maher, B., Hughes, H. B., 3rd, et al., 2003b. Genome-wide linkage survey for genetic loci that influence the development of depressive disorders in families with recurrent, early-onset, major depression. Am J Med Genet B Neuropsychiatr Genet 123Bl, 1-18.

8. CURRICULUM VITAE

Laura Carlberg, MD

1. HIGHER EDUCATION

2004 – 2011 Medical training, MUV

10/2011 - 10/2016 Doctoral Program of Applied Medical Science N790, "Mental Health and Behavioral Medicine", Medical University Vienna (MUV)

PhD Thesis: „Brain Derived Neurotrophic Factor - Epigenetic regulations in Major Depressive Disorder“

Supervisor: Prim. Priv.-Doz. Dr. Alexandra Schosser, PhD. MBA

Department of Psychiatry und Psychotherapy, MUV

Zentren für seelische Gesundheit Wien, BBRZ-Med, Vienna

2. AWARDS

2011 **Young Investigator Award,** (Laura Carlberg, Martin Uffmann, Sarah Poetter-Lang, Michael Weber, Peter Homolka, Cornelia Schaefer- Prokop) “Dual Readout CR Chest Radiography: Effect of Tube Voltage on Image Quality”

Radiation Protection Society, Annual Meeting 2011, Salzburg, Austria

3. DOCTORAL TRAINING

Current Position

07/2019 - present Clinical training, Resident, Ordination Dr. Bräuer und Dr. Weber, Gießaufgasse 4/12, 1050 Vienna

10/2018 - 03/2019 Clinical training, Resident, Department of Internal Medicine, Otto-Wagner-Hospital, Vienna, Chair: Primaria Dr. Heidemarie Abrahamian

04/2018 - 09/2018 Clinical training, Resident, Department of Neurology, Otto-Wagner-Hospital, Vienna, Chair: Prim. Assoz.-Prof. Priv.-Doz. Dr. Peter Lackner

12/2017 - 04/2018 and 04/2019 - 06/2019 Clinical training, Resident, 4th Department of Psychiatry, Otto-Wagner-Hospital/Krankenhaus Nord, Vienna, Chair: Primarius Dr. Michael Ertl

04/2013 - 09/2016 Clinical training, Resident, Department of Psychiatry and Psychotherapy, Medical University of Vienna, Chair: O.Univ.Prof. Dr.med. Dr.hc.mult. Siegfried Kasper

4. PUBLICATIONS

4.1 Articles - First Authorship

4.1.1 **Carlberg L**, Scheibelreiter J, Hassler M, Schloeglhofer M, Schmoeger M, Ludwig B, Kasper S, Aschauer H, Egger G, Schosser A. (2014) Brain-Derived Neurotropic Factor (BDNF) – Epigenetic regulation in unipolar and bipolar affective disorder. J Affect Disord. 2014 Oct;168:399-406. doi: 10.1016/j.jad.2014.07.022. Epub 2014 Jul 19. PMID: 25106037

4.1.2 **Carlberg L**, Schosser A, Calati R, Serretti A, Massat I, Papageorgiou K, Linotte S, Mendlewicz J, Souery D, Zohar J, Montgomery S, Kasper S. (2013) Hint for gender-specific association of creb1 and a history of suicide attempts in MDD: Results from a european multicenter study on treatment resistant depression. Int J Neurosci. 2014 Jun 23:1-20. PMID: 24955721.

4.2 Articles - Co-Authorship

4.2.1 Genomic Relationships, Novel Loci, and Pleiotropic Mechanisms across Eight Psychiatric Disorders. **Cross-Disorder Group of the Psychiatric Genomics Consortium.** Electronic address: plee0@mgh.harvard.edu; Cross-Disorder Group of the Psychiatric Genomics Consortium. Cell. 2019 Dec 12;179(7):1469-1482.e11. doi: 10.1016/j.cell.2019.11.020.

4.2.2 Ludwig B, Kienesberger K, **Carlberg L**, Swoboda P, Bernegger A, Koller R, Wang Q, Inaner M, Zotter M, Kapusta ND, Haslacher H, Aigner M, Kasper S, Schosser A. (2018) Influence of CRHR1 Polymorphisms and Childhood Abuse on Suicide Attempts in Affective

Disorders: A GxE Approach. Front Psychiatry. 2018 Apr 26;9:165. doi: 10.3389/fpsyt.2018.00165. eCollection 2018. PMID: 29755375

4.2.3 Bernegger A, Kienesberger K, **Carlberg L**, Swoboda P, Ludwig B, Koller R, Inaner M, Zotter M, Kapusta N, Aigner M, Haslacher H, Kasper S, Schosser A. (2018). The Impact of COMT and Childhood Maltreatment on Suicidal Behaviour in Affective Disorders. Sci Rep. 2018 Jan 12;8(1):692. doi: 10.1038/s41598-017-19040-z. PMID: 29330410

4.2.4 Schosser A, **Carlberg L**, Calati R, Serretti A, Massat I, Papageorgiou K, Linotte S, Mendlewicz J, Souery D, Zohar J, Montgomery S, Kasper S. (2017) The impact of BDNF gene polymorphisms on suicidality in treatment resistant major depressive disorder – A European Multicenter Study. Int J Neuropsychopharmacol. 2017 Oct 1;20(10):782-787. doi: 10.1093/ijnp/pyx028. PMID: 28977521

4.2.5 Kraus C, Rabl U, Vanicek T, **Carlberg L**, Popovic A, Spies M, Bartova L, Gryglewski G, Papageorgiou K, Lanzenberger R, Willeit M, Winkler D, Rybakowski JK, Kasper S. Administration of ketamine for unipolar and bipolar depression. (2017) Int J Psychiatry Clin Pract. 2017 Mar;21(1):2-12. doi: 10.1080/13651501.2016.1254802. Epub 2017 Jan 18. Review. PMID: 28097909

4.2.6 Hinney A, Kesselmeier M, Jall S, Volckmar AL, Föcker M, Antel J; GCAN; WTCCC3, Heid IM, Winkler TW; GIANT, Grant SF; EGG, Guo Y, Bergen AW, Kaye W, Berrettini W, Hakonarson H; Price Foundation Collaborative Group; Children's Hospital of Philadelphia/Price Foundation, Herpertz-Dahlmann B, de Zwaan M, Herzog W, Ehrlich S, Zipfel S, Egberts KM, Adan R, Brandys M, van Elburg A, Boraska Perica V, Franklin CS, Tschöp MH, Zeggini E, Bulik CM, Collier D, Scherag A, Müller TD, Hebebrand J. (2016) Evidence for three genetic loci involved in both anorexia nervosa risk and variation of body mass index. Mol Psychiatry. 2016 May 17. doi: 10.1038/mp.2016.71. PMID: 27184124

4.2.7 Bernegger A, Kienesberger K, **Carlberg L**, Swoboda P, Ludwig B, Koller R, Kapusta ND, Aigner M, Haslacher H, Schmöger M, Kasper S, Schosser A. Influence of Sex on Suicidal Phenotypes in Affective Disorder Patients with Traumatic Childhood Experiences. (2015) PLoS One; 10(9): e0137763.

4.2.8 The Wellcome Trust Case Control Consortium 3. (2014) A genome-wide association study of anorexia nervosa. Mol Psychiatry. 2014 Oct;19(10):1085-94. doi: 10.1038/mp.2013.187. Epub 2014 Feb 11. PMID: 24514567

4.2.9 GCAN. (2014) Using ancestry-informative markers to identify fine structure across 15 populations of European origin. Eur J Hum Genet. 2014 Oct;22(10):1190-200. doi: 10.1038/ejhg.2014.1. Epub 2014 Feb 19. PMID: 24549058

4.2.10 Ludwig B, **Carlberg L**, Kienesberger K, Swoboda P, Mitschek MM, Bernegger A, Koller R, Inaner M, Senft B, Meisner L, Fischer-Hansal D, Affenzeller A, Huber J, Schoenthaler S, Kapusta ND, Haslacher H, Aigner M, Weinhaeusel A, Kasper S, Schosser A. Sex-specific MAOA Gene Single-Nucleotide Polymorphisms and Methylation Status and the Risk of Violent Suicide Attempts in Affective Disorder Patients (in Revision)

4.3 Articles in German Language Publications

4.3.1 **Carlberg L**, Schosser A. (2012) Epigenetik in der Psychiatrie; CliniCum neuropsy 1/2012, S. 40-43.

4.3.2 **Carlberg L**, Ludwig B, Kasper S, Winkler D. (2015) Die Generalisierte Angststörung; CliniCum neuropsy 5/2015, S. 14-20.

4.4. Abstracts

4.4.1. Carlberg L, Uffmann M, Poetter-Lang S, Weber M, Homolka P, Schaefer-Prokop C. (2010) Welchen Einfluß hat die Aufnahmespannung auf die Bildqualität von digitalen Thoraxaufnahmen? Oesterreichisch-Bayerischer Röntgenkongress, Linz, Austria

4.4.2 Carlberg L, Uffmann M, Poetter-Lang S, Weber M, Homolka P, Schaefer-Prokop C. (2009) Dual Readout CR Chest Radiography: Effect of Tube Voltage on Image Quality Oral presentation: Annual Meeting 2009 - Radiological Society of North America, Chicago, USA

4.5 Posters

4.5.1 Carlberg L, Schosser A, Calati R, Serretti A, Massat I, Papageorgiou K, Linotte S, Mendlewicz J, Souery D, Zohar J, Montgomery S, Kasper S. Hint for gender-specific association of creb1 and a history of suicide attempts in MDD: Results from a european multicenter study on treatment resistant depression

Poster: 12th World Congress of Psychiatric Genetics (WCPG), Hamburg, Germany, 10/2012

4.5.2 Schosser A, Schloegelhofer M, Zeiler J, Schmoeger M, Carlberg L, Knabl R, Olajossy-Hilkesberger L, Kaufmann R, Aschauer HN. Preliminary results of a BICC1 and NLGN1 association study in MDD – an attempt to replicate previous GWAS findings.

Poster: 12th World Congress of Psychiatric Genetics (WCPG), Hamburg, Germany, 10/2012

4.5.3 Carlberg L, Scheibelreiter J, Hassler M, Schloeglhofer M, Schmoeger M, Ludwig B, Kasper S, Aschauer H, Egger G, Schosser A. Epigenetic regulation of the BDNF gene in Major Depressive Disorder and Bipolar Affective Disorder

Poster: 13th World Congress of Psychiatric Genetics (WCPG), Boston, United States of America, 10/2013

4.5.4 Carlberg L, Scheibelreiter J, Hassler M, Schloeglhofer M, Schmoeger M, Ludwig B, Kasper S, Aschauer H, Egger G, Schosser A. DNA methylation of the BDNF gene in schizophrenia.

Poster: 26th European College of Neuropsychopharmacology (ECNP), Barcelona, Spain, 10/2013

4.5.4 Carlberg L, Kienesberger K, Swoboda P, Ludwig B, Bernegger A, Koller R, Kapusta ND, Aigner M, Haslacher H, Schmöger M, Kasper S, Schosser A. Effect of BDNF Val66Met on suicidal behaviour in affective disorders.

Poster: The German Association for Psychiatry, Psychotherapy and Psychosomatics (DGPPN), Berlin, Germany, 11/2015

5. TEACHING

2012 - 2015 Medical Diploma thesis co-supervision, Influence of CRHR1 and childhood trauma on suicidal behaviour: a gene-environment approach.
Dr.med.univ. Birgit Ludwig B.A., B.Sc.