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COORDINATORE: Prof.ssa Lorenza Trabalzini

Variation of circulating levels of BDNF neurotrophin in depression: correlations with symptom severity, metabolic status and inflammatory indices

Dottorando: Dott.ssa Valentina Falaschi

Tutor: Prof. Gino Giannaccini

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Summary

Introduction

In these last two decades, the biochemical and molecular research of neuropsychiatric disorders has greatly expanded thanks to the attained technological and methodological advances that have become available to scientists and clinicians. Given the pathophysiological complexity of mental disorders, now also seen as real neuroendocrine and systemic dysfunctions, current research in the neuropsychiatric field is no longer focusing on a single substrate or neurotransmitter system but rather tends to integrate multiple parameters (Maes et al, 2009, Strawbridge et al, 2017). In the case of Mood Disorders, recent studies show how Major Depression can be considered as a systemic pathology, which involves primarily the brain, but also the whole organism, accordingly to a two-way communication model.

There is currently a large literature on the neuroanatomical, neurophysiological and neuroendrocrinological correlates of Major Depression, although no laboratory test has been sufficiently sensitive and specific as a diagnostic tool for the disorder so far. Numerous studies indicate, in addition to neurotransmitters and neuroendocrine markers, the involvement of the inflammatory response and, more generally, of the immune system, energy/redox metabolism and growth factors in depression, implying the presence of altered biochemical patterns/networks

rather than disturbances of single and separate parameters (Strawberidge et al, 2017).

The search of valuable biomarkers and correlates of depression has acquired an ever-increasing consent among neuropsychiatrists with the aim at differentiating clinical subtypes and improving patients' clinical care. In fact, despite a high range of treatment options for major depression and the considerable therapeutic progress achieved, about one third of patients reach a full remission after initial treatment and the probability of non-response increases with the number of episodes experienced (Gaynes et al, 2009). This is probably due to the heterogeneity of the illness: not all patients show the same clinical presentation and, presumably, the same pathogenetic molecular substrates (Kauer-Sant'Anna et al, 2009). It is therefore necessary to ameliorate and even personalize treatments dedicated to this psychiatric illness, in order to possibly prevent its tendency to become chronic.

Numerous evidences have accumulated over time in favor of the presence of altered inflammation patterns in depression, related to an impaired activity of the neuroendocrine hypothalamus-pituitary-adrenal (HPA) axis and the system of neurotrophins, in particular of the Brain-Derived Neurotrophic Factor (BDNF). Under an integrated vision of the pathogenesis of depression, this illness would be defined by variable disturbances of these systems, known to reciprocally interact with the

functionality of the serotonin (5-HT) and other neurotransmitter signaling pathways. It is noteworthy that both the HPA responses and BDNF signalings work to maintain the homeostasis of metabolic processes during the occurrence of stressful events (Pitsillou et al, 2020, Levy et al, 2018). Specifically, an impaired expression of BDNF has been reported in some types of depressed patients, so that the administration of this neurotrophin has been thought as one of the most promising therapeutic strategies for the development of innovative antidepressant drugs, even if first data are not encouraging. Indeed, a main problem of this approach consists in the possible induction of a too rapid change of BDNF in the body that seems to increase the risk at developing tumors, due to the angiogenic properties of this neurotrophin. For this reason, some biological drugs capable of regulating the neurotrophin expression at the level of its gene transcription are currently under study (Radin et al, 2017). It is also important to understand how the neurotrophin system and BDNF interact with the inflammatory response in the pathogenesis of mood disorders. In support to the integrated hypothesis of depression, BDNF and its TrkB receptor, originally discovered in the brain, have been also detected in many peripheral tissues and in the bloodstream, suggesting their widespread, whole-body physiological role, linked to cell survival and differentiation (Serra-Millàs, 2016). Thus, the measurement of peripheral and circulating BDNF in depressed patients under different clinical conditions and

therapeutic approaches is still a challenging topic for the improvement of patients' clinical care, even as a potential index of the risk or presence of specific psychiatric or somatic co-morbidities. Moreover, upgrading current knowledge on the regulatory mechanisms of gene/protein expression and post-translational/conformational modifications of BDNF, together the full understanding of its mechanisms of release or uptake in different cells and tissues, would help to explain the pathogenetic significance of an altered neurotrophic signaling within body districts. One of the first steps towards these goals consists, by consequence, in evaluating the circulating amount of BDNF and its correlation with patients' clinical features (Hashimoto et al, 2010).

Taking into account these premises, the main objectives of this work were therefore:

1) to estimate the peripheral, bloodstream counterpart of BDNF in Major Depressive Episode through its measurement in plasma and platelet samples obtained from a well-defined group of patients;

2) to search possible correlations with patients' clinical symptoms and results at routine laboratory tests, these last including the main blood metabolic, cellular and inflammation parameters.

CHAPTER 1: Depression and pathophysiological mechanisms

1.1 Depressive episodes in mood disorders-Implications of monoaminergic neurotransmission

Mood Disorders include different conditions in which the most severe and common forms are Major Depressive Disorder (MDD) and Bipolar Disorders (BDs). Different depressive episodes can occur in the life of patients so a major depressive episode can be ascribed to a bipolar or a unipolar disorder. Furthermore, many definitions of types of major depression have been described in the years and then various specifiers in DSM-5, for more appropriate therapeutic strategies.

The Diagnostic and Statistical Manual of Mental Disorders (DSM) is one of the nosographic systems for mental or psychopathological disorders most used by psychiatrists, psychologists and physicians from all over the world, both in clinical practice and in research. Over the years, the manual, now in its 5-th edition, has been drafted taking into account the current development and results of psychological and psychiatric research in numerous fields, modifying and introducing new definitions of mental disorders. The first version dates back to 1952 (DSM-I) and was written by the American Psychiatric Association (APA), since then there have been further editions: in 1968 the DSM-II, in 1980 the DSM-III, in 1987 the

DSM-III-R (revised edition), in 1994 the DSM-IV, in 2000 the DSM-IV-TR (revised text) and in 2013 the DSM-5.

Unlike the DSM-IV-TR, in DSM-5 the chapter "Depressive disorders" has been separated from the chapter "Bipolar and related disorders".

The common features of Depressive Disorders (Figure 1) are the presence of a sad, empty or irritable mood, accompanied by somatic and cognitive changes that significantly affect the individual's ability to function. What differs among them are issues of duration, timing, or presumed etiology.

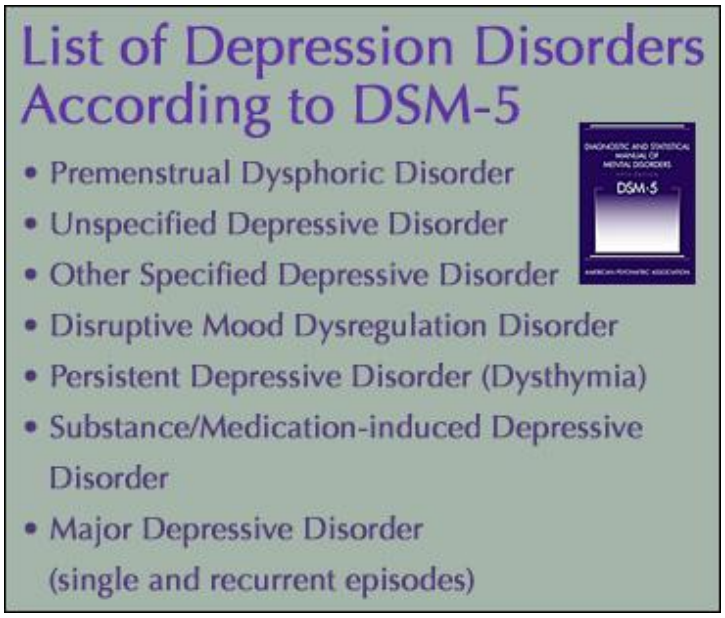


Figure 1: Classification of depressive disorders accordingly to the DSM-5. Major Depressive Disorder (MDD) is defined by single and recurrent episodes (DSM-5)

The DSM-5 describes “Major depressive Disorder” (MDD) as a complex disorder characterized by at least five symptoms present over a two-week period, at least one of which consists of depressed mood or loss of interest or pleasure. Symptoms may consist of weight loss or gain, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or lack of energy, feelings of worthlessness or guilt, impaired ability to think or concentrate, recurring thoughts of death, all causing a clinically significant distress and a relevant impairment in social, occupational, or other important areas of functioning (American Psychiatric Association, 2013).

Major depressive disorder is a chronic and highly debilitating syndrome, one of the currently four main disorders affecting the world's population, with a lifetime probability of 10-30% in women and 7-15% in men (Kessler et al, 2003; Bromet et al, 2011; Hasin et al, 2018).

Furthermore, it is estimated that the prevalence of depressive episodes could continue to rise until they will become the second cause of illness among the World Health Organization (WHO) member states (Smith K. et al, 2014, Mathers and Locar 2006). In addition to provoking a significant decrease in social and physical functioning, MDD can lead to such discomfort to the point of causing a complete disability and/or even increasing the risk of suicide.

There is a high comorbidity with other neuropsychiatric disorders such as panic, obsessive-compulsive, eating and borderline-personality disorders,

substance abuse, but also with physical illnesses and pain (American Psychiatric Association 2013).

Major Depressive disorder has a variety of socio-economic consequences that include low education, job and emotional instability and poor job performance (Kessler and Bromet 2013).

Furthermore, this illness is tightly linked to a high rate of mortality, associated not only with suicide: depressed patients are more likely to develop coronary artery disease and type-2 diabetes (De Burgos-Lunar et al 2012, Mondal and Fatima 2018). Thus, depression continues to increase the global burden of disease and disability (Ahern et al, 2011).

The DSM-5 has described several specifiers that can be attributed to the single Major Depressive Episode with clinical and therapeutic implications. In fact, the specifiers for a depressive episode can be of different types: with anxiety, with mixed characteristics, melancholic, atypical or psychotic, with onset in the peri-partum and with a seasonal trend.

Depressive episodes can arise not only in the context of a diagnosis of unipolar depression, but also in patients with bipolar disorder, when, in the latter case, counter-polar (manic or hypomanic) episodes occur alternately with the depressive ones. According to DSM-5 in Bipolar I disorder, there are episodes that fully meet the criteria for a manic episode that can be preceded or followed by hypomanic or major depressive episodes

throughout life. Bipolar II disorder requires the experience of at least one major depressive episode and one hypomanic episode. In Figure 2 are reported the mood profiles occurring in MDD as compared to other mood disorders.

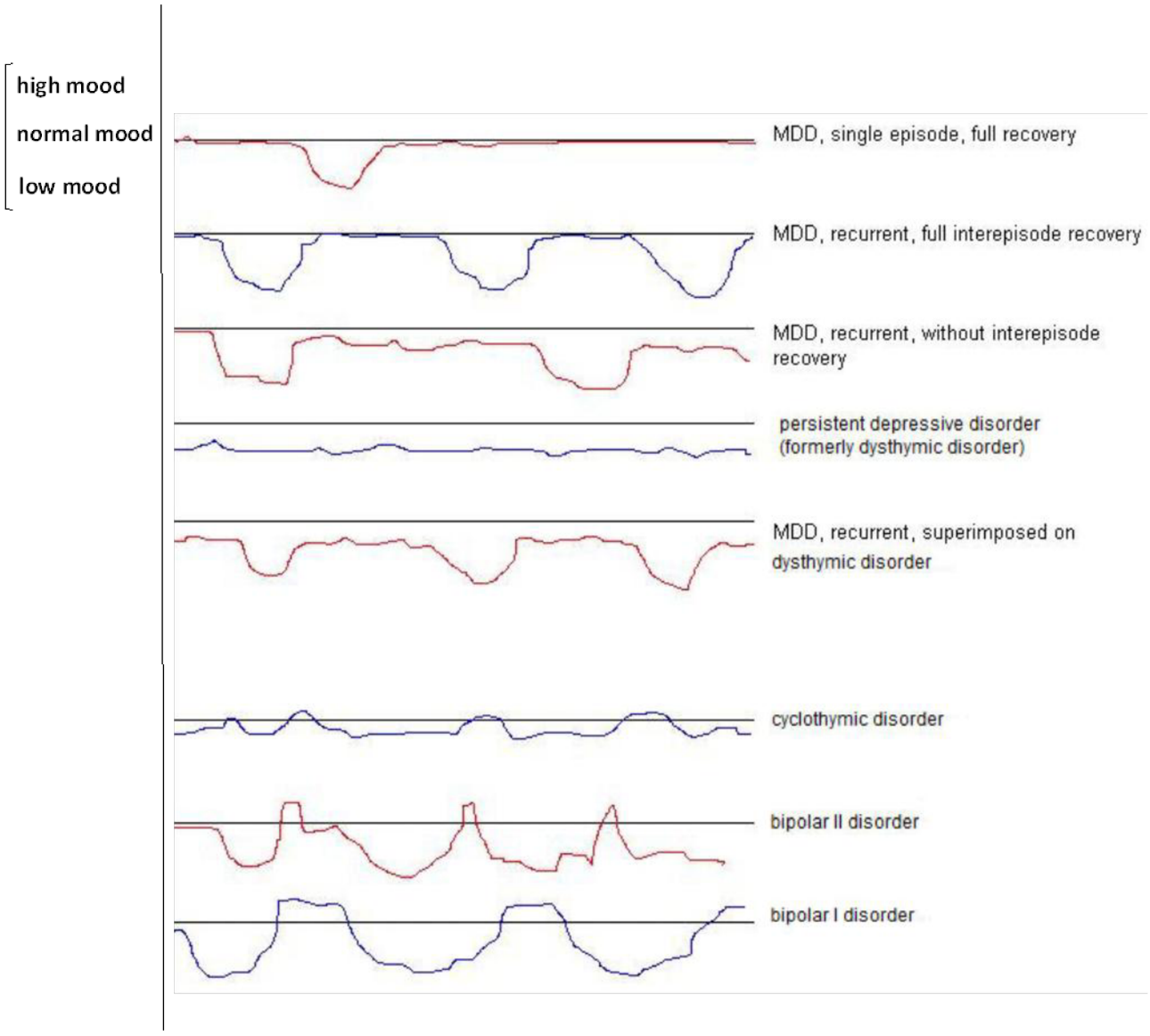


Figure 2. Profiles of mood in MDD, single or recurrent depressive episodes, in comparison with those occurring in the other mood disorders (modified from tpb.psy-ohio state.edu)

Therefore, Major Depression is a heterogeneous disorder both in the transverse that in the longitudinal course. Some authors have hypotized that unipolar and bipolar depression may represent distint nosological entities, possibly related to different biological substrates, but in years it has developed also a dimensional approach (mood spectrum model). So different authors and clinicians tend to identify Major Depressive Disorder (MDD) as a part of a continuum (bipolar spectrum), encompassing depressive and manic symptomatology of different severity levels along a longitudinal course (Akiskal et al, 1999).

Despite of the considerable impact of major depression on health, knowledge on the pathophysiology of this mental illness is very poor in respect to other common chronic diseases. Because of the complexity and heterogeneous nature of depressive episodes, current treatment outcome is suboptimal. From the most recent studies, depression is considered a multifactorial and multifaceted mental illness, related to variably combined genetic, biochemical, and non-genetic factors and triggers, such as stress, affective trauma, viral infection and abnormality in neurodevelopment which have more impact with the increased complexity of the pathogenesis of the disease (Chen, et al 2007, Ugo 2008). However, the precise pathogenic causes have not been defined yet.

The etiology of major depression can involve in fact both genetic and environmental factors (Lesch et al, 2004, Saveanu et al, 2012, Subbarao et

al, 2008). Environmental, lifestyle or transitional changes in the course of life, in relation to genetic and acquired vulnerabilities, define a variety of biological endophenotypes and clinical symptoms (Leuchter et al, 2014). After the discovery of the first antidepressants, (ADs), as monoamine oxidase inhibitors (MAOIs) and tricyclic ADs (TCA), the most relevant pathophysiological mechanism of MDD was considered the diminished monoamine neurotrasmission (Dell'Osso et al, 2016). The effectiveness of the first ADs consisted indeed in the rise of the extra-cellular levels of monoamines, in particular serotonin (5-hydroxytryptamine, 5HT) and norepinephrine (NE), through the blockade of their presynaptic catabolic enzymes MAO or respective re-uptake mechanism (membrane-bound transporters).

The classical monoamine theory, formulated in the '60s, thus suggests that, at the origin of mood symptom presentations, stress or genetic or non-genetic vulnerabilities, lead to the reduction of biogenic monoamines as 5-HT, NE, and dopamine (DA) (Shilldkraut et al, 1965).

This theory was also formulated by the observation that reserpine, an antihypertension drug showing also antipsychotic properties, can provoke CNS monoamine depletion, precipitating depressive symptoms (Everett and Toman, 1959) (Figure 3).

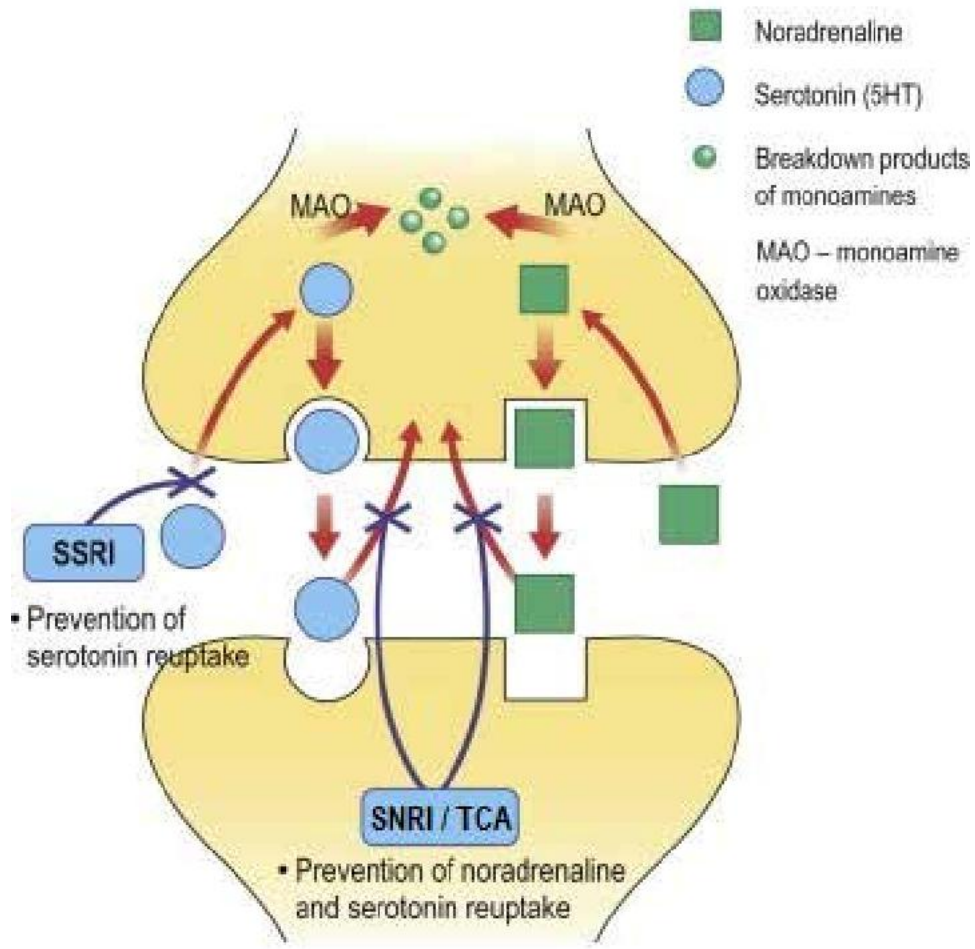


Figure 3. Mechanism of action of antidepressant drug: selective serotonin re-uptake inhibitors (SSRIs), tricyclic (TCA) and selective noradrenaline reuptake inhibitors (SNRIs) (Van Rensburg and Reuter, 2019).

Tricyclics are a class of AD drugs so called due to their characteristic chemical structure formed by three condensed rings.

Although not all tricyclics share the same mechanism of action, they generally act through the inhibition of monoamine neurotransmitter reuptake and as antagonists of different receptors synaptic receptors,

particularly muscarinic cholinergic, serotonin and histamine receptors, causing various side effects. Toxicity occurs at approximately 10 times normal dosages (the therapeutic index is relatively low); these drugs are often fatal if taken in overdose, since they can cause ventricular arrhythmias, delirium and convulsions. Although they are still prescribed under special conditions, because they are known to be effective, in recent times their use has been largely replaced in the clinic by the most recent second-generation ADs (SGAs) as selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline (SNRIs), which generally have fewer side effects (Feighner et al, 1999).

The importance of the monoamine hypothesis consisted in the introduction of the concept of "biochemical lesion" in depression (Leonard, 2000). Meanwhile, the monoamine hypothesis of depression has received much sustenance from neuroanatomical, brain imaging and functional studies conducted in animals and humans (Cummings, 1993; Arango et al, 2002). These investigations have shown a possible link between the onset of depression and brain areas as basal ganglia, mesencephalic nuclei, cortical and limbic districts (in particular, the amygdala and hippocampus), regions that have all been tightly associated with the control of mood tonus, emotion, psychomotor abilities, sleep, appetite and a variety of other stress-related functions. These regions have also shown high densities of monoamine biosynthesis enzymes (mesencephalic nuclei) and differential

expression patterns of monoamine receptor subtypes (Charney and Leger, 2010).

However, the monoamine hypothesis over time has been increasingly considered simplistic and incomplete, because it cannot fully explain complex clinical presentations (Dell'Osso et al, 2016, Catena dell'Osso et al, 2013), as well as the limited effectiveness of ADs that may arise in these and other cases.

Furthermore, currently available AD drugs are effective about 3 weeks after the beginning of the pharmacological treatment. Thus, if current ADs are surely golden treatments for the relief of severe depression, many pharmacological investigations are now more deeply considering their mechanisms of action in order to improve the AD efficacy. In particular, the complexity of monoamine receptors, their sensitization states or specific coupling to signal transduction pathways (Hamon and Blier, 2013), together the activity of some intracellular kinase pathways, are regarded as possible new targets to advance patients' pharmacological care (Yuan et al, 2016). In the meantime, other SGAs have been developed and introduced into the clinical practice that have much improved the flexibility of the applied treatments in respect to specific symptom presentations. These relatively new ADs are the atypical ones, also named multifunctional ADs, such as venlafaxine, bupropion, mirtazapine, trazodone, nefazodone and agomelatine. Atypical/multifunctional compounds are characterized by

their ability at interacting with multiple recognition sites. Precisely, they can block the reuptake sites of 5-HT and/or NE, and/or behave as agonists, antagonists or partial agonists on specific 5-HT and NE receptor subtypes (5-HT1AR, 5-HT2R, alpha adrenergic receptors) (Stahl, 2009a; 2009b), as well as on receptors for neurotransmitters as glutamate, melatonin, dopamine, histamine or others (Hamon and Blier, 2013, Metts et al, 2019).

1.2. Other pathogenetic mechanisms of depression

Despite the previously reported advancements in the neurobiology and pharmacology of depression, the molecular bases of this invalidating illness, including the number of susceptible and resistant genes involved in its development, are still elusive. Moreover, drug resistance and relapses of episodes continue to be a clinical defy. Thus, the implication of other systems besides monoamines or other neurotransmitters and signal molecules, as melatonin and glutamate/GABA, has been postulated, being under intense investigation. Over years of research, additional metabolic and biochemical pathways have been thought to act in an orchestrated manner with neurotransmission for the pathogenesis of mood disorders. Among these, we mention tryptophan fates, cytokines, abnormalities of lipid/phospholipid metabolism, homocysteine or abnormalities of purinergic system and adenylate cyclase turnover (Raison et al, 2006; Folstein et al., 2007; Duman and Voleti, 2012; Danzer, 2017; Lener et al,

2017; Bartoli et al, 2020). In particular, some studies on ADs have shown that the prolonged exposure to these drugs can activate genes involved in the expression of trophic factors (Levy et al 2018). Furthermore, it has been demonstrated that ADs can influence the plastic properties of neurons associated with morphological changes and synapse re-modelling or dendritic spine formation (Cavez-Castillo et al, 2019, Cai et al 2015). Experimental studies also showed the phenomenon of "shrinking" on rats subjected to chronic stress: animals displayed a reduction in the density and volume of dendritic spines and synapse arborization concerning neurons of the *nucleus accumbens*, hippocampus and prefrontal cortex (Tang et al 2019). This condition, improved by ADs, was also observed in depressed patients and in people subjected to chronic stress (Sapolski et al, 2001).

These studies have led to the neurotrophic hypothesis of depression. Among neurotrophic factors that have attracted more attention in depression research there are the *Brain-Derived Neurotrophic Factor* (BDNF, also called abrineurin) and the neurotrophin-related *Glial Cell-Derived Neurotrophic Factor* (GDNF). Indeed, an ever-growing evidence has shown neurotrophic alterations in mood disorders, particularly in depression (Castren, 2014). Many studies have focused in particular on BDNF, a protein of the neurotrophic family, widely expressed in the human brain, which increases and maintain neuroplasticity favoring

neurogenesis, neurite arborization, synaptogenesis and synapse re-modelling (Colle et al, 2017). BDNF has been found mostly expressed in the hippocampus and cerebral cortex, the main brain areas that control cognition, mood and emotion (Mattson et al, 2008).

Current neurotrophic hypotheses show that depression is associated with a reduced expression and deregulation of neurotrophins accompanied by the aberrant neurogenesis of discrete brain regions (Duman et al, 2006). Accordingly, the disturbance of BDNF and GDNF expression/functionality affects the survival and plasticity of dopaminergic, GABA-ergic, glutamatergic, cholinergic and serotoninergic neurons in the central nervous system (CNS), disrupting monoamine neurotransmission (Martinowich et al 2008). The neurotrophic and monoamine hypotheses of depression are thus interlocked.

Beside the neurotrophic hypothesis of depression, other investigations have shown the involvement of the hyperactivity of the neuroendocrine stress response, as the hypothalamic-pituitary-adrenal axis (HPA), revealing how this dysfunction can be implicated in melancholy, psychotic characteristics and suicide risk. Concurrently, other cellular and molecular studies have shown the implication of inflammation and immune response in the pathogenesis of depression, together with the finding of the association of genetic variants of peripheral factors as pro-inflammatory cytokines in patients (Strawberidge et al, 2017).

It has been also hypothesized that HPA axis, for genetic and environmental reasons, is dysfunctional and hyperactive in depression due to the loss of the negative feedback exerted on the hypothalamus and pituitary gland by Glucocorticoid/Cortisol Receptors (GRs). Indeed, it has been extensively reported that a percentage of depressed patients have increased levels of cortisol in urine, plasma and saliva, with pituitary and adrenal glands increased in volume (Nemeroff and Vale, 2005; Pariante, 2006). The presence of HPA axis alterations, hypercortisolemia, desensitized GRs, the increase of circulating cytokines, the stimulation of specific leukocyte subpopulations and the alteration of flogosis indexes as well as blood cardiovascular risk factors, such as Erythrocyte Sedimentation Rate (ESR), C Reactive Protein (CRP) or platelet reactivity, could be part of the pathogenetic mechanisms occurring in the depressed patient. Receptors for mineralocorticoids or MRs are also investigated for their role in the HPA axis impairment present in depressed patients, suggesting that the loss of HPA feedback inhibition can occur at multiple levels in the depressive pathology (Pariante and Lightman, 2008). In summary, the monoamine dysfunction remains one of the main pieces of the puzzle that defines the pathogenesis of depression but more and more researchers suppose that it is not always the only determinant as was once believed. Essentially, we have here briefly introduced and pointed out those medical hypotheses that sustain the complexity of the neurobiology of depression and, more

broadly, of mood disorders. These severe psychiatric conditions presumably result from and/or delineate malfunctioning brain-body communication networks, variably involving the physiology and plasticity of some brain centers, stress-related neuroendocrine cascades and cellular and/or molecular effectors at the crossroad with the immune system, inflammation components as well as energy/redox metabolism. Neurotrophins and BDNF are considered main players in such an integrated vision of depression.

Before dealing in more depth with the involvement of BDNF in the pathogenetic mechanisms of depression, we will first present the main biochemical characteristics of this protein and its receptor.

1.3. Neurotrophins and BDNF

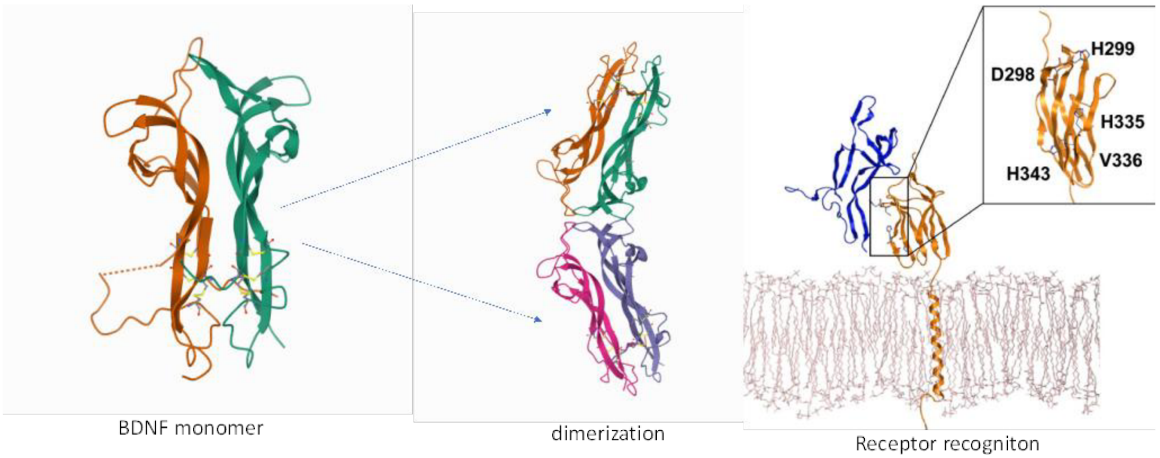


Figure 4. Tridimensional structure of BDNF: BDNF dimerization and receptor recognition (modified from Cell Press)

Neurotrophins are a family of closely related secretory proteins, originally identified as growth factors, which mainly display their action at the level of neuronal survival and plasticity in both the CNS and PNS. These proteins include the Nerve Growth Factor (NGF), the Brain-Derived Neurotrophic Factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Sahai et al, 2017). Here we describe the structural, transcriptional, translational and functional features of the neurotrophin BDNF, due to its presumed implication in the pathogenesis of mood disorders (Hashimoto et al, 2004). The physiological active isoform of the

human BDNF is an approximately 27 kDa homodimeric secretory protein (Radziejewski et al, 1992) encoded by the *BDNF* gene located on the chromosome position 11*p*14.1 (Silva de Olivera et al, 2019). Comparably to other neurotrophins, BDNF is characterized by a 55% homology of amino acid sequence with the other components of this protein family, including 6 cysteine residues involved in the intra-chain disulphide bridge formation (Isaacs, 1995). BDNF is a small protein, rich of basic amino acid residues, that can be separated both as a monomeric form (a polypeptide of about 120 aminoacidic residues) and as the physiologically active homodimer (Figure 4) or as a heterodimer, composed by a BDNF monomer and a monomer of another neurotrophin (eg. the BDNF/NT3 heterodimer) (Junbluth et al, 1993). The structural assembly of BDNF monomers, as for the other neurotrophins, is thought to be part of the complex BDNF signaling. The possible biological activity and physiological role of these neurotrophin heterodimers is under investigation, even at the pharmacological point of view (Dechant and Newman, 2013). From the comparison of the crystal structures of the different neurotrophins, as NGF (McDonald et al., 1991; 1993), monomers assume a typical arrangement of their β-folds and disulphide bridges, the so-called cystine knot motif (Isaacs, 1995). Interestingly, neurotrophins share the cystine knot structure with other secretory proteins and growth

factors, as the multifunctional cytokine Transforming Growth Factor- β2 (TGF- β2) and platelet-derived growth factor (PDGF) (Isaacs, 1995). The cystine-knot protein conformation is known to contribute to the formation of the non-covalent dimeric form of neurotrophins and BDNF, presumably attributing a greater number of specific functional features to these molecules as well as to their interaction with specific receptors. It should be also mentioned that if BDNF shares structural similarities with the other neurotrophins, each neurotrophin is uniquely characterized by a specific pattern of charged (basic or acidic) residues exposed on its surface. Differences between the individual neurotrophins are particularly manifest within those loops extended from the β-sheet core of the molecules that define the interaction with the specific receptor (Dechant and Neumann, 2013). These structural properties make neurotrophins endowed with a mix of hydrophilic and hydrophobic interactions together a limited capacity to diffuse and a tendency to stick to membranes, surfaces and other possible bonding sites (Dechant and Neumann, 2013). Beside its structural features, BDNF presents also a typical organization at the gene locus level. The human BDNF monomer is encoded by the *BDNF* gene, firstly described by the research group of Tonis Timmusk, Department of Gene Technology of the University of Tallinn (Estonia). The *BDNF* gene spans about 70 Kb and is composed of 11 exons and 9 promoters (Pruunsil

et al, 2007), being able to generate a variety of BDNF transcripts in relation to the cell type.

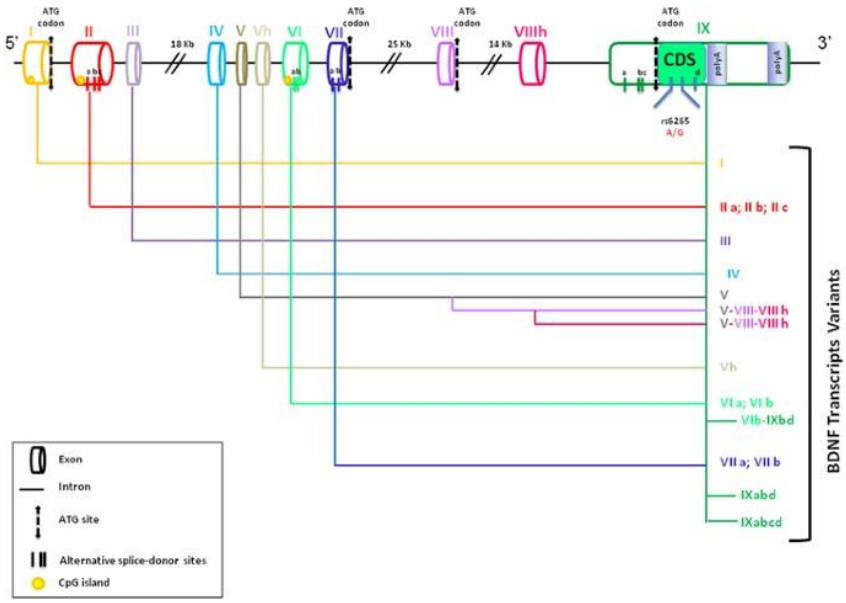


Figure 5. The human BDNF gene, its possible transcription initiation sites and the corresponding different transcripts (from Cattaneo et al., 2016).

Indeed, transcript variance is not only area-dependent, but it is also present within a same brain area, and even within neurons, as reported in cortical neurons (Kaneko et al, 2012; Hempstead, 2015). Moreover, the RNA messengers containing exons II, III, IV, V and VI are prevalent in the CNS level, while the others being rather transcribed in periphery (Pruunsild et al., 2007).

In Figure 5 is reported the *BDNF* gene organization and all the possible coding transcripts that can be produced by *BDNF* gene expression mechanisms. All transcripts are similar for their 3' encoding region in exon IXd but are distinguishable by 5' UTR sequences (untranslated region).

Substantially, the 3′exon encodes all or most of the protein, depending on the 5′exon used. The most recognized transcription mechanism consists in the conjugation of exon IX with another upstream exon (I-VIIIh) generating mature transcripts including only the downstream region of exon IX, or the IXd region. Exons I, VII and VIII display internal ATG codons used as possible translation initiation sites, forming a protein product with a longer N-terminal portion. Inside exons II, V and VI are also present splicing sites that produce transcripts similar for the coding region but differing for the 5'UTR size. Such a great number of exons, transcription initiation sites, different promoters and splicing sites imply the complex regulation activity of this gene locus. Moreover, the human *BDNF* gene is capable to produce natural antisense mRNA or non-coding anti-*BDNF* complementary to the sense *BDNF* transcript, a mechanism representing an additional post-transcriptional way to control BDNF protein expression (Pruunsild et al., 2007). These *BDNF*-anti-*BDNF* mRNA duplexes and these bidirectional transcription mechanisms play a main role in regulating protein expression in the brain (Pruunsild et al, 2007). The *BDNF* gene transcription is also regulated by CREB signaling, epigenetic mechanisms and microRNAs (Tao et al, 1998; Lubin et al, 2008; Caputo et al, 2011; Cattaneo et al, 2016; Khani-Habibabadi et al, 2019).

At the translational level, the BDNF protein, as other secretory proteins, is firstly synthesized in the endoplasmic reticulum, leading to a precursor

protein, the pre-pro-BDNF, a peptide with a molecular weight of about 27 kDa (Cattaneo et al, 2016). Then, the 18 amino acids pre-domain or signal peptide is immediately cleaved to produce pro-BDNF, the precursor form of the neurotrophin consisting of a pro-domain of 129 amino acids and a mature domain of 118 amino acids (Hempstead, 2015). The pro-BDNF is subsequently transported to the cis cisterns of the Golgi apparatus complex and then to the trans-Golgi through the vesicular transport (Figure 6). In the trans-Golgi the pro-BDNF is sorted into either the constitutive or the regulated secretory pathways.

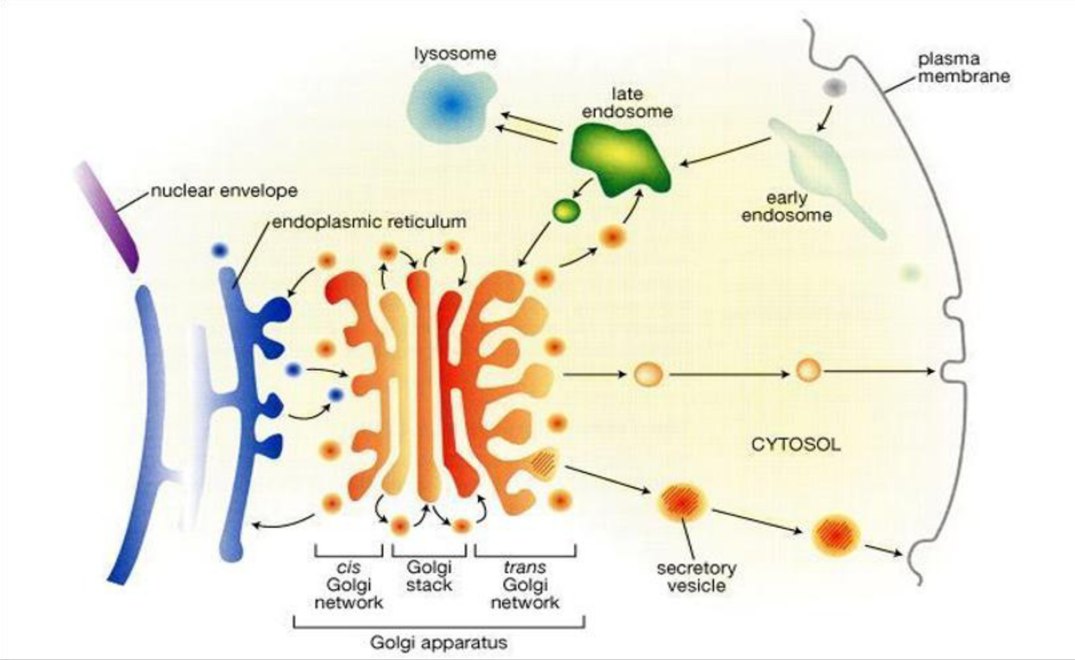


Figure 6. Intracellular organization of the Golgi apparatus.

For the constitutive secretion, the pro-BDNF signal peptide is cleaved by specific proteins, including endoproteases as furin, belonging to the trans-

Golgi network. The mature form (about 14KDa) is internalized into vesicles transported to the plasma membrane for exocytosis and secretion. For the regulated secretion, the signal peptide cleavage occurs later: pro-BDNF is internalized in granules that are transported from the Trans-Golgi to the plasma membrane. At this stage, proteolitic enzymes, as pro-protein convertases, transform pro-BDNF into the mature BDNF form (Cattaneo et al, 2016). The mature form then accumulates at the membrane level and BDNF is secreted only after specific stimuli (Lessmann et al., 2009). Otherwise, released pro-BDNF can be cleaved extracellularly by plasmin, matrix metalloproteinases and other factors. Vesicular secretion can involve both pro-BDNF and mBDNF, with the amount of secreted mBDNF or pro-BDNF depending on the type and activity of convertase involved (Mowla et al, 2001). The mature form is the one recognized and bound by the TrKB receptor, a class of tyrosine kinase receptors involved in the regulation of CNS plasticity (Serra-Millàs, 2016). Neurotrophins and their receptors have a very high potential to contribute to the CNS and PNS repair after injury and damage (Meeker and Williams, 2015).

The membrane receptors for neurotrophins comprise three receptor tyrosine kinases belonging to the tropomyosin receptor kinases (Trk) family, which autophosphorylate after activation, and the p75 neurotrophin receptor, $p75^{NTR}$. The $p75^{NTR}$ is a molecule that groups within the large family of homologs of death-domain containing receptors for the cytokine

Tumor Necrosis Factor (TNF), which do not display an intrinsic catalytic activity. Each homodimeric form of each neurotrophin binds to a specific Trk receptor: NGF activates TrkA, mature BDNF and NT-4/5 activate TrkB whereas NT-3 activates TrkC (Kaplan e Miller, 2000); as concerns the p75 receptors, many in vitro investigations have shown the binding preferential affinity and specificity for secreted pro-BDNF (Kowiański et al. 2018) (Figure 7).

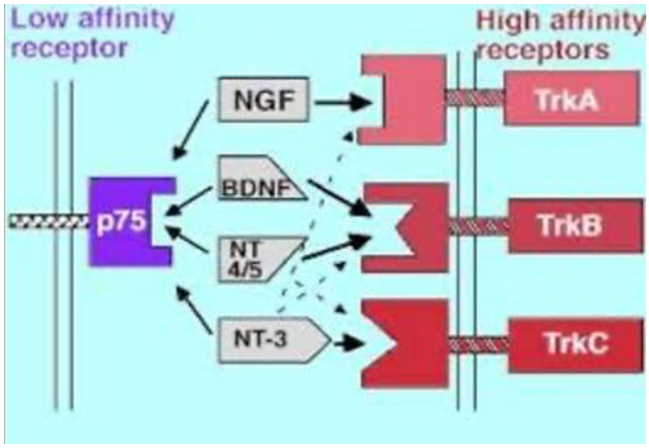


Figure 7. Affinity and specificity of neurotrophins for their receptors.

Each neurotrophin binds to its TrK receptor provoking the receptor dimerization and then triggering the tyrosin-kinase activity. There are 10 conserved tyrosine residues in the intra-cytoplasmic domain of each Trk, receptor, 3 of them present inside the autoregulated loop of the enzyme kinase activity domain. The phosphorylation of these amino acids promotes the further activation of the kinase, while the phosphorylation of the other

residues transduces the signal to protein adapters coupled to intracellular cascades, as the Ras/ERK (extracellular signal regulated kinase), the PI3K/Akt (Phosphatidylinositol-3-OH kinase) and PLC-γ1 (phospholipase C) pathways (Kaplan and Miller, 1997; Pawson e Nash, 2000).

These signal transduction pathways are responsible of the biological function of each neurotrophin through the activation of transcription factors and the modulation of cell gene expression. The molecular relationship between Trk and p75 receptors is complex and incompletely understood, but it is known that the resulting cellular effects are highly ligand and cell specific. Under physiological conditions the BDNF binding to Trks or p75 receptors regulates cell survival. Indeed, p75 receptors have been found linked to both pro-apoptotic and anti-apoptotic features. Under physiological conditions, the anti-apoptotic signal deriving from the neurotrophin-TrK receptor complex antagonizes the pro-apoptotic action originating from the binding to the p75 receptors, synergistically acting with the p75 anti-apoptotic signal (Mazzoni et al., 1999; Aloyz et al., 1998; Maggirwar et al., 1998; Hamanoue et al., 1999). Under pathological conditions, the pro-apoptotic effect deriving from the interaction with p75 is not counteracted by the anti-apoptotic counterpart (pro/anti-apoptotic disequilibrium). The p75 neurotrophin receptor triggers signaling by non-covalent binding to intracellular molecules and the resulting cellular effects

of this signaling cascade are diverse. Moreover, if the p75 receptor function has not been still fully understood, it appears ever clearer that the biochemical and functional interactions between the Trk and p75 receptors belong to the fine-tuning of neurotrophin-receptor interactions determining distinct cellular responses and adaptation to different stimuli (Dechant and Neumann, 2013).

1.3.1 BDNF in the CNS

In the CNS, BDNF is the most widespread neurotrophic factor. It has been overall localized in neurons (Murer et al., 2001), but glial cells can express this neurotrophin under metabolic stress (Ceccatelli et al., 1991; Batchelor et al., 1999). Nakajima and coauthors (1998) have reported that glial BDNF is taken from the extracellular milieu through a truncated form of the TrkB receptor located in the plasma membrane that is capable to internalize BDNF without activate ensuing signal transduction pathways. The highest levels of CNS BDNF have been found in the neocortex, striatum, pro-encephalic nuclei, hippocampus, hypothalamus, brain stem and cerebellum (Murer et al., 2001). The expression pattern of BDNF mRNA is similar to that of the protein, even if the complexity of its transcriptional and translational mechanisms has brought to report the protein presence without appreciable amounts of its transcripts (Altar et al., 1997; Baquet et al., 2004). The neurotrophin BDNF has been linked to

both anterograde and retrograde transport in the CNS. The cortical-striatal network has been linked to the anterograde transport of BDNF from the cortex (II-III e IV-V layers) (Fusco et al., 2003; Baquet et al., 2004). The BDNF retrograde transport has been also observed (Altar et al., 1998), starting from dendrites and axons to the soma, a mechanism mediated by TrkB receptors present at the pre-synaptic level (Heerssen et al., 2004). The retrograde transport of BDNF seems to exert protective actions on BDNF-producing neurons by means of nuclear interactions linked to cell survival and counteraction against toxic stimuli (Lindholm et al., 1994; Heerssen et al., 2004).

BDNF central function has been linked to neuronal plasticity, learning, memory and Long-Term Potentiation (LTP) (Schinder et al., 2000; Schinder et al., 2002). The neurotrophin is also under investigation for its glial expression, as aforementioned, and therefore for its potential myelinization properties (Fletcher et al, 2018). The tight interaction observed between monoaminergic neurotransmission and BDNF in the brain is thought to correlate with a main role of this neurotrophin in neuropsychiatry disorders (Altar et al, 1998; Martinovich and Lu, 2008). The impaired BDNF signaling can be a vulnerability factor of these pathologies, by impacting neuroplasticity, inflammation patterns or hypothalamic–pituitary–adrenal axis action that are altered in psychiatric disorders (Cattaneo et al, 2016).

BDNF has been found also at the peripheral level. The physiology and distribution of peripheral BDNF is still under investigation, since many peripheral tissues have been found to produce BDNF and to express its receptor, implying that circulating levels of this neurotrophin cannot exclusively derive from the brain. At the same time, even if there is not a complete consensus on this assumption, neurotrophins can cross the blood-brain barrier (BBB), and the brain and serum levels of these proteins have been found positively correlated to each other (Numakawa et al, 2010).

1.3.2 Peripheral and platelet BDNF

The BDNF and TrkB mRNAs are expressed in several non-neuronal tissues, including muscle, thymus, heart, liver, vascular smooth muscle cells, lung and spleen (Nakahashi et al, 2000). BDNF is also produced in monocytes, lymphocytes and eosinophils (Elding et al, 2004). The latter cells produce BDNF *via* the autocrine system and utilize it to evoke and extend the allergic reaction (Nockher et al, 2005). BDNF has been shown to play a pivotal role in the growth, survival and chemoresistance of tumor cells in various types of cancers, including Hodgkin lymphoma, myeloma, hepatocellular carcinoma and neuroblastoma (Yang et al, 2006). BDNF also mediates the survival and activation of endothelial cells through its interaction with TrkB (Kermani et al, 2005), suggesting its potential role in angiogenesis. Many non-neuronal cells, such as smooth muscle cells,

fibroblasts and astrocytes, may not express the molecular components of the regulated secretory pathway and therefore only secrete neurotrophins constitutively.

Furthermore, platelet BDNF represents probably the most relevant component of the peripheral neurotrophin: platelets are indeed the main storage source of BDNF secreted from all other tissues (Bus et al, 2011). Platelets are small un-nucleated blood cells with a size of approximately 3 μm deriving from megakaryocytes (MK) in the bone marrow, from which they are released into the bloodstream. Resting platelets have a rounded-discoid shape, while relevantly changing their morphology upon activation. They circulate for an average of 7-10 days and act to stop bleeding. Platelet surface proteins enable them to adhere to each other and to blood vessel walls. They possess several important organelles, microtubules, mitochondria, endoplasmic reticulum, the Golgi apparatus and 3 types of secretory granules: 1) α-granules - the most abundant; 2) dense granules; 3) lysosomes. Moreover, platelets also have an open canalicular system (OCS) connecting microtubules and the plasma membrane that is attracting a great attention for the physiology of these blood cells (Selvadurai and Hamilton, 2018).

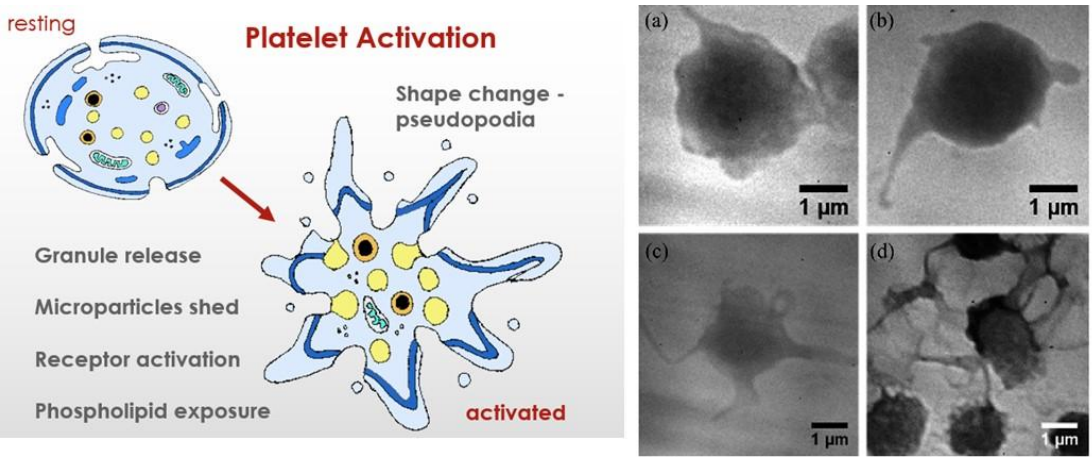


Figure 8: Resting and activated platelets. Left panel: platelet rearrangements with activation (from *memorang: the physiology of coagulation*); right panel: the different grade of platelet shape changes during progressive activation (from Yang et al, 2014).

Under a variety of precise stimuli, as thrombin, ADP, arachidonic acid, serotonin and epinephrine, platelets can change their metabolism and shape, undergoing a more or less pronounced activation state, progressively becoming sticky and releasing factors from the different granules (Serra-Millàs, 2016) (Figure 8). The activation state of platelets induces not only deep morphological changes but also intense biochemical variations and rearrangements (Giannaccini et al, 2010). Platelets have been primarily related to coagulation, vascular repair functions and blood homeostasis, but there are more and more evidence indicating their active participation to inflammation and immune response (Serra-Millàs, 2016). Indeed, they can release vascular permeability factors and promote

chemotaxis of neutrophil granulocytes. Platelets release numerous inflammatory mediators that are not involved in hemostasis, as IL1, P-selectin, Platelet Factor 4 (PF4 or CXCL4) and MIP-1α (CCL3) (Thomas and Storey, 2015). Most of these mediators are stored in platelet α−granules, modifying, after release, leukocyte and endothelial-mediated responses, while activating bridges and networks between platelets, leukocytes and endothelium (Thomas and Storey, 2015). This suggests a role of platelets as active modulators of the interactions between monocytes, neutrophils, lymphocytes and the endothelium, in the framework of inflammation and both innate and adaptive immune responses (Semple et al, 2011). In should be also pointed out that platelets are under continuous investigation since years in the field of biological psychiatry, as they are considered a kind of "window to the brain", containing enzymes, carrier proteins, neurotrophins, neurotransmitters and receptors also active in the CNS and synapses (Stahl, 1977; Da Prada et al, 1998; Leiter and Walker, 2019).

Ninety percent or more of blood BDNF is stored in platelets and their α-granules (Fujimura et al, 2002). A close relationship has been indeed found between BDNF and platelets under physiological conditions. In addition, there is an approximately 100- to 200-fold estimated difference between the plasma and serum levels of BDNF because platelets release BDNF

during the clotting process (Lommatzsch et al, 2005). It has been demonstrated that the amount of BDNF in serum is nearly identical to that found in washed platelet lysates (Fujimura et al, 2002). Thus, the difference between the serum and plasma BDNF levels seems to reflect the amount of BDNF stored in circulating platelets, and the BDNF in platelets might serve as a reservoir for circulating BDNF. The BDNF in platelets may play a role during tissue trauma or nerve injury, releasing their contents into the circulation at the site of the injury (Radka et al, 1996).

In the first studies, BDNF was not expressed in or produced from the megakaryocyte precursor cell of the mature platelets, in which protein synthesis is generally absent, but was sequestered from the circulation (Nakahashi et al, 2000). Recently, one study found that BDNF is present in the cytoplasm of platelets and in α-granules, suggesting that BDNF is either produced in platelets or passed down from MK (Tamura et al, 2011). A second study found that a megakaryocyte (MK) progenitor line, the MEG-01 cell line, produces BDNF upon thrombopoietin stimulation (Kaushansky et al, 2006), and the levels of BDNF in MEG-01 cells increased in a time-dependent manner (Tamura et al, 2012). This was the first report of the production of BDNF in a megakaryocyte cell line, a finding that led to the hypothesis of BDNF as a cell proliferation agent of the megakaryocyte lineage *in vivo*. It is also likely possible that there is a receptor for BDNF on the MEG-01 cell surface, but the TrkB receptor has

not been detected yet in MEG-01 cells or human platelets (Burnouf et al, 2012). Therefore, there should be an unidentified novel receptor in MKs or platelets. Some agonists, such as thrombin, collagen, Ca^{2+} and shear stress, could induce a rapid release of BDNF from platelets. Even with agonist stimulation, only approximately half of the BDNF in platelets is secreted, which suggests that platelets maintain a stable pool of BDNF as a buffer system (Bus et al, 2011). Anyway, there is still little knowledge about the relationship between BDNF and platelets: only a few studies have assessed issues such as the platelet activation mechanism that allows the release of BDNF and BDNF localization within platelets. The rate of BDNF release parallels the secretion of 5-HT from the dense granules and PF4 from the α-granules, although a greater proportion (90%) of the total 5-HT and PF4 are released compared to BDNF. Because only 40%-60% of the total content of platelet BDNF is released by maximal platelet activation, some authors postulated that platelets either have a non-releasable pool of BDNF, or that the released BDNF is sequestered by binding to a transporter or receptor on the platelet surface (Fujimura et al, 2002). Such binding could promote the internalization of BDNF by the platelets, as has been reported for 5-HT (Rendu et al, 2001) and for the BDNF astrocyte recycling (Alderson et al, 2000). The binding of BDNF to washed platelets was confirmed by FACS (cytofluorometric) analysis, microscopy as well as confocal microscopy, suggesting that platelets bind exogenous BDNF

(Fujimura et al, 2002). However, a recent study found two different locations of BDNF intra-platelet storage: the α-granules and the cytoplasm. Using immunoelectron microscopy, BDNF was clearly detected in the same fractions of P-selectin, a α-granule marker, and protein kinase C, a cytoplasmic marker (Tamura et al, 2011). BDNF is predominantly released from platelets through the activation of protease-activated receptor 1 (PAR1) during thrombin stimulation, along with the stimulation of vascular endothelial growth factor but not endostatin. Platelets stimulated with concentrations of the PAR1-activator peptide have shown a dose-response curve of BDNF release, exhibiting a two-phase pattern. The first phase is a drastic release phase occurring at a low-level of activation, which is completely inhibited by a pretreatment with Prostaglandin (PGE1), suggesting that this phase depends on calcium mobilization. The second phase is a mild release phase arising at a high-level of activation, which is not affected by the PGE1 pretreatment, suggesting that this step depends on the activation provoked by a PGE1-independent signal. BDNF response curve was found similar to that of PF4 (Tamura et al, 2011). There was no significant difference in BDNF release between the non-stimulated and the PAR4-AP-stimulated cells. PAR1 activation promotes the release of proangiogenic factors and these results support the action of BDNF as a proangiogenic factor (Italiano et al, 2009). Moreover, the α-granule BDNF component is released upon platelet activation, whereas the cytoplasmic

BDNF is not (Tamura et al, 2011). The maximum BDNF release is approximately 30%-40% with stimulation and the remaining 70% of BDNF is equivalent to that found in the cytoplasm, which is not released (Fujimura et al, 2002).

1.4 BDNF and the pathogenesis of depression

Many investigations have suggested BDNF as a main player in the pathophysiology of depression. Reduced BDNF gene expression and protein levels have been reported in many animal models of depression as well as in patients. The investigation of rodent models of depression, represented by exposition to a variety of stressful conditions and stimuli as social defeat, maternal deprivation or prenatal stress exposure, has shown that the induced depressive-like behavior is related to a reduced gene expression of BDNF mRNA in different brain regions, including the hippocampus and cortex, whereas AD drugs are able to upregulate BDNF expression in animal models (Cattaneo et al, 2016). These BDNF alterations have been linked to the reduction of the hippocampus size, a key region in the control of emotions in humans, providing the basis of the neurotrophin hypothesis of depression (Duman 2006). Results on animal models have been paralleled by investigations conducted in depressed patients, revealing the BDNF reduction both in post-mortem brains and in peripheral blood samples (Pandey et al, 2008; Sen et al, 2008).

Electroconvulsive therapy as well AD treatments have shown to increase BDNF in blood samples from patients suffering of severe depression (Polyakova et al, 2015; Cattaneo et al, 2016).

From preclinical studies a reduced BDNF activity, increased apoptosis and decreased hippocampal neurogenesis were found in association with depression and exposure to chronic mild stress (Filho et al, 2015). Besides, many other studies have appraised BDNF expression and amounts as possible peripheral biomarker of depression (Rana et al, 2020).

Some data show that depressed patients have lower BDNF plasma levels than controls suggesting that plasma BDNF may represent central BDNF and may be associated with clinical features of major depressive disorder (Polyakova et al, 2015; Klein et al, 2011; Dell'Osso et al, 2010).

Recent meta-analyzes have also shown that BDNF is more pronouncedly reduced in severe depression and increased during AD treatment even in the absence of clinical remission (Molendijk et al, 2014).

Some studies have shown low BDNF levels associated with more severe melancholic characteristics, psychomotor retardation or slowdown and insomnia (Alves et al, 2018; Monteiro et al, 2017).

However, if none of defined subsets of symptoms of depression were yet significantly associated with circulating peripheral BDNF, a negative correlation between BDNF (plasma or serum BDNF) and the number of

depressive episodes with melancholic features was found (Kotan et al 2012 Ceroleo 2019).

Low levels of BDNF have been observed in the brain of suicide subjects, depressed patients and stressed animals (Allen et al, 2015, Banerjee et al 2013). Negative environmental effects like psychological stress, chronic foot shocks and chronic social defeats also decrease BDNF levels in the hippocampus (Jiang et al, 2015). Additionally, as aforementioned, the administration of ADs increases BDNF levels, confirming a pathogenetic role of this protein in depression (Yoshikimura et a, 2010). Moreover, this hypothesis is corroborated by the finding that a high baseline BDNF level is associated to a positive response to ADs (Kurita et al, 2012).

Another hypothesis supports the fluctuation of peripheral BDNF levels in relation to different patterns of symptoms defining depressive episodes: for instance, an increase of circulating BDNF has been also associated with depression familiarity (Knorr et al, 2017) and mixed state episodes (Piccinni et al, 2015). According to DSM-5 in major depressive episodes the specifier with mixed characteristics is characterized by the presence of at least three manic/hypomanic symptoms present almost every day in most days of a depressive episode: high mood, expanded mood, hypertrophic self-esteem or grandiosity, increased talkativeness, flight of ideas, involvement in activities with the potential for harmful consequences, decreased need for sleep.

A recent report has shown the ability of the tricyclic AD imipramine to significantly increase the hippocampal BDNF levels in a rat model of depression induced by chronic unpredictable stress (Hazra et al, 2017). Yoshimura and co-workers revealed that a treatment with ADs or atypical antipsychotics at low doses can increase the plasma BDNF levels in patients with MDD or bipolar disorder (Yoshimura et al, 2010)

From these and many other investigations, peripheral BDNF was proposed as a potential diagnostic, prognostic, and therapeutic biomarker for mood disorders, particularly associated with disease severity and response to AD treatments (Dimitriadis et al, 2019). However, results are still controversial, non-conclusive and needing further investigation, and this protein is being actually rather considered a generic biomarker of depression (Poliakova et al, 2015).

It appears clear that the relationships between neuronal BDNF and its peripheral counterpart still require further investigation. In any case, the link between neuroendocrine systems, HPA axis and the immune/inflammatory response can be a key aspect to understand the regulation of BDNF release and storage into the bloodstream. This hypothesis deserves confirmation, in order to clarify if the BDNF platelet reservoir and BDNF extracellular levels can reflect, at different levels, the functionality of brain-periphery cross-talks actives in adaptation to stressors. Since these paths are supposed to be altered in depression,

peripheral BDNF variations in patients could be illness indices, allowing a more careful patients' monitoring.

1.5 Inflammation in depression and correlation with BDNF

Several studies have showed that inflammation may interfere with the physiological 5-HT signaling, the neurotrophin synthesis, and the HPA axis functioning (Miller and Raison, 2016, Amodeo et al, 2018). Recent studies highlighted the possible role of proteins defined as "inflammasome", a cytosolic protein complex, usually generated in response to infection, but also to many other factors, and involved in a pro-inflammatory state, in oxidative stress and in the onset of depressive symptoms (Uint et al, 2019, Alcocer et al, 2014).

Recent studies have found increased levels of various pro-inflammatory markers in blood and cerebrospinal fluid (CSF) of depressed patients, including interleukin 1(IL-1), IL-6, interferon- (IFN), tumor necrosis factor alpha (TNF), CRP and neurotoxic factors such as superoxide anion and nitric oxide (NO) (Rosenblat JD et al, 2017, Muller et al, 2011).

Studies show that IL-6 and CRP appear frequently and reliably elevated in depression (Haapakoski, et al 2015). IL-8 has been reported elevated in subjects with severe depression, while the immunomodulatory IL-4 and IL-2 have decreased in line with symptom remission (Baune et al, 2012).

TNF-alpha may only reduce with treatment in responders (Strawberidge et al, 2015). From these studies, it has been suggested that inflammatory responses appear aberrant in approximately one-third of patient with depression (Krishnadas et al, 2015, Raison et al, 2011). The inflammatory system, however is extremely complex and there are numerous biomarkers representing different aspects of its functionality and activation. Recently additional novel cytokines and chemokines have been found abnormally expressed in depression. Amongst these factors, we mention IL-1α, IL-1β, IL-7, IL-5, IL-16, IL-17 and IL-12p70.

It is worthy to mention that also traditional inflammation parameters as serum levels of C-Reactive Protein (CRP) have been related to decreased motivation and psychomotor retardation, but also to symptoms of anxiety in psychiatric patients (Miller, 2020). The presence of inflammation seems to reduce the response to ADs, as observed in a recent study in which almost half of resistant subjects, with failure response to conventional treatments, showed a CRP level >3 mg/L (with inflammatory status) (Arteaga-Henriquez et al, 2019). High CRP levels were correlated with a positive history of depression and treatment resistance, child abuse, or other comorbid medical diseases and metabolic syndromes (Arteaga-Henriquez et al, 2019). Even some polymorphisms of pro-inflammatory

genes, including genes encoding for IL-1, TNF, and CRP, have been linked to depression and treatment response (Lezheiko et al, 2018).

T-cells seem to be able to protect laboratory animals from stress and depression (Maes et al, 1999). By transferring T-cells to chronically stressed animals, an AD-taking phenotype was obtained. It was associated with the activity of pro-inflammatory cytokines released by T-cells in the meningeal space, particularly IL-4. Indeed, IL-4 levels were related to the stimulation of BDNF production by astrocytes and to the shift of microglia immune responses towards a neuroprotective M2 phenotype, together with an increase in hippocampal neurogenesis. Interestingly, regulatory T (T-reg) cells might also modulate inflammatory pathways and ensuring neuronal support during stress (Amodeo et al, 2018).

Moreover, according to the monoamine hypothesis of MDD, different factors can cause an alteration in monoamine activity, such as decreased plasma levels of the essential amino acid L-tryptophan (L-TRP). Low plasma L-TRP levels have been related to an increased production of IL-1β, TNF-α, and INF-γ, which would promote the degradation pathways of L-TRP named kynurenine shunt (Rosenblat et al, 2017). Other studies have shown that inflammatory patterns, impaired tryptophan metabolism and oxidative stress are related to HPA axis malfunctioning and altered cortisolemia in depression (Czarny et al, 2018). Neuroendocrine

dysfunctions in depressive patients can increase circulating cytokines, stimulate specific leukocyte populations and impair cardiovascular risk factors (as CRP) and platelet reactivity, possibly reflecting shared pathogenetic mechanisms between depression and inflammation (Figure 9). Moreover, many studies have shown that inflammation is involved in the pathophysiology of depression thanks to empirical evidence. First, inflammatory markers and HPA axis dysfunction are frequent in major depression, together the increase of medical conditions with predominant inflammation features, as multiple sclerosis or rheumatologic conditions including rheumatoid arthritis, chronic fatigue syndrome and fibromyalgia.

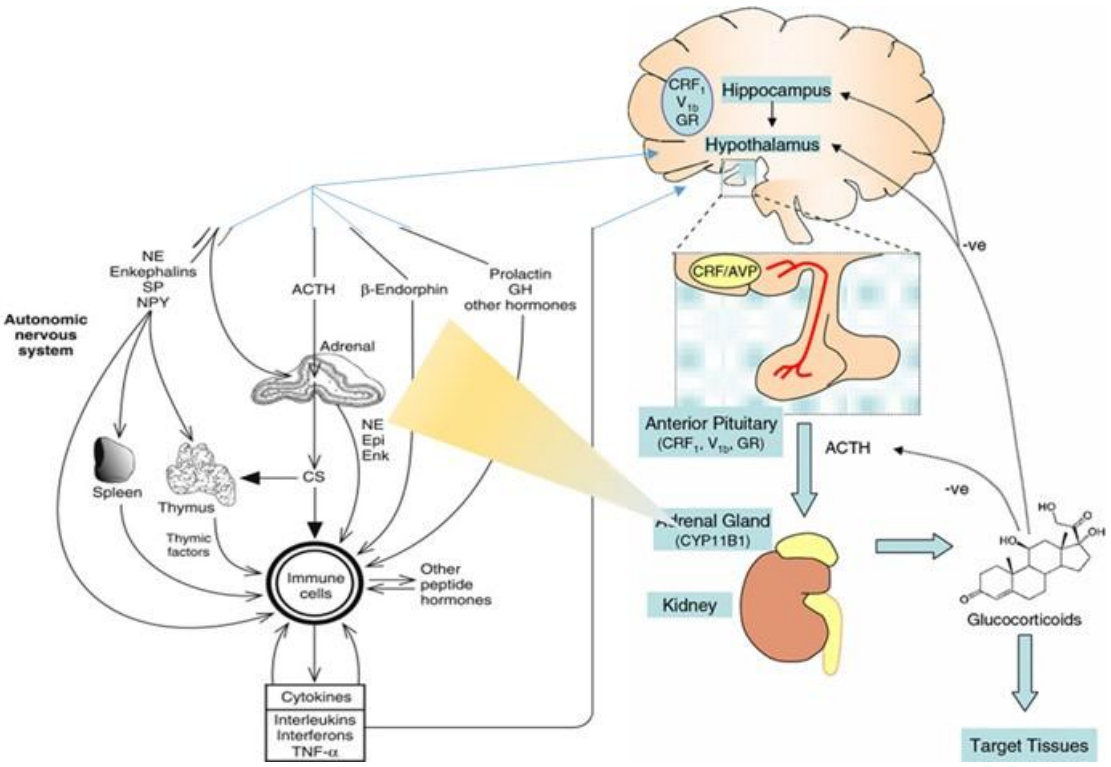


Figure 9: Hypothalamus-hypophysis-adrenal (HPA) axis and inflammation (modified from Thomson and Craighead, 2008; Dunn et al, 2007)

Then, the observation of depressogenic effects produced by cytokine immunotherapy such as interferon-α in those subjects undergoing these treatments (Capuron et al, 2011, Howren et al, 2009, Krishnadas et al, 2012, Muthuramalingam et al, 2016).

It has also been shown that physical symptoms of depression are related to inflammation, in particular the "sickness behavior" (malaise, fatigue, loss of appetite, muscle and joint pain) effect of pro-inflammatory cytokines in the brain (Danzter et al, 2009) .

Studies on C reactive protein show that higher levels are associated with greater severity of symptoms, cognitive symptoms and suicidality, especially in women (Kölher-Forsberg et al, 2017a).

Furthermore, high levels of inflammation are associated with a subtype of depression, particularly atypical depression, which is also associated with the metabolic syndrome (Strawbridge et al, 2017).

Moreover, there is a correlation with BDNF. The effects of stress on BDNF mRNA expression in the hippocampus appears to be dependent on several factors: the type of stressor and its intensity, frequency, duration, number of exposures. Stress increases plasma corticosterone levels (Haque et al, 2013) and several studies reported an inverse correlation between corticosterone levels and BDNF expression in the hippocampus. Also, corticosterone administration has been reported to decrease BDNF levels in hippocampus

(Lee et al, 2014). If sleep deprivation, which is one of the symptoms of depression, appears to be associated with dysregulated levels of BDNF (Alzoubi et al, 2013), on the other hand training and ECT in depressed patients has been shown to increase or even restore BDNF levels.

Recent findings reported that BDNF is a key regulator in the neuroimmune axis regulation (Jin et al, 2019). Stress and its associated activation of inflammatory cytokines might have a negative effect on neurogenesis and neuroplasticity (Wohleb et al, 2016). The reduction in BDNF in hippocampus and cerebral cortex, caused by the administration of pro-inflammatory cytokines or the cytokine-inducer lipopolysaccharide (LPS) or INF-α, was found to produce anxious and depressive symptoms (Fruhauf-Perez et al, 2018). Inflammation inhibits the BDNF/trkB expression, while pro-inflammatory cytokines influence the phosphorylation of the BDNF receptor (TrkB) interfering with BDNF signaling (Cortese et al, 2011).

In rat models, the injection of pro-inflammatory substances led to increased IL- 1, IL-6, and TNF-α expression and decreased BDNF-mRNA expression, particularly in the hippocampus (Gibney et al, 2013). IL-1 is a pro-inflammatory cytokines that seems to influence hippocampal cytogenes and neurogenesis by interacting with its IL-1R1 receptor, by activating NF-

kB, and by stimulating the glucocorticoid secretion in response to environmental stress (Pariante et al, 2017).

In humans, patients under treatment with IFN-γ showed reduced systemic BDNF levels in combination with increased levels of the cytokines IL-1 and IL-2 (Lotrich et al, 2013). Some studies suggested that an increase in IL-1 concentrations in depressed patients can be associated with a decrease in BDNF concentrations and also BDNF signaling pathways, reporting an altered BDNF-TrKB receptor efficiency, causing BDNF resistance despite its normal or high levels (Carlos et al, 2017).

This could explain the opposite results obtained on the relation between inflammation and neurotrophins. Additionally, it can explicate why in some cases of depression, despite drug treatment, there are high levels of BDNF.

According to a recent study, even lipoproteins could decrease brain BDNF levels in the prefrontal regions and in the hippocampus, while increasing them in the *nucleus accumbens* (Kauppi et al, 2014).

Moreover, an increased brain microglia was observed in depressed patients. Microglia could regulate the release of BDNF and reduce the expression of BDNF and its affinity to the tyrosine receptor kinase B (TrkB) (Jin et al, 2019).

Conversely, BDNF can promote glia growth and proliferation (Zhang et al, 2017, Tu et al, 2017) thus contributing to the chronic inflammatory state of

the brain and neurotoxicity observed in several mood disorders (Muller et al, 2019).

A recent study (Jeenger at al, 2018) have shown that patients with a first depressive episode and recurrent depressive disorder had significant lower serum concentrations of BDNF together higher levels of IL-2 in comparison with the control group, while no significant difference was reported for CRP levels. CRP levels were found significantly higher in patients with 2 or more stressful life events.

However, despite several ongoing studies, there are still no reliable data on the correlation between inflammatory events and the expression of specific BDNF genes (Kauppi et al, 2014).

1.6 Depression and metabolic status

In the framework of the search of depression biomarkers, we cannot forget that obesity and metabolic syndrome are conditions often associated with inflammation and depressive disorders, especially major depression. Obesity and metabolic syndrome can increase the risk of major depression and vice-versa. The production of cytokines in adipose tissue (TNF-α and IL-6) gives rise to a pro-inflammatory state in obesity (Luppino et al, Pan et al 2012)

The major biological markers associated with metabolic syndrome include leptin, adiponectin, ghrelin, triglycerides, high-density lipoprotin (HDL),

glucose, insulin and albumin. Associations of many of these markers with depression have been studied: for example, leptin and ghrelin can be reduced in depression compared to controls and may increase during AD treatment or in remission (Lu et al, 2007, Wittekind et al, 2015). Insulin resistance can be increased in depression (Kan et al, 2013).

The lipid profile, including HDL cholesterol, can be altered in many patients with depression, including those without comorbid physical illnesses (Liu et al, 2016).

In addition, associations between depression and hyperglycemia and hypoalbuminemia have also been reported (Lustman et al, 2000).

Some studies show how the increase in appetite in the context of a depressive episode is positively associated with BMI, waist circumference, metabolic markers, C reactive protein and TNF-α. This feature is characteristic of a form of immune-metabolic depression, in particular the atypical depression (Lamers et al, 2017). Atypical depression according to DSM-5 involves the presence of mood reactivity plus at least two of the following symptoms: increased appetite or weight gain, hypersomnia, leaden paralysis and interpersonal rejection sensitivity. A study showed how women with atypical depression were more likely to have a higher fat mass than controls and, in geriatric depression, those with atypical forms had most metabolic dysregulation (Vogelzangs et al, 2014). Other studies

have found a low-grade inflammation profile in the atypical depression subtype only (Lames et al, 2013, 2016), revealing significantly higher levels of inflammatory markers, BMI, waist circumference and triglycerides and lower high-density lipid cholesterol (Lamers 2013). It is thus possible that the potential biological subtype of MDD, characterized by inflammation and metabolic syndrome patterns, may overlap with the clinical subtype of MDD defined by atypical depressive symptoms.

The implications of the prevalence of inflammation and metabolic syndrome can explain the high-risk rates of medical comorbidities and poor health outcomes in person with depression. These features would also outline poorer treatment outcomes, being indicative of specific biological subtypes of depression. A recent research has begun to identify potential novel treatment options for depression with elevated inflammation and suggest that infliximab, a TNF antagonist, may be efficacious in those patients showing elevated inflammation indexes (as CRP levels greater than 5.0 mg/L). Genetic transcription factors related to glucose and lipid metabolism were predictive of treatment response (Rethorst et al, 2014).

Depression is associated with a significantly increased risk of developing type 2 diabetes and cardiovascular disease. These adverse effects have been mainly attributed to poor lifestyle behaviors including: increased caloric intake and reduced rates of physical exercise (Vallence et al, 2011). However, it should be evidenced that even when these factors have been

controlled, the association between depression and increased risk of diabetes or cardiovascular diseases has persisted (Kan et al, 2013, Gan et al, 2014). The casual pathways linking depression with metabolic dysregulation have not been fully elucidated, but the presence of insulin resistance, low-grade inflammation and leptin secretion have been frequently reported in patients (Morris et al, 2012). More severe cases of depression have been associated with the activation of HPA axis and sympathetic nervous system, which lead to an increased catecholamine release, which in turn inhibits insulin-induced uptake of glucose in adipocytes (Holsboer et al, 2000). Studies show the positive association between depression and levels of pro-inflammatory cytokines (TNF, IL-6), but also the association with an altered leptin homeostasis, in particular the presence of high circulating leptin, a marker of leptin resistance (Kraus et al, 2001). Despite these results, the association between depression and leptin dysfunction remain unclear because of divergent reports that have associated depression with both hypo and hyper-leptinemia (Hafner et al, 2011, Cordsa et al, 2015). Most studies analyzing depression and metabolic dysfunction have not considered the impact of gender. The prevalence of depression differs with sex, with Kessler and colleagues reporting a 21,3% prevalence in women compared with 12.7% in men, although the risk of mortality from major chronic diseases is higher in depressed men than in women (Warnke et al, 2016). Some studies show that women tended to

show elevated biomarkers related to an increased risk of type 2 diabetes (insulin resistance, leptin and TNF-α), whereas men showed an increase of CRP, a biomarker related to the risk of cardiovascular disease (Webb et al, 2017).

Furthermore, BDNF levels may also be associated with metabolic markers in major depression (Skibinska et al 2017). Low plasma BDNF levels may be associated with higher BMI, low HDL cholesterol, high triglycerides and hyperglycemia (Malgorzata et al, 2013). Different studies have also shown altered levels of oxidative stress markers in depression, such as reduced concentrations of non-enzymatic and enzymatic antioxidants, which can be normalized by AD treatment (Solleiro-Villavicencio et al, 2018). It has been hypothesized that monoaminergic, neurotrophic, and HPA axis dysfunctions may alter metabolic and redox mechanisms in depression. Moreover, a dysfunctional response to stressors may be related to inadequate lifestyle and dietary habits, with consequences on the anti-oxidant response and metabolic or nutritional conditions of depressed patients (Czarny et al, 2018). The antioxidant system consist of two main components: non-enzymatic antioxidants, such as glutathione, thiols (R-SH), plasma proteins, uric acid (UA), vitamin C, vitamin E, zinc and coenzyme Q10, and enzymatic antioxidants, such as superoxide dismutase (SOD), catalase, glutathione peroxidase and reductase, and the

thioredoxin-peroxiredoxin system (DE Melo et al, 2017)]. The main components that protect against brain damage caused by free radicals are antioxidant enzymes, expressed in both peripheral organs and the brain (De Melo et al, 2017). In any case, together with an altered immune-inflammatory response, the induction of oxidative stress, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), appears to play a crucial role in aging and in the development of severe diseases, such as cancer, cardiovascular, and neurodegenerative disorders, and psychiatric disorders, including MDD (Liguori et al, 2018). This is possibly due to the high vulnerability of the CNS to oxidative damage (Siwek et al, 2013). A series of studies reported lipid oxidative damage in depression. A study showed a decrease in poly-unsaturated fatty acids (PUFAs) of red blood cell lipid membranes in depressed patients, indicating an increase in long-chain fatty acid degradation by peroxidation in depression (Maiuolo et al, 2016).

As part of the purine system, UA, the final compound of purines catabolism, seems to play a leading role in many cell functions, regulating sleep/wake cycle, appetite, cognitive ability, memory, seizure threshold, social interaction, and impulsiveness (Black et al, 2018). In physiological conditions, there is a balance between the synthesis and degradation of purines (Maiuolo et al, 2016).

Over-production of UA has proven to play an emerging role in different human diseases, including neuropsychiatric disorders (Maiuolo et al, 2016).

Some studies have investigated the peripheral levels of potential biomarkers of the purinergic system, particularly UA, in subjects affected by mood disorders.

BD and MDD patients showed altered levels of serum UA, with higher concentrations during the manic phase (Kesebir et al, 2013) and lower during depressive phases. Furthermore, UA levels in depressed patients seem to normalize after a treatment with ADs and mood stabilizers as lithium and carbamazepine (Wen et al, 2012). In addition, genetic studies suggest that some purinergic receptors subtypes could be involved in mood dysregulation and many altered behaviors.

Some single-nucleotide polymorphisms (SNPs) of purinergic system effectors showed a significant association with an increased risk of mood disorders (Karve et al, 2013). In agreement with this finding, a recent study showed that purinergic modulators seem to rapidly improve the clinical picture of mood disorders (Ortiz et al, 2015).

In vitro studies showed that ATP and adenosine could be potential targets for future treatments of mood disorders, and two inhibitors of the xanthine oxidase (XO) enzyme, allopurinol and febuxostat, have shown an AD

effect, similar to fluoxetine, a selective serotonin reuptake inhibitor (SSRI) AD, in animal models (Karve et al, 2013).

Furthermore, in the view of a unified pathogenetic model of mood disorders, it is currently well known that the purinergic system can also takes part in the neuro-inflammation and neurotoxicity mechanisms of depression (Bishnoi et al, 2014).

CHAPTER 2. Study Aims

Major depression is one of the most prevalent psychiatric conditions in the general population, being among the furthermost disabling mental illness not only for patients themselves, but also even for their families and the whole community. Despite years of intense research and despite the availability to clinicians of a number of compounds at effective AD and mood stabilizer activity, the etiopathogenesis of mood disorders remains not precisely defined so far, while the applied treatments being not always able to prevent relapses, chronicity and suicidality. More effort is thus required to fight these disorders. The evaluation of suitable biomarkers is considered a valuable tool to better outline patients' diagnosis and clinical care management (Hashimoto et al, 2004; 2010). Under the light of what previously reported, several preclinical studies have suggested that BDNF

plays a relevant role in the pathophysiology of mood disorders (Hashimoto et al, 2010). Furthermore, accordingly to the integrated hypothesis of depression (Maletic et al, 2007; Leonard 2010; Dean and Keshavar, 2017), the measurement of peripheral BDNF in easily available samples such as plasma, serum, blood cells and saliva could be one of the most promising biological correlates of mood disorders, reflecting the impairment of brain circuits and brain-body neuro-immuno-endocrine pathways (Dantzer, 2018). However, there are still controversies as concerns results on peripheral BDNF as a biomarker of depression. Methodological issues, regarding both the technical procedures and the biological matrices used to measure peripheral BDNF as well as the inclusion/exclusion criteria adopted for patients' recruitment, have been suggested as probable biases leading to discrepant results among different surveys. Regarding this last concern, we must remember that mood disorders display a heterogeneous clinical presentation. At the same time, peripheral BDNF, similarly but also distinctively in respect to its brain counterpart, undergoes a complex transcriptional and post-translational regulation. As well, peripheral BDNF is principally stored in blood platelets and its bloodstream concentrations are under the influence of many factors and inducers, while its biosynthesis has been hypothesized during megakaryocytic differentiation. Despite these analytical difficulties, the assessment of blood BDNF remains of current interest in the monitoring of depression (Rana et al, 2020).

The main purpose of this experimental study was thus to evaluate, in a group of patients with a major depressive episode both their inflammation and metabolic states and both the plasma and intra-platelet levels of BDNF, together their possible reciprocal relationships and correlations with symptoms' severity and other clinical features. Essentially, the design of the present investigation was meant to further verify the possible use of the measure of two blood BDNF components as a correlate of the severity of depressive symptoms and drug resistance, in relation to the presence of certain inflammatory, metabolic and clinical patterns.

Clinical symptoms were assessed by the following tools: the "Hamilton Depression Rating Scale "(HRSD or HAM-D) (Hamilton, 1960); the "Young Mania Rating Scale" (YMRS) (Young et al, 1978); the "Clinical Global Impression-severity (CGI-s) (Guy and, William, 1976) and the "Global Assessment of Functioning "(GAF).

The metabolic and inflammation parameters, assessed through routine clinical-chemical analyses, were CRP, ESR, the lipid profile, glucose, uric acid (purinergic and antioxidant system), bilirubin, the blood cell count and derived inflammatory markers as the neutrophils/lymphocytes (NLR), platelets/lymphocytes (PLR) and monocytes/lymphocytes (MPL) ratios.

In fact, recent evidences have reported that hematological indexes, as the above reported ratios, can vary in depression with respect to specific features (Mazza et al, 2018). These investigations are showing that the

immune/inflammatory system may be involved also in terms of cell reactivity in the pathogenesis of mood disorders. The interest in measuring these indices, NLR, PLR and MLR, easily obtainable from routine laboratory analyses, specifically consists in the possible identification of clinical subtypes of depression and vulnerabilities at developing somatic co-morbidities (Mazza et al, 2018).

The appraisal of the biochemical and hematochemical and immune/inflammatory fingerprints of the pathogenesis of depression could have a relevant impact on the therapy and monitoring of the most resistant and insidious forms of this disease, potentially contributing to pave the way towards the investigation of new drug targets for attaining a faster, more adapted and better tolerated AD action (Mendlewitcz et al, 2006; Savitz et al,2018).

CHAPTER 3. Materials and Methods

3.1. Subjects

All subjects participating to the study were recruited from inpatient or outpatient healthcare settings where they were assisted for the treatment of a major depressive episode.

Precisely, all subjects were enrolled at the Psychiatry Unit of the Santa Chiara Hospital, Department of Clinical and Experimental Medicine, Pisa Hospital-University Center AOUP - University of Pisa, by skilled psychiatrists, with at least 5 years of post-specialization clinic practice, during one year, from January 2019 to March 2020.

The major depressive episode occurred in the context of a lifetime diagnosis of Major Depressive Disorder, Bipolar II or Bipolar I disorder, in agreement with the criteria of the DMS-5,

The adopted inclusion criteria were:

• Age between 18 and 80 years;

• History and diagnosis of major depressive episodes according to the classification criteria of the DSM-5;

• recruitment at the first visit and with or without psychopharmacological therapies;

• having read and accepted the research protocol and signed the informed

consent about the study approved by the Ethics Committee of the University of Pisa.

The exclusion criteria were as follows:

• past or present personal history of neurological, rheumatic, infectious, tumor and metabolic disorders.
• cognitive alterations and inability to sign the informed consent of the study;
• pregnancy
• alcohol abuse in the past 6 months;
• substance abuse in the past 6 months.

Patients could decide to leave the study:
• in the presence or onset of clinical signs incompatible with the inclusion and exclusion criteria of the investigation;
• due to the onset of serious internal pathology;
• for withdrawal from informed consent.

All patients included in the study underwent clinical psychiatric evaluations and biochemical investigations based on the experimental design.

3.2 Psychiatric evaluation

All patients who met the inclusion and exclusion criteria were evaluated by the staff of the Psychiatry Unit with appropriate tools in order to determine the presence of depressive, manic, anxiety symptoms and suicidal ideation in order to allow psychiatrists to define their precise clinical picture.

The psychiatric rating scales applied to enrolled patients consisted in standardized, validated scales used worldwide in the mental illness clinical practice. The diagnostic and symptomatology assessments were performed with the following tools:

•the Structured Clinical interview for DSM-5, SCID 5 (First, M.B. et al.2015)

•The semi-structured Interview for Mood Disorders (SIMD) (Cassano et al.1988)

• the Hamilton Depression Rating Scale (HRSD or HAM-D) (Hamilton, 1960);

• the Young Mania Rating Scale (YMRS) (Young et al., 1978);

• the Clinical Global Impression-severity, improvement or change and therapeutic response (CGI) (Guy, 1976);

• the Global Assessment of Functioning (GAF)

1) The Structured Clinical Interview for DSM-5, SCID 5: this interview is used for the diagnosis of disorders according to the DSM-5 criteria. It consists in ten modules containing each question to investigate the presence of symptoms-criteria for different diagnostic categories. Each module is independent and can be used separately from the other according to specific searches. The interview is organized according to the diagnostic categories of DSM-5: the sequence of questions follows the structure of this manual and the different items explore the diagnostic criteria.

For this study the form relating to Mood Disorders was mainly used in order to adequately recruit patients with Major Depressive Episode as well as to estimate different episode's specifiers: "*with anxious distress*", "*with melancholic features*", "*psychotic features*", "*with peri-partum onset*", "*with seasonal pattern*", "*with atypical features*" *and the severity/course specifier".*

2) the Semi-structured for Mood Disorder (SIMD): was used to collect information on different familiar, demographics, medical history and clinical aspects. This interview has been developed to gather systematic information on family history, previous episode number and polarity, suicide attempts and psychotic symptoms of any polarity.

3) The Hamilton Depression Rating Scale (HRSD or HAM-D) provides an easy way to quantitatively assess the severity of the depressive condition shown by patient and to document changes, taking into account both the extent symptoms and their frequency.

The HAM-D consists of 21 items. Generally, the first 17 items are considered the "nuclear" ones and, more precisely, the reported scores define the cut-off severity as follows (Hamilton M., 1960):

- Scores > 25 = severe depression
- Scores between 18-24 = moderate depression
- Scores between 8-17 = mild depression
- Scores < 7 = no depression.

4) The Young Mania Rating Scale (YMRS) is an 11-item scale that explores key symptoms of mania, generally present throughout the course, from the most modest symptoms to more serious ones. The MRS resembles HRSD in its structure and must be applied by an experienced clinician. The assessment of severity is subjective, based on what the patient reports about his/her condition in the past 48 hours and on the observation of his/her behavior by the doctor during the interview.

The scale consists of 11 elements. There are four items that are graded on a 0 to 8 scale (irritability, speech, thought content, and disruptive/aggressive behavior), while the remaining seven items are graded on a 0 to 4 scale.

These four items are given twice, in a way that the weight of the others can compensate for poor cooperation in case of severely ill patients (Young et al, 1978).

5) The Clinical Global Impression (CGI) is a three-item scale, the first measures the severity of symptoms in patients with psychiatric disorders (CGI-s). It consists of 7 levels of increasing severity, according to the following scores:

1- Healthy patient

2- Borderline

3- Slightly ill

4- Moderately ill

5- Markedly ill

6- Seriously ill

7- Very high severity level

The second item measures the overall improvement (7 maximum points for worsening, decreasing) or change in symptoms; the third item measures the therapeutic response (Guy, 1976).

6) The Global Assessment of functioning (GAF) is a global assessment scale of functioning and is used to evaluate how much symptoms are present and affect daily life based on a scale from 0 to 100. The lowest scores

correspond to a higher impairment of daily activities.

Scoring can help to understand what level of care is required and how much specific treatments may be effective.

According to the current guidelines the scores are:

-100-91 = No symptoms.

-90-81 = Absent or minimal symptoms.

-80-71= If symptoms are present, they are transient and expectable reactions to psychosocial stressors.

-70 -61 =Some mild symptoms or some difficulty in social, occupational, or school functioning but generally the patient functions pretty well, with some meaningful interpersonal relationships.

-60-51= Moderate symptoms or moderate difficulty in social, occupational, or school functioning.

-50-41 = Serious symptoms or any serious impairment in social, occupational, or school functioning.

-40-31= Some impairment in reality testing or communication or major impairment in several areas, such as work or school, family relations, judgment, thinking, or mood.

-30-21= Behavior is considerably influenced by delusions or hallucinations or serious impairment in communication or judgment or inability to function in almost all areas.

-20-11= Some danger of hurting self or others or occasionally fails to maintain minimal personal hygiene or gross impairment in communication.

-10-1 = Persistent danger of severely hurting self or others or persistent inability to maintain minimal personal hygiene or serious suicidal act with clear expectation of death.

-0 = Inadequate information.

3.3. Determination of the hematological/hematochemical parameters and circulating BDNF

3.3.1. Chemicals, reagents and instruments

All chemicals and reagents used in the study were of the best analytical grade. A milli-Q, ultrapure HPLC-gradient grade distilled water (18 MΩ cm^{-1} resistivity), obtained by a Simplicity Millipore Apparatus (Figure 10) equipped with a 0.2micron filter and an UV lamp to avoid water contamination from particles and bacteria, was employed for preparing all required solutions. For absorbance measurements, a 96-well plate spectrophotometer (Multi-Scan FC ThermoScientific, Thermofisher Scientific, Waltham, MA, USA), (Figure 10) was used; uric acid assay was appraised by the multimodal Enspire reader (ThermoFisher Scientific, Walthman, Mass, USA).

Figure 10: The Multiscan Fc Spectrophotometer and the Simplicity Millipore Apparatus

3.3.2 Blood sampling, sample treatments and storage conditions

About 20 ml of peripheral venous blood were collected from each patient introduced into the study by the skilled nursing staff of the Psychiatry Unit of the Santa Chiara Hospital of Pisa, Department of Clinical and Experimental Medicine - AOUP, University-Hospital of Pisa. Blood withdraws were carried out avoiding hemolysis, as part of routine blood tests, accordingly to the guidelines established by the Ethical Committee of the University of Pisa for this study. All patients were fasting since the previous evening and blood samplings were always carried out between 8.00-9.00 *a.m.* in order to circumvent possible variations related to circadian rhythms. Precisely, patients' blood was collected in different vacutainer tubes, marked and predisposed for two different sets of analyses:

- about 8 ml were sent to the Clinical Chemical Laboratory of the Cisanello Hospital, Department of Laboratory Medicine, AOUP, University-Hospital of Pisa, for the determination of routine hematological and hematochemical parameters;
- about 12 ml, collected in vacutainer tubes containing K_3EDTA as the anticoagulant, were sent to the Biochemistry Laboratory of the Department of Pharmacy, University of Pisa, for the determination of plasma and intra-platelet BDNF.

At the Clinical Chemical Laboratory of the Cisanello Hospital, all main follow-up examinations were performed, including the assessment of the hematological and inflammation indices of our interest, such as: the concentration of leukocyte populations, platelet count, mean platelet volume, CRP, ESR, uric acid, bilirubin and the main metabolic indices. For logistical reasons, part of the uric acid determinations was carried out at the Biochemistry Laboratory of the Department of Pharmacy.

For evaluating the plasma and intra-platelet BDNF components, a first procedure was carried out at the Biochemistry Laboratory of the Department of Pharmacy to obtain two distinct samples separately, plasma and whole platelets (Betti et al, 2018). Briefly, no more than 30 min after collection, blood samples from the Psychiatry Unit were low-speed centrifuged, 150 x g for 15 min at room temperature (RT), in order to precipitate erythrocytes and leucocytes. Ensuing supernatants, S1, or the

platelet-rich plasma (PRP), were then divided in two 15ml-Falcon tubes/patient and centrifuged at 1,500 x g for 15 minutes. The resulting two S2 supernatants/patient containing platelet-poor plasma (PPP) and the two P1 pellets/patient containing the whole platelets were taken as separate assay samples:

1) PPP was aliquoted (about 200 µl/tube) in high-quality, low-binding protein

Eppendorf test tubes and frozen at -80°C, until the time of the **PPP-BDNF** assay;

2) P1 pellets were directly stored at -80°C, until the analysis of the intra-platelet **PLT-BDNF** levels.

In summary, for each patient, 2 aliquots of platelet pellets and approximately 10 aliquots of PPP were obtained and stored at -80°C.

Prior to freezing, both PPP and platelet P1 tubes were duly marked with patient's codes, date and sample type. In the case of platelets, the initial volume of PRP from which P1 pellets derived was saved into the biological database for calculations of intra-platelet BDNF.

3.3.3. Preparation of platelet soluble fractions for the analysis of BDNF

The day of BDNF assay, the first step was the preparation of the soluble, cytosolic fractions of whole platelet pellets (P1).

For this procedure, one P1 aliquot/patient was removed from -80 °C, thawed and immediately placed on ice. Then, each sample was homogenized by an ultrasound mechanical technique using a hypotonic buffer, followed by subcellular fractionation (Betti et al, 2018). In more detail: each P1/patient was suspended in 6 ml of ice-cold lysis buffer solution, 10mM Tris-HCl, pH=7.9, containing a mixture of protease inhibitors (Protease Inhibitor Cocktail, Sigma Aldrich, code: P8340), diluted 1: 500 (*v/v*). At that point, each platelet sample was homogenized by sonication on ice for 60" using a cell sonicator (Vibra-Cell Ultrasonic Liquid Processor, Sonics & Materials) (Figure 11). The homogenate was subsequently transferred into Eppendorf tubes and centrifuged at 15,000 x g for 5 min at 4°C by a microfuge. The resulting supernatant, or soluble fraction of platelets, was used for the BDNF assay.

Figure 11: The sonication device employed to homogenize whole platelets

3.3.4. Plasma and intra-platelet BDNF assay by sandwich ELISA

To evaluate BDNF concentrations in our patients' samples, a specific and sensitive methodology was used, the immune-enzyme ELISA technique, which exploits a double biological specificity, that of antibodies for antigens and that of the enzyme catalytic site for its substrate. This technique is also highly versatile: a variety of different approaches and strategies are indeed applicable to the different biological samples and to the different type of analyte/s under consideration. The ELISA methodology enables to easily appraise almost any kind of biological matrix, including cell culture supernatants, serum, plasma, saliva, urine and tissue extract specimens. Other advantages of the immune-enzymatic analytical methods applied to clinical-chemical laboratory evaluations are linked to their ability to process many subjects' samples all at once, and, for each subject, to appraise more types of biological matrices (e.g. plasma, platelets, tissue extracts) simultaneously. ELISA is the acronym of the expression "Enzyme-Linked Immuno-Sorbent Assay", since it consists in an immuno-absorbent assay linked to a revealing system consisting in an antibody or other binding-specific proteins, as the streptavidin-biotin complex, conjugated to an enzyme. Competitive, non-competitive and sandwich ELISA protocols are available for clinicians, researchers and technicians.

More in detail, each of them comprises an immunological analysis that permits to detect the presence and the amount of a substance using one or more antibodies, one of them directly or indirectly coupled to an enzyme. The enzyme represents the revelation device for the quantitative analysis, catalyzing a reaction that transforms a chromogenic/fluorogenic substrate into a colored/fluorescent/chemioluminescent product, measurable by a spectrophotometer or a multimodal detection system. The limitations of the ELISA techniques are linked to the possibility of antibody cross-reactions and the loss of analytical specificity, together the inability of the methodology to separate analytes from the other sample components or to directly localize them when present inside cells. These limitations can lead to the so-called matrix effects, which can disturb the analytical accuracy. Anyway, when many samples have to be appraised for a same analyte, the use of 96-wells microtiter plates in ELISA is a much valuable alternative solution to the high-throughput HPLC/U(H)PLC separation techniques. For our samples we used a Biosensis ELISA kit developed for the preferential determination of the mature form of BDNF (Biosensis, mature BDNF Rapid^{TM}, Thebarton, Australia), which allows the colorimetric identification and quantitation of the neutrotrophin levels in several types of biological samples and cell/tissue extracts. It is an *ad hoc* method, sensitive and specific enough, to measure mature BDNF by using a sandwich-type antibody system. Moreover, the kit is validated against analytical

interferences and it is certificated for its quantitative performances, attained in less than 3 hours. The antibodies used in this ELISA kit recognize epitopes of the mature BDNF form only, reducing cross-reactions with the proBDNF protein. Moreover, the test sensitivity is high, enabling to detect concentrations of BDNF as low as 7 pg/ml, also showing a high specificity: less than 3% of the other neurotrophins, as NT3, NT4 and NGF, can be "captured" by the first monoclonal antibody.

The kit comprises a pre-coated monoclonal anti-mature BDNF capture antibody, a biotinylated anti-mature BDNF detection second antibody and a horseradish peroxidase (HRP)-conjugated streptavidin detection complex. The addition of the HRP substrate 3',3'5,5'-tetramethylbenzidine, TMB, yields a blue colored reaction product which is proportional to the concentration of mature BDNF present in samples and BDNF standards. The kit provides a validated human recombinant standard BDNF and a Quality Control sample that serves as a BDNF positive control at a defined concentration. Prior to assay, the lyophilized BDNF standard provided by the kit must be diluted in 1 ml of Sample Diluent Buffer (SDB) (also provided by the kit) to a final concentration of 1ng/ml (1000 pg/ml). This first solution is then serially diluted in SDB to obtain seven calibration solutions, with BDNF amounts ranging from 7.8 to 500 pg/ml. The SDB contains "blocking" components avoiding the formation of non-specific

binding to the bottom of each plate well and consequent high background values at the end of the assay.

The day of BDNF assay, all steps were performed at an ambient temperature ranging between 22-25°C. In substance, one aliquot/patient of thawed PPP (platelet-poor plasma) and one aliquot/patient of freshly prepared soluble fractions from P1 were properly diluted in SDB, accordingly to the assay guidelines. Subsequently, 100 µl of diluted mature BDNF standards, Quality Control Sample, patients' samples (diluted PPP and soluble platelet fractions) and blanks (SDB only) were added to the pre-coated microplate wells. The plate was then sealed with a plate sealer and incubated for 45 min. at 25 °C on a plate thermo-shaker (PST-60 HL, Biosan, Riga, Latvia,) at very low shaking speed (Figure 12).

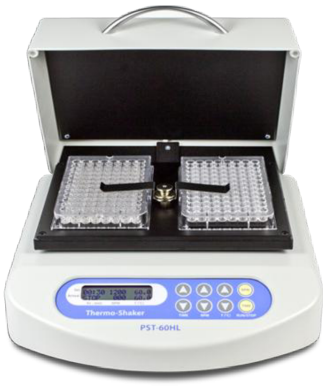


Figure 12: The Thermo-shaker used for all ELISA incubation steps

After this incubation, all solutions inside plate wells were discarded and 5 successive vigorous washes were carried out, by adding 200 μl of washing buffer (provided by the kit). Then, a sequential incubation for 30 min at 25 °C and very low shaking speed, was performed after the addition to each well of 100 μl of biotinylated anti-mature BDNF antibody, diluted 1:100 in SDB. After the incubation, well contents were discarded again and the microplate washed as indicated afore. 100μl of Streptavidin-HRP complex, diluted 1:100 in SDB, were then added into each well, a procedure followed by another incubation at 25 °C at low shaking speed for 30 min. After a supplementary washing step, 100 μl of the HRP substrate TMB were added to each plate well and the reaction was stopped after about 6-7 min by adding 100 μl of the stop solution (a concentrated solution of strong acid). The formed blue colored HRP reaction product become yellow in acidic conditions: the amount of BDNF was proportional to the intensity of color, generated in the redox enzymatic reaction. Afterwards, absorbance was read at 450 nm by the Multi-Scan spectrophotometer. In figure 13 is depicted the whole assay procedure of the adopted ELISA sandwich technique.

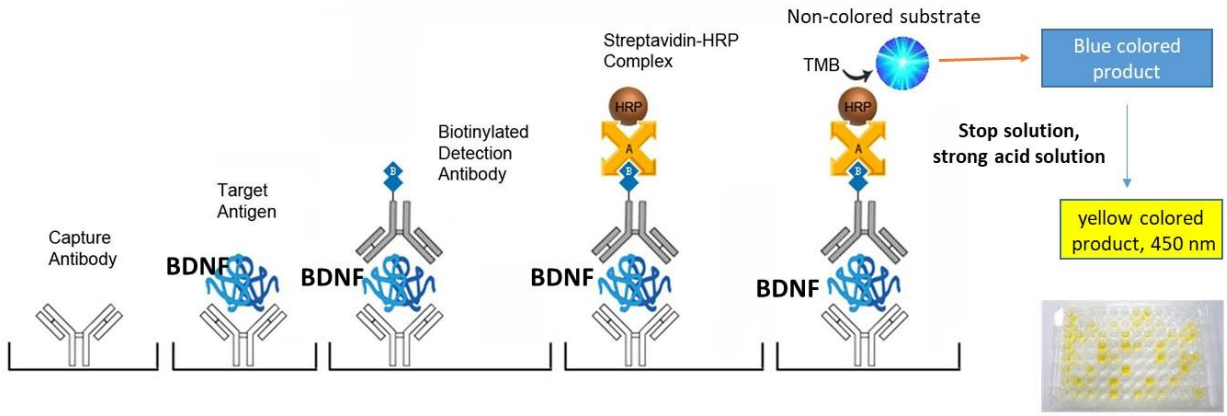


Figure 13: The mature BDNF assay procedure (modified from LSbio.com)

For the calculation of BDNF unknowns, blank absorbance was subtracted from standard and sample absorbance. Then, a calibration curve was built using a 4PL logistic non-linear regression equation by means of the Graph-Pad Prism Software (version 7.0, San Diego, CA, USA; Figure 14).

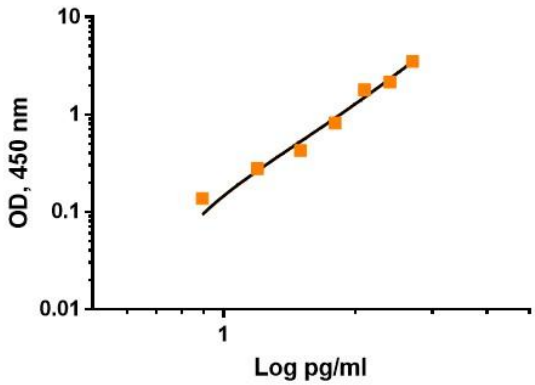


Figure 14: BDNF assay calibration curve, obtained by a 4PL logistic non-linear regression analysis

BDNF values, interpolated as log(pg/ml), were then transformed into pg/ml ($Y=l0^y$) and multiplied by dilution factors to obtain the final concentrations of patients' PPP-BDNF and intra-platelet BDNF as ng/ml.

3.3.5. Total protein determination by the Bradford's method

Due to the high inter- and intra-individual variability of platelet counts, as well as to take into account possible different yields of platelets and proteins after the centrifugation steps and platelet extraction procedures, intra-platelet BDNF contents (ng/ml) were normalized for the quantity of total proteins present in each final platelet soluble fraction. Precisely, we assumed that, for each sample, the protein content in soluble fractions was proportional to both the platelet number separated by the PRP centrifugation as well as to the final recovery after the whole preparation. For the purpose, the colorimetric Bradford's method, an ease-to-use procedure also chosen for its high sensibility and rapidity (Bradford, 1976), was employed, using γ-globulins as the standard. This method is also easy to adapt to very low protein concentrations (<25 µg/ml; 1–20 µg total) as well as for rapid determinations in 96-well microplates. A single step is necessary: the Bradford dye, Coomassie Brilliant Blue G-250, is added to previously distilled water-diluted samples and protein standards (20:80 *v/v*), followed by the immediate spectrophotometric measure at 595nm. The Bradford dye in acidic conditions forms complexes with basic and aromatic amino acids of sample proteins, which shift the dye's max λ absorbance from 465 to 595 nm,

promoting a blue color proportional to the protein content (Figure 15). This complex is quite stable, permitting to repeat measures or to perform the analysis within 1 h from the addition of the Bradford dye.

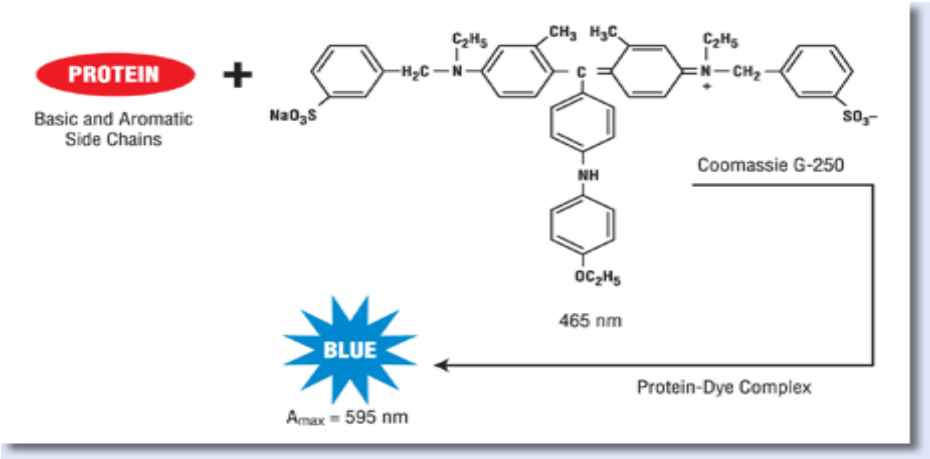


Figure 15: The reaction scheme of the Bradford's method (Bradford, 1976)

Positively charged basic amino acids and hydrophobic, aromatic amino acids in proteins bind to the Bradford dye by electrostatic and hydrophobic non-covalent interactions. Essentially, the day of assay a γ-globulin solution of 0.1 mg/ml was milliQ water-diluted to obtain the standard curve concentrations. As well, platelet soluble fractions were diluted 1:10-1:50 in milliQ distilled water. After the addition of the Bradford dye, samples and standards were gently mixed and immediately read at 595 nm by the Multi-Scan FC ThermoFisher Spectrophotometer. Protein content was calculated from the calibration line (Figure 16), multiplied for dilution factors and reported as mg/ml.

The intra-platelet BDNF values, expressed as ng/ml, were then normalized for the protein content, mg/ml, and finally reported as ng/mg proteins, obtaining

the PLT-BDNF component.

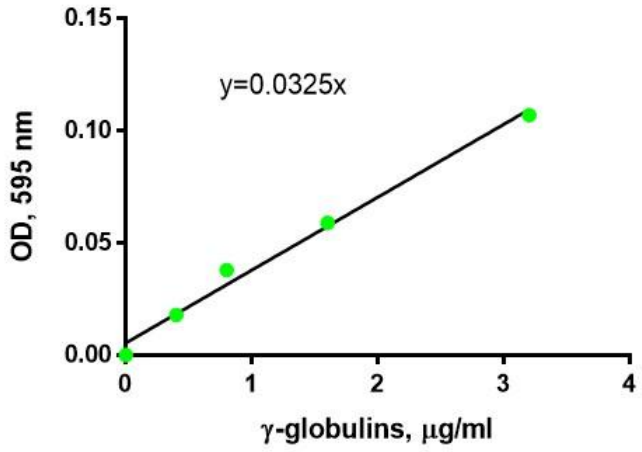


Figure 16: Bradford's method of protein quantitation: calibration line from linear regression analysis.

3.3.6. Uric Acid assay

A fluorometric assay kit purchased from Cayman Chemicals (Ann Arbor, MI, USA) was used to determine uric acid in some platelet-poor plasma (PPP) samples stored at the laboratory of Biochemistry. The principle of the method applied is based upon the reaction of the enzyme uricase (EC 1.7.3.3) which transforms sample uric acid into allantoin and H_2O_2. The amount of H_2O_2 formed is then put to react with 10-acetyl-3,7-dihydroxyfenoxazine (ADHP) in the presence of horseradish peroxidase (HPR), generating a tricyclic fluorescent compound, resorufin. Briefly, for assay, a range of uric acid dilutions at known concentration (standards: 0.5-10 μM) were prepared, while PPP samples were thawed and diluted in the assay buffer provided. A 96-well microplate was used for the dosage: in a final volume of 150 μl/well, blanks

(buffer only), diluted samples and standard solutions were incubated at RT for 15 min in the presence of ADHP and a cocktail enzyme mix, containing uricase and peroxidase. Fluorescence, proportional to the formed resorufin and, therefore, to the amount of uric acid present in samples, was read within no more than 30 min, by means of the Enspire reader in mode fluorescence, preset at the λ_{ex}=535 nm and λ_{em}=590 nm. Mean blank fluorescence was subtracted from standard and samples fluorescence. A "best-fit" standard curve was then calculated and plasma uric acid values interpolated as μmol/l (μM). Final uric acid concentration was then calculated as mmol/l, after correction for samples' dilution factor. Prior to statistical analyses and to uniform results, uric acid levels obtained from the Cisanello Hospital Laboratory, reported as mg/100 ml (mg/dl), were converted into mmol/l using the compound's molecular weight.

3.4. Statistical analysis

All demographic, clinical and laboratory data were presented as the mean ±standard deviation (SD) and value ranges (min and max values). In some graphs data were presented as the mean ± SEM (standard error of the mean) for the sake of clarity.

For the calculation of the calibration curves and the "best-fit" regression analysis for each assay used in the study, the GraphPad Prism® software was used (Version 7.0.2, San Diego, USA).

Correlations between BDNF values, chemical-clinical tests, clinical characteristics of patients and the scores on the psychopathological scales, were conducted by means of the non-parametric Spearman test, always using the GraphPad Prism® 7.0.2 software, which allowed us to calculate the correlation coefficient rho (r) and the probability index, P. For comparisons between patients' BDNF components expressed as ng/ml, or for appraising differences in patients' subgroups (e.g., in case of non-continuous variables), the non-parametric Wilcoxon test (paired data) and Mann-Whitney U-test (unpaired data) were employed, respectively, using the same statistical software. A preliminary attempt to apply multiple linear regression model was also performed for possibly identifying predictive independent clinical and biological variables of BDNF levels by using the Statistical Package for Social Sciences (SSPS), (version 23, IBM SPSS Statistics). The two-tailed statistical threshold was preset at $P = 0.05$ and statistical significance at $P< 0.05$. Given the cross-sectional nature of this study and the initial state of the investigation, P values close to the significance threshold, comprised between 0.05 and 0.1 ($0.05 \leq P \leq 0.1$), were considered as "trend to significance" results.

CHAPTER 4: Results and Discussion

At the end of the recruitment phase of the study, a total of 39 patients was enrolled, after a long, careful selection carried out on the basis of established inclusion and exclusion criteria. Concomitantly, the recruited 39 patients underwent clinical psychiatric examinations together the determination of circulating levels of mature BDNF, precisely both the PPP-BDNF and PLT-BDNF components. Among them, 10 patients could not complete the study for the assessment of hematochemical and inflammation parameters, for logistical reasons.

4.1 Clinical evaluation

Tables 1-4 present the demographic and clinical characteristics of the whole group of patients introduced into the study.

Patients	Age, years	Education degree, years	Marital status	Occupational status
M, n= 12	57 ± 19	12 ± 3	1.3 ± 0.5	2 ± 0.7
F, n= 27	52 ± 14	10 ± 4	1.6 ± 0.8	1.9 ± 0.7
Total, n=39	**54 ± 16 (18-81)**	**11 ± 3.8 (5-18)**	**1.5 ± 0.76 (1-3)**	**1.9 ± 0.7 (1-3)**

Table 1. Demographic data, (mean ± SD) in 39 patients with Major Depressive Episode, with ranges. Numeric criteria for marital status were: 1= married or in couple, 2= unmarried, 3= widower or separated; for occupational status, they were: 1= occupied, 2= student, housewife or retired, 3 = unemployed.

Patients were all recruited during a Major Depressive Episode and were on prevalence women, displaying a female-to-male ratio of about 2:1, reflecting the typical gender frequency of this mental illness (Hasin et al, 2018). They were also overall middle-aged, mean age 54, resulting on average non-occupied, middle-school educated, widower or unmarried, with high social and occupational maladjustment (Table 1).

Patients	BMI, Kg m^{-2}	Diagnosis	Age at onset, years	Illness duration, years	Lifetime episode numbers
M, n= 12	28 ± 6 (20-37)	1.75 ± 0.75 (1-3)	29 ± 15 (12-62)	28 ± 16 (6-56)	11 ± 6 (4-20)
F, n= 27	27 ± 5 (19-37)	2 ± 0.6 (1-3)	30 ± 12 (6-61)	22 ± 11 (2-47)	7 ± 5 (1-21)
Total, n=39	**27 ± 5 (19-37)**	**2 ± 0.6 (1-3)**	**30 ± 13 (6-62)**	**24 ± 13 (2-56)**	**8 ± 5 (1-21)**

Table 2. Patients' diagnosis and main illness features. Numeric criteria for diagnosis were: 1 = Bipolar Disorder type I (BD-I), 2= Bipolar Disorder of type 2 (BD-II), 3= Major Depressive Disorder (MDD).

Several important features of the patients' illness can be gathered from Table 2: first, the recruited group was prevalently composed of overweight subjects (mean BMI = 27 Kg m^{-2}) with a Major depressive Episode, mostly presenting a lifetime diagnosis of bipolar disorder of type II (BD-II). The diagnosis had been done about twenty years earlier, at a mean age of 30 years old, indicating that these patients were suffering of a chronic form of the disease, with recurrent episodes. Indeed, no patient was at his/her first

episode at the time of the study (mean illness duration = 24 years) and they had experienced on average 8 lifetime episodes.

Further and more detailed information on patients, not shown in Table 2, was: 61.5% of patients had a diagnosis of BD-II, 20.5% of Major Depressive Disorder (MDD) and 18% of BD-I. In addition, the prevalent comorbidity was Panic Disorder (64%), while comorbidity with Generalized Anxiety Disorder and Obsessive Compulsive Disorder was present at the 10% and 7% respectively. About the specifiers of Major Depressive Episode according to DSM-5: a) "*with anxious distress*", b) "*with mixed features*", c) "*with melancholic features*", d) "*with psychotic features*", e) "*with atypical features*" and f) "*with seasonal pattern*", were present at the a) 79.4%, b) 35.8%, c) 33%, d) 15.3%, e) 10.2% and f) 7.6%, respectively. "*Current Severity*" according to DSM-5 criteria enabled to identify a severe episode in 59% of patients and a moderate one in 41% of them. Table 3 reports the DSM-5 severity estimation given by psychiatrists and based on the number of criterion symptoms, the severity of those symptoms, and the degree of functional disability. Most of the patients (92%) had been hospitalized during periods of illness, while 31% of the sample had attempted suicide and the 15.3% of this 31% attended suicide more than once.

As shown in Table 3, enrolled mood disorder patients had a prevalent familiarity for depression with a first episode polarity of the depressive type, implying their high vulnerability to this illness.

Patients	DSM-5 Severity	Familiarity	Polarity first episode
M, n= 12	3.6 ± 0.5 (3-4)	2 ± 0.6 (1-3)	1.3± 0.9 (1-4)
F, n= 27	3.6 ± 0.5 (3-4)	2 ± 0.7 (1-4)	1.4 ± 0.8 (1-4)
Total, n=39	**3.6 ± 0.5 (3-4)**	**2 ± 0.7 (1-4)**	**1.4 ± 0.4 (1-4)**

Table 3. Patients' diagnosis and main illness features. Numeric criteria for episode's severity were: 1 = absent, 2= mild, 3= moderate; 4= severe; for familiarity were: 1=absent, 2= fam. for depression, 3 = fam. for BD, 4 = fam. for anxiety, 5 = fam. for psychosis; for polarity of first episode were: 1 = depressive, 2=manic, 3 = mixed manic, 4 = mixed depressive.

Table 4 reports results obtained by means of the psychiatric evaluations of selected patients. Patients had particularly severe depressive symptoms, with scores on the HAM-D scale≥ 17, and a mild manic component. They also presented a high-to-moderate severity of the depressive episode, accompanied by a relevant impairment of daily activities, as detected through the CGI-s scale displaying average scores ≥ 4 and the GAF scale, with values comprised between 30 and 60.

Most patients had a tendency to relapse and to a poor response to treatments. At the time of recruitment and request of health care, they were

following a pharmacological therapy with one or more ADs, associated with first or second generation antipsychotics, benzodiazepines, and/or mood stabilizers.

In summary, from this first presentation of clinical data, it can be noted that subjects introduced into the study constituted a relatively homogeneous group, most patients presented a Major depressive episode "*with anxious distress*" specifier in the context of a diagnosis of BD, implying that applied selection criteria were able to contain the marked heterogeneity that characterizes mood disorders. At the same time, the evaluation of patients' symptomatology revealed the presence of different features in depressive episodes. This aspect evidences the intrinsic and peculiar difficulty at attaining a full uniformity of clinical pictures in surveys on different subtypes of depressive episodes, associated also to the challenging therapeutic management and follow up of these patients.

Patients	HAM-D	YMRS	CGI-s	GAF
M, n= 12	26 ± 4 (20-32)	3 ± 3 (0-9)	4.9 ± 0.5 (4-6)	47 ± 5 (41-58)
F, n= 27	25 ± 5 (17-36)	3 ± 3 (0-12)	5 ± 0.6 (4-6)	44 ± 9 (30-60)
Total, n=39	**25 ± 5 (17-36)**	**3 ± 3 (0-12)**	**5 ± 0.6 (4-6)**	**45 ± 8 (30-60)**

Table 4. Patients' scores at psychiatric rating scales.

To further describe the selected group of depressed patients under investigation, Spearman correlations were then carried out in respect to

clinical-demographic features and psychiatric evaluation scores. Also, Mann-Whitney subgroup-comparisons were conducted in respect to illness features or the presence of episode's specifiers (expressed as absence=1 or presence=2), when considered appropriate. Results of these analyses reported a positive significant correlation between the duration of the disease and the number of episodes (r=0.384, p=0.016). A significantly shorter duration of disease was observed in suicide attempters in respect to patients without suicide attempts (Mann-Whitney test, p=0.016). This indicates a greater number of episodes in patients displaying more chronic forms, but a greater number of suicide attempts in the earlier illness phases. Patients who attempted suicide had also a significant lower number of experienced episodes (Mann-Whitney, p= 0.036). Furthermore, significant negative correlations were found between the GAF and HAM-D scales (r= -0.436; P = 0.006) as well as between the GAF and CGI-s scores (r=-0.363, p=0.023). These results reveal that a more severe state of depression is linked to higher social and occupational impairments. Furthermore, YMRS scores were found positively correlated with the number of lifetime episodes experienced by patients (r=0.5, p= 0.001).

No significant or trend-to-significant correlation was found between body mass index (BMI, Kg m^{-2}) and clinical scales, nor between BMI and demographic characteristics of the patients analyzed.

4.2 Evaluation of hematochemical parameters, pro-inflammatory indices and metabolism substrates in patients with Major Depressive Episode

Table 5 shows results recorded at the chemical-clinical analyses conducted at the Laboratory of the Cisanello Hospital. As previously indicated, these evaluations were completed in 29 of the 39 patients. Patients had mean CRP values slightly higher than the normal threshold (0.9 mg/100ml vs. highest normal value, 0.5 mg/100 ml). Precisely, 34.5% of patients (n=10) had CRP < 0.1 mg/100ml, 45 % had $0.1 \leq$ CRP ≤ 0.5 mg/100ml (n=13) and 20.5 % levels > 0.5 mg/100ml (n=6) (Figure 17). Five of them reported abnormally elevated levels, ranging from 1.8 to 12.7 mg/100ml.

Conversely, ESR mean levels were within normal ranges, with 6 patients displaying values over the physiological limit (25 mm/h). Four patients displayed both CRP and ESR measures above normal limits. This is in agreement with the reported presence of a mild, CRP-related inflammatory state in treatment-resistant depression, linked in turn to inflammasome activation (Chamberlain et al, 2019; Osimo et al, 2019).

	Mean ± SD (range)
CRP (mg/100ml)	0.9 ± 2.4 (<0.01-12.7)
ESR (mm/h)	15 ± 17 (2-76)
Total Cholesterol (mg/100ml)	178 ± 39 (65-225)
LDL (mg/100ml)	116 ± 33 (34-180)
HDL (mg/100ml)	49 ± 17 (24-100)
Triglycerides (mg/100ml)	129 ± 53 (46-280)
Glucose (mg/100ml)	90 ± 23 (67-193)
Uric Acid (mmol/l))	0.29 ± 0.08 (0.16-0.47)
Total bilirubin (mg/100ml)	0.5 ± 0.22 (0.11-0.97)
Platelets (cells^10^3/µl)	229 ± 65 (120-389)
Medium Platelet Volume (MPV)	11 ± 0.9 (9.4-12.8)
Leukocytes (cells/µl)	6830 ± 2086 (3260-11250)
Neutrophil granulocytes (cells/µl)	4022 ± 1719 (1400-7750)
Basophil granulocytes (cells/µl)	23 ± 16 (0-80)
Eosinophil granulocytes (cells/µl)	190 ± 119 (0-440)
Lymphocytes (cells/µl)	2060 ± 676 (1030-4100)
Monocytes (cells/µl)	516 ± 159 (310-910)
NLR (neutrophil/lymphocyte ratio)	2.09 ± 1 (0.77-4.45)
MLR /monocyte/lymphocyte ratio)	0.275 ± 0.12 (0.12-0.6)
PLR (platelet/lymphocyte ratio)	119 ± 41 (65-209)

Table 5. Hematochemical results and pro-inflammatory indices in 29 of the 39 enrolled depressed patients

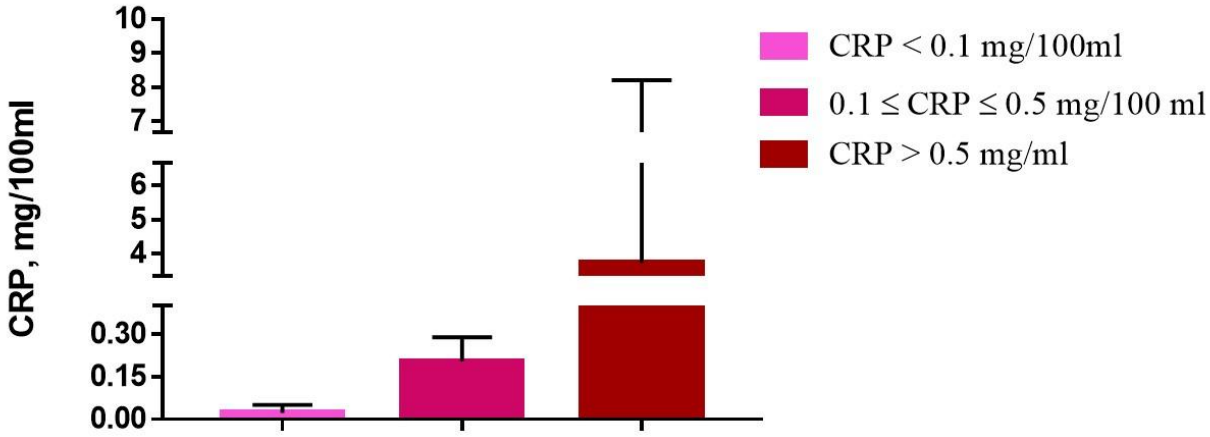


Figure 17. C-reactive protein (CRP) levels in recruited depressed patients

The patients' metabolic profile reported normal glucose, uric acid, bilirubin, triglyceride and total cholesterol mean values, with however mean HDL and LDL levels at their physiological limits. At least 40% of patients had HDL levels < 45 mg/100ml and LDL values > 115 mg/100ml, thus revealing an altered cholesterol metabolism. Impaired lipid profiles have been also linked to depression, also as risk factors for the development of cardiovascular diseases and metabolic syndrome in patients (Joynt et al, 2003; Marazziti et al, 2014; Dhar and Barton, 2016).

White blood cell counts were within the physiological ranges. Only few patients had slightly higher values of leukocytes/µl and neutrophils/µl. Of note, some red blood cell parameters were found lower in a fair number of recruited patients: 58.6% of them had erythrocyte counts and hematocrit

values under the physiological limits (considering gender differences of physiological range thresholds) (data not shown).

4.3 Correlations and comparisons of of hematochemical-inflammatory parameters and clinical features of illness

The main scope of this investigation was to possibly identify associations and correlations between clinical-demographic features of depressive patients, their different components of metabolism or inflammation/immunity and their levels of different bloodstream BDNF pools. In this paragraph are presented the most relevant significant or nearly significant correlations/comparisons obtained by the analyses of hematochemical, immune-inflammatory and clinical-demographic indices. As regards the inflammatory indices, only CRP values were found to significantly and positively correlate with BMI (Kg m^{-2}) ($r=0.377$; $p=0.04$) (data not shown); CRP levels did not correlate with episode's severity or clinical scores, but there was a mild increase of this parameter in both moderate and severe depressive episodes (HAM-D scores$\geq$ 17) also associated with anxious symptoms as already reported (Vogelzangs et al, 2013). In agreement with this finding, higher CRP and altered peripheral inflammation profiles have been already determined in concurrent depression and obesity (Ambròsio et al, 2018). White blood cell parameters were instead found related to patients' clinical scorings. Figure 18 depicts

that HAM-D scale positively correlated with leukocytes (r=0.375, p=0.045), neutrophil granulocytes (r=0.406, p=0.029) and NLR (r=0.364, p=0.052), while YMRS tended to positively correlate with PLR (r=0.328, p=0.082) (Figure 22). Interestingly, a trend to positive correlation between VES and duration of disease (r=0.395, p=0.056) was also found (data not shown). Finally, significantly higher values of NLR (p=0.017), PLR (p=0.033), MLR (p=0.003) and monocytes (p=0.039) were reported in patients with severe episodes in respect to moderate episodes (Mann-Whitney test comparison, data not shown).

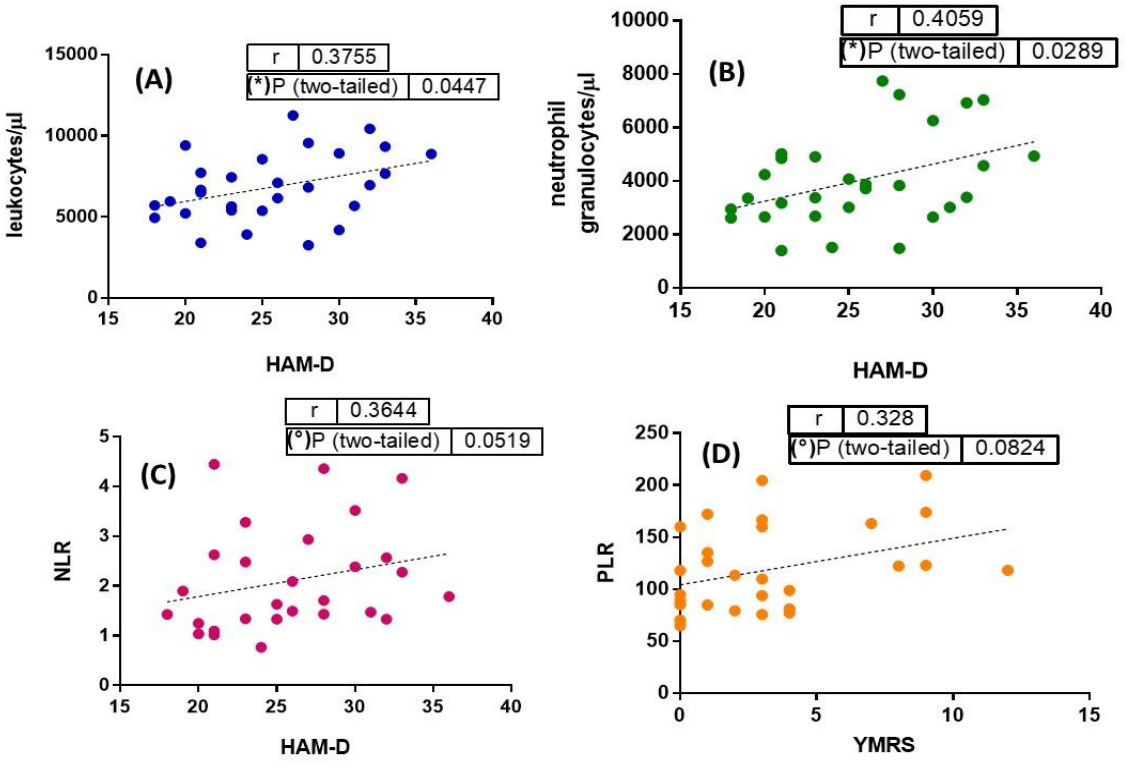
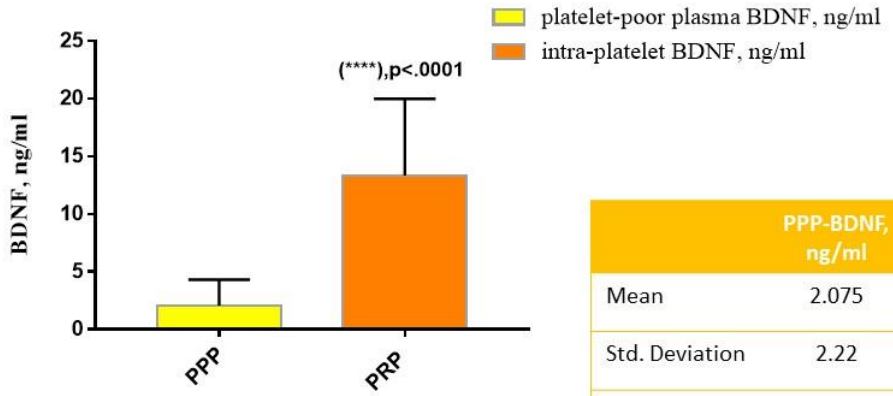


Figure 18. Significant positive correlations between HAM-D scores and leukocyte count (A), neutrophil granulocytes (B); nearly significant positive correlations between HAM-D and NLR (C) as well as between YMRS results and PLR (D).

These results indicate the presence of relationships between white blood cell counts or inflammatory indices and depressive/manic symptoms, duration of disease as well as severity of the episode in the recruited sample of patients. Our patients seem thus featured by impaired white blood cell indices, as reported in other surveys conducted on depressed patients, which also highlight their significance in relation to cardiovascular risk in depression (Demir et al, 2015; Aydin Sunbul et al, 2016; Köler et al, 2018; Mazza et al, 2018). Other investigations have reported higher NLR in first episode depressed elderly subjects rather than in patients with recurrent episodes as in the present study (Abraska et al, 2018), or in geriatric women patients (Liang et al, 2020). Some other studies have instead found low leukocyte counts in patients at their first depression diagnosis (Köler-Forsberg et al., 2017b). Taken together, these findings suggest that mood disorders are linked to altered leukocytes' formulas, while displaying different characteristics in respect to the presence or not of inflammatory patterns (Brinn et al, 2020). The various indices, as NLR or PLR, could help clinicians to identify those patients with altered inflammatory networks, needing personalized treatments.

4.4. Determination of PPP-BDNF and intra-platelet BDNF (ng/ml)

To our knowledge, the present work is the first which has concurrently evaluated both extracellular (platelet-poor plasma, PPP) and intra-platelet (PLT) components of mature BDNF in mood disorder patients with a relapse of depressive episode, with a current major depressive episode associated with anxious symptomatology.



	PPP-BDNF, ng/ml	PRP-BDNF, ng/ml
Mean	2.075	13.34
Std. Deviation	2.22	6.65
Min. value	0.11	2.5
Max. value	8.95	40.72

Figure 19: BDNF levels obtained in PPP and in platelet soluble fractions both presented as ng/ml (mean ± SD) in 39 patients. (****), highly significant Wilcoxon test.

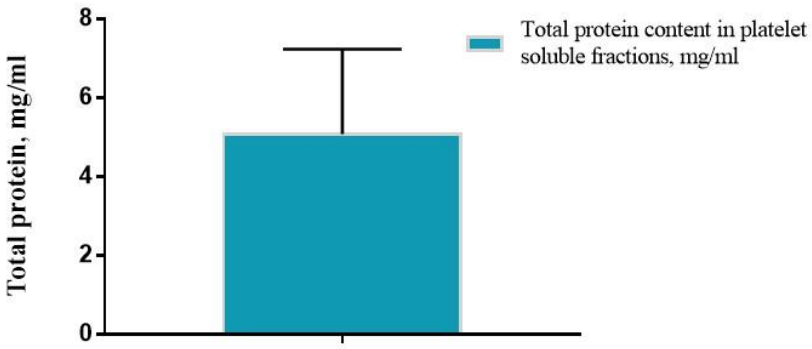
Figure 19 reports BDNF values measured in PPP and in soluble fractions of platelet pellets (P1), obtained from platelet-rich plasma (PRP). The plasma values of this neurotrophin were markedly and significantly lower than those determined in platelets, showing a ratio between platelet and plasma

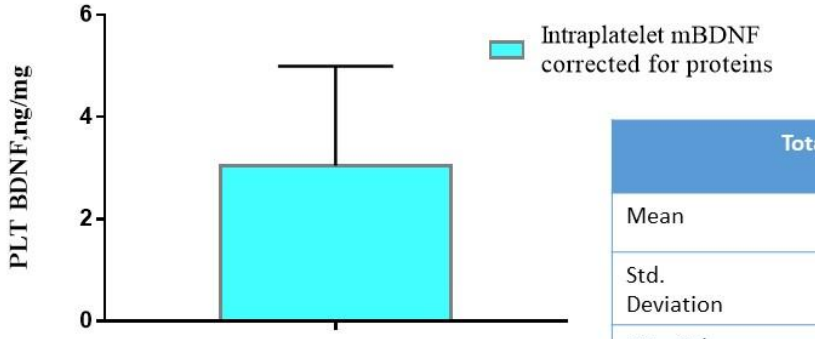
components accounting for by, on average, a value of 15, ranging from 1.2 to 99. In more detail, in our study, the mean plasma BDNF values in depressed patients were 2.1 ± 2.2 ng /ml (mean ± SD) , more or less in the range of those reported in previous literature (Karege et al, 2005; Lee et al, 2007; Serra-Millàs et al, 2011; Piccinni et al, 2008; 2015;. Dell'Osso et al, 2010; Haas et al, 2010; Pluchino et al, 2009; Colle et al, 2017; Aldoghachi et al, 2019; Buselli et al, 2019). Differences observed among the different papers were considerable in some cases. This observation, surprising at first sight, is however compatible with physiological variations already observed for other "adaptive" secretory proteins and peptides, such as cytokines, the quantity of which can remarkably vary depending on the degree of activation and release by the various types of blood cells and many other factors, including stress response effectors (Dowlati et al, 2010; Leonard, 2010; Gejl et al, 2019). In the case of the BDNF protein, it should be also considered that the peripheral component of this neurotrophin can be found linked to membranes, surfaces or other bonding proteins (Okragly and Haak-Frendsho, 1997). Therefore, the use of sample preparation procedures, such as plasma acidification to detach the BDNF bound to proteins and membranes not performed here, allows to obtain the total amount (bound + free) of neurotrophin (Serra-Millàs, 2016). This could contribute to determine higher plasma values. The evaluation of "drug-naive" patients compared to patients who are taking AD therapies may represent a factor of

additive variability, since BDNF levels significantly increase during treatment with these drugs (Gervasoni et al, 2005; Piccinni et al, 2008; Bocchio-Chiavetto et al, 2010; Aldoghachi et al, 2019). Overall, we can assume that, beside the functional variability, the application of acidification or patients' drug treatment, discrepancies in plasma levels can be attributed to other important factors. Among these, we mention: 1) the type of separation and preparation of the plasma sample (anticoagulants, temperature, direct separation of plasma or preliminary separation of PRP to obtain PPP, Serra-Millàs, 2016; Gejl et al, 2019); 2) the ELISA method used, showing a variable degree of cross-reactivity of the "capture" antibody for BDNF or pro-BDNF, or displaying a different sensitivity/reactivity of the detection system (Polacchini et al, 2015; Aldoghachi et al., 2019; Gejl et al, 2019); 3) the patients' inclusion/exclusion criteria and the diagnostic tools used for enrollment, together the different intrinsic clinical features of recruited subjects, a factor that could impact the neurotrophin secretion in the bloodstream. As far as the intra-platelet BDNF levels are concerned, in this study they measured: 13.3 ± 6.6 ng/ml (Figure 19). Figure 20 presents the correction of intra-platelet BDNF (ng/ml) for the quantity of total protein of platelet soluble fraction (mg/ml), allowing to obtain PLT-BDNF expressed as ng/mg proteins (3.09 ± 1.95 ng/mg), a step performed to restrain the platelet count inter-individual variability and to normalize for platelet/protein yields during the separation and extraction procedures.

In the case of PLT-BDNF levels, comparisons with other studies are more difficult than PPP-BDNF, since most investigations used acidification and have normalized intra-platelet BDNF by platelet number rather than the concentration of total proteins contained in platelet soluble fractions, as in the present work (Betti et al, 2018). For platelet samples, beside acidification procedures, ELISA antibodies and the other afore-mentioned variability factors reported for plasma BDNF measurements, results can also derive from the adopted homogenization and fractionation methods, leading to appraise more or less distinct components of this neurotrophin (Serra-Millàs et al, 2011). Diverse platelet BDNF pools/components could have been evaluated in the different works, probably with a diverse biological significance, specifically in relation to the degree of inflammation, megakaryocyte production (Chacòn-Fernàndez et al, 2016) and BDNF destination into different intracellular compartment, e.g. α-granules or cytosol (Serra-Millàs, 2016). These variability factors could also explain the quite discrepant ratios of s-BDNF (serum)/p-BDNF (plasma) observed in the literature focused on this topic. All these methodological considerations can help to understand which BDNF component can be better identified and measured under a precise experimental condition, together its presumed physiological worth: the levels of free BDNF reported herein in platelets and plasma, or the PPP-BDNF and the PLT-BDNF, represent the residual intra-platelet cytosolic unbound fraction, before its

destination as storage or releasing peptide (PLT-BDNF) and the extracellular unoccupied amount, still available for TrkB receptor binding and activation (PPP-BDNF).





	Total protein content, mg/ml	PLT-BDNF, ng/mg
Mean	5.08	3.05
Std. Deviation	2.15	1.95
Min. Value	1.23	0.73
Max. Value	8.56	8.73

Figure 20: Total protein amount (mean ± SD, mg/ml), obtained in platelet soluble fractions (superior panel); concentrations of intra-platelet BDNF normalized for the total protein amount (mean ± SD, ng/mg) (inferior panel).

4.5. Correlations and comparisons of PPP-BDNF (ng/ml) and PLT-BDNF (ng/mg) with hematochemical-inflammatory parameters and clinical features of illness

According to the study's aims, in this section are presented the main associations/comparisons obtained between the clinical-demographic features, hematochemical, immune-inflammatory or metabolic parameters and the two distinct bloodstream BDNF components here investigated in the recruited patients with a major depressive episode. Figure 21 shows the negative correlation of PLT-BDNF levels and HAM-D scores, attaining the statistical threshold ($r = -0.332$; $P = 0.055$), reported by this study, which implies a reduction of intra-platelet, cytosolic, free BDNF in mood disorder patients showing the most severe depressive episode symptoms.

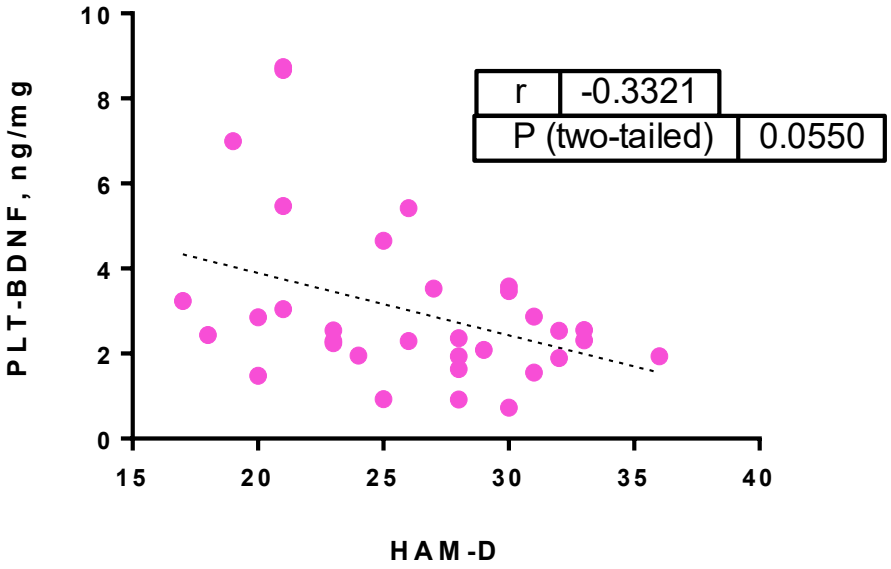


Figure 21. Correlation of Intra-platelet free BDNF and HAM-D depressive scoring

Additionally, among all variables, HAM-D scores were found to be a negative predictive independent variable of PLT-BDNF, or the cytosol reserve of free mature BDNF inside patients' platelets (Table 6).

	Unstandardized coefficients		Standardized coefficients		
	B	St. Error	Beta	t	Sig
HAM-D	-,146	,063	-,382	-2,338	,026

Table 6. Regression analysis, predictive independent variable HAM-D, PLT-BDNF dependent variable (p<.026)

Conversely, more complex results were reported for the PPP-BDNF pool. Indeed, PPP-BDNF levels significantly and positively correlated with PLT-BDNF (r=0.37, p=0.029), showing however only a weak trend for lower

values in respect to a higher HAM-D scoring (r=-0.26, p=0.1), while revealing a tendency towards higher amounts in patients with severe vs. moderate episodes (p=0.08); furthermore, there were also significantly higher levels of PPP-BDNF in suicide attempters than those measured in non-attempters ($p < 0.04$) (Figure 22).

These results seem to reflect that PPP-BDNF can fluctuate under the influence of more factors than PLT-BDNF. Indeed, BDNF can be released into the bloodstream by other cells and tissues. Figure 22 results would also mirror the effect of other clinical features on plasma BDNF extracellular levels.

Unlike PLT-BDNF, PPP-BDNF was found to correlate with white blood cell cells and inflammatory parameters. Figure 23 depicts the significant or nearly significant positive correlations obtained between plasma BDNF and these blood cell components.

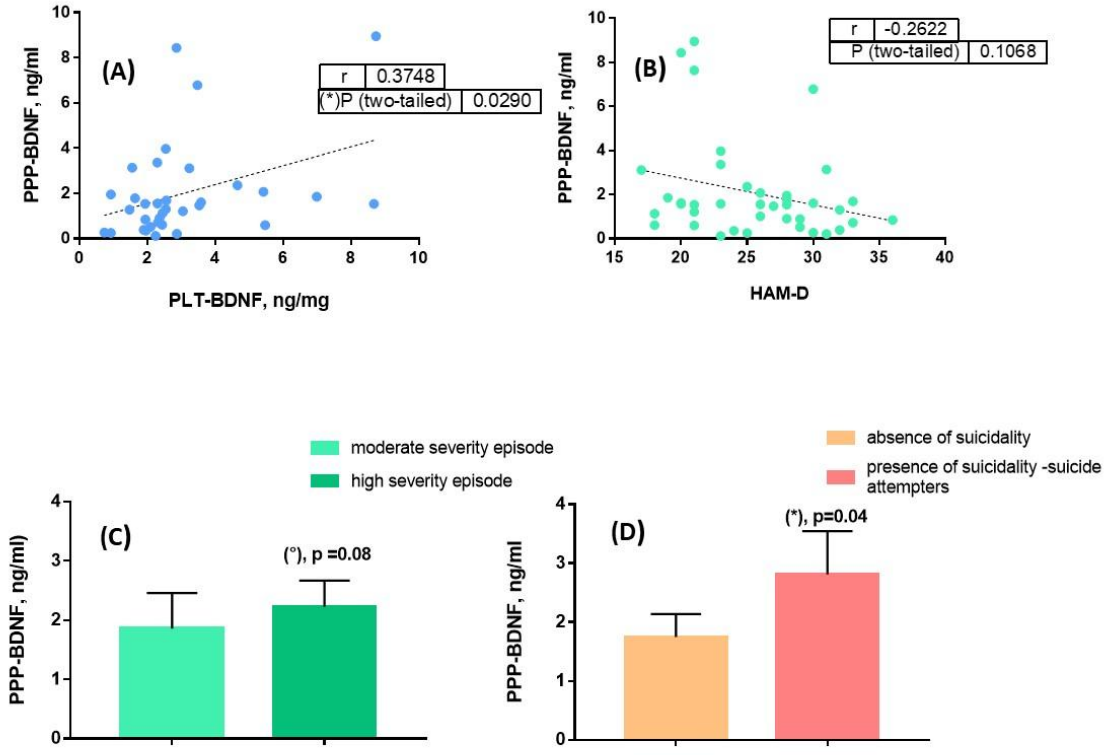


Figure 22. Plasma PPP-BDNF correlations with PLT-BDNF (A) and HAM-D (B). (C) Comparison between PPP-BDNF levels in moderate vs. severe depressive episode, (°): M-W test, p=0.08; (D) comparison between PPP-BDNF levels in patients without suicide attempts vs suicide attempters, (*): M-W test, p< 0.05. Data in (C) and (D) are depicted as the mean ± SEM.

In more detail, PPP-BDNF was found to positively and significantly correlate with leukocytes (r=0.377, p=0.044) and neutrophil granulocytes (r=0.450, p=0.014).

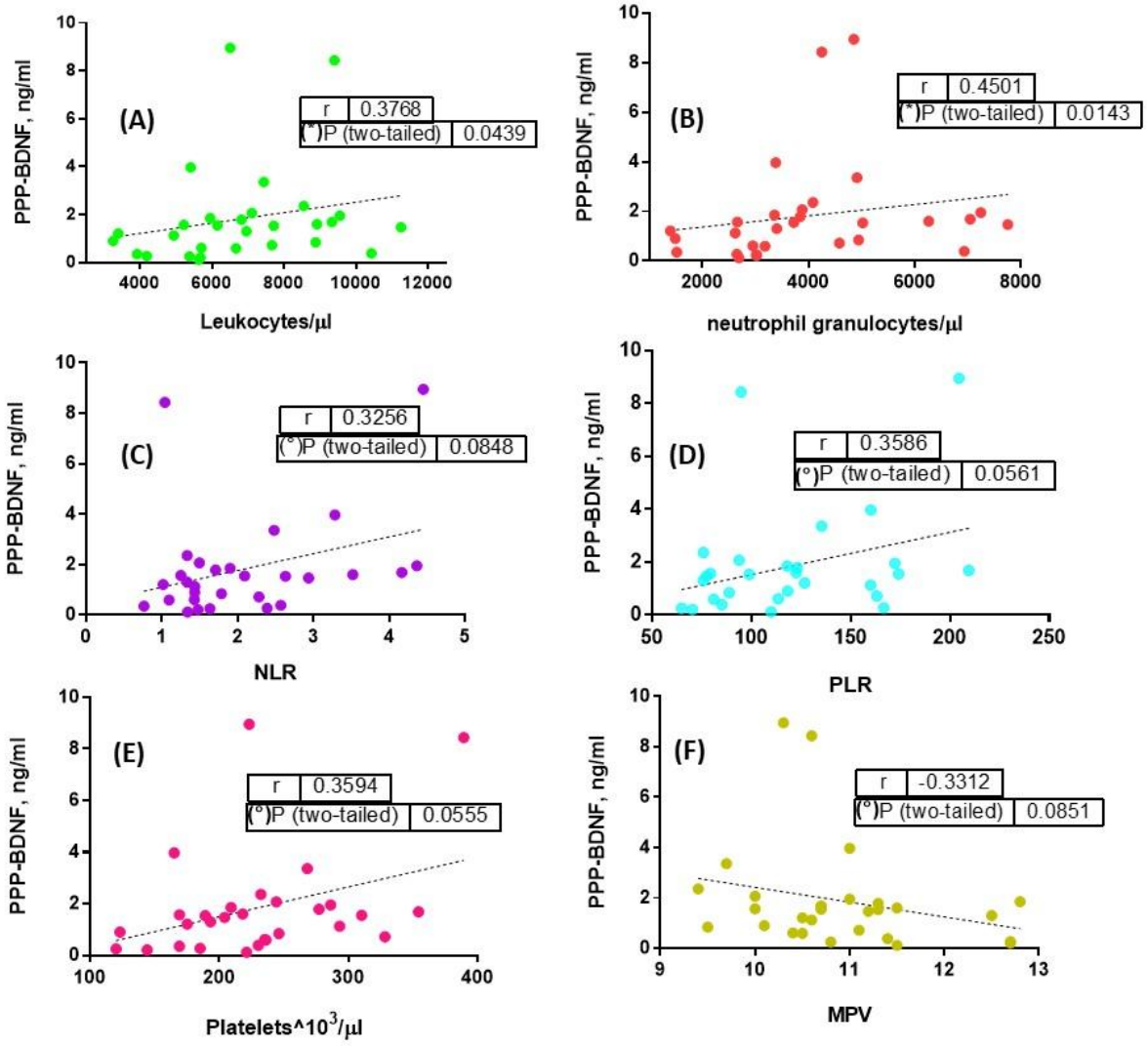


Figure 23. Plasma PPP-BDNF significant positive correlations with leukocyte count (A) and neutrophil granulocytes (B); nearly significant positive correlations between PPP-BDNF and NLR (C), PLR (D) and platelet count (E); nearly significant correlation between PPP-BDNF and mean platelet volume (MPV) (F)

Positive correlations, close to the threshold of statistical significance, were also reported between PPP-BDNF and NLR (neutrophils/lymphocytes) (r=0.326, p=0.085) or PLR (platelets/lymphocytes) (r=0.359, p=0.056). Thus, higher levels of plasma BDNF corresponded to a higher number of

leukocytes, neutrophil granulocytes as well as tended to have greater NLR and PLR values, which are considered inflammatory indices in psychiatric disorders and, particularly, in depression (Mazza et al, 2018; Öztürki et al, 2019).

Also, PPP-BDNF showed a positive correlation at the statistical threshold with platelet number ($r = 0.359$; $P = 0.055$), together a trend for a negative correlation with the mean platelet volume (MPV) ($r = -0.331$; $P = 0.085$), suggesting, with due caution, that platelet activation seems not directly linked to PPP-BDNF release into the bloodstream (Karege et al, 2005). Since PPP-BDNF was found to be rather related to the above reported hematological indices compared to the scores of the HAM-D and YMRS scales, this would indicate that its release into the bloodstream would be primarily linked to the presence of a mild inflammatory state in severe depressive episode. This hypothesis can be supported by the significant (or tending to significant) correlations of scale's scores with white blood cell parameters previously shown in Figure 18. Higher PPP-BDNF levels would be also linked to suicide attempters among these patients.

These data appear in contrast to results obtained in those previous studies showing the reduction of BDNF in both plasma and serum of depressed patients (Gervasoni et al, 2005; Karege et al, 2005). However, the present BDNF extracellular component is not fully comparable to that reported in the works of Gervasoni or Karege and coauthors (Serra-Millàs et al, 2016;

Gejl et al, 2019). Indeed, serum BDNF is the platelet quota released from α-granules whereas plasma BDNF extracellular level seems much to depend not only on the release from platelets but also from other blood cells and tissues; moreover, different experimental conditions are supposed to detect distinct extracellular pools (Serra-Millàs et al, 2016; Gejl et al, 2019).

In present conditions, we carefully controlled blood separation timing, by rapidly processing blood samples after withdraw (30 min), as suggested to avoid relevant artefacts linked to abnormal BDNF release after blood collection (Zuccato et al, 2011), thus excluding in principle the obtaining of plasma biased results due to a nonspecific neurotrophin release during sample separation. The explanation of our findings, which do not support a direct link between PPP-BDNF and HAM-D scores, in contrast to platelet BDNF, are not simple, require more investigation (Jin et al, 2019) and additional methodological resolution (Betti et al, 2018). Moreover, in the case of PPP-BDNF, the regression analysis model was not able to precisely estimate which variable, among leukocytes, neutrophil granulocytes, platelets, NLR or PLR was predictive of the neurotrophin plasma levels, presumably due to the co-linearity of some of these variables or to the possible reciprocal influence of one of them on the other as well as to the too small sample size.

Basically, present findings suggest complex associations of circulating BDNF in mood disorder patients with a major depressive episode. The

unreleased, free mature BDNF inside platelets is lower in patients with higher depression scores, suggesting a loss of peripheral BDNF reserve in such a clinical condition. Conversely, the extracellular free PPP-BDNF amount seems to fluctuate, in this type of depressive patients, in respect to subsets of white blood cells and inflammatory indices. If in the majority of depression studies, serum and plasma BDNF levels were both found reduced (Bocchio-Chiavetto et al, 2010), elevated plasma patterns of the neurotrophin have been also reported (Serra-Millàs et al, 2011). In fact, an increase of circulating BDNF was observed in relation to a family history of depression (Knorr et al, 2017) or in patients with mixed-state episode compared to patients with a depressive episode (Piccinni et al, 2015), in pain perception disturbances (Haas et al, 2010; Bazzichi et al, 2013) and adaptation disorders (Buselli et al, 2019). It must be also considered that patients enrolled so far in this clinical study represented a peculiar group in respect to other studies. They had a prevalent diagnosis of BD and a current anxious depressive episode, which is believed to display distinct neurobiological substrates from non-anxious depression (Ionescu et al, 2013). Furthermore, they had a tendency to relapse and to scarcely respond to treatments. In this framework and for these peculiar aspects, results reported in a recent study should be considered: depressed patients with a diagnosis of bipolar disorder were found to display a concomitant increase of plasma BDNF and IL1-β in relation to their resistance to ADs and

cardiovascular risk (Uint et al, 2019). Therefore, this work provides further support to the notion that complex interactions exist between circulating BDNF, inflammation and cytokine networks, in the presence of specific symptomatological pictures (Lee et al, 2007; Han, 2015; Piccinni et al, 2015; Serra-Millàs, 2016; Jin et al, 2019).

This study presents the main limitation, as already mentioned, of the restricted number of enrolled patients in respect to the elevated number of variables considered, a factor that much reduces its statistical power and robustness. Gender differences were not appraised for this reason. Other limitations are the lack of information on nutritional and other lifestyle habits, as smoking behavior.

5. Conclusions

At the current state of the survey, we have evaluated a group of patients with a Major Depressive Episode, mostly with anxious distress specifier and diagnosis of BD. Moreover, patients enrolled had a clinical laboratory profile rather related to a mild-inflammatory state, and moderately increased CRP mean values, while showing an overall normal metabolic profile, except for the cholesterol pattern. As far as circulating BDNF is concerned, the results of this study allow to believe that plasma and platelet neurotrophin levels are distinct components that might underlie peculiar networks related to inflammation, symptom presentation and severity of symptoms: in particular, the association found between reduced platelet contents of BDNF and higher scores on the HAM-D scale would indicate an impaired cytosolic reserve of this neurotrophin in platelets and the presence of a severe depressive symptomatology in this type of patients. HAM-D scores were also associated with higher NRL, neutrophil and leukocytes, featuring the occurrence of inflammatory cell patterns in the depressive episode. The present study provides forerunner results to further investigate these and other white blood cell parameters in different depressive episodes. Higher levels of the plasma extracellular BDNF pool were instead found linked to the presence of this mild inflammatory condition in patients, rather than being a direct index of depression. Higher PPP-BDNF levels were also

found in suicide attempters among the investigated mood disorder patients. This circulating BDNF component is supposed to better indicate, at least in our experimental conditions, the link between the secretion of specific neurotrophic factors and inflammatory effectors in depression, possibly defining clinical subtypes also in respect to episode specifiers and propensity to attempt suicide. Furthermore, this evaluation permits to well define how to continue this survey. First, the increase in the sample size of patients is needed in order to: 1) compare their clinical, biochemical and clinical chemistry variables with control subjects, according to previous experimental models (Gervasoni et al, 2005; Piccinni et al, 2008; Bazzichi et al, 2013); 2) allow sample stratification in respect to biological, biochemical and clinical variables; 3) apply more robust statistics, including multivariate models, and confirm present findings; 4) consider the effect of variables such as gender, smoking behavior and diet. Then, it can be envisaged to additionally determine the levels of pro-BDNF in patients' platelet and plasma, permitting to appraise of the relationship between the mature and immature forms of this neurotrophin, an index considered clinically useful (De Vincenti et al, 2019). Moreover, the measure of white blood cell parameters and indexes must be integrated to the appraise of pro-inflammatory cytokines, such as IL1 or IL6, and immunomodulatory cytokines, as IL4 or IL10, in patients' plasma. As well, these results encourage the search of other inflammatory mediators that could better

explain the here observed link between plasma BDNF variations and white blood cell indices, with the aim at applying these patterns as predictive biological indices of tendency to relapse, severity of depressive episode or responsiveness to drugs.

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