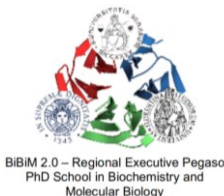

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Peripheral biomarkers and clinical correlates of post-traumatic stress disorder (PTSD) and major depressive episode in patients with bipolar disorder versus healthy controls

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Abstract

Background: Trauma related and post-traumatic stress syndromes have been subject of increasing attention in psychiatric research in most recent decades. Post-Traumatic Stress Disorder (PTSD) is a severe psychiatric disorder typically occurring after exposure to a traumatic event, causing chronic psychological suffering and leading to an often chronic and invalid course. According to the current DSM-5 classification, PTSD is characterized by 4 clusters of psychiatric symptoms, namely intrusion (criterion B), avoidance (criterion C), negative alterations in cognitions and mood (criterion D), alterations in arousal and reactivity (criterion E). Some neurobiological mechanisms might play a significant role on the development of PTSD after traumatic experiences in these subjects. The biological heterogeneity and variable symptoms presentation of PTSD suggest the need for biomarkers that reflect multiple biological measures. The sequential responses to recurrent and chronic stress by the hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system are considered to play a significant role in the onset and progression of PTSD. Decreased activity of the HPA axis and parasympathetic nervous system, along with increased activity of the sympathetic nervous system, have been observed in PTSD, which may lead to increased levels of proinflammatory cytokines. Such heightened activity of the immune system may cause alterations in the structure and function of brain regions through changes in levels of serotonin metabolites, direct neurotoxic effects of cytokines, oxidative stress, and decreasing some specific neurotrophins (as Brain Derived Neurotrophic Factor – BDNF). Furthermore, proinflammatory cytokines induced by stress and traumatic event exposure have been also implicated in the upregulation of the indoleamine 2,3-dioxygenase (IDO), which is a crucial enzyme in the kynurenine (KYN) pathway, a metabolite of tryptophan (TRP) degradation. The activation of IDO can result in the decrease of serotonin (5-HT) concentration and the impairment of TRP metabolic fluxes, including those related to KYN and quinolinic acid (QUIN), that have been involved in the NMDA neurotransmission and possible neurotoxicity. Clinical and epidemiological studies have often remarked the strong association between PTSD and other mental disorders, particularly bipolar disorder (BD), with increased PTSD prevalence rates among these patients with respect to the general population and consequent greater symptoms severity, number of hospitalizations and worsening in quality of life. Patients with BD have been reported to be particularly exposed to lifetime traumatic events as well as being more prone to report post-traumatic stress symptoms across the lifespan even when exposed to minor, even if often multiple, lifetime potentially traumatic events. On the counterparts, the same biochemical parameters mentioned above have also been investigated in patients with mood disorders, particularly major depression, with findings highlighting dysregulation in HPA axis, immune and oxidative stress systems, TRP metabolism and neurotrophins. Scant and conflicting data are currently available in the literature about potential biomarkers of PTSD, and even less on possible comparisons of PTSD and depressive state biomarkers in subjects affected by BD.

Aims: The aim of the present investigation was to evaluate potential biochemical markers of PTSD in a group of BD subjects in euthymic phase (PTSD group), compared with a group of subjects with BD with a major depressive episode (DEP group) and a healthy control group (CTL group). In particular, we aimed to analyze, among the different groups of subjects, the circulating concentrations of: 1) 5-HT; 2) TRP, KYN, and QUIN, in order to evaluate the main TRP metabolic pathways; 3) melatonin (particularly its urinary metabolite 6-OH-melatonin-S); 4) plasmatic (PPP) and intraplatelet (PLT) BDNF; 5) IL-6 and IL-1β, as the most investigated and frequently reported altered cytokines in PTSD; 6) superoxide dismutase (SOD), catalase (CAT) and total thiols, as a potential marker of oxidative-stress and impaired antioxidant processes in this population; 7) cortisol, as principal effector of HPA axis that is involved in stress-related reactions. Moreover, we aimed at evaluating the possible correlations between clinical features, as measured by the psychometric scales, and biochemical parameters.

Methods: A sample of patients with BD in euthymic phase with PTSD (PTSD group) or with a major depressive episode (DEP group) were recruited among patients followed at the Psychiatric Clinic of the University of Pisa, whereas unrelated controls (CTL group) were recruited on a voluntary basis. All subjects underwent a psychiatric assessment and concomitant biological samplings. The psychometric instruments included: the Structured Clinical Interview for DSM-5 (SCID-5), the Hamilton Depression Rating Scale (HAM-D), the Young Mania Rating Scale (YMRS), the Impact of Event Scale-Revised (IES-R), the Mood Spectrum-Self-Report lifetime version (MOODS-SR), the Trauma and Loss Spectrum-Self Report lifetime version (TALS-SR), and the Work and Social Adjustment Scale (WSAS). A sample of peripheral venous blood, besides a sample of nighttime and first morning urines were withdrawn from all the subjects and then processed for obtaining the different analytical specimen for biochemical assessments: the platelet poor plasma (PPP), platelet pellets, serum and sediment-free urines. All biological parameters under investigation were measured by means of dedicated Enzyme-linked immunosorbent assays (ELISA) or other clinical chemistry procedures. Biochemical evaluations were performed at the Laboratory of Biochemistry of Department of Pharmacy, University of Pisa.

Results: Our results showed significantly lower TRP levels in the DEP group than in the CTL one. Both PTSD and DEP groups exhibited lower 5-HT levels when compared with CTL group, and DEP group reported lower 5-HT levels than PTSD ones. Further, DEP group showed significantly higher QUIN concentrations and lower 6-OH-Melatonin-S levels than the CTL group. The intraplatelet (PLT) BDNF levels resulted significantly lower in the DEP group only when compared with the CTL one. Furthermore, both PTSD and DEP groups showed lower SOD and Total Thiols levels than CTL ones, while PTSD patients reported also lower CAT levels with respect the CTL group. IL-6 levels were found significantly higher in the PTSD and DEP groups with respect to the CTL one. Urinary cortisol levels resulted significantly higher in the PTSD group when compared with DEP and CTL ones. Finally, no differences emerged in the IL-1β, KYN and plasmatic (PPP) BDNF levels among groups. Specific patterns of association were found among acute and lifetime post-traumatic stress or mood symptoms and biochemical variables. Finally, discriminant and logistic regression analyses contributed to identify a subset of biochemical variables more associated to both groups of BD patients with respect to the CTL one, whereas specific biological variables were found more closely associated to the PTSD group than the DEP one.

Conclusions: To the best of our knowledge, this is the first study investigating and comparing possible peripheral biomarkers of PTSD and major depressive episode in patients with BD versus healthy controls. Our results suggest the key role of a chronic low-grade inflammatory state in BD patients, both with comorbid PTSD and in a major depressive episode, probably related to a dysregulation in HPA axis and cortisol release, with an increase in proinflammatory cytokines including IL-6. This seems to be predominant in patients with BD with comorbid PTSD, with a higher oxidative distress as measured by the decrease in circulating CAT, SOD and total thiols. Conversely, 5-HT and TRP metabolic pathway, including both KYN shunt and melatonin production, seem to be more markedly altered in patients with BD in a major depressive episode. Our findings point out the role of neurobiological substrates in BD patients, with specific state biomarkers of mental disorder, related to the cross-sectional symptomatology (PTSD and major depressive episode), suggesting peculiar neurobiological pathway in PTSD and depression. This is fundamental to allow the development of new specific tailored psychopharmacological treatments for these psychopathological conditions.

SSD: BIO/10, BIO/11, MED/25.

1. Introduction

1.1 Post-Traumatic Stress Disorder (PTSD)

Post-Traumatic Stress Disorder (PTSD) is a severe psychiatric disorder typically occurring after exposure to a traumatic event, causing chronic psychological suffering and also leading to an often chronic and invalid course (Brady et al., 2000; Howard and Hopwood 2003; Hamner et al., 2004; Peleg and Shalev 2006; McHugh and Treisman, 2007; Javidi and Yadollahie, 2012). The lifetime prevalence of PTSD varies across studies upon the instruments adopted for the assessments, and it is estimated around 3.9% in the worldwide with double rates in female rather than male subjects (10–12% vs. 5–6%) (Passos et al., 2015). PTSD definition and its biological and psychic aspects have only recently been understood. The 1970s represented a turning point for the psychiatric nosography of PTSD. It was indeed outlined by several studies that the Vietnam war, on a psychopathological level, was one of the most devastating for the militaries and veterans who supported it. The war provided a large sample of subjects which allowed to perform multiple studies that have been indispensable for reaching the current diagnosis of PTSD (Snow et al., 1988; Dohrenwend et al., 20008; Koenen et al., 2008). In 1980, with the publishing of the third edition of the Diagnostic and Statistical Manual for Mental Disorders (DSM III) by the American Psychiatric Association (APA), the first definition of Post-Traumatic Stress Disorder came into being, with the aim of isolating and defining all mental illness which appeared after a trauma with similar symptomatic characteristics. For the first time a mental disorder that arose as a result of exposure to a traumatic event in healthy and unprepared subjects was introduced in the psychopathological panorama, therefore considering an external factor to the individual, not an intrinsic weakness of the person, as etiopathogenetic for a psychiatric disorder.

In DSM-5 (APA, 2013) and DSM-5-TR (APA, 2022), the latest editions of the diagnostic manual published to date, PTSD no longer falls into anxiety disorders but has acquired its own nosographic

dignity, and has been included in a specific chapter dedicated to "Disorders related to Traumatic and Stressful Events", which includes mental illnesses in which exposure to a traumatic or stressful event is explicitly listed as a diagnostic criterion. Indeed, among these mental disorders, we find the Reactive Attachment Disorder, the Uninhibited Social Engagement Disorder and the Acute Stress Disorder and the Attachment Disorders. This important modification is mainly based on the presence of phenotypes in which the most evident characteristics are anhedonia, dysphoria, anger and aggression or even dissociative symptoms, rather than aspects of strong anxiety or fear. Furthermore, a difference has been introduced between the Acute Stress Disorder, in which symptoms immediately following exposure to traumas are rather considered, and a chronic stress disorder, thus considering the diagnosis of PTSD possible only if the symptomatic picture exceeds the duration of a month (Friedman et al., 2011). As regards the PTSD diagnostic criteria, the most significant innovations are:

- the division of criterion C into two distinct sections, namely active avoidance and negative alterations of thought and emotions;
- the inclusion of the E2 criterion, which includes the so-called maladaptive behaviors, eliminating the presence of double diagnoses (previously many of these patients were diagnosed with PTSD in conjunction with a Substance-Use Disorder);
- the new definition of criterion A, eliminating the aspect of feelings of intense fear, helplessness and terror, following various studies which have highlighted their secondary importance for diagnostic purposes;
- the delineation of the concept of trauma, to make a more targeted selection on which events were actually to be considered traumatic and which instead were to be excluded (for example, it is specified that medical accidents are qualified as traumatic only if they are "events sudden and catastrophic").

PTSD is characterized by the development of a typical symptomatology in the aftermath of the exposure to one or more traumatic events, from which derives a disproportionate psychological

suffering. It is resistant to pharmacological treatments, with a disabling clinical picture, often characterized by a chronic course and associated with a significative deterioration in the quality of life and a high suicidal risk (Brady et al., 2000; Howard et al., 2003). Yehuda et al. in 2000 described the disorder as the body's inability to reestablish homeostasis conditions prior to exposure to the stressor. In the current list of mental disorders, the diagnosis of PTSD consists of 8 criteria that must be simultaneously met. The first criterion (A) concerns with the exposure to the traumatic event, criteria B, C, D and E instead describe the main symptomatic characteristics, while the last three criteria, F, G and H, respectively explain the time threshold that must be exceeded, the significant discomfort that must be caused by the pathology and the exclusion of other possible causes to which the clinical picture could be attributed.

A) Exposure to actual or threatened death, serious injury, or sexual violence in ≥1 of the following way(s):

   1. Directly experiencing the event
   2. Witnessing the event in person as it occurred to others
   3. Learning that the traumatic event occurred to a close family member/close friend; if actual/threatened death of a family member or friend, event must have been violent or accidental
   4. Experiencing repeated or extreme exposure to aversive details of the traumatic event (e.g. first responders collecting human remains or police officers repeatedly exposed to details of child abuse)

B) Presence of ≥1 of the following intrusion symptoms associated with the traumatic event, beginning after the traumatic event occurred:

   1. Recurrent, involuntary, and intrusive distressing memories of the traumatic event
   2. Recurrent distressing dreams where content and/or affect of dream are related to the

traumatic event

3. Dissociative reactions, e.g. flashbacks, where individual feels or acts as if the traumatic event were recurring

4. Intense or prolonged psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event

5. Marked physiological reactions to internal or external cues that symbolize or resemble an aspect of the traumatic event

C) Persistent avoidance of stimuli associated with the traumatic event, beginning after the traumatic event occurred, as evidenced by ≥1 of the following:

1. Avoidance of (or efforts to avoid) distressing memories, thoughts, or feelings about or closely associated with the traumatic event

2. Avoidance of (or efforts to avoid) external reminders (e.g. people, places, conversations, activities, objects, situations) that arouse distressing memories, thoughts, or feelings about or closely associated with the traumatic event

D) Negative alterations in cognitions and mood associated with the traumatic event, beginning or worsening after the traumatic event occurred, as evidenced by ≥2 of the following:

1. Inability to remember an important aspect of the traumatic event (typically due to dissociative amnesia and not to other factors such as head injury, alcohol, or drugs)

2. Persistent and exaggerated negative beliefs or expectations about oneself, others, or the world

3. Persistent, distorted cognitions about the cause or consequences of the traumatic event that lead the individual to blame themselves or others

4. Persistent negative emotional state

5. Markedly diminished interest or participation in significant activities
6. Feelings of detachment or estrangement from others
7. Persistent inability to experience positive emotions

E) Marked alterations in arousal and reactivity associated with the traumatic event, beginning or worsening after the traumatic event occurred, as evidenced by ≥2 of the following:

1. Irritable behavior and angry outbursts (with little or no provocation) typically expressed as verbal or physical aggression toward people or objects
2. Reckless or self-destructive behavior
3. Hypervigilance
4. Exaggerated startle response
5. Problems with concentration
6. Sleep disturbance

F) Duration of the disturbance (criteria B, C, D, E) is >1 month

G) The disturbance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning

H) The disturbance is not attributable to the physiological effects of a substance (e.g. medication, alcohol) or another medical condition.

The DSM-5 also allows the possibility of specifying if the typical PTSD symptomatology is associated with dissociative symptoms: in some cases, individuals experience depersonalization and/or derealization. Depersonalization consists in persistent and recurrent feelings of detachment from the body as if patients were external observer of themselves, whereas derealization is the persistent and recurrent perception of unreality of the surrounding environment. Finally, there is the

specifier "with delayed expression" concerning the late onset of the symptomatologic frameworks, used if the diagnostic criteria are not fully satisfied within six months from the event, although the onset and expression of some symptoms may be immediate.

Increasing evidence suggests that bipolar disorder (BD) is frequently associated to a history of traumatic experiences and PTSD, leading to a consequent greater symptoms' severity, number of hospitalizations and worsening in quality of life. Some neurobiological mechanisms might play a significant role on the development of PTSD after traumatic experiences in these subjects (Carmassi et al., 2020a; 2020b; 2023).

1.2 The link between PTSD, inflammation and immune system alterations

PTSD is a heterogeneous disorder and multiple pathophysiology pathways are hypothesized to contribute to its onset and endurance. In recent years, the scientific community has explored the possible biological pathways underlying the disorder, highlighting a probable linkage with dysregulations of the hypothalamic-pituitary-adrenal (HPA) axis, autonomic nervous system and monoaminergic transmission system, as well as with impaired inflammatory and immune responses. Particularly, the HPA axis has been widely investigated in PTSD: stress exposure causes the release of corticotropin-releasing factor (CRF) and vasopressin from the paraventricular nucleus of the hypothalamus to stimulate the anterior pituitary gland, which in turn secretes adrenocorticotropic hormone (ACTH) into the systemic circulation. The ACTH induces the release of glucocorticoids, especially cortisol, from the cortical part of adrenal glands; at the same time, stressing situations induces the release of catecholamines (epinephrine and norepinephrine) from the medulla of the adrenal glands. ACTH, cortisol and catecholamines responses depend on the type and intensity of the stressor (Herman et al., 2016; Kim et al., 2019; Palego et al, 2021).
Increasing evidence has been reported on the possible role of inflammation and immunological dysregulations in PTSD pathogenesis, so that pro-inflammatory cytokines are thought to relevantly contribute to the illness presentations, for instance, through the activation of the NF-κB and P38MAPK signal path. Particularly, alterations of interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) as well as interferon-γ (INF-γ) have been implicated in impaired processes of synaptic plasticity and neuroinflammation paths, underlying functional and cognitive anomalies related to PTSD (Levin et al., 2017). Moreover, the imbalance between reactive oxygen species (ROS) damages and the activity of antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD), have been described (Karanikas et al., 2021). Similarly, a link between PTSD and an increased risk of physical diseases, such as cardiovascular diseases and autoimmune diseases has

been described, suggesting that a dysregulated inflammatory component can be a common subset of the condition (Levine et al., 2014).

Furthermore, proinflammatory cytokines induced by stress and traumatic event exposure have been also implicated in the upregulation of the indoleamine 2,3-dioxygenase (IDO), which is a crucial enzyme in the kynurenine shunt, a main pathway of tryptophan degradation. The activation of IDO results, at least in acute, in the decrease of tryptophan concentration and the increase of several metabolites, including kynurenic and quinolinic acids, that have been involved in the NMDA neurotransmission and possible neurotoxicity (Hori et al., 2019; Kim et al., 2019; Katrinli et al. 2022) (see **Figure I1**).

Although the existing literature indicates possible changes of immune biomarkers in PTSD, the results in human models are still conflicting (Sbisa et al., 2023). As a consequence, there is no full consensus on their use as biomarkers in clinical practice.

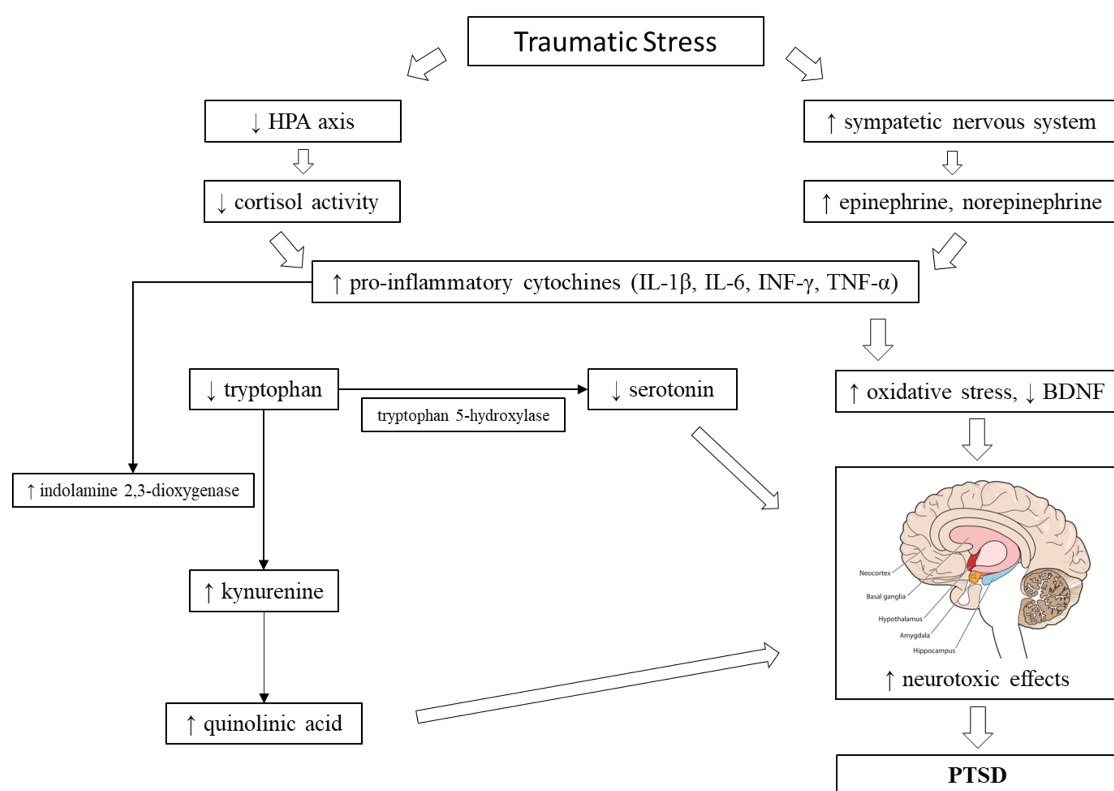


Fig. I1. Schematic representation of the alterations in the HPA axis, autonomic nervous system, and immune system in response to traumatic events, which lead to alterations in structure and function of the amygdala, hippocampus, medial prefrontal cortex, anterior cingulate cortex and insula in PTSD. Adapted from *Kim et al., 2015*.

1.2.1 PTSD and cortisol

During acute stress, the neurons in the paraventricular nucleus of the hypothalamus secretes CRF under the influence of serotonin from the amygdala (Stalder et al., 2017), norepinephrine and indirect limbic inputs and medial prefrontal cortex (Herman et al., 2016). CRF stimulates the pituitary gland to release ACTH, which causes the adrenal cortex to produce cortisol, which can influence and inhibit many immune reactions, neuronal defensive and metabolic mechanisms (Morris et al., 2012). Cortisol, as the primary effector molecule of the HPA axis, induces a variety of effects throughout the body to support the stress response, including suppressing insulin action and enhancing glucose availability, regulating immune system functions, impacting electrolyte balance (Myers et al., 2014) and mood and sexual behaviour (Sapolsky et al., 2000). Particularly, cortisol is the primary endogenous glucocorticoid in humans. It is a non-polar steroid molecule, that easily crosses the cell membrane where it can bind with the glucocorticoid receptors molecular complex. Glucocorticoids receptors are present in all body tissues, including most brain regions, where they contribute to brain energetics and synaptic physiology (Myers et al., 2014). At the cellular level, glucocorticoids receptors reside primarily in the cytosol, where they process the slow, genomic effects of cortisol. Within the cytosol, upon binding cortisol, the complex undergoes a conformation change and initiates transactivation, involving homodimerization and translocation to the nucleus, where it binds to glucocorticoid response elements to activate transcription of specific genes (Grad and Picard, 2007). Cortisol is both the primary molecule that enables the stress response as well as the primary inhibitor of ongoing HPA axis activity, by a negative feedback signal (Dunlop and Wong, 2019).

Cortisol levels typically follow a strong diurnal rhythm: particularly, levels are high on waking, rise an average of 50–60% in the 30–40 min after waking, fall rapidly in subsequent few hours after the awakening raise and then fall more slowly until reaching a nadir at around bedtime (Pruessner et al., 1997; Adam and Kumari, 2009). Variation in cortisol levels as a function of time of day is substantial (Adam et al., 2017). Healthy adults produce about 10 mg of cortisol daily from the adrenal glands,

though the dynamic range of HPA responsiveness is demonstrated under periods of intense stress, when up to 400 mg may be produced in a day (Esteban et al., 1991; Lamberts et al., 1997). Diurnal cortisol rhythm changes with emotional and psychosocial stress (Adam and Gunnar, 2001; Adam et al., 2006; Doane and Adam, 2010), being related to health outcomes (Sephton et al., 2000; Matthews et al., 2006; Kumari et al., 2009; Doane et al., 2013). Diurnal cortisol rhythm also varies in respect to both adverse experience and worse health, being associated with a flattened glucocorticoid release across the waking day. It has therefore been proposed that it may be one mechanism by which stress influences negative health outcomes (Sephton and Spiegel, 2003). Cortisol's diurnal variation may be an important element of its regulatory actions. Cortisol is one pathway by which central circadian rhythms are signalled to multiple peripheral biological systems (Bass and Lazar, 2016; Man et al., 2016). Disruption of cortisol's circadian pattern and signalling may affect the functioning of a diverse set of central and peripheral systems, with these effects cascading over time to contribute to a wide variety of negative health outcomes. Associations between flattened cortisol release and depression were reported (Doane et al., 2013).

There are various biological matrices such as plasma, serum, saliva, hair, cerebrospinal fluid and urine that could be used to measure cortisol (Klaassens et al., 2012; Meewisse et al., 2007; Steudte-Schmiedgen et al., 2015). Nevertheless, because of their non-invasive nature, saliva and urine have always been chosen as the preferred sources of samples for cortisol measurement (Wahbeh and Oken, 2013; Wingenfeld et al. 2015; Pan et al., 2018). The diurnal cortisol rhythm has been divided into several key components which provide complementary information, and the more frequently examined are: the average level of cortisol across the day, the size of the post-awakening surge, called the cortisol awakening response, and the diurnal cortisol slope, the degree of change in cortisol from morning to evening over the waking day (Adam and Kumari, 2009). It is generally believed that 24-h urinary cortisol levels provide an integrated measure that is more trustworthy than that obtained from random salivary samples (Wingenfeld et al., 2007). A recent meta-analysis comparing the 24-h urinary cortisol levels data between PTSD patients and the controls showed lower concentrations of

24-h urinary cortisol in patients with PTSD than in controls (Pan et al., 2020). Further, nocturnal urinary cortisol may represent a non-invasive good estimate of the cortisol circadian rhythm because reflecting its production levels from the nadir (at the bedtime) to the awakening peak.

Individuals with PTSD often exhibit dysregulated HPA function in the form of enhanced glucocorticoid negative feedback (Morris et al., 2012; Yehuda, 2002) and lower circulating cortisol levels compared to non-traumatized controls (Meewisse et al., 2007, Miller et al., 2007, Morris et al., 2012; Klaassens et al., 2012). However, it remains unclear whether HPA dysregulation is present in the immediate aftermath of trauma and contributes to elevated risk for developing PTSD (Delahanty and Nugent, 2006, Morris and Rao, 2013). Trauma-exposed individuals without PTSD also exhibit enhanced HPA negative feedback, like those with PTSD, but do not differ from non-traumatized controls in their daily cortisol output (Morris et al., 2012). Prospective studies indicate that individuals vulnerable to developing PTSD are characterized by dysregulations at different levels of the glucocorticoid signalling cascade (van Zuiden et al., 2013), including lower circulating cortisol levels prior to (Steudte-Schmiedgen et al., 2015) and in the early aftermath of trauma exposure (Luo et al., 2012, van Zuiden et al., 2012). Glucocorticoid receptors signalling also participate in the suppression of inflammatory molecules, including interleukin-6, interleukin-1β, tumor necrosis factor-α and interferon-γ, which are often elevated in PTSD (Dimitrov et al., 2004; Passos et al., 2015). The complexity of glucocorticoid receptor signalling presents a significant challenge to identify the pathophysiological consequences of altered HPA axis function in patients with PTSD and other psychiatric illnesses (Morris et al., 2016). However, those studies that have examined acute posttraumatic cortisol levels as risk biomarkers of PTSD appear to be influenced by a variety of moderators including the timing of PTSD assessment, timing of cortisol measurement, gender, and developmental stage (Delahanty and Nugent, 2006, van Zuiden et al., 2013). HPA axis' dysregulation in PTSD in summarized in **Figure I2**.

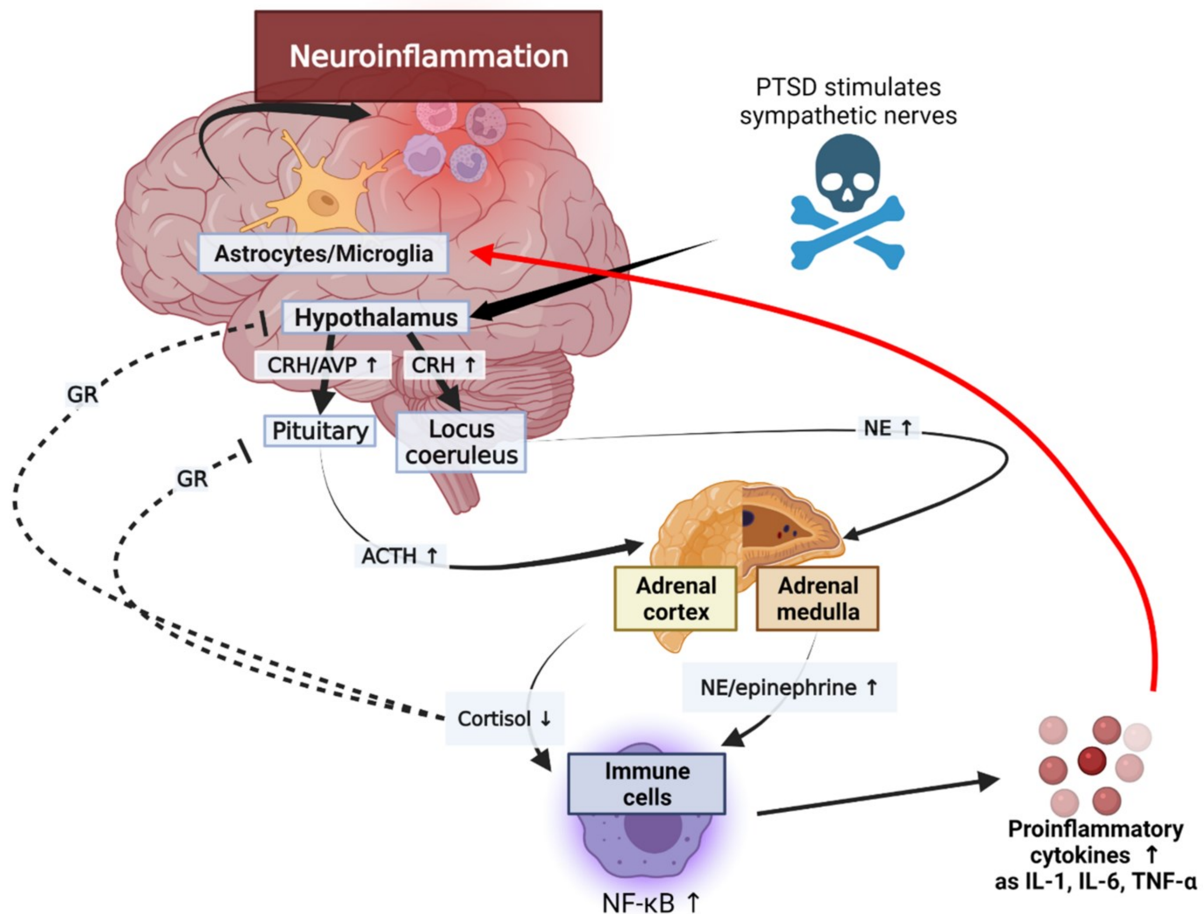


Figure I2: Dysregulated HPA axis and immune system in PTSD. From *Lee et al., 2022*.

1.2.2 PTSD, proinflammatory cytokine and oxidative stress

Peripheral pro-inflammatory cytokines could produce neuroinflammation by crossing the blood-brain barrier (BBB) (Banks et al., 2002), leading to a blockade in the neurogenesis mechanism, as reported in human studies showing a volume reduction of some regions within the central nervous system (CNS), as the hippocampus, in PTSD subjects versus controls (Kuhn and Gallinat, 2013; Monje et al., 2003). Several studies have showed significantly higher levels of pro-inflammatory cytokines in PTSD subjects (Bruenig et al., 2017; Lindqvist et al., 2017), probably due to the hyperactivity of the sympathetic nervous system and to the alteration of the HPA axis function (Bersani et al., 2016). Under this condition, the secretion of pro-inflammatory cytokines is stimulated through the release of catecholaminergic neurotransmitters and the activation of their adrenergic-β receptor (Tan et al.,

2007). PTSD patients showed indeed an increased expression of adrenergic-β2 receptor together a raise of norepinephrine release (Ressler and Nemeroff, 1999; Gurguis et al., 1999). The dysregulation of HPA axis, expressed by a decreased secretion of glucocorticoids (cortisol) and a glucocorticoid receptor resistance promote the pro-inflammatory cytokines secretion in PTSD (Yang and Jiang, 2020). Further, inflammatory cytokines, including interleukin IL-6, IL-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, are elevated in PTSD patients with respect to healthy controls, as emerged in a recent meta-analysis (Tursich et al., 2020). Another meta-analysis study found the levels of inflammatory markers in the PTSD were increased also in subgroup without comorbid MDD (Passos et al., 2015).

Those results suggest that inflammation could promote the development of mental diseases, as major depression, BD, schizophrenia and PTSD (Dowlati et al., 2010, Köhler et al., 2017), and that PTSD patients are in a state of “low grade” systemic pro-inflammation (Speer et al., 2018). In this regard, chronic stress stimulates pro-inflammatory biochemical imbalances in the CNS, also increasing oxidative stress damage (Liu et al., 2017; Savic et al, 2015). Free radicals, as reactive oxygen species (ROS), have also been shown to play a significant role in the pathogenesis of depression and PTSD by inducing protein oxidation. They include several radical species such as superoxide ($O2^-$), hydroxyl (HO•), hydroperoxyl (HOO•), alkoxyl (RO•), peroxyl (ROO•) and nonradical species such as hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HClO) and peroxynitrite ($ONOO^-$) (Salim, 2017). When they are massively produced or the enzymatic and nonenzymatic antioxidant scavenger systems are inefficient, cellular injury or apoptosis are activated (Stadtman, 1992). Excessive production of ROS plays an important role in the promotion of permanent degradation of equilibrium among antioxidants and prooxidant processes in cells, leading to damaging proteins, lipids and DNA in the cell, also triggering apoptosis and cell death (Palta et al., 2014; Siwek et al., 2013). The damage caused by oxidative stress activates inflammatory processes in the CNS and contributes to increase the levels of proinflammatory cytokines as well as to decrease

neurogenesis, thus promoting neurodegeneration processes, mitochondrial alterations and HPA axis dysfunction (Schiavone et al., 2013).

ROS are produced in cells and tissues not only during ischemia and trauma, but also through many different processes and metabolic routes that occur physiologically. Among these, there are principally the activation of the general/innate immune system and stimulation of phagocytes, the electron transport activity in mitochondria, lipid peroxidation, cytochrome P-450 oxidase activity, and purine metabolism through the xanthine oxidase reaction (Gutteridge, 1995). Multiple ROS-generating and ROS-eliminating systems actively maintain the intracellular redox state, which serves to mediate redox signalling and regulate cellular functions (Lennike and Cochemé, 2021). The redox status can be indirectly measured by appraising the content and the activity of some antioxidant enzyme levels, such as the so-called first line antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as by measuring the levels of plasmatic total thiols (Tezcan et al., 2003). A reduction of these antioxidant systems and chemical species can be related to oxidative stress and impaired ROS scavenging.

Both depression and PTSD are characterized by high oxidative stress pathways that results from an overload of free radicals. The overproduction of ROS decreases the antioxidant concentrations (Palta et al., 2014; Bakunina et al., 2015). If ROS can induce antioxidant defences, the persistence of oxidative stress inactivates and modifies antioxidant systems (Anderson and Maes 2014; Moskovitz and Oien, 2010). Oxidative stress damage in proteins encompass the formation of carbonyl groups and the oxidation of thiol cysteine groups.

Superoxide dismutase (SOD), as an important antioxidant enzyme, can scavenge superoxide anion radical (O_2^-) to produce hydrogen peroxide (H_2O_2) and decompose it into water and oxygen (Sakamoto and Imai, 2017). SOD includes three kinds of isoforms: the copper-zinc isoform (SOD_1), prevalently expressed in the cytoplasm, the extracellular isoform (SOD_3), and the manganese isoform (SOD_2) present in mitochondria, which are one of the main sources of body ROS though the oxidative

phosphorylation cascade (Robinson 1998). Few studies explored SOD concentration in PTSD samples: particularly, one study showed a lower serum SOD concentration in PTSD patients than controls, while no significant plasma SOD activity difference between the two groups was reported in another one (Dell'Oste et al., 2023).

Catalase (CAT) is one of the major intracellular oxidoreductase and antioxidant enzymes that forms the antioxidant system against radical-mediated damage (Tsai et al, 2016). Catalase decomposes hydrogen peroxide to H_2O, thus limiting the accumulation of the peroxide; meantime, CAT can also lead to the formation of free radicals (Lindemer et al., 2013), implying its direct participation to the hydrogen peroxide chemical redox signalling in the body (Veal et al., 2007; Di Marzo et al., 2018). There is a relationship between depression and polymorphisms in genes that are involved in oxidative pathways such as CAT (Herringa et al., 2012). Some studies reported an increase in CAT concentrations in depressed subjects with respect to controls, suggesting an overcompensation mechanism (Gałecki et al., 2009; Szuster-Ciesielska et al., 2008). Conflicting results were reported in PTSD samples (Dell'Oste et al., 2023).

Finally, to prevent oxidative stress overload and to adapt the protein-thiol redox status, glutathione peroxidase/reductase and other related enzyme activities are also active. So, thiol/disulfide homeostasis is a further method for evaluating oxidative processes. The ratio of thiol/disulfide specplays a critical role in antioxidant protection, detoxification, and signal transduction, regulation of enzymatic activity, apoptosis and cellular signalling mechanisms (Circu and Aw, 2010; Ozyazici et al., 2016). Thiols contain a sulfhydryl group (-SH) that is composed of hydrogen and sulphur atoms attached to a carbon atom. Thiols can respond to free radicals for protecting tissues and cells from the damage caused by organic compounds containing reactive oxygen products (Kemp et al., 2008). The reduction of total thiols may be a sign of oxidative status (Biswas et al., 2006; Incecik et al., 2019), as reported in schizophrenia, neurodegenerative disorders, and BD (Erzin et al., 2018; Topcuoglu et

al., 2017; Erzin et al., 2020). However, no data is still available in literature on thiols levels in PTSD (Dell'Oste et al., 2023).

In conclusion, the evaluation of redox components and first-line antioxidant enzymes in PTSD is of currently and challenging interest.

1.2.3 Tryptophan metabolism and the kynurenine pathway

Tryptophan (TRP) is an essential large neutral amino acid with an indole ring structure, which is a precursor of the neurotransmitter serotonin (5-HT) and its derivate melatonin. Diet has a strong impact on TRP endogenous levels and metabolism (Palego et al. 2016; Badawy, 2017), that can also be modulated by the gut microbiota (Gao et al., 2020). Several foods, as meat, fish, cereals, milk, chocolate and bananas are rich source of TRP (Kałużna-Czaplińska et al. 2017). Plasmatic tryptophan commonly ranges from 40 to 80 $\mu mol*L^{-1}$ (Eynard et al., 1993). Serotonin is synthesized from about 1% of the available tryptophan in the body: 80-90% by the enterochromaffin cells in the gut and 10–20% in the brain after crossing the BBB. The central availability of tryptophan depends on competition by the large amino acids for transport across the BBB (Myint, 2012). TRP can pass the BBB through a competitive transport carrier, suggesting that peripheral TRP levels may also reflect the availability of this amino acid for 5-HT and melatonin synthesis in the CNS, for which TRP is the principal source (Kałużna-Czaplińska et al. 2017; Savino et al. 2020; Brown et al., 2021). Details on melatonin synthesis from TRP and 5-HT through the methoxyindole pathway are illustrated in **Figure I3**.

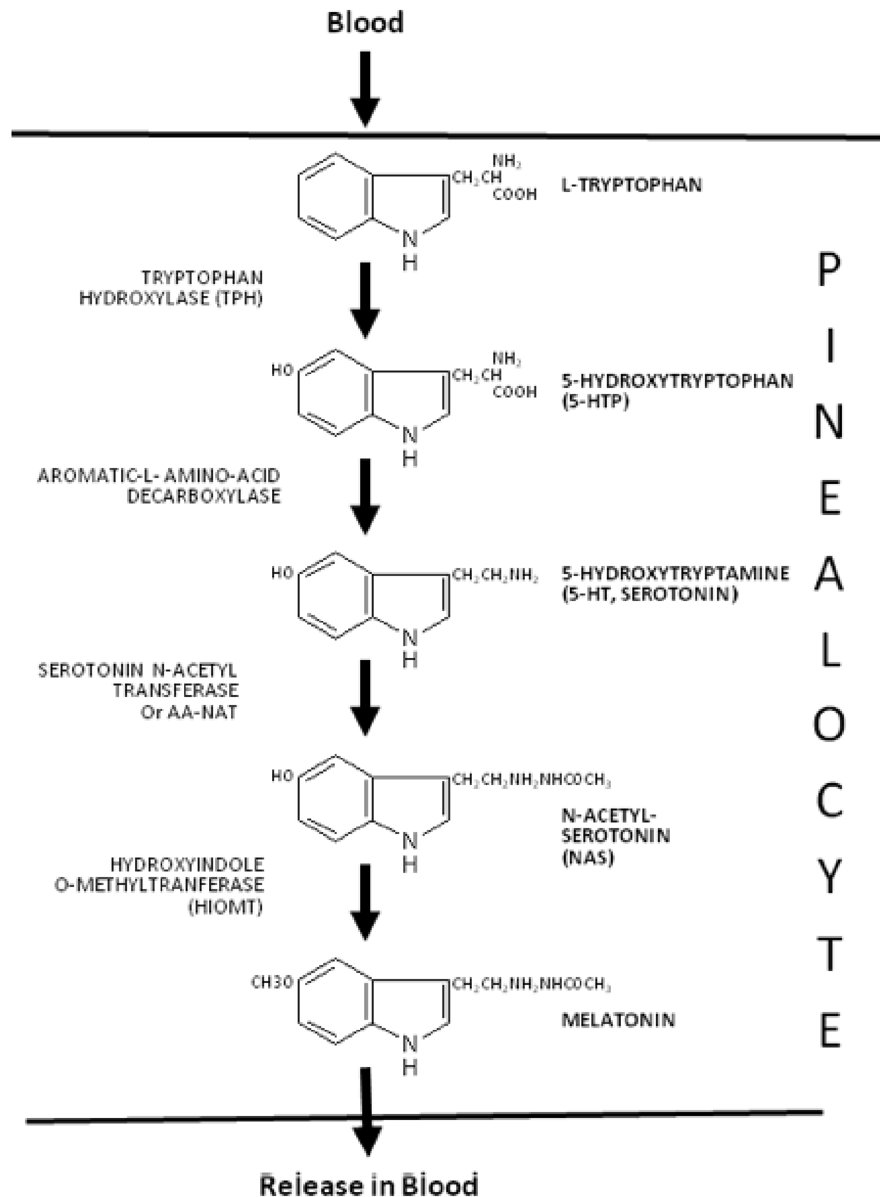


Figure I3: The TRP methoxyindole pathway (in the pineal gland). Adapted from *Norman and Henry, 2015*. The TRP-hydroxylase isoform TPH1 is active in other tissues than the neuronal ones, where the TPH2 isoform is instead rather expressed.

However, TRP metabolism is not limited to the 5-HT pathway: in fact, other TRP-derived metabolites play a crucial role in many additional processes, such as the immune and inflammatory responses, oxidative stress regulation, being key mediators between the immune and neuroendocrine systems (Kałużna-Czaplińska et al. 2017; Savino et al. 2020; Brown et al., 2021). The amount of TRP metabolized into 5-HT through the methoxyindole pathway is about only 1-2 % of the total one, since

the main biotransformation of this essential amino acid is represented by the kynurenine (KYN) shunt, also known as the TRP catabolite (TRYCAT) pathway (see **Figure I4**).

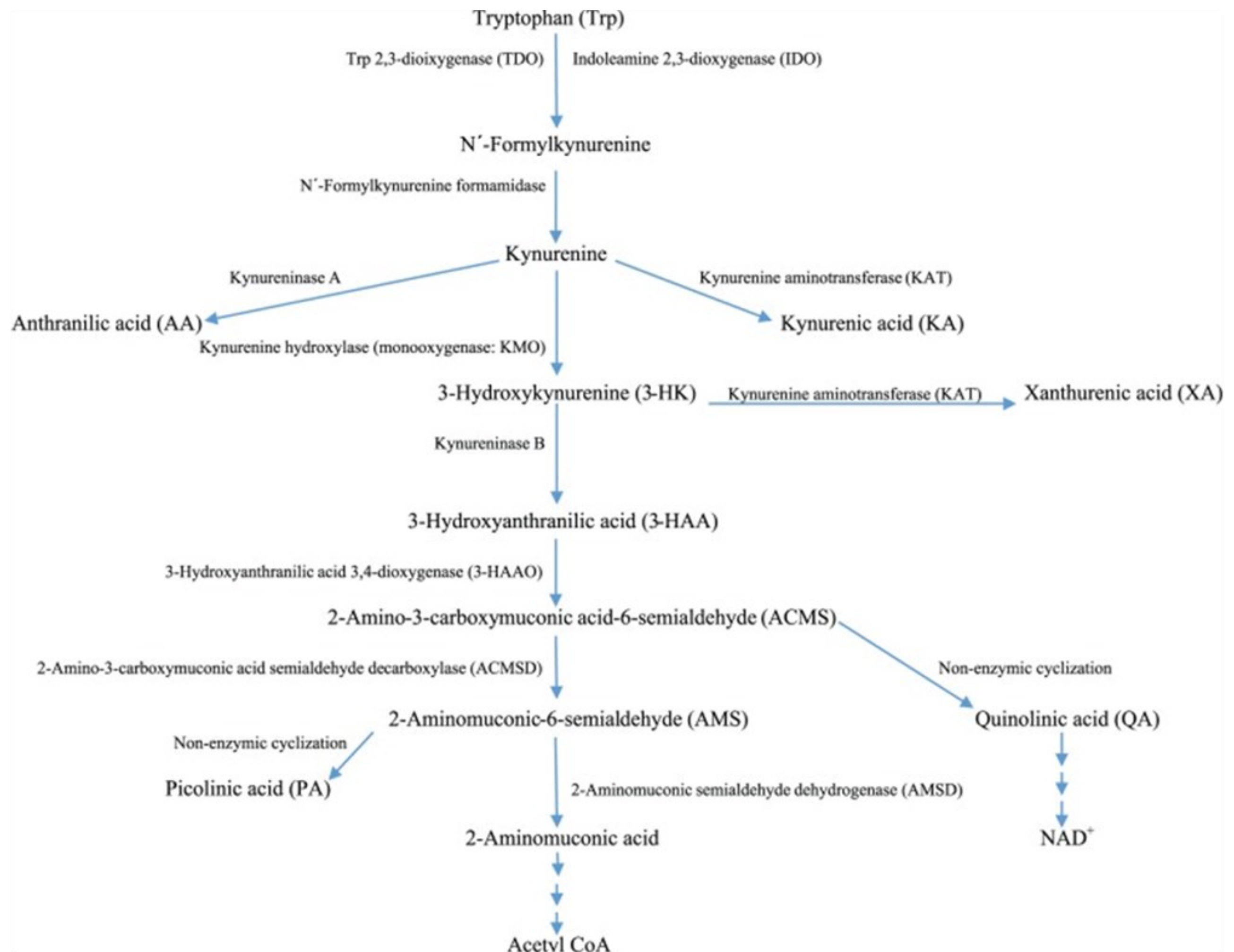


Figure I4: The tryptophan-deriving kynurenine (KYN) shunt with its main metabolic branches. From *Badawy, 2017.*

About 99% of tryptophan is metabolized to N-formyl KYN by the indole-2,3-dioxygenase (IDO) or, in a minor quote and mainly in the liver and kidneys, by the TRP-2,3-dioxygenase (TDO). TDO activity is mainly controlled by the tryptophan level itself and therefore its activity is generally stable (Watanabe et al., 1980). It is supposed that only the 0.5 % of total TRP in the body would be excreted unchanged (Kałużna-Czaplińska et al. 2017). Subsequently, the KYN formamidase enzyme metabolizes N-formyl KYN into L-KYN. After that, the pathway further splits in different possible

ways. Starting from KYN, it can lead to the production of quinolinic acid (QUIN), or also of picolinic acid (PA), through the production of 3-hydroxy-L-KYN (3-HK) and 3-hydroxyanthranilic acid (3-HAA). Moreover, QUIN can be further transformed, by QUIN phosphoribosyl transferase (QPRT), into NAD^+. This way is known as the "neurotoxic branch": the catabolism proceeds in the liver either into the complete oxidation pathway and ATP formation or into the production of quinolinic acid (QUIN), which is finally degraded into NAD^+. Under physiological conditions, the catabolism goes mainly to ATP formation and only a minor portion proceed towardso NAD^+ formation (Myint, 2012). Since ATP formation in the cells is dependent on NAD^+, depletion of NAD^+ is fatal to the cells especially if they are under stress. In the normal state, to get normal NAD^+ requirements, QUIN synthesis occurs only transiently in the liver and QUIN does not accumulate inside hepatocytes (Bender, 1989). The other path originating from KYN is known as the "neuroprotective branch", through the transformation of this metabolite into kynurenic acid (KYNA) by the enzyme KYN aminotransferase (KAT) (Savino et al. 2020, Brown, 2021). This metabolism in the liver is more or less stable and age and gender have an influence on it (Leklem, 1971). The KYN pathway also plays a role in glucose metabolism. For instance, while 3-HAA formed from this pathway inhibit the mitochondrial respiratory sistem (Quagliariello et al., 1964), KYN, KYN-metabolites and overall QUIN inhibit gluconeogenesis (Lardy, 1971), implying a potential modulatory effect of these substrates on carbohydrate metabolism during stress response.

The TRP metabolism has a possible impact on neuroinflammation by affecting the balance between excitatory and inhibitory processes (Lim et al. 2016; Brown et al., 2021). An impaired balance between glutamate-linked excitatory transmission and gamma-aminobutyric acid (GABA) inhibitory action may indeed generate excitotoxic effects (Myint, 2012). TRP and KYN can be transported by the BBB (Bryn et al. 2017). It is estimated that 60% of brain KYN derives from the periphery (Gal and Sherman, 1980). In the CNS, while KYNA is known to exert a neuroprotective effect, the QUIN pathway has been linked to potentially neurotoxic effects. In particular, KYNA is an antagonist of N-methyl-D-aspartate (NMDA) receptors and modulates excitotoxic processes linked to glutamate

transmission (Savino et al. 2020; Brown et al., 2021). KYNA is also an antagonist of kainate and α-7 nicotinic acetyl choline receptors, whereas QUIN is instead an agonist of them (Brown et al., 2021; Bilgiç et al. 2022). Other potentially protective functions of KYNA are represented by its action as an inductor of the amyloid degrading enzyme, and its role as a free radical scavenger and antioxidant molecule. Moreover, KYNA showed an anti-inflammatory action as an agonist of G-protein coupled receptor (GPR35), that regulates the production of cyclic adenosine monophosphate (c-AMP) and the release of cytokines (Bilgiç et al. 2022; Brown et al., 2021). However, the excess of inhibition of NMDA and cholinergic receptors caused by increased KYNA levels seems to lead to detrimental effects (Brown et al., 2021). Conversely, QUIN has been reported to activate NMDA receptors, distributed in specific brain regions such as the striatum and the hippocampus (Lugo-Huitrón R et al. 2013; Savino et al. 2020). The physiological role of this molecule seems to be linked to its action as a glutamatergic excitotoxin produced in response to inflammation, in order to provide more energy to cells by increasing NAD^+ levels (Savino et al. 2020; Brown et al., 2021). QUIN is also involved in free radicals and oxidative stress generation, including the increase of ROS, decreased glutathione levels and SOD activity (Lugo-Huitrón R et al. 2013; Savino et al. 2020; Brown et al., 2021). In addition, QUIN is involved in the neural apoptosis due to an intracellular calcium imbalance (Williams et al. 2017; Savino et al. 2020) (See **Figure I5**).

Thus, QUIN, as glutamate, exerts important CNS roles but is also endowed of excitotoxic and neurotoxic effects, following a kind of physiological paradox; it is therefore conceivable to think that a dynamic and adapted balance between KYNA and quinolinic acid production is required, without a remarkable accumulation or loss of these two substrates over time.

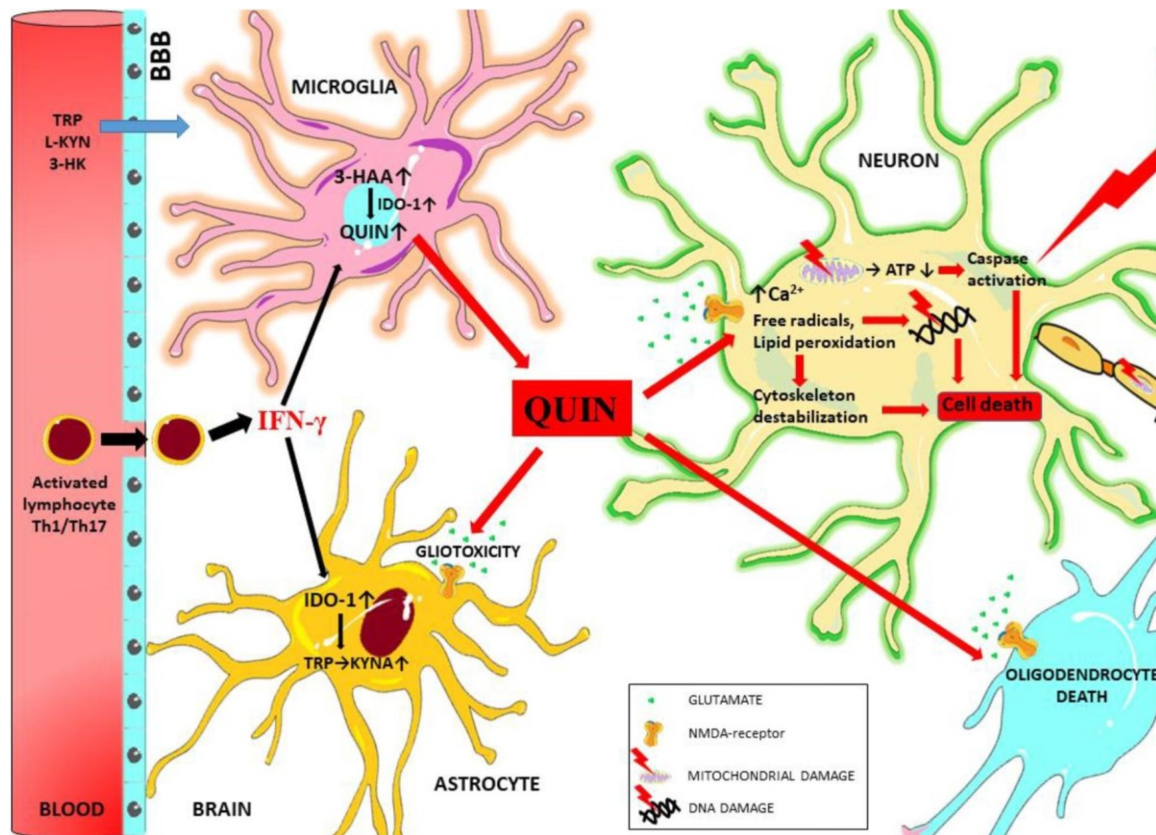


Figure I5: Summary of the neurotoxic mechanisms involving QUIN. Adapted from *Pukoli et al., 2021*.

Inflammation, infection or oxidative stress induce the limiting enzyme IDO and the KYN shunt: cortisol and several cytokines, such as IL-6, IL-1β or TNF-α may activate the limiting enzymes of the KYN pathway (Tordjman et al. 2014, Savino et al. 2020), specifically shifting it towards the production of QUIN. Further, the activation of the KYN pathway will lead not only to an increased synthesis of its metabolites, but also to TRP depletion and to the reduction of TRP metabolism through the methoxyindole route (Bilgiç et al. 2022; Brown et al., 2021). It should be also pointed out that melatonin, the hormone regulating circadian rhythmicity and sleep derived by the 5-HT metabolism, has been found to regulate the shift between its own synthesis and the KYN shunt, since this molecule is able to induce IDO activity (Li et al. 2017; Savino et al. 2020). This suggests that melatonin is capable of modulating both molecular circadian drives and metabolic homeostasis.

Tryptophan degradation across the kynurenine pathway and the release of neurotoxic metabolites is reported to be implicated in stress-related psychiatric disorders (Myint, 2012). Reduced neurogenesis

and a lack of neurotrophic support, such as that reflected in reduced plasma brain-derived neurotrophic factor (BDNF) levels, as well as increased stress hormones, are consistent findings in stress-related disorders, including Multiple sclerosis, Alzheimer's and Parkinson's disease, psychosis and schizophrenia, anxiety, somatic symptoms disorder, BD and depression (Abdallah et al., 2018; Ormstad et al. 2018; Songtachalert et al., 2018; Bilgiç et al. 2022; Brown et al., 2021; Marx et al., 2021). Lower peripheral levels of TRP, and KYN were reported in meta-analyses on mood disorders and schizophrenia (Marx et al. 2021; Hebbrecht et al. 2021). Furthermore, a higher production of QUIN with respect to KYNA were reported only in mood disorders than schizophrenia (Marx et al. 2020). Lower KYNA/QUIN ratio would be found only in mood disorders, while lower KYNA/KYN and increased KYN/TRP would be detected in both Schizophrenia and Major depressive disorder (Marx et al. 2020; Kindler et al. 2020; Hebbrecht et al. 2021). Increased QUIN and KYN levels have also been associated with suicidal ideation/behaviors (Erhardt et al. 2013; Sublette et al. 2011; Lim et al. 2016; Bryn et al. 2017).

Future studies should be performed for both therapeutic purposes (Muneer, 2020) and for estimating the possible use of the various metabolites of the KYN pathway as biomarkers of psychiatric disorders. Finally, research on TRP metabolism in PTSD, particularly on the KYN pathway, is still limited, showing a specific lack of studies. A recent systematic review showed only one study analysing the serum kynurenine/tryptophan ratio, but no statistically significant differences between PTSD patients and healthy controls emerged (Dell'Oste et al., 2023). Further studies on this field are needed.

1.2.4 BDNF in mood disorders and PTSD

Neurotrophins represent a family of regulatory factors capable of mediate neuronal differentiation and survival, as well as modulate the signal transmission and synapse plasticity (Patapoutian and Reichardt, 2001; Poo, 2001). Five neurotrophins have been isolated: the factor of nerve growth (Nerve

Growth Factor, NGF), the neurotrophic factor of brain origin (Brain-Derived Neurotrophic Factor, BDNF), neurotrophin-3 (NT3), neurotrophin-4/5 (NT4/5) and neurotrophin-6 (NT6) (Patapoutian and Reichardt, 2001). These different proteins share numerous characteristics, such as a similar molecular weight (13.2-15.9 kDa), a sequence homology of about 50% and a similar receptor specificity (Mowla et al., 2001). Neurotrophins are secretory proteins generated by proteolytic processes starting from precursors of higher molecular weight (31-35 kDa), a process which has been studied for NGF (Edwards et al., 1988; Seidah et al., 1996) and also for the BDNF (Mowla et al., 2001). They bind to and activate tropomyosin-related kinase (Trks) receptors belonging to the tyrosine kinase receptor family (TrkA, TrkB and TrkC) and a p75 pan-neurotrophin receptor (Patapoutian and Reichardt 2001). Proneurotrophins have been reported to bind preferentially to the receptor p75 while the mature forms interact with Trk receptors promoting survival neuronal (Lee et al., 2001). The p75 receptor performs several functions: it delivers NGF to the receptor TrkA, directly activates intracellular signaling pathways and promotes rather than inhibits neuronal death in Trk receptor-deprived cells. The NTs can be secreted stably or transiently, and often in activity-dependent modality. They are secreted locally by dendrites and generally act retrogradely towards the presynaptic endings to induce long-lasting alterations. Besides their classic role in neuronal differentiation and survival, NTs have also been implicated in axonal growth and synaptic plasticity (Thoenen, 1995). NTs have been shown to increase length and complexity of dendritic trees in cortical neurons (McAllister et al., 1995). In the family of neurotrophins a relevant role belongs to BDNF, a protein effective in ensuring neuronal survival and implicated in nervous system diseases such as Parkinson's disease, Alzheimer's dementia, peripheral diabetic neuropathy, and last but not least mood disorders (Smith et al., 1995; Duman et al., 1997; Salehi et al., 1998; 2009; Malberg et al., 2000).

BDNF is the most abundant neurotrophin in the brain (Lindsay et al., 1994) and plays an important role in promoting survival and differentiation of different neuronal populations in the prenatal period (Snider, 1994). It is a small dimeric protein of 27 KDa whose synthesis is encoded in short arm of chromosome 11, locus 13 (11p13) (Maisonpierre et al., 1991). The BDNF gene encodes a precursor

of neurotrophin called pro-BDNF. The BDNF gene transcription is regulated by cAMP response element-binding protein (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). Pro-BDNF is transported to the Golgi apparatus and converted into mature BDNF by internal Golgi endoproteases or by convertases located in the granules of immature secretion, or by extracellular proteases (Cattaneo et al, 2016; Hempstead et al., 2015). Recent evidence suggests that proBDNF is also biologically active, by exercising an action opposite to that of BDNF on neuroplasticity: proBDNF would enhance the neuronal apoptotic process through the p75 receptor, for which has higher affinity (Mowla et al., 2001; Lee et al., 2001; Pang et al., 2004; Woo et al., 2005; Teng et al., 2005; Hashimoto et al., 2007; Greenberg et al., 2009). The signaling of p75 exerts a double action, as it can, on the one hand, promote the cell survival, on the other, trigger neuronal apoptosis. This is because the binding of neurotrophins to the p75 receptor activates the factor NF-kB transcript, Jun-kinase and acidic sphingomyelinase. NF-kB promotes the cell survival, while Jun-kinase and sphingomyelinase promote the apoptosis. Trks receptor-mediated signaling suppresses Junkinase and sphingomyelinase activation, but not NF-kB induction. Under physiological conditions, the binding of mature neurotrophins to Trks receptors and p75 promotes cell survival, as the survival signal mediated by Trk suppresses the pro-apoptotic signal from p75 and acts synergistically with the anti-apoptotic signal from p75 itself (Bamji et al., 1998). Apoptosis is triggered when there is no longer a balance between the two receptors, either because p75 is up-regulated or because the concentration of the pro-NT, which, as demonstrated by in vitro studies, preferentially binds and activates p75. This occurs in disease states: p75 up-regulation is documented in cases of ischemia, epileptic attacks and in conditions of excitotoxicity (Dechant e Barde, 2002). 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 noncovalent binding to intracellular molecules, generating an ensuing signaling cascade able to promote various effects. 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. 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 promotes both long- and short-term effects in the adult nervous system (McAllister et al., 1999): on the one hand it acts as a powerful excitatory neurotransmitter by determining the rapid depolarization of the postsynaptic membranes, even at low concentrations (Kafitz et al., 1999; Hartmann et al., 2001), on the other it determines long-term modifications of synaptic plasticity as well as of the production of neurotransmitters and neuropeptides (Kang et al., 1995; Li e coll., 1998; Carter et al., 2002). The BDNF also produces acute effects on synaptic plasticity and neurotransmitter release and facilitates the release of glutamate, γ-aminobutyric acid (GABA), dopamine and serotonin (Schinder et al., 2000). It is believed to have long lasting effects which are involved in processes such as memory, learning and adaptive behaviour (Fujimura et al., 2002; Egan et al., 2003). In favour of this correlation, it has been reported that the expression of BDNF and his TrkB receptor is particularly elevated in the hippocampus (Hofer et al., 1990), brain region critically involved in these cognitive processes. BDNF plays an important role in the underlying mechanisms of the consolidation of the visuo-spatial memory, through the activation at the level of the hippocampus of the TrkB/PI3-K cascade (Yamada and Nabeshima, 2003), and has myelinization properties (Fletcher et al, 2018). Details on the BDNF/TrkB signalling pathway are reported in **Figure I6**.

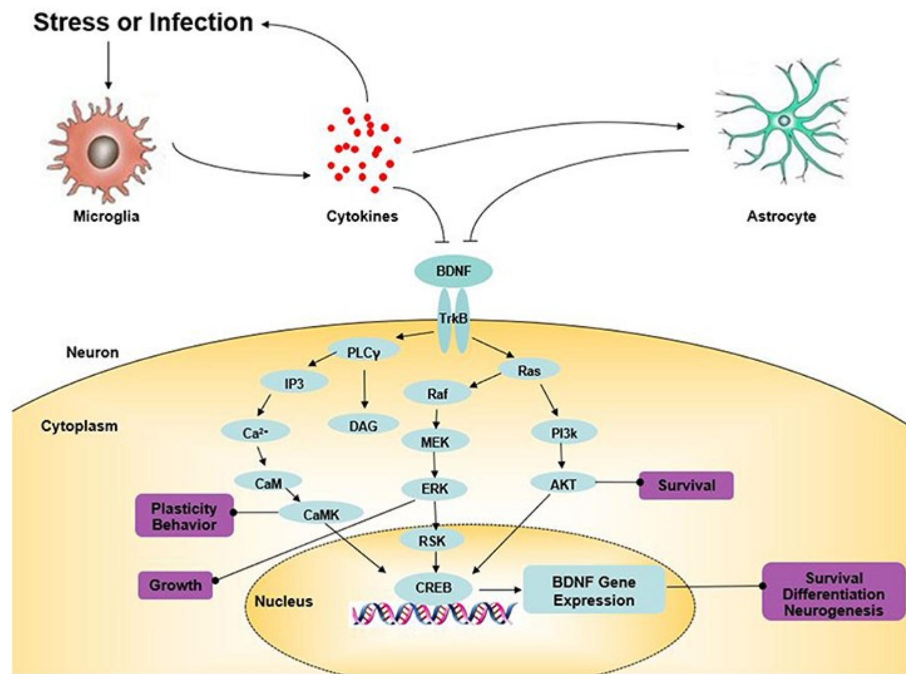


Figure I6: BDNF/TrkB signalling pathway and its neurotrophic effects. Akt, serine/threonine protein kinase; BDNF, brain-derived neurotrophic factor; CaM, calmodulin; CaMK, calcium-calmodulin-dependent protein kinase; CREB, cAMP response element-binding protein; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; IP3, inositol 1,4,5-trisphosphate; MEK, mitogen-activated extracellular signal-regulated kinase; PKC, protein kinase C; PI3K, PI-3 kinase; PLC-g, phospholipase-Cg; RSK, ribosomal S6 kinase; TrkB, tyrosine kinase B. From *Jin et al., 2019*.

BDNF has been found also at the peripheral level. Indeed, there is a body of evidence in the literature that highlights how BDNF also acts at the level of the peripheral nervous system, modulating the proliferation, the differentiation and activity of specific neuronal populations (Lommatzsch e coll., 1999; Hartmann et al., 2001). Experimental tests on mice have shown how BDNF mRNA is not only synthesized at the neuronal level but also in many other peripheral tissues and organs, such as aortic walls, endothelium, heart, kidneys, submaxillary glands, ovaries, dorsal ganglia, muscles and lungs (Fujimura et al., 2002). The BDNF is released in these sites from the target cells of the neurons, acting as survival factor of the same. Rosenfeld and colleagues (1995) found the presence of BDNF also at the blood level. Plasma BDNF is probably produced by the endothelium, by the smooth musculature and by activated macrophages and lymphocytes. However, the contribution of these cells to the

plasma concentration of BDNF is considered marginal under basal conditions, compared to the amount released by platelets (Karege et al., 2005). It is not yet certain whether there is a contribution from central sources (CNS neurons and glial cells) at the blood concentration of BDNF extension. However, the ability of BDNF to cross the BBB has been described (Pan et al., 1998) and a study by Karege et al. (2002) reports that brain and serum levels of BDNF in rats undergo parallel changes during growth and development processes of rats. In addition, other peripheral growth factors, such as VEGF and IGF-1, can enter the brain (Pan et al., 1998; Trejo and coll., 2001; Fabel et al., 2003). The role of intraplatelet BDNF levels are not yet known; probably it has a specific function in tissue trauma, injury nerves and hemorrhages. Indeed, when platelets are activated, they release the BDNF which may play a reparative role in inflammation and cell proliferation of smooth muscle.

As reported above, BDNF is found in peripheral blood (Rosenfeld et al., 1995; Radka et al., 1996; Fujimura et al., 2002), where it is about ten times more concentrated in serum than plasma (Rosenfeld et al., 1995), since it tends to collect in platelets, which contain the majority of blood BDNF (Pliego-Rivero et al., 1997; Fujimura et al., 2002). There is conflicting evidence regarding the passage of BDNF across the BBB (Poduslo and Curran, 1996; Pan et al., 1998; Pardridge, 2002), but if we consider that neutrophin can cross it in both directions, it can also be said that the cerebral BDNF concentrations are in equilibrium with its concentrations in the plasma, more than in the serum, where the neurotrophin levels are closely related to the platelet release.

As aforementioned, BDNF levels are approximately tenfold higher in serum compared to plasma, probably because platelets release large amounts of BDNF when enabled. In fact, platelets can capture the neurotrophin from plasma through a mechanism not yet known (Fujimura et al., 2002). 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. The activation state of platelets induces not only deep morphological changes but also intense biochemical variations and rearrangements (Giannaccini et al, 2010), beside the release of factors from their different granules (Serra-Millàs, 2016). Platelets, when activated, 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). As aforementioned, ninety percent or more of blood BDNF is stored in platelets and their α-granules (Fujimura et al, 2002). The difference between the serum and plasma BDNF levels seems to reflect the amount of BDNF stored in circulating platelets, and the BDNF content in platelets might serve as a reservoir for circulating BDNF. A MK progenitor line (MEG-01), precursor cell of the mature platelets, produces BDNF upon thrombopoietin stimulation (Kaushansky et al, 2006), and the levels of BDNF in MEG-01 cells have been found to increase in a time-dependent manner (Tamura et al, 2012). 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). It should be mentioned here that, more recently, some authors have reported that human MK can instead produce BDNF, suggesting that platelets can have their own neurotrophin reserve (Chacòn-Férnandez et al., 2016). It is thus conceivable to think that there is a complex equilibrium among all the diverse intra- and extra-platelet BDNF components (Serra-Millàs, 2016; Le Blanc et al, 2020).

In recent years increasing evidence demonstrated the role of BDNF in the pathophysiology of stress-related and mood disorders and in the context of the complex relationships between stressful life

events, acute or chronic, and the onset or exacerbation of mood disorders (Gold and Chrousos, 2002; Brown et al., 2003). Stress reduces the synthesis of BDNF at the level of dentate gyrus cells as well as of CA1 and CA3 pyramidal cells of the hippocampus (Smith et al., 1995; Cattaneo et al, 2016); this results in a reduced protection against those apoptotic processes that BDNF controls, activating Bcl-2 (anti-apoptotic gene) and inhibiting Bad (pro-apoptotic gene) (Finkbeiner, 2000). The down-regulation of BDNF occurring in response to stress may contribute to the pathological changes observed in PTSD (Bremner, 2003) and in depression (Mervaala et al., 2000). This assumption is supported by the observation of how both disorders are sensitive to stressful experiences (Breslau et al., 1995; Kendler et al., 2000). Reduced CREB concentrations were observed in the temporal cortex of suicidal depressed patients; further, the levels of this protein increased in the patients who received antidepressant treatment (Dowlatshahi et al., 1998). Some studies conducted on adult animals showed how stress can determine atrophy, cell death (McEwen, 1999; Sapolsky, 2000) and reduced hippocampal neurogenesis. Several post-mortem human studies have highlighted a significant reduction in volume (Manji et al., 2000; Rajkowska, 2000) and in the number of neurons and glial cells (Ongur et al., 1998; Rajkowska, 2000) in the hippocampus and other regions of the brain cortex of depressed patients. An important aspect is that the number of glial cells has been found reduced and not increased, as would be expected in the necrotic process, which is characterized by inflammation and increased in glia (gliosis). Therefore, we can reasonably assume that neuronal death from stress can occur by apoptosis. Stress related to depression or PTSD can reduce the levels of BDNF by inducing excessive production of glucocorticoids interfering with BDNF transcription processes (Smith et al., 1995; Vaidya et al., 1997). Antidepressants, on the other hand, increase the concentrations of serotonin (5-HT) and noradrenaline (NA) which, by binding to their respective receptors, determine a cytoplasmic increase of cAMP which induces the activation of PKA (Protein Kinase A); this protein phosphorylates and activates CREB, which ultimately promotes transcription of the BDNF gene (D'Sa and Duman, 2002). The increase in the concentrations of BDNF support the mechanisms of the long-term potentiation (potentiation of synaptic activity following electrical

stimulation repeated at high frequency, which causes a lasting increase in potential postsynaptic excitatory) and synaptic plasticity (Patterson and coll., 1996; Kang et al., 1997). Chronic treatment with lithium and valproate induces the expression of antiapoptotic proteins (such as Bcl-2) and determines an increase in the expression levels of BDNF in the hippocampus and in the cerebral cortex. The mechanisms described above may explain why a certain delay is necessary to be able to appreciate the clinical improvements induced by the antidepressants: this latency time would be the one required for the synthesis of sufficient amounts of BDNF so that it can exert its neurotrophic effects (Björkholm and Monteggia, 2016). All these findings support the neurotrophic hypothesis of depression, according to which the depressive pathology is linked to a deficit of neurotrophic factors, which can resolve with antidepressant therapy (Duman et al., 1997; Castrén and Monteggia, 2021; Duman et al., 2021). Recently, it has been demonstrated that the transmembrane domain of tyrosine kinase receptor 2 (Trkb), the BDNF receptor that promotes neuronal plasticity and antidepressant responses, has a cholesterol-sensing function that mediates synaptic effects of cholesterol (Casarotto et al., 2021). Antidepressants seem to directly bind to TRKB, thereby facilitating synaptic localization of TRKB and its activation by BDNF (Casarotto et al., 2021), thus implying a relevant role of the BDNF-Trkb conformational status in the antidepressant response.

Many other studies have appraised BDNF expression and amounts as possible peripheral biomarker of depression or stress-related disorders (Rana et al, 2021). 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-analyses have also shown that BDNF is more pronouncedly reduced in severe depression and increased during antidepressant treatment even in the absence of clinical remission (Molendijk et al, 2014). Other studies reported low BDNF levels to be related to more severe melancholic characteristics, psychomotor retardation or slowdown and insomnia (Primo de Carvalho Alves & Sica da Rocha, 2018; Monteiro et al, 2017). Furthermore, a negative correlation between BDNF (plasma or serum BDNF) and the number of depressive episodes

with melancholic features was found (Kotan et al., 2012). 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). Environmental stressors like psychological stress, chronic foot shocks and chronic social defeats also decrease BDNF levels in the hippocampus (Jiang et al, 2015). Additionally, a decrease of circulating BDNF has been also associated with mood episodes in BD, including mixed state episodes, though with slightly less reduced values than in depressed patients (Piccinni et al, 2015).

Regarding PTSD, animal models exhibiting extremely disturbed behaviour from threat exposure reported a significant down-regulation of BDNF mRNA expression and up-regulation of TrkB mRNA in the hippocampal CA1 region compared to animal groups minimally or partially disturbed and to the unexposed control group. The prolonged effect of the traumatic event on the expression of BDNF and its receptor, associated with severe behavioural alterations, it has been suggested to be associated with chronic psychopathological stress-induced processes that occur mainly in the hippocampus, such as changes of neuronal plasticity and synapse functionality, which could be responsible for the clinical manifestations of PTSD (Kozlovsky et al., 2007). Mitoma et al. (2008) looked for a correlation between stress workload and serum BDNF levels in a sample of human healthy volunteers, by administering to the subjects the Stress and Arousal Check List (s-SACL). They found a statistically significant negative correlation between stress and serum concentrations of BDNF, and a positive correlation between stress and plasma concentrations of IL-6 and 3-methoxy,4-hydroxyphenylglycol (MHPG), a metabolite of norepinephrine degradation. Several neurobiological models on reduced BDNF function in PTSD have been proposed, including the contribute of the single-nucleotide polymorphisms of the BDNF gene (particularly $BDNF^{Val66Met}$ polymorphism), besides trauma-induced epigenetic regulation and current stress (Rakofsky et al., 2012). However, data on BDNF in PTSD patients are conflicting in the literature. Studies aimed at evaluating possible correlations between $BDNF^{Val66Met}$ polymorphism in patients diagnosed with PTSD did not show any clear associations (Zhang et al., 2006, Lee et al., 2006). Surprisingly, a recent systematic review on twenty studies on serum BDNF levels showed increased levels in PTSD patients with respect to

healthy controls (Mojtabavi et al., 2020). In this framework, it should be mentioned that increased serum or plasma BDNF levels have been even reported in mood-disorder patients under peculiar states, in pain disturbances and in adaptive disorders, implying the need at further investigating this parameter in different clinical presentations (Bazzichi et al., 2013; Serra-Millàs et al, 2011; Piccinni et al, 2015; Serra-Millàs, 2016; Buselli et al., 2019; Uint et al., 2019).

In sum, several studies have showed that inflammation may interfere with the physiological 5-HT signalling, the neurotrophin synthesis, and the HPA axis functioning (Miller and Raison, 2016; Amodeo et al., 2018). Recent studies highlighted the possible role of a chronic pro-inflammatory state, characterizing mood disorders and post-traumatic stress disorder (Uint et al, 2019, Alcocer et al, 2014; Miller, 2020) (see **Figure I7**). Recent studies have found increased levels of various pro-inflammatory markers in blood and cerebrospinal fluid (CSF) of depressed and PTSD patients, including interleukin 1 (IL-1), IL-6, interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-alpha) and other neurotoxic factors (Muller et al., 2011; Rosenblat and McIntyre, 2017). Studies show that IL-6 appears frequently elevated in depression and PTSD (Haapakoski et al., 2015, Passos et al., 2015). Further, IL-8 has been reported elevated in subjects with severe depression, while the immunomodulatory IL-4, IL- 2 and TNF-α have decreased when symptom remitted in treatment responders (Baune et al., 2012; Strawberidge et al., 2015). Inflammatory response may result aberrant in approximately one-third of patient with depression (Krishnadas and Cavanagh, 2015; Raison and Miller, 2011). However, results are still controversial, non-conclusive and needing further investigation, before this protein could be considered a valid biomarker of PTSD or mood disorders' phases (Polyakova et al., 2015, Aspesi and Pinna, 2018; Dimitriadis et al., 2019).

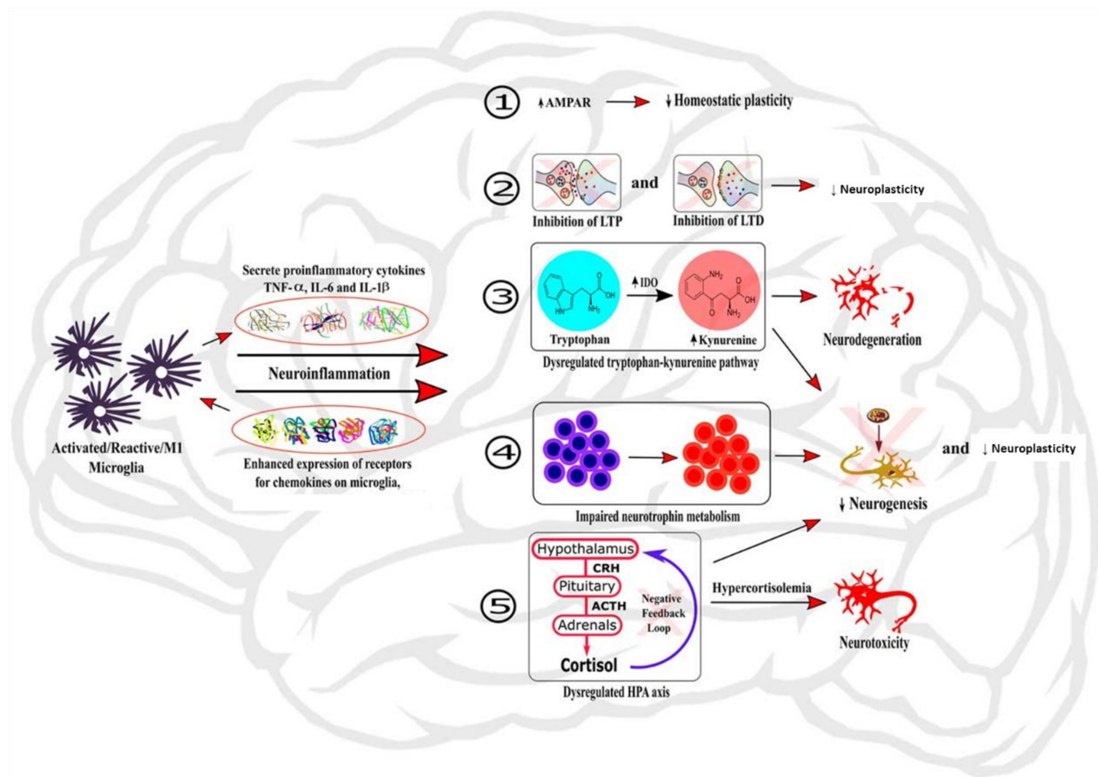


Fig I7: Neuroinflammation hypothesis in the dysregulation of neurobiological processes underlying psychiatric disorders, supposed to play a key role also in PTSD. The activation of microglia within the brain induces neuroinflammation through the secretion of local pro-inflammatory cytokines and the enhanced expression of chemokine receptors on microglia with an impairment in neurotrophin metabolism and in the neurogenesis/neuroplasticity processes. LTP: Long term potentiation; LTD: Long term depression. Adapted from *Fourrier et al., 2019*.

2. Aims of the study

As presented in the introduction section, several neurobiological mechanisms have been supposed to be implicated in the pathophysiology of PTSD, including HPA axis, monoaminergic transmitter system, immune system, oxidative-stress reactions and specific neural circuits (Michopoulos et al., 2015). However, there are no generally accepted PTSD biomarkers for use in clinical practice (Kim et al., 2019).

Further, clinical and epidemiological studies have often remarked the strong association between PTSD and other psychiatric disorders, including depression and BD (Kessler et al., 1995; Breslau et al., 1998; Rytwinski et al., 2013). This has led to consider comorbidity in PTSD to be the rule rather than the exception (Brady et al., 2000; Flory and Yehuda, 2015; Longo et al., 2019), determining a significantly poorer outcome, higher symptom burden, lower quality of life, higher percentage of further concurrent psychiatric disorders, and higher suicide risk (Bajor et al., 2013; Hernandez et al., 2013; Passos et al., 2016; Carmassi et al., 2020b). Some studies also stressed the possible relevance of some symptom similarities between PTSD diagnostic criteria and BD, suggesting a possible partial wide symptom overlap (negative affect, sleep problems, irritability and anxiety). Conversely, a huge number of clinical and epidemiological studies emphasized different psychopathological and neurobiological hypotheses underling PTSD and BD, proposing peculiar pathways for these disorders. Scant and conflicting data are currently available in literature about potential biomarkers of PTSD, and even less on possible comparisons of PTSD and depressive state biomarkers in subjects affected by BD.

The aim of the present investigation was to evaluate potential biochemical markers of PTSD in a group of BD subjects in euthymic phase (PTSD group), compared with a group of BD subjects with major depressive episode (DEP group) and a healthy control group (CTL group).

In particular, we aimed to analyse among different groups the circulating concentrations of: 1) 5-HT; 2) TRP, KYN, and QUIN, in order to evaluate the main TRP metabolic pathways; 3) melatonin, the hormone regulating sleep and circadian rhythms; 4) plasmatic (PPP) and intraplatelet (PLT) BDNF, as the main neurotrophin, synaptic modulator agent and peripheral inflammation mediator; 5) IL-6 and IL-1β, as the most investigated and frequently reported altered cytokines in PTSD; 6) SOD, CAT and total thiols, as a potential marker of oxidative-stress and impaired antioxidant processes in this population; 7) cortisol, as the principal effector of HPA axis that is involved in stress-related reactions. Moreover, we aimed to evaluate eventual correlations among these biochemical parameters and the mood and post-traumatic stress symptoms' severity, and other clinical features as measured by the psychometric scales most frequently employed in this field. Our results may shed light also on possible biochemical correlates of dimensional symptoms, conceived as a psychopathological continuum, from sub-threshold to full-blown mood and post-traumatic stress symptoms.

From a clinical point of view, improving our knowledge about possible biochemical correlates of PTSD could aid clinicians to identify individuals at high risk for PTSD, as well as to promote early diagnosis and a prompt initiation of treatment, in addition to contributing to develop new target for a tailored treatment and assist in monitoring the response of therapy (Schmidt et al., 2013).

3. Methods

3.1 Sample recruitment and assessment

The study involved the enrolment of a sample of 1) BD patients with a current PTSD diagnosis in euthymic phase (PTSD group) and 2) BD patients with a current Major Depression Episode diagnosis (DEP group), both recruited among adult out-patients or in-patients treated at the Psychiatric Clinic of the University of Pisa; 3) voluntary Healthy Controls (CTL group).

Inclusion Criteria:

• Age, between 18 and 65 years old

• History and diagnosis of a Bipolar Disorder (BD), according to the classification criteria of the DSM-5, in euthymic phase (only for the PTSD group) or with Major Depression Episode (only for the DEP group)

• History and diagnosis of current PTSD according to the classification criteria of the DSM-5 (only for the PTSD group)

• Acceptance of the protocol and signature of informed consent.

Exclusion criteria:

• Patients under the age of 18 and over 65 years old

• History and diagnosis of relevant neurological or medical diseases

• Inability to sign an informed consent to the study

• Alcohol or substance abuse in the last six months

• Pregnancy

• Having received a diagnosis of a psychiatric disorder according to DSM-5 (only for CTL group)

All the recruited subjects were assessed by trained psychiatrists by means of a structured clinical interview and psychometric scales. Blood and first-morning urine samples were also collected from all participants to perform the biochemical evaluations. Biochemical evaluations were accomplished at the Laboratory of Biochemistry and Pharmacy of the University of Pisa (tutor: Prof. Gino Giannaccini). All participants received clear information about the study and had the opportunity to ask questions before providing a written informed consent. All data were treated according to Italian and European Privacy laws and rules. The study was conducted in accordance with the declaration of Helsinki, and all procedures were approved by the local ethical committee (19299/21).

3.2 Psychometric instruments

The psychopathological evaluation was performed by trained psychiatrists with a clinical interview and a psychometric investigation, by using also some self-reported psychometric questionnaires. Socio-demographic and clinical information were also registered in a case report form.

Participants were investigated by means of the *Structured Clinical Interview for DSM-5 disorders* (SCID-5) in order to evaluate the presence a current psychiatric diagnosis (First et al., 2015); the *Hamilton Depression Rating Scale* (HAM-D), to assess the actual severity of the depressive symptoms (Hamilton, 1960); the *Young Mania Rating Scale* (YMRS), to assess the actual severity of the manic symptoms (Young et al., 1978); the *Impact of Event Scale-Revised* (IES-R), to assess the actual severity of post-traumatic stress symptoms (Weiss and Marmar, 1997); the *Mood Spectrum-Self-Report* lifetime version (MOODS-SR), to investigate mood spectrum symptoms lifetime, in a dimensional perspective (Fagiolini et al., 1999; Dell'Osso et al., 2002); the *Trauma and Loss Spectrum-Self Report* lifetime version (TALS-SR), to investigate posttraumatic stress spectrum symptoms lifetime in a dimensional perspective (Dell'Osso et al., 2008; Dell'Osso et al., 2009a); the *Work and Social Adjustment Scale* (WSAS) to examine the impact of symptoms on global functioning

of these subjects (Mundt et al., 2002).

The *Structured Clinical Interview for DSM-5 disorders* (SCID-5), according to the DSM-5 criteria, is a diagnostic instrument used by clinical psychiatrists or trained mental health professionals who are familiar with the DSM-5 system to make psychiatric diagnoses through a semi-structured interviewing process (First et al., 2015). It is composed of ten independent modules; the sequence of questions follows the order of the related diagnostic manual (DSM-5) and the different items of each module guide the interviewer through the evaluation of the presence of symptoms that may satisfy the diagnostic criteria.

The *Hamilton Depression Rating Scale* (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 contribute to the total score. The reported scores define the cut-off severity as follows: score > 25 = severe depression; score between 18-24 = moderate depression; score between 8-17 = mild depression; score < 8 no depression (Hamilton, 1960).

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 YMRS resembles HAM-D 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).

The *Impact of Event Scale-Revised* (IES-R) is a 22-item scale measuring three core features of PTSD (re-experiencing of traumatic events, avoidance, and hyperarousal) and thus items, coded on a 0–4 scale, are divided into three subscales: intrusion, avoidance, and hyperarousal. All items refer to the last week prior to the assessment. The questionnaire has an adequate internal consistency (alpha = 0.80–0.93 for the intrusion; alpha = 0.73–0.84 for avoidance) and high test–retest reliability (r = 0.93). The mean score of the items of each subscale determines the subscale score. The IES-r total score is calculated adding the score of each item. A score over 32 represents a cut-off for PTSD (Weiss and Marmar, 1997).

The *Mood Spectrum-Self-Report* (MOODS-SR) and the *Trauma and Loss Spectrum-Self Report* (TALS-SR) are both instruments developed according to a spectrum model of psychopathology, in the framework of the international research network called Spectrum Project (Cassano et al., 1999; Dell'Osso et al., 2011a, 2017; Frank et al.,1998). The primary aim of spectrum instruments is not to verify if a mental disorder, according to DSM, is present. Rather, they are meant to detect lifetime sub-threshold symptoms related to a certain psychiatric dimension, by assessing its wide range of typical and atypical manifestations. Sub-threshold and atypical symptoms, as well as personality characteristics, may co-occur with the full-blown presentation of a psychiatric condition but, interestingly, can be also found in subjects without a diagnosis of a mental disorder.

The MOODS-SR is a 140-item questionnaire exploring mood spectrum symptoms, coded dichotomously, as present or absent, for one or more periods of at least 3–5 days. Items are organized into three manic and three depressive domains, exploring "mood," "energy," and "cognition," besides a rhythmicity and vegetative functions domain. The number of the mood-, energy- and cognition-manic items endorsed by subjects makes up the manic component (62 items) while the sum of the mood-, energy- and cognition-depressive items constitutes the depressive component (63 items). The rhythmicity and vegetative functions domain explores alterations in the circadian rhythms and vegetative functions, including changes in energy; physical well-being; mental and physical

efficiency related to the weather and season; and changes in appetite, sleep, and sexual activities across 29 items. Mood Spectrum Self-Report–Lifetime Version, the self-report version of SCI-MOODS, presented good intraclass correlation coefficients (from 0.88 to 0.97) with the interview format (SCI-MOODS). SCI-MOODS, the interview version for assessing mood symptomatology, had a good internal consistency (Cronbach α ranged between 0.72 and 0.92) (Fagiolini et al., 1999; Dell'Osso et al., 2002).

The TALS-SR is composed by 116 dichotomous questions (yes/no) grouped in nine domains: loss events (I), grief reactions (II), potentially traumatic events (III), reactions to losses or upsetting events (IV), re-experiencing (V), avoidance and numbing (VI), maladaptive coping (VII), arousal (VIII), personal characteristics/risk factors (IX). The questionnaire investigates both a comprehensive set of traumatic and loss events that may occur in the lifetime and a broad spectrum of symptoms related to these events. The post-traumatic symptomatology explored by the instrument represents an approach along two dimensions: (1) the dimension of acute and peritraumatic reactions and (2) the dimension of the post-traumatic psychopathology, including symptoms described in DSM criteria for Post-Traumatic Stress Disorder (PTSD) but also sub-threshold and atypical manifestations eventually arisen after the event(s). Moreover, events themselves listed in the questionnaire are meant along a wide dimension (3), since they include traumatic and loss events as conceptualized by the DSM-5 in the criterion A for PTSD, but also lower-severity life events, which may turn out to be traumatic for the subject. So, in the framework of a dimensional approach, the questionnaire is able to highlight also the wide spectrum of trauma- and stress- related manifestations that may follow different kinds of stressful experiences across the lifetime. In the validation study, all Kuder-Richardson coefficients for TALS-SR exceeded the minimum standard of 0.50, and the instrument demonstrated positive correlations between its domains (Pearson's r ranging from 0.46 to 0.76) (Dell'Osso et al., 2008, 2009).

The *Work and Social Adjustment Scale* (WSAS) is a test used to evaluate and measure the work and

social adjustment. It includes five items that assess the individual’s ability to perform the activities of everyday life and how these are affected in the week prior to the assessment. The first item investigates the work ability of the subject. The second item assesses the ability to cope with household chores such as cleaning the house, looking after the children, and doing the shopping. The third item assesses private recreational activities carried out by the patient, such as going to the cinema, visiting museums, and reading. The fourth and fifth items investigate the family interaction and relationship: in particular, the fourth item investigates the social activities carried out exclusively with people who are not part of the family and includes activities such as parties, tours of pleasure, going clubbing, or going on romantic dates. The fifth item analyzes only the relations with family members with whom the person lives, and whether any problems of the subject under examination have interfered with this type of relationship. Each of the five items is rated on a nine-point scale ranging from 0 (not at all) to 8 (severe interference), so that the total scores are between 0 and 40. The internal consistency of the instrument varies from 0.70 to 0.94 (Cronbach's alpha) and the reliability of the test-retest is 0.73 (Mundt et al., 2002).

3.3. Biochemical measurements

3.3.1. ***Instruments, chemicals and reagents***

All reagents and chemicals used for the study were of the best quality and purity, in agreement with the gold-standard guidelines of analytical laboratories. All required solutions were prepared using an ultrapure HPLC gradient-grade milli-Q water (18 MΩ cm^{-1} resistivity), obtained by means of a Simplicity Millipore Apparatus equipped with a UV lamp and a 0.2-micron filter to prevent contamination by particles and biological agents (**Figure M1-A**). A PST-60HL plate thermo-shaker (Biosan, Riga, Lativia) was used for sample incubations during ELISA procedures and chemical assays when required (**Figure M1-B**). Absorbance at 450, 540 and 595 nm was appraised using a 96-well plate spectrophotometer (MultiSkan FC ThermoScientific, Thermofisher Scientific, Waltham, MA, USA) (**Figure M1-C**). For absorbance at 412 nm and for fluorometric detection of total thiols the multimodal Enspire reader (Perkin-Elmer, Waltham, MA, USA) was employed (**Figure M1-D**).

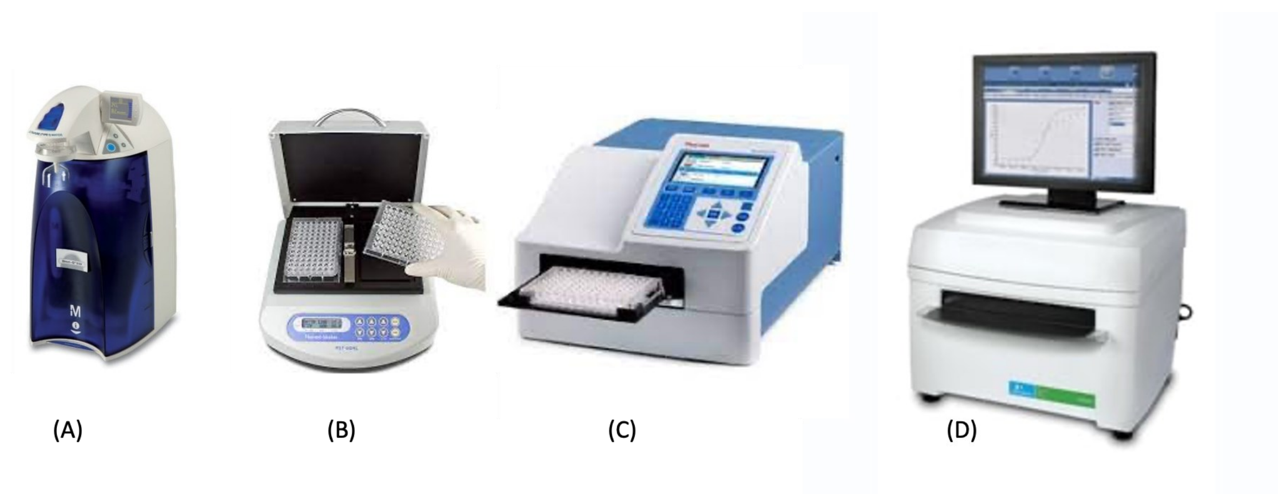


Figure M1: A) The Milli Q Simplicity apparatus; B) the plate thermo-shaker; C) the multiscan spectrophotometer; D) The multimodal Enspire reader.

3.3.2. *Blood sampling, preparation and storage procedures*

3.3.2.1. Blood collection

All blood samples were accurately collected avoiding hemolysis by the skilled and authorized nursing staff of the “Azienda Ospedaliera Universitaria Pisana” (AOUP), Psychiatric Unit, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy. 15 mL of peripheral venous blood were withdrawn from all recruited subjects, fasting from previous evening and at least for 12 hours. Blood withdraws were carried out between 9 and 10 *a.m.*, to avoid circadian rhythm interferences, as part of routine-blood tests, accordingly to the local Ethical Committee guidelines for this study.

Different blood collections were carried out to obtain the separation of suitable biological matrices for the measure of the biochemical parameters under investigation:

- 9 mL were gathered in 3 vacutainer tubes containing K_3EDTA as the anticoagulant, for separating the platelet-poor-plasma (PPP) from platelets: TRP, KYN, 5-HT, PPP-BDNF and IL-6 were measured in PPP; PLT-BDNF was measured in platelet extracts;

- 3 mL were collected in 1 vacutainer tube containing lithium-heparin for plasma separation; QUIN, SOD, CAT, total thiols and IL1-β were measured in heparin-plasma;

- 3 mL were collected in 1 vacutainer tube without anticoagulant and with a clot activator for serum separation; QUIN, SOD and IL-6 were measured in some serum samples for comparison with heparin- or K_3EDTA-plasma.

Vacutainer tubes were then immediately transported in thermostatic containers by authorized medical personnel of the Psychiatry Unit to the laboratory of Biochemistry of the Department of Pharmacy, University of Pisa, for sample preparation procedures and proper storage.

3.3.2.2. Preparation and storage of platelet poor-plasma (PPP), whole platelet pellets, heparin-plasma and serum

For PPP and platelet preparation, the 3 K_3EDTA vacutainer tubes were centrifuged within 30 minutes from withdrawal. All centrifugations were performed at room temperature (RT). The first centrifugation was conducted at low speed (150 g for 15 minutes) in order to separate the platelet-rich-plasma (PRP) from the other cellular elements (Betti et al. 2018). Subsequently, the PRP volume was measured, transferred in 2 Falcon tubes (capacity = 15 mL) marked with the PRP volume and centrifuged again, together with lithium heparin and clot activator tubes, at 1,500 g for 15 minutes. After this last centrifugation, the PRP resulted divided in two different phases, collected as separate samples: 1) the supernatant containing the K_3EDTA-PPP; 2) the whole-platelet pellets. This centrifugation also allowed to obtain heparinized plasma from the lithium heparin tube and serum from the clot activator one. All these specimens were then separately aliquoted in high quality, low-binding protein Eppendorf Safe-Lock tubes (Sigma-Aldrich, St. Louis, Mo, USA), while whole-platelet pellets were maintained in the Falcon tubes. In particular, for each patient, two aliquots of platelet pellets and a total of about 17 aliquots of plasma/serum were obtained. The PRP volume, from which PPP and whole-platelet pellets were derived, was registered and saved in the database for the calculation of intraplatelet 5-HT levels.

Subjects' codes, dates and sample types were recorded on Low-binding protein Eppendorf tubes and all blood-derived sample aliquots were stored at -80°C until assays (**Figure M2**).

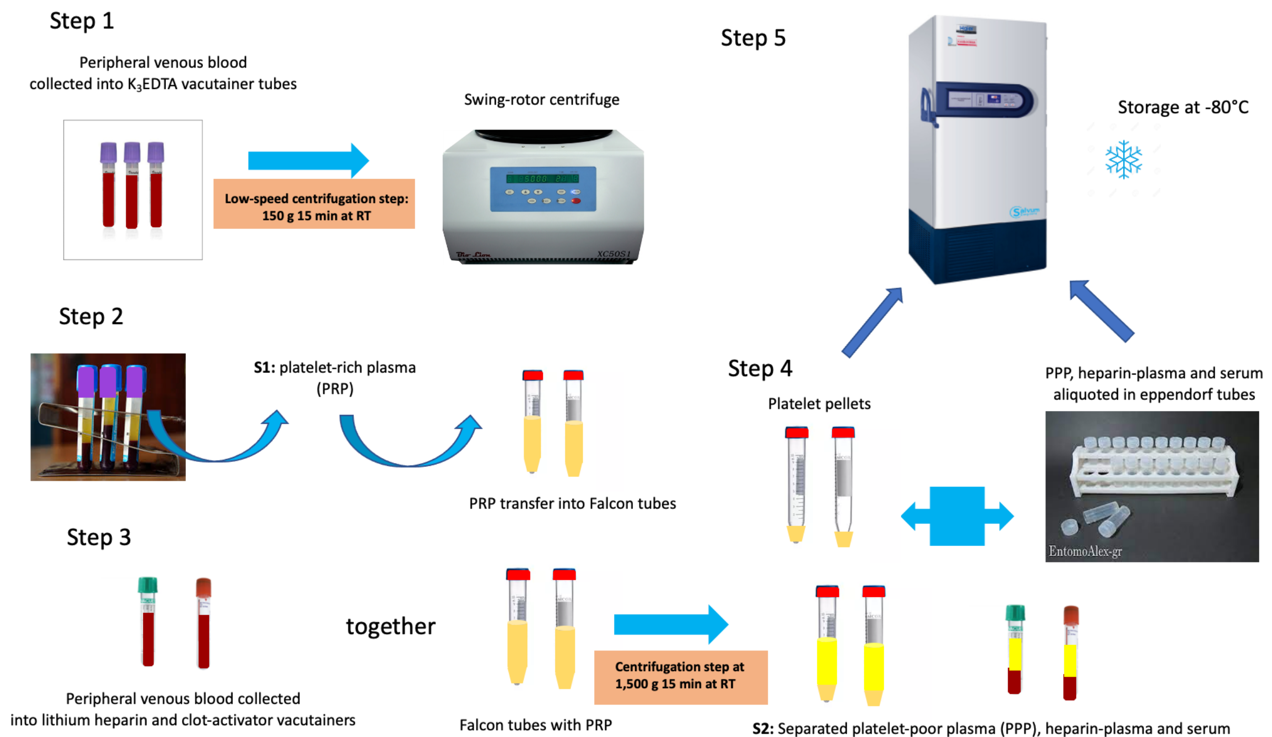


Figure M2: The different steps of blood treatment to obtain the various fractions: the platelet-poor plasma (PPP), the platelet pellets, the heparin-plasma and serum.

3.3.3. *Urine sample collection, preparation and storage*

In this work, cortisol and 6-OH-MLT-sulfate (6-OH-MLT-S) were investigated in urine samples. Even if the measure of cortisol in 24-h urine samples is considered a gold-standard methodology and a non-invasive procedure, this method can be the source of analytical inaccuracy (Mann and Gerber, 2019), especially in the case of sample collection from psychiatric outpatients. For these reasons, these two biochemical parameters were both appraised in nighttime and first morning urines, which are much easier to collect. A graduated big container and a smaller flask were given to each outpatient and control subject, who had been previously instructed in detail on the precise procedure of urine collection at home: the night before the urine specimen delivery at the Psychiatric Unit, each subject had to cast urine before sleeping without collecting anything; the subject then had to gather the early morning urines upon waking in the graduated container; if, during the night the subject/patient woke up and got up for micturition, he/she had to collect and keep urines in the same graduated container

where they also had to collect the subsequent early-morning urine, at the time of the last awakening; at the end of the collection, each enrolled subject had to measure the total volume and decant an aliquot of urine into the delivered flask, after marking the total volume and his/her own number code on this same flask (**Figure M3**). The flask was then transported to the Psychiatric Unit where blood collection also took place. The procedure adopted was the same for inpatients, under the support of the nursing staff. The time of collection started the night before at about 10-11 *pm* and was completed the next morning at 6-7 *am*, thus lasting about 8 hours.

After delivery to the Psychiatry Unit, urine samples were transported to the laboratory of biochemistry of the Department of Pharmacy together blood vacutainer tubes, as afore indicated.

Figure M3: The graduated container for the nighttime/first morning urine sample collection; the sample flask used for urine delivery to the Psychiatry Unit.

At the laboratory of Biochemistry, a complete urine analysis was carried out by means of an automated device, the Arkray Auction-micro spectrophotometer (Shiga, Japan - Menarini diagnostics, Florence, Italy), using Aution sticks (Arkray factory, Shiga, Japan - Menarini diagnostics, Florence, Italy) for the measure of 10 parameters: glucose, proteins, bilirubin, urobilinogen, pH, specific gravity, blood, ketone bodies, nitrites and leukocytes (**Figure M4**). After analysis, urines were centrifuged at 1,500 g for 5 min at RT, aliquoted in Safe-Lock Eppendorf test and stored at -80°C until assay.

Figure M4: Urine analysis device and Aution sticks.

3.3.4. *Panels of biochemical assays*

For the sake of clarity, the methods adopted for determining the thirteen peripheral biochemical parameters under investigation are described herein by dividing them into 5 distinct panels, on the basis of the respective physiological roles of each analyte:

panel 1 - tryptophan and kynurenine metabolism (plasma TRP, KYN and QUIN);

panel 2 - the methoxyindole pathway (plasma 5-HT, urinary 6-OH-MLT-sulfate);

panel 3 - the neurotrophin signaling (PPP-BDNF, PLT-BDNF);

panel 4 - oxidative stress markers (plasma SOD, CAT and total thiols);

panel 5 - HPA, inflammation and cytokines (urinary cortisol, plasma IL6 and IL1-β).

3.3.4.1. ELISA assays for panel 1 determination - tryptophan and kynurenine metabolism

In order to evaluate the concentrations of TRP, KYN and QUIN in our sample we used the Enzyme-linked Immuno-sorbent Assay (ELISA) technique. ELISA is a specific and sensitive methodology which brings together the specificity of the antigen-antibody reaction and that of the enzymes for their substrates. ELISA is an immuno-sorbent assay performed in a solid phase, a 96-wells microplate

in polystyrene plastic. Competitive, non-competitive and sandwich ELISA protocols are available. Each of them includes an immunological analysis which allows the qualitative and quantitative determination of the analyte by the use of one or more antibodies, with one of them being directly or indirectly associated with an enzyme; in some type of competitive ELISA protocols fixed analytes at known amount are conjugated with the enzyme. In any case, the conjugated enzyme is part of the revelation device for the quantitative analysis by catalyzing a reaction which transforms a chromogenic/fluorogenic substrate into a colored/fluorescent/chemiluminescent product, which can be measured by a spectrophotometer or by a multimodal detection system. The ELISA methodology comprises highly versatile techniques, allowing the measurement of different kinds of specimen and analytes, thus offering a simple method to measure the chosen analytes in almost any kind of biological sample, such as serum, plasma, saliva, urine, tissue extract specimens or cell culture supernatants. Moreover, by means of ELISA methods, several samples can be measured simultaneously, in relatively short times. However, there are also some limitations linked to the use of this method. In particular, ELISA can be affected by possible antibody cross-reactions and loss of the specificity for the analytes; moreover, the assay is unable to separate the analytes from the other sample components or to localize them within cells, potentially leading to a loss of analytical accuracy through the phenomenon known as "matrix effects" (Selby, 1999). However, due to the use of 96-well microtiter plates, ELISA methods remain valuable alternatives to HPLC or Ultra-HPLC separation techniques which are more accurate but also time consuming, since they involve several different chromatograms for each subject and each analyte.

In the present work, for the determination of TRP, KYN and QUIN, we used commercially available competitive ELISA kits, produced by ImmuSmol (Bordeaux, France). The provided technical instructions for each kit were accurately followed during the analysis. TRP and KYN were measured in thawed PPP samples, QUIN was measured in thawed heparin-plasma and serum (some samples). For the quantitative analysis of these parameters, the immune-enzymatic assays were developed for the accurate measure of low molecular-weight analytes through a derivatization step. Derivatization

is a chemical reaction which modifies the analyzed compound in order to better identify and quantify it by specific antibodies. In the ELISA kit used for TRP evaluation, the derivatization was conducted with a non-specified patent-protected reagent, while for KYN and QUIN the derivatization consisted in an acylation reaction. Each ELISA kit featured specific initial steps. For TRP, samples were treated in Eppendorf tubes with an acidic solution in order to precipitate proteins, then removed by centrifugation, to obtain free TRP. Once extracted, samples were derivatized prior to ELISA assay. For KYN and QUIN, a derivatization reaction through acylation was performed, without any extraction/deproteinization procedure. TRP, KYN and QUIN kits were provided with 6 ready-to-use standard solutions at known concentrations for the quantitative analysis: these ranged from 0 to 1,220 μM for TRP; from 0 to 10,000 ng/mL for KYN; from 0 to 2,032 ng/mL for QUIN. All these kits also provided two control samples at known validated concentration, a low-concentrated and a high-concentrated sample, to check the quality of the entire procedure. All the kits also provided two kinds of 96-well microtiter plates: one plate to be used for the analyte derivatization and the other for performing the actual ELISA assay.

The derivatization step was necessary for TRP, KYN and QUIN assays, in both standard and samples, although, for each analyte, there was some variation, such as incubation times and the use of the thermo-agitator: for instance, TRP was derivatized for 2 hours at 25°C on the shaker (600 rpm); KYN for 90 min at 37°C without shaking; QUIN for 2 hours at 37°C without shaking. After derivatization, a same volume of standards, samples and controls (the volume varies depending from the kit/analyte) was added to the second micro-plate wells in order to carry out the ELISA dosage. The ELISA procedure featured several steps. The first step consisted in a competitive reaction and an overnight incubation. The assay micro-plate contained, pre-adhered in the bottom of each well (coating), the same fixe amount of the derivatized analyte. To realize the competitive reaction, derivatized analytes competed with the pre-adhered derivatized analyte for a specific antibody which was also added in equal volume to all the microplate wells. The overnight incubation time for this reaction was about 15-20 hours, at 4°C. After this incubation, the second step consisted in the washing procedures, in

order to eliminate the unbound excess of reagents: 3 washes with 300 μL of washing buffer were carried out. Finally, the detection reaction was started, adding a secondary antibody linked to the horseradish peroxidase (HRP) enzyme. Through this procedure, which is of the indirectly competitive type, the second antibody associated with the enzyme is bound depending on the quantity of the first antibody immunocomplex which previously adhered on the bottom of the well (see **Figure M5**).

After further washing steps, the 3,3′,5,5′-Tetramethylbenzidine (TMB), a HRP substrate, was added to the wells avoiding light exposure: the incubation time for this enzymatic reaction was 25-30 min at 25 °C in the thermo-agitator (600 rpm).

After this incubation, the reaction was stopped through precipitation and inactivation of HRP by adding a stop solution containing concentrated sulfuric acid (H_2SO_4). Due to the acidic pH, the blue product deriving from the enzymatic reaction changed color, becoming yellow. Thus, in this competitive ELISA assay, the color intensity decreased at the increasing of the analyte concentration. The final absorbance in each micro-well was measured by the plate reader spectrophotometer at λ = 450 nm within 10 min from the adding of the stop solution.

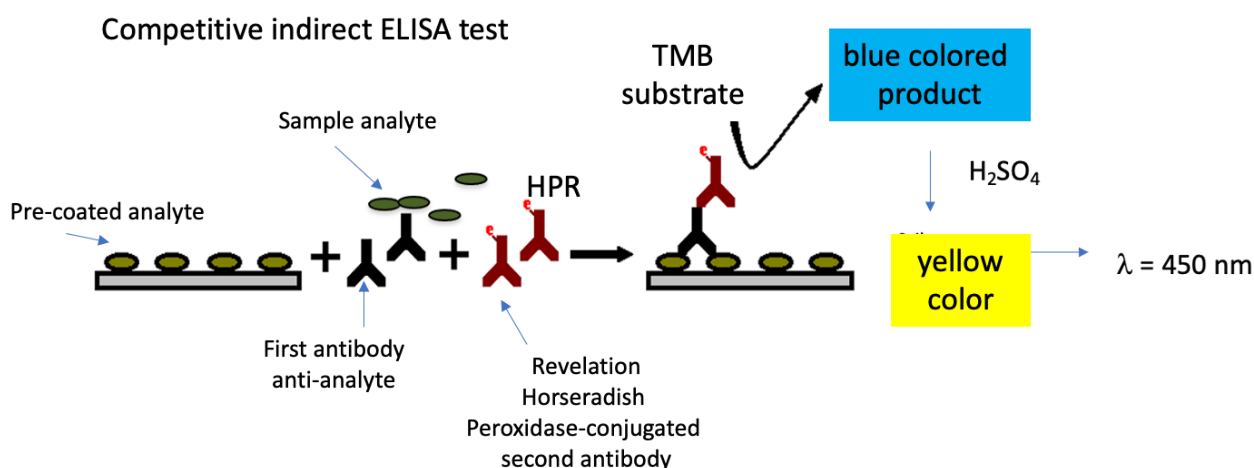


Figure M5: Schema of the indirect competitive ELISA protocol. TMB= Tetramethylbenzidine.

The calibration curve for the quantitative analysis of each analyte was then calculated by evaluating the absorbance values of STDs through a non-linear 4-logistic parameters regression (4-PL regression). The regression had a negative slope value due to the inverse proportion between the

analyte concentration and the color intensity. The final concentrations of the analytes in the samples were then interpolated from the calibration curve, considering the dilution factor when necessary. TRP amounts were reported as μM, whereas KYN and QUIN as ng/mL.

3.3.4.2. ELISA tests for panel 2 determination – the methoxyindole pathway

Serotonin (5-HT) was measured in defrosted PPP samples using an ELISA indirect competitive assay purchased from ImmuSmol (Bordeaux, France). The assay procedure was very similar to that employed for TRP, KYN and QUIN. No deproteinization procedure was necessary prior to 5-HT derivatization, which consisted into the analyte acylation. The Assay Buffer provided by the kit contained 1% stabilizing agent (final concentration) to avoid 5-HT oxidation. All PPP samples, STD solutions and control quality samples were diluted in the Assay Buffer prior to derivatization: PPP-samples were diluted 1:10 (*v:v*), while STDs and controls 1:1000 (*v:v*). Diluted 5-HT STD solutions ranged from 0 to 2.5 ng/ml. Acylation was carried out at 25°C for 30 min on the shaker (600 rpm). The subsequent steps were almost similar to the TRP, KYN and QUIN assays. 5-HT plasma levels were interpolated by a 4-PL non-linear regression analysis and, after correction of the dilution factor, presented as ng/mL.

The other component of the TRP methoxyindole pathway, the main MLT catabolite 6-OH-MLT sulfate, was determined in the nighttime/first morning urine samples by means of a competitive ELISA kit purchased from TECAN Italia (Milan, Italy), developed by IBL International GMBH (Hamburg, Germany). 6-OH-MLT sulfate STD solutions ranged from 0 to 420 ng/ml. The kit provided a control sample for assay quality test. The day of assay, thawed urine samples, STDs and the control were all diluted 1:50 (v:v) in the Assay Buffer, containing Tris, Bovine Serum Albumin (BSA) and 0.01% thimerosal. The 96-well microtiter plate was pre-coated with a polyclonal antibody, an anti-rabbit IgG goat antibody. Diluted urine samples, STDs and control were added to the pre-coated microplate together a fixed amount of 6-OH-MLT sulfate conjugated with the HPR enzyme and a rabbit anti-6-OH-MLT-sulfate antibody. This latter competed for the unlabeled 6-OH-MLT

sulfate contained in samples, STDs and control as well as for the HRP-conjugated 6-OH-MLT sulfate. The labeled or the unlabeled immunocomplex formed was then retained into the well by reacting with the coated anti-IgG antibody. This reaction was carried out for 2 hours at 25°C on the shaker (500 rpm). After this incubation, the microplate was washed 4 times and then the TMB substrate was added for 30 min at 25°C on the shaker (500 rpm). The stop solution containing a strong acid solution was then added and absorbance read at λ= 450 nm by the MultiSkan FC Spectrophotometer. A 4PL logistic equation was then applied. The regression had a negative slope value due to the inverse proportion between the analyte concentration and the color intensity. To correct for the interindividual variation due to urine dilution and glomerular excretion rate the Levine equation was used, which employs urine specific gravity (Giannaccini et al, 2016) and data presented as ng/mL.

3.3.4.3. ELISA tests for panel 3 determination - the peripheral neurotrophin signaling - PPP-BDNF and PLT-BDNF

The day of the BDNF assay, whole-platelet pellets were thawed and homogenized to obtain platelets' soluble fractions: platelets were immediately placed on ice and homogenized in 6ml ice-cold lysis buffer containing protease inhibitors (1:500, *v:v*), by means of an ultrasound mechanical device, and centrifuged as previously described (Betti et al, 2018). The resulting supernatant was used for the PLT-BDNF assay. PPP-BDNF was instead apprised in thawed PPP samples.

For BDNF dosages, a sandwich ELISA kit provided by Biosensis, developed for the preferential determination of the human BDNF mature form (Biosensis, mature BDNF RapidTM, Thebarton, Australia) was used. This kit allowed the colorimetric identification and quantification of BDNF levels in different kinds of biological specimens. It is validated to prevent analytical interferences and its quantitative performances are certified. The antibodies used in the kit react only with the mature form of BDNF, avoiding cross-reactions with the precursor pro-BDNF. The test has a high sensitivity, allowing the detection of BDNF concentration as low as 7 pg/mL, and a high specificity, with less than 3 % of other neurotrophins (such as NT-3, NT-4 and NGF) that can be bound by the first

monoclonal anti-BDNF “capture” antibody. The kit thus includes this first monoclonal antibody pre-coated on the bottom of the 96-well micro-plate, a second biotinylated detection antibody directed towards another BDNF epitope, and a streptavidin-biotin detection complex conjugated with HRP. By adding the TMB substrate, a blue reaction product is generated, proportional to the BDNF concentrations in standards and samples. The kit also provides a validated human recombinant lyophilized BDNF standard at a certified concentration, together with a quality control sample which functions as a positive control of BDNF at a validated concentration range. Before the test, the lyophilized BDNF standard must be diluted in 1mL of sample diluent buffer in order to reach a final concentration of 1 ng/mL (1000 pg/mL). This first solution is then serially diluted in the sample diluent buffer in order to obtain 7 calibration solutions, resulting into BDNF concentrations ranging from 7.8 to 500 pg/mL. The sample diluent buffer includes some blocking components, which allow preventing the formation, on the bottom of the micro-plate wells, of non-specific bindings and the consequent possible high background values (noise) at the end of the test. The aliquots of defrosted PPP and the freshly prepared platelet fractions were diluted in the sample diluent buffer as requested by the kit guidelines. Subsequently, diluted mature BDNF standards, diluted quality control sample, diluted PPP aliquots or platelet soluble fractions and “blanks” (containing only the sample diluent buffer) were added to all micro-plate wells. The plate was then covered with an appropriate plate sealer film for the incubation on a thermos-plate agitator at low shaking speed (about 200 rpm). After the incubation, the procedure featured 5 washing steps. Then, diluted biotinylated anti-mature BDNF antibody was added in each well, and the plate underwent a further incubation on the thermo-agitator. Subsequently, after a further washing phase, diluted streptadivin-HRP complex was added to all the wells and then the plate was incubated again at very low shaking speed. To reveal the immunocomplex formation, TMB, the HRP substrate, was added in each well: this reaction was stopped after around 6-7 min at RT by adding the stop acidic solution. As reported for the other ELISA dosages, this latter step converts the blue color due to the HRP reaction product into a yellow color, yielding, in this case, an intensity directly proportional to BDNF concentrations (**Figure M6**).

Micro-well absorbance was then measured by the plate reader spectrophotometer, pre-set at λ = 450 nm.

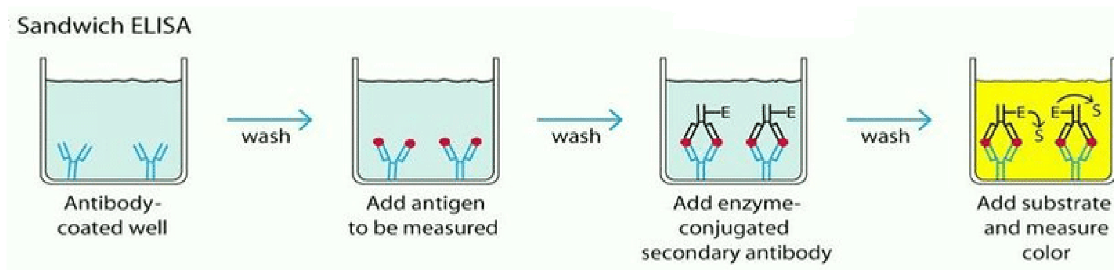


Figure M6: Schematization of the sandwich ELISA procedure (Figure from Alahi and Mukhopadhyay 2017).

For calculating BDNF values, the blank absorbance (Abs-450 BDNF= 0 pg/mL) was subtracted from both standard and sample absorbances. The BDNF values were then interpolated from the calibration curve and multiplied by dilution factor permitting to obtain the final BDNF amounts in the PPP and soluble platelet fractions as ng/mL. For determining PLT-BDNF contents, values reported as ng/mL were normalized for the total protein amount (mg/ml) present in each final platelet soluble fraction and converted into ng/mg proteins. This was done to restrain interindividual variability of platelet number and to contain the variable loss of proteins during the soluble fraction preparation. Protein amount was measured by the Bradford method (Bradford, 1976), using γ-globulins as the standard (**Figure M7**). PLT-BDNF was thus presented as ng/mg proteins.

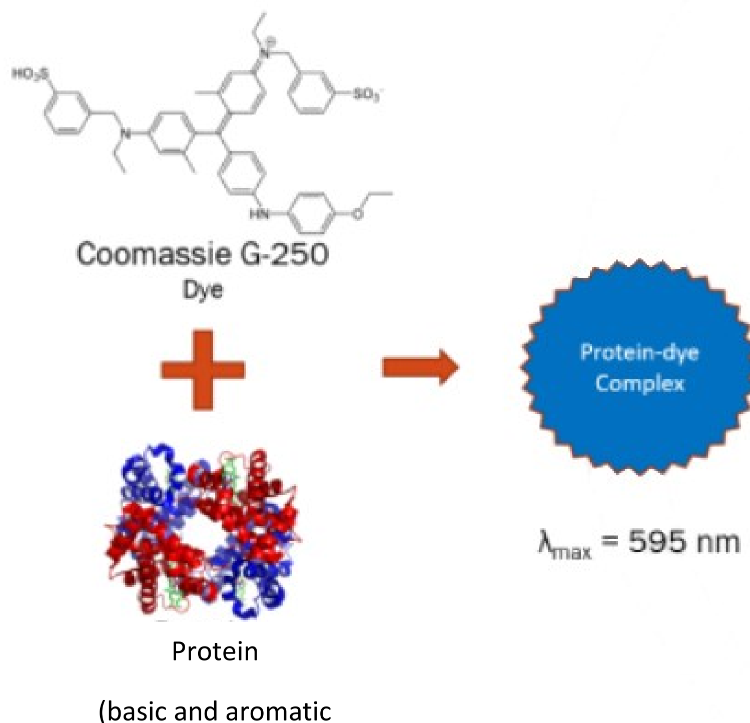


Figure M7: Schematization of Bradford's method for determination of total proteins in platelet fractions (Bradford 1976). Figure obtained from https://tipbiosystems.com/.

3.3.4.4. Chemical chemistry tests for panel 4 determination - oxidative stress markers SOD, CAT and total thiols

Oxidative stress biomarkers were mainly investigated in heparin-plasma samples using dedicated chemical chemistry assays.

3.3.4.4.1. Superoxide dismutase (SOD) assay

To evaluate SOD in plasma and serum samples from enrolled subjects, a competitive colorimetric assay was employed, accordingly to previous works (Peskin and Winterbourne, 2000; Zhou and Prognon, 2006). The assay uses also as an ancillary enzyme, xanthine oxidase (XO), which produces superoxide ions O_2^-, which, in the presence of tetrazolium salt, form in turn a colored compound, formazan, which absorbs at 450nm. In the presence of SOD in plasma, O_2^-, anions are dismutated in H_2O_2 and O_2, thus avoiding the reduction of tetrazolium salt to formazan dye. Thus, higher Abs are

obtained in lower concentrated SOD samples while lower Abs in the higher concentrated ones. In this study, we used a commercial kit provided by Cayman Chemicals (Ann Arbor, MI, USA) (**Figure M8**).

The day of assay, the standard curve was carried out using bovine erythrocyte SOD: 7 solutions of this enzyme at known increasing concentrations (from 0 to 0.050 U/ml) were prepared. Plasma and serum were also thawed and diluted 1:5 in sample buffer (50mM di Tris- HCl, pH 8). Then, 1) 200µl of a tetrazolium salt solution (radical detector), previously diluted in the assay buffer (50 mM Tris-HCl, pH 8, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine), 2) 10 µl of SOD (Cu/Zn) standards and 3) 10 µl of each diluted plasma sample were added to a 96 -well microtiter plate. The reaction was initiated by adding 20 µl XO to each well. After 30 min incubation at RT on a plate shaker, absorbance was read at λ=450nm.

For calculation, the average absorbance of each standard and sample was obtained.

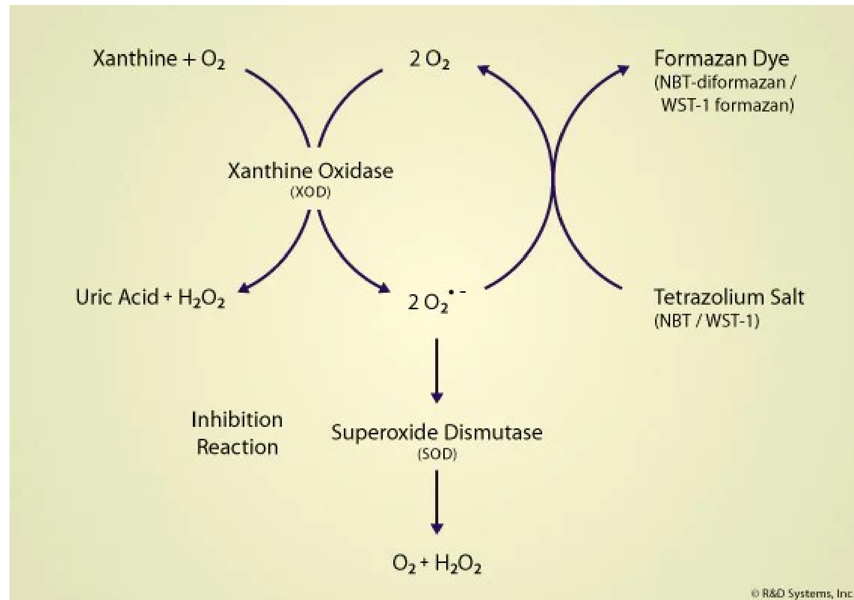


Figure M8: Superoxide dismutase (SOD) procedure. Competitive colorimetric assay. Figure obtained from Cayman Chem (Ann Arbor, MI, USA).

Then, the blank Abs was divided for itself and for each standard absorbance, obtaining the linearized rate (LR). LR was used to perform the linear regression analysis.

The same was done for sample absorbance. SOD activity was calculated from the following formula and linear equation parameters:

SOD U mL^{-1} = [(sample LR -y intercept/slope) x 0.23/0.01] x sample dilution.

One Unit of SOD is defined as the total enzyme required to dismutate the 50% of the O_2^- radical.

3.3.4.4.2. Colorimetric determination of catalase (CAT) activity

As reported previously (Tsai et al, 2016), CAT is one of the main enzymatic antioxidant mechanisms in the body, together the glutathione peroxidase-reductase and thioredoxin activities, essentially devoted to restrain the O_2^--derived H_2O_2 accumulation in tissues, participating to the balance of cell redox state. The assay employed herein to appraise plasma CAT in patients and controls is based on the method proposed by Johansson and Borg (1988). The principle of this method considers that CAT ability to catalyze 2 types of reactions in the body: the well-known transformation of 2 molecules of H_2O_2 to molecular oxygen and 2 molecules of H_2O (catalytic activity) and another redox reaction, named peroxidatic activity, in which low-molecular, aliphatic weight alcohols act as electron donors, yielding the corresponding aldehyde and H_2O.

The method of Johansson and Borg uses the peroxidatic function of CAT: in the presence of methanol and suitable amounts of H_2O_2, CAT produces formaldehyde which can be measured using the compound 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen for colorimetric detection.

Indeed, Purpald reacts specifically with aldehydes forming a bicyclic heterocycle which in turn, under oxidation conditions in the presence of K/Na-periodate ($NaKIO_4$), gives a purple-colored compound (Jendral et al, 2011) (**Figure M9**).

In this study, a commercial kit purchased from Cayman Chemicals (Ann Arbor, MI, USA) was used. The day of assay, buffers were properly diluted as indicated: assay buffer was a 100mM potassium phosphate buffer, pH 7, while sample buffer was a 25mM potassium phosphate, pH 7.5, containing 1mM EDTA and 0.1% BSA (Bovine serum albumin). The calibration curve was prepared using formaldehyde at increasing concentrations as the standard, ranging from 0 to 150μM.

Plasma samples were then thawed and a microtiter 96-wells plate used for the reaction. 100 μl of assay buffer, 30 μl of methanol and 20 μl of each formaldehyde concentration point or 20 μl of each plasma sample were added in duplicate to each corresponding well.

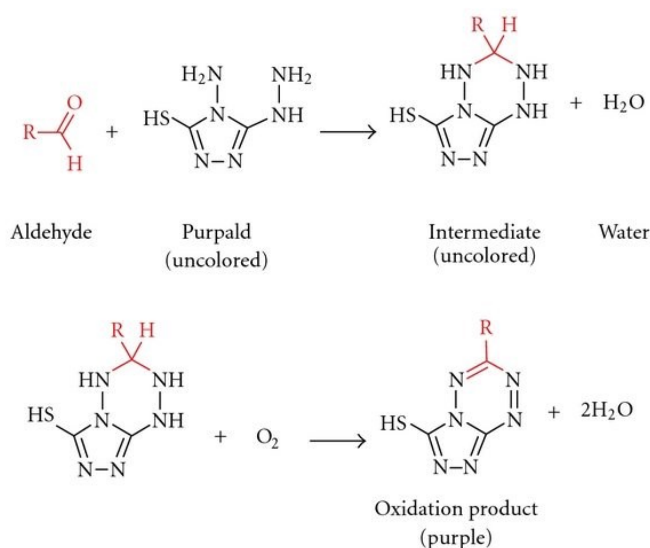


Figure M9: Principle of the CAT method based on the method of Johnsson and Borg (1988), adapted from Jendral et al, 2011.

The reaction was started by adding 20 μl of H_2O_2 to each well. After 20 min of incubation on a plate shaker (500 rpm) at RT, 30μl of a solution of KOH were added to each well to stop the reaction, followed by the addition of 30μl of the reagent Purpald, provided by the kit. After 10 min of incubation on a plate shaker (500 rpm) at RT, 10 μl of a solution of KIO_4 were added to each well, followed by 5 min of incubation under the same conditions. The absorbance was read at 540 nm using the MultiSkan spectrophotometer.

For calculation of CAT activity, average absorbance of each standard and sample was obtained; then, the 0µM formaldehyde absorbance was subtracted from itself and all other standards and samples; the resulting values were plotted as a function of the formaldehyde concentration (µM). After a linear regression analysis, the CAT equation was obtained and CAT activity from each sample was calculating using these formulas:

1) Formaldehyde (µM): [Sample A540nm – (y-intercept)/slope] x 0.17/0.02

2) CAT activity: (µM of sample/20 min) x Sample dilution = nanomoles min^{-1} mL^{-1}.

3.3.4.4.3. Total thiols fluorometric assay

Total thiols in plasma represents a direct evaluation of the redox state of patients. Total thiols are one of the antioxidant components present in biological fluids, together other parameters as, for instance, plasma uric acid. Due to their sulfhydryl group, these compounds, R-SHs, are endowed of free radical scavenging activities. In plasma there are several thiols, including protein cysteines, free cysteine, glutathione, homocysteine and others. The more relevant component in plasma is represented by the albumin cysteine residues (**Figure M10**). A reduction of thiols in plasma can be related to a loss of circulating ROS scavenging actions and lower antioxidant functions. In this study, a rapid fluorometric assay purchased from Cayman Chemicals (Ann Arbor, MI, USA) was employed. This method is a valuable and high-sensitive alternative to the colorimetric procedure which uses the Ellman's reagent (Hu, 1994).

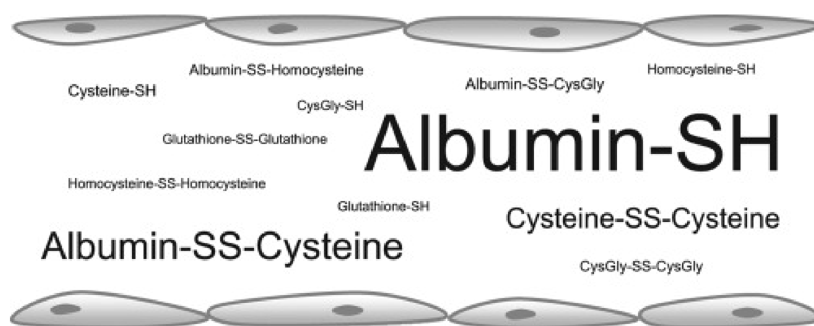


Figure M10: Plasma thiols. Figure obtained from Turell et al, 2013.

The principle is very simple and based on the incubation of plasma and standard solutions with a proprietary fluorometric detector that specifically reacts with thiols to emit a strong fluorescent signal which can be detected using excitation λ between 380-390 nm and emission λ between 510-520 nm. The day of assay, heparin-plasma samples were thawed and diluted 1:5 (*v:v*) with Assay buffer, a solution composed of 100 mM Potassium Phosphate, pH 7.4, containing 1 mM EDTA.

Standard solutions were prepared by using cysteine as the calibrator compound: cysteine powder was weighted and diluted to a concentration of 2 μM in Assay buffer. This solution was then diluted to prepare the STD calibration curve, consisting in 8 cysteine concentrations ranging from 0 to 1000 nM. The thiol assay was started by incubating 50μl of diluted plasma and STD solutions into a black 96-well microplate for fluorometric detection with 50μl of fluorometric detector avoiding light exposure. After 5 min, fluorescence was read by the Enspire multimodal reader, using an excitation λ = 385 nm and an emission λ = 515 nm.

Thiol concentration in samples was calculating by first subtracting the blank fluorescence from that of each STD and sample, and then by plotting STD fluorescence vs. cysteine concentrations. Plasma thiol concentrations were interpolated as μM from the STD linear regression analysis, after correcting for the dilution factor.

3.3.4.5. ELISA tests for panel 5 determination – HPA and inflammatory markers- cortisol, IL-6 and IL1-β

3.3.4.5.1. Measurement of urinary cortisol

Cortisol was measured in nighttime and first morning urine samples. The assay kit employed herein was based on an ELISA direct competitive procedure purchased from Cayman Chemicals (Ann Arbor, MI, USA). This assay is based on the competition between free cortisol and a cortisol-acetylcholinesterase (AChE) conjugate (the Cortisol Express-AChE Tracer) for a fixed and limited number of cortisol monoclonal antibody binding sites. Because the concentration of the Cortisol Express-AChE Tracer is held constant while the concentration of free cortisol varies, the amount of Cortisol Express-AChE Tracer that is able to bind to the cortisol monoclonal antibody will be inversely proportional to the concentration of free cortisol in the well. This antibody-cortisol complex binds to goat anti-mouse IgG attached to pre-coated microplate wells. The plate is washed to remove any unbound reagents and Ellman's Reagent containing the substrate of AChE, is added to the well (**Figure M11**).

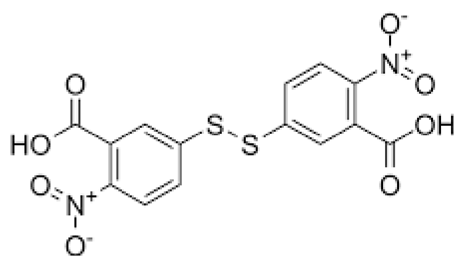


Figure M11: The Ellman reagent or 5,5′-disulfanediyl-bis-(2-nitrobenzoic acid) (DTNB), the substrate of acetylcholinesterase (AChE).

The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Cortisol Express-AChE Tracer bound to the well, which is inversely proportional to the amount of free cortisol present in the well during the incubation. The day of assay, standard curve was prepared from a 500 ng/mL cortisol stock solution, serially diluted in the ELISA assay buffer to obtain 9 increasing cortisol concentrations ranging from 0 to 5,000 pg/mL. Urine samples were thawed and properly diluted in the Assay buffer. The competition was started by incubating at RT cortisol standards, diluted urine samples, the Cortisol Express-AChE Tracer and the anti-cortisol monoclonal antibody. The reaction was conducted for 2 hours at RT on the shaker (600 rpm). After incubations the microplate was emptied and washed five times with the washing buffer containing polysorbate. The Ellman reagent was then added to each well and incubated in the dark for 80-90 min. Absorbance was read at 412 nm by the Enspire multimodal reader. To calculate cortisol concentrations in urine samples a 4-PL non-linear regression analysis was conducted by log transforming cortisol standard concentrations and plotting the % B/B_0 value, where B/B_0 was the sample or standard bound/maximum bound. The interpolated concentrations in samples were transformed into pg/ml. Final concentrations of cortisol in nighttime/first morning urines was the calculated by multiplying results for the dilution factor, by applying the Levine formula, as already done for 6-OH-MLT sulfate determination, by transforming pg/mL into μg/mL and multiplying for the urine's total volume. Urinary nighttime/first morning cortisol levels were presented and analyzed as total cortisol μg excreted during the 8 hours of monitoring.

3.3.4.5.2. Plasma IL-6 ELISA assay

IL-6 concentrations were measured in PPP specimens by means of a sandwich ELISA kit (Cayman Chem IL-6 assay, MI, USA). In more detail, the kit utilizes a first monoclonal antibody, in this case an anti-human-IL-6 antibody, then a second biotinylated antibody, as well as a complex of streptavidin-biotin-peroxidase as the signal-to-noise ratio amplifier (see **Figure M12**).

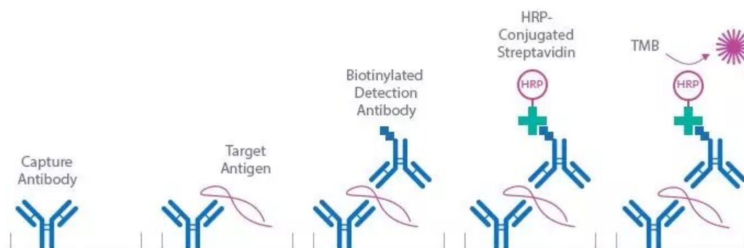


Figure M12: Schema of an ELISA sandwich procedure similar to that employed for IL-6 assay. Figure obtained from https://www.bmgrp.com/

The IL-6 concentrations used for the calibration curve ranged from 3.9 to 250 pg/mL, prepared by serial 1: 2 (*v:v*) dilutions in Assay buffer. For assay, STD solutions and samples were added to the 96-well microplate pre-coated with the "capture" anti-IL-6 antibody and blocked by proprietary proteins to avoid non-specific binding. In figure xxx is depicted the schema of the IL-6 ELISA sandwich assay. Plasma and serum samples were evaluated without a previous dilution. Unknowns were interpolated from the calibration line using a linear regression analysis and IL-6 levels presented as pg/mL.

3.3.4.5.3. Plasma IL-1β ELISA assay

The IL-1β assay was a sandwich ELISA kit purchased from Cayman Chemical (Ann Arbor, MI, USA). The method is rapid and easy to perform: the 96-well microplate is pre-coated with a capture monoclonal antibody which specifically recognize human IL-1β; thus, this antibody will recognize and attach any IL-1β molecule added to each well, as IL-1β deriving from STD solutions, control and plasma samples. STD solutions were prepared by diluting the STD IL-1β stock solution (5 ng/mL) in a sample matrix blank (SMB) provided by the kit, or an IL-1β–free human plasma. This was done to avoid any sample matrix effect. Dilutions were then carried out as indicated for the IL-6 ELISA sandwich assay, by serially diluting the 5ng/mL stock solution in SMB to yield 8 decreasing concentrations ranging from 0 to 250 pg/mL. To avoid interferences due to the presence of

heterophilic antibodies (Grebenchtchikov et al, 2002), plasma samples and STD dilutions were treated with a non-specific mouse serum (NSMS) also provided by the kit: 20 µl of NSMS were added to 400 µl of STD or plasma sample prior to its loading into the assay microplate. After the addition of blanks, STDs, samples, and the second antibody conjugated with AChE, the microplate was incubated at 4°C overnight (about 16 hours). After this overnight incubation, the microplate wells were emptied and washed five times with a washing buffer containing polysorbate. Soon after 200 µl of Ellman reagent were added and the microplate was incubated in the thermo-shaker at RT under shaking (500 rpm) for at least 120 min. Absorbance was read at 412 nm using the Enspire multimodal reader. Unknowns were interpolated from linear regression analysis and IL-1β results shown as pg/mL.

3.4. Calculations and statistical analyses

All data are presented as the mean ± SD and the mean rank.

All biochemical assays were conducted at least in duplicate. For some biological parameters investigated here, a 4-PL non-linear regression analysis was conducted. In this case, the final concentrations in plasma, serum or urines were interpolated as follows: the STD concentrations of the analyte, reported in the corresponding unit of measure, were log transformed and a semi-log calibration curve (y vs. logx) was built by means of the 4-PL non-linear regression equation. After interpolating the analyte concentration in unknowns, resulting log values were then retransformed into the non-logarithmic measures ($X= X^{10}$).

All calibration curves for biochemical dosages were carried out using the GraphPad Prism software (Version 7.0, San Diego, USA).

Due to the limited number of subjects recruited in this study and considering that normality tests and variance homoscedasticity were not respected for some variables in our sample, we applied non-parametric analyses for elaborating our data. A Kruskal-Wallis one way analysis of variance was used in order to compare continuous socio-demographic variables, psychometric instrument scores and biochemical parameter concentrations among the three groups, followed by Dunn test for post-hoc comparisons. For comparisons of continuous nonparametric variables between the two groups of BD patients, the Mann-Whitney U-test was used. Chi-square tests were performed in order to compare categorical socio-demographic variables among groups.

We performed a Spearman's correlation coefficient (r) in order to evaluate the relationships between biochemical variables themselves and between biochemical variables and the psychometric instrument scores. We also performed a discriminant analysis to evaluate which biochemical variables discriminated each group. Finally, a bivariate logistic regression analysis was performed within BD patients to evaluate which biochemical parameters were most statistically predictive of being in the PTSD group with respect to the DEP one.

All the analyses were performed using SPSS version 28 (IBM Corp. 2021). For all the statistical analyses carried out in this study, the statistical threshold was set at $p \leq .05$.

4. Results and Discussion

4.1 Socio-demographic features and psychometric scales

Results

Our sample was composed of 20 BD subjects in euthymic phase with PTSD (PTSD group), 20 BD subjects with a Major Depressive Episode (DEP group) and 24 healthy controls (CTL group). The mean age in the total sample was 41.38±14.98 years, 42.10±15.11 years for the PTSD group, 50.35±16.41 years for the DEP group and 33.29±8.05 years for the CTL group. The total sample included 42 (65,6%) females and 22 (34.4%) males. The PTSD group included 15 (75.0%) females and 5 (25.0%) males, the DEP group 12 (60.0%) females and 8 (40.0%) males and the CTL group 15 (62.5%) females and 9 (37.5%) males. No statistically significant gender differences emerged in the three groups.

The mean Body Max Index (BMI) in the total sample was 25.23±6.05 Kg/m^2, 27.14±7.29 in the PTSD group, 31.83±6.14 in the DEP group and 23.02±3.92 in the CTL group. Subjects in the DEP group showed a significantly higher BMI than those in the CTL group (p=.014).

Among patients, 11 subjects (27.5%) were at the first contact with a psychiatric service, while 29 (72.5%) were at a follow-up visit; particularly, 30 (75.0%) were inpatients while 10 (25.0%) were outpatients. All patients had a BD diagnosis lifetime: particularly, in the PTSD group five subjects (25.0%) had a BD type I diagnosis and 15 (75.0%) had a BD type II diagnosis, while in the DEP group 8 (40.0%) and 12 (60.0%) had BD type I and type II diagnosis, respectively. Thirty-four (85.0%) patients received an antidepressant treatment, 33 (82.5%) a mood stabilizer, 30 (75.0%) an antipsychotic and 24 (60.0%) a benzodiazepine drug. Further, the mean value of year of illness in patients was 15.18±12.75 years.

In the PTSD group, 13 (65.0%) subjects reported to had been exposed to actual or threatened death, 4 (20.0%) to serious physical injury and 3 (15.0%) to sexual violence. Particularly, 13 (65.0%)

reported to had directly experienced the traumatic event, while 7 (35.0%) had witnessed, in person, the event as it occurred to others. The mean value of years after the traumatic event in patients with PTSD was 7.37±12.52 years, with 12 subjects (60%) reporting a traumatic event occurred in the last year. Finally, 7 (35.0%) reported a traumatic event related to COVID-19 pandemic.

In the DEP group, although none reported ongoing post-traumatic symptoms or a "major" traumatic event, the following lifetime "minor" potentially traumatic events were reported when fulfilling the IES-R and TALS-SR symptomatological domains: interpersonal violence (7, 35.0%), the breakup of a romantic relationship (4, 20.0%), a grief-related event (3, 15.0%), a chronic illness (3, 15.0%), being bullied (3, 15.0%).

We also performed a comparison of the scores reported at the psychometric scales among groups. PTSD and DEP groups reported higher scores than CTL group on all domains for each instrument (MOOD-SR Spectrum, TALS-SR spectrum, WSAS). Further, PTSD group showed higher scores than DEP group on *Avoidance and numbing* TALS-SR domain.

Details are reported in **Table 1**.

Table 1: Comparison of psychometric instrument scores among groups.

	PTSD (n=20) *(Mean±SD, Mean rank)*	**DEP (n=20) *(Mean±SD, Mean rank)***	**CTL (n=24) *(Mean±SD, Mean rank)***	**H**	**p**	**post-hoc**
Moods-SR Spectrum lifetime version						
Mood Depressive	13.10±6.17, 38.78	16.00±4.28, 46.61	4.13±3.34, 14.79	36.07	<.001	*PTSD, DEP>CTL*
Mood Maniac	12.80±5.85, 43.90	11.10±5.82, 40.03	3.00±2.99, 15.73	31.11	<.001	*PTSD, DEP>CTL*
Energy Depressive	5.05±2.65, 37.45	6.16±3.34, 44.21	1.25±1.29, 17.79	25.07	<.001	*PTSD, DEP>CTL*
Energy Maniac	5.55±3.75, 41.45	5.32±2.21, 43.16	1.08±1.35, 15.29	32.82	<.001	*PTSD, DEP>CTL*
Cognition Depressive	12.55±7.96, 40.13	13.84±4.65, 44.68	2.41±2.32, 15.19	33.39	<.001	*PTSD, DEP>CTL*
Cognition Maniac	7.25±4.39, 41.38	6.11±4.63, 37.50	2.04±2.24, 19.83	17.71	<.001	*PTSD, DEP>CTL*
Rhythmicity	13.55±6.22, 40.80	13.32±4.11, 41.58	5.54±4.30, 17.04	25.77	<.001	*PTSD, DEP>CTL*
Total Depressive score	30.70±15.57, 40.08	36.00±8.16, 45.97	7.79±5.75, 14.21	37.59	<.001	*PTSD, DEP>CTL*

Total Manic score	25.60±12.88, 43.13	22.53±11.05, 41.13	6.13±5.74, 15.50	31.59	<.001	*PTSD, DEP>CTL*
TALS-SR Spectrum lifetime version						
Loss events	5.15±1.90, 41.00	4.79±1.90, 37.47	2.88±1.45, 20.17	16.98	<.001	*PTSD, DEP>CTL*
Grief reactions	15.35±4.18, 46.13	12.68±4.66, 36.13	5.96±4.84, 16.96	29.10	<.001	*PTSD, DEP>CTL*
Potentially traumatic events	6.15±3.42, 42.80	4.94±3.13, 37.86	1.58±1.38, 17.31	25.32	<.001	*PTSD, DEP>CTL*
Reactions to losses or upsetting events	10.20±3.62, 42.03	8.89±4.36, 37.71	3.88±4.60, 19.13	19.79	<.001	*PTSD, DEP>CTL*
Re-experiencing	5.45±2.14, 43.68	4.16±2.43, 35.37	1.83±2.39, 19.60	20.07	<.001	*PTSD, DEP>CTL*
Avoidance and numbing	7.70±1.49, 47.65	5.16±3.22, 36.03	0.88±2.42, 15.77	35.67	<.001	*PTSD>DEP, CTL*
Maladaptive coping	3.70±2.32, 43.93	2.63±2.34, 36.79	0.54±1.56, 18.27	25.13	<.001	*PTSD, DEP>CTL*
Arousal	3.25±1.16, 46.18	2.32±1.57, 37.42	0.33±1.05, 15.90	34.19	<.001	*PTSD, DEP>CTL*
Personal characteristics/risk factors	3.40±1.60, 44.48	2.58±1.74, 37.11	0.71±1.12, 17.56	26.58	<.001	*PTSD, DEP>CTL*
WSAS						
Work	5.15±2.60, 41.05	6.05±2.06, 45.63	0.38±0.71, 14.44	38.61	<.001	*PTSD, DEP>CTL*
Home management	4.20±2.50, 40.35	5.20±2.17, 46.48	0.29±0.62, 14.31	39.43	<.001	*PTSD, DEP>CTL*
Social leisure activities	3.90±2.40, 39.00	5.50±2.28, 47.70	0.21±0.51, 14.42	40.73	<.001	*PTSD, DEP>CTL*
Private leisure activities	4.60±2.62, 39.73	5.70±2.27, 47.18	0.13±0.34, 14.25	41.14	<.001	*PTSD, DEP>CTL*
Close relationships	4.70±2.49, 41.63	5.40±2.19, 45.43	0.08±0.28, 14.13	40.54	<.001	*PTSD, DEP>CTL*
IES				**z**	**p**	
Intrusion	2.39±0.90, 29.63	0.51±0.36, 10.85	-	-5.156	<.001	*PTSD>DEP*
Avoidance	2.14±0.69, 29.61	0.53±0.40, 10.88	-	-5.135	<.001	*PTSD>DEP*
Hyperarousal	2.37±0.75, 29.42	0.54±0.37, 11.05	-	-.5.047	<.001	*PTSD>DEP*
Total score	50.42±11.18, 30.00	11.55±6.99, 10.50	-	-5.344	<.001	*PTSD>DEP*
HAM-D				**z**	**p**	
Total score	8.40±2.41, 10.50	18.40±3.55, 30.50	-	-5.433	<.001	*DEP>PTSD*
YMRS				**z**	**p**	
Total score	6.60, 20.93	6.25, 20.08	-	-.232	.736	-

Moreover, DEP group presented higher HAM-D total score than PTSD group (18.40±3.55 vs 8.40±2.41, *p*<.001), while PTSD group exhibited higher IES-R total (50.42±11.18 vs 11.55±6.99, *p*<.001) and *Intrusion* (2.39±0.90 vs 0.51±0.36, *p*<.001), *Avoidance* (2.14±0.69 vs 0.53±0.40,

p<.001), and *Hyperarousal* (2.37±0.75 vs 0.54±0.37, *p*<.001) IES-R domain scores. No differences in the YMRS total score emerged in the two groups (*p*=.736).

Discussion

Our data showed a higher frequency of female subjects versus males in the PTSD group, that is in line with the evidence in the field. The Epidemiologic Catchment Area (ECA), the first U.S. study on the general population in the late 1980s, found a double incidence in the female gender (Helzer et al., 1987). Norris (1992) showed that two thirds of his survey group of 1000 participants, equally sorted by gender, race and age, had experienced at least one traumatic event. Moreover, the same author (Norris, 1992) showed that among the various types of traumas encountered, the most frequently associated with the development of PTSD was sexual violence, while the most stressful episode was the sudden and catastrophic death of a close person. Breslau et al., 1998, in a study on 2181 USA subjects focused on which types of traumatic events were most experienced in everyday life and which of them were most related to the symptoms of PTSD, reported a 9.2% risk of PTSD in subjects who had experienced at least one lifetime trauma and a greater risk of development related to episodes of physical violence (risk of PTSD accounting for by 20.9%). In addition, the most experienced traumatic event was the sudden death of a close person (60% of the traumas), with a risk of PTSD of 14.3%. This is in line with the results of the present study, showing that the most common trauma in PTSD subjects was the exposition to actual or threatened death, directly experienced or happened to a close person. In the Random Community Survey (Breslau et al., 1991) the rates of PTSD showed a lifetime prevalence of PTSD in the general population of 9.2%, with a double prevalence in the female gender compared to the male one (11.3% vs 6%). In the National Comorbidity Survey study (Kessler et al., 1995), however, different PTSD rates were found, with a lifetime prevalence of the disorder of 7.8%, which remained double in females compared to males (10.2% vs. 5%). For what concern Italian data, the European Study of the Epidemiology of Mental Disorders (ESEMeD) found lifetime

prevalence rates of PTSD of 2.3%, 0.8% at twelve months and 0.4% one month after the traumatic event (De Girolamo et al., 2006), with a 56.1% exposure rate to trauma in the Italian population (Carmassi et al., 2014a). Among the events most encountered as traumatic in Italian citizens there were: the "relational events" (29.4%); the sudden death of a close person (24.1%); having witnessed atrocities (18.2%); war trauma (12.2%); sexual violence (0.8%). In conclusion, multiple studies have shown a double prevalence in women compared to men (Kessler et al., 1995; Breslau et al., 1998; Tolin et al., 2006; Dell'Osso et al., 2011; Carmassi et al., 2014b). The greater PTSD prevalence in women seemed to be related to an increased exposure to traumatic events such as abuse, rape and violence in general (Kessler et al., 1995). However, double PTSD prevalence rates have been found in females also in mass trauma, so it has been concluded that there is a female gender predisposition for PTSD (Dell'Osso et al., 2011; Carmassi et al., 2014b). Also, DEP group was more represented by female (65.5%) with respect to male (34.5%) subjects, reflecting the typical gender frequency of this mental illness (Hasin et al, 2018).

It is important to note that, in our sample, PTSD subject presented a BD in comorbidity, that was in euthymic phase during the enrolment. The presence of other psychiatric disorders and some personality traits represent significantly relevant PTSD risk factors, such as Mood Disorders, Agoraphobia, Social Phobia and Antisocial Personality Disorder (Mellman et al., 1992). In addition, several studies showed that other comorbid psychiatric disorders influenced the prognosis of PTSD. Unfavourable PTSD prognoses seem, indeed, to be related to comorbidity with mood disorders, substance use disorders, anxiety disorders and panic-agoraphobic spectrum disorders, which seem to be implicated in the chronicity of the course of PTSD symptoms (Breslau et al., 1991; Zlotnick et al., 1999). A greater rate of suicidal behaviours in subjects who present the comorbidity between PTSD and Major Depression has been also highlighted, compared to individuals who present the two disorders disjunct (Marshall et al., 2001; Oquendo et al., 2003; Oquendo et al., 2005).

Finally, it is important to note that 35.0% of patients with PTSD reported a traumatic event related to COVID-19 pandemic. The medical, social, economic, and cultural consequences of the COVID-19 pandemic probably represent the most critical and complex global event of the recent history (Adhanom Ghebreyesus, 2020; Holmes et al., 2020). This worldwide emergency, in fact, may imply exposure to a wide sort of stressful or traumatic events (as social isolation, being quarantined or infected, loss of a loved-one, economic difficulties), suggesting the key role of individual vulnerability for the development of mental health consequences and the role of previous mental illness (Carmassi et al., 2022a; 2022b).

4.2 Comparison of biochemical parameters among groups

Results

First of all, plasma and serum amount of QUIN, SOD and IL-6 measured in some samples yielded comparable results indicating that these two biological matrices were both valuable for the measure of these parameters. For what concerns TRP and the metabolites of the KYN pathways, significant differences were found for TRP. 5-HT, QUIN and 6-OH-Melatonin-S levels, while no difference was found in KYN levels among groups. In particular, TRP levels were significantly lower in the DEP group than in the CTL one. Both PTSD and DEP groups exhibited lower 5-HT levels when compared with CTL group, and DEP group reported lower 5-HT levels than PTSD one. Further, DEP group showed significantly higher QUIN concentrations and lower 6-OH-Melatonin-S levels than the CTL group. In order to better evaluate the TRP-metabolism fluxes, we also compared the ratio between parameters among groups. The 5-HT/TRP ratio was significantly lower in both PTSD and DEP groups when compared with CTL subjects. Moreover, KYN/TRP and QUIN/TRP ratios were significantly higher in the DEP group when compared with the other two groups.

The PLT-BDNF levels resulted significantly lower in the DEP group only when compared with the CTL one. No differences emerged in the PPP-BDNF levels among the three groups.

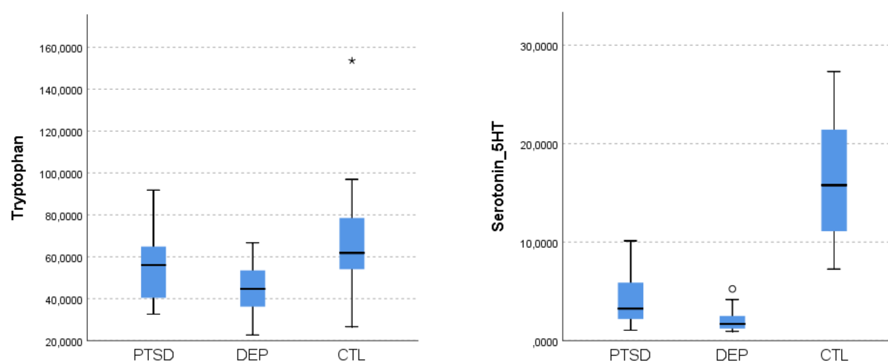
Furthermore, both PTSD and DEP groups showed lower SOD and Total Thiols levels than CTL one, while PTSD group reported also lower CAT levels with respect the CTL one. IL-6 levels were found significantly higher in the PTSD and DEP groups with respect to the CTL one. No differences emerged in the IL-1β levels among groups. Finally, cortisol levels resulted significantly higher in PTSD group when compared with DEP and CTL ones.

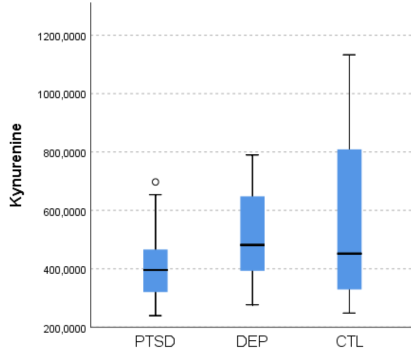
Details are reported in **Table 2** and **Figure 1**.

Table 2: Comparison of biochemical parameters among groups.

	PTSD (n=20) *(Mean±SD, Mean rank)*	DEP (n=20) *(Mean±SD, Mean rank)*	CTL (n=24) *(Mean±SD, Mean rank)*	H	p	post-hoc
TRP, μM	56.43±17.43, 33.15	44.55±12.61, 21.35	67.17±25.70, 41.25	12.50	.002	*DEP<CTL*
5-HT, ng/mL	4.03±2.32, 25.38	2.00±1.14, 13.14	16.39±6.27, 46.82	39.50	<.001	*DEP<PTSD, CTL; PTSD<CTL*
KYN, ng/mL	405.80±124.96, 25.28	508.98±148.95, 37.35	563.58±304.10, 34.48	4.64	.098	-
QUIN, ng/mL	80.43±58.29, 31.84	103.24±59.86, 42.42	53.28±26.70, 22.58	12.83	.002	*DEP>CTL*
BDNF (PPP), ng/mL	3.37±1.70, 33.40	3.98±4.67, 27.05	4.93±5.49, 36.29	2.76	.252	-
BDNF (PLT), ng/mg proteins	2.05±1.76, 27.05	1.27±0.78, 18.80	2.34±1.00, 35.00	9.11	.011	*DEP<CTL*
SOD, U/ml	1.20±0.31, 21.45	1.28±0.32, 24.73	1.95±0.78, 42.58	17.32	<.001	*PTSD, DEP<CTL*
CAT, nmol/min/ml	33.60±16.84, 20.78	45.46±19.01, 31.98	57.57±28.32, 37.63	9.79	.008	*PTSD<CTL*
Total Thiols, μM	0.37±0.06, 23.70	0.37±0.04, 25.15	0.43±0.06, 42.65	14.60	.001	*PTSD, DEP<CTL*
IL-6, pg/mL	6.20±1.06, 35.29	5.87±0.69, 31.44	5.38±0.19, 18.92	10.49	.005	*PTSD, DEP>CTL*
IL-1β, pg/mL	1.55±0.68, 30.40	1.59±0.70, 31.25	1.99±2.46, 26.56	0.83	.662	-
6-OH-MLT-S (urine), ng/ml	37.80±37.85, 30.58	14.49±5.93, 18.65	51.28±46.14, 32.61	8.01	.018	*DEP<CTL*
Cortisol (urine), μg/8h	70.45±70.29, 35.39	24.78±11.90, 21.39	25.97±10.47, 24.06	8.30	.016	*PTSD>DEP, CTL*
5-HT/TRP	0.01±0.00, 24.10	0.01±0.01, 16.11	0.00±0.00, 45.18	30.64	<.001	*DEP, PTSD<CTL*
KYN/TRP	0.04±0.02, 27.65	0.06±0.02, 43.60	0.05±0.05, 27.29	10.34	.006	*DEP>PTSD, CTL*
QUIN/TRP	0.01±0.00, 29.94	0.01±0.01, 44.74	0.00±0.00, 20.92	19.18	<.001	*DEP>PTSD, CTL*

Figure 1. Comparison of biochemical parameters

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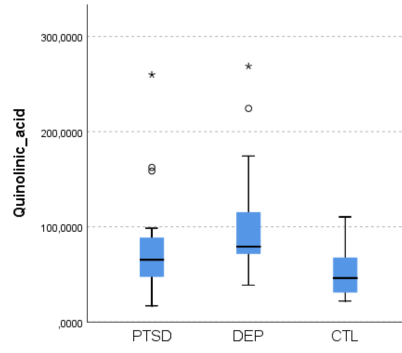
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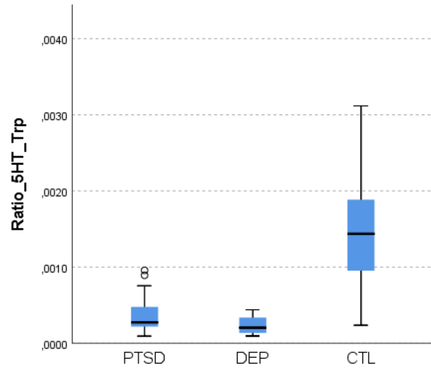
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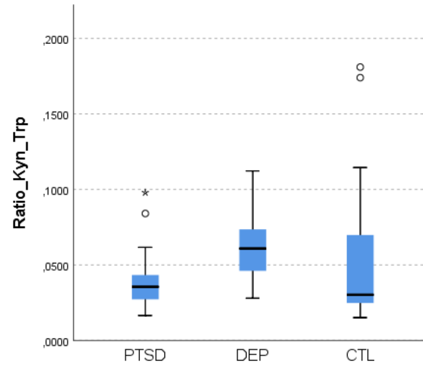
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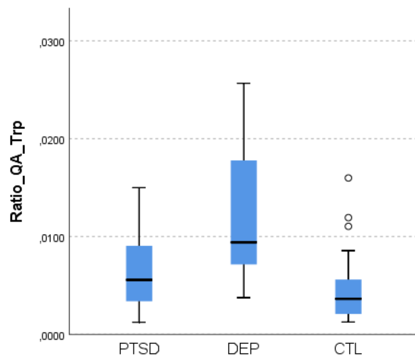
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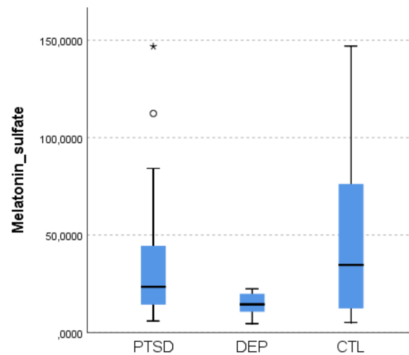
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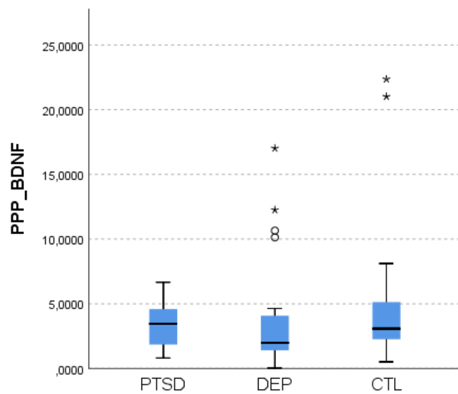
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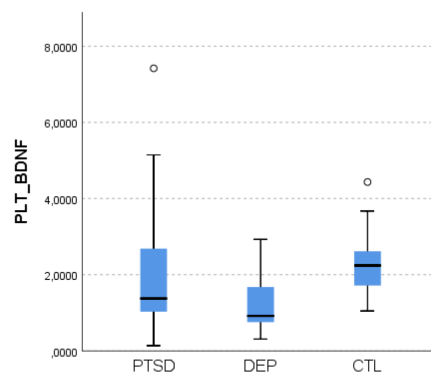
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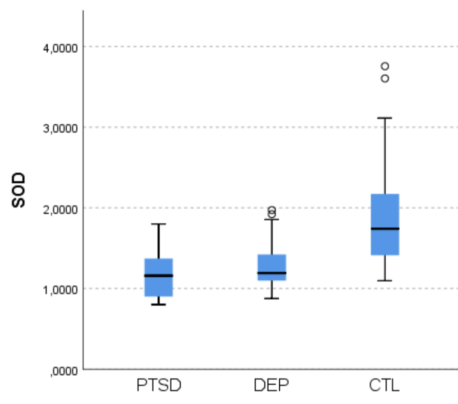
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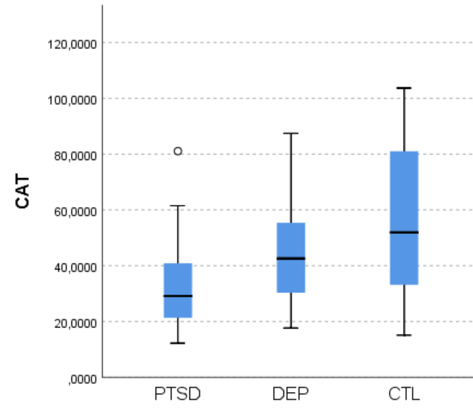
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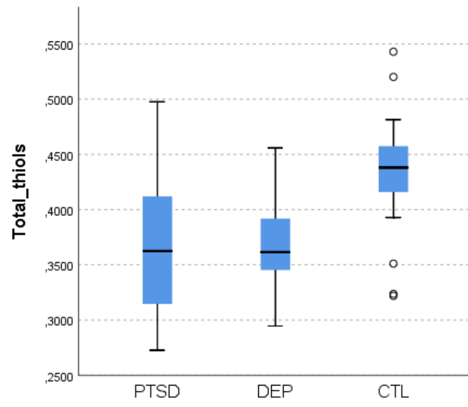
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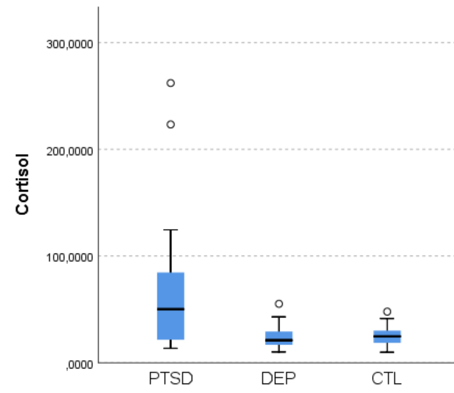
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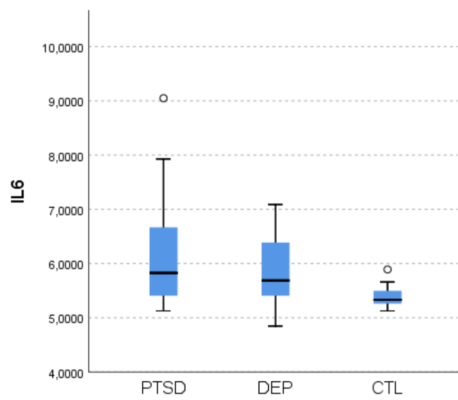
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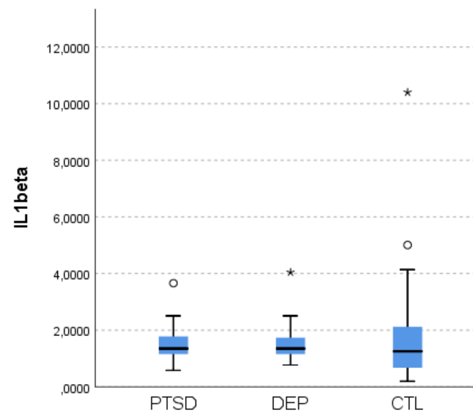
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CTL

Discussion

To the best of our knowledge, this is the first study investigating and comparing possible peripheral biomarkers of PTSD and major depressive episode in patients with BD versus healthy controls. Our data showed lower levels of 5-HT and its precursor TRP in BD patients with major depressive episode (DEP group). This is in line with the classical monoamine theory, suggesting as "biochemical lesion" in depression the reduction of biogenic monoamines as 5-HT, Norepinephrine and Dopamine at the origin of mood symptom presentations (Leonard, 2010). 5-HT is produced from the essential amino acid tryptophan (TRP), which cannot be synthesized by the human body and must be introduced through the diet. During the process, TRP is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH), which is available in two isoforms: one is active in the periphery or in the pineal gland (TPH1), while the other (TPH2) works in the CNS. Finally, the ubiquitous aromatic acid decarboxylase (AADC) converts 5-HTP in 5-HT (Harrington et al. 2013). Studies have widely shown a possible link between the onset of depression and the reduction of 5-HT transmission in several brain areas as basal ganglia, mesencephalic nuclei, cortical and limbic districts (in particular, the amygdala and hippocampus), leading to alterations in the control of mood tonus, emotion, psychomotor abilities, sleep, appetite and a variety of other stress-related functions (Charney and Leger, 2010). The depletion of 5-HT could be explained by the upregulation of the indoleamine 2,3-dioxygenase (IDO), the crucial enzyme in the kynurenine shunt, a main pathway of TRP degradation, resulting in the decrease of TRP concentration and the increase of several metabolites, including KYN and QUIN (Kałużna-Czaplińska et al. 2017; Savino et al. 2020, Brown et al., 2021). Our data support this hypothesis in DEP group, as demonstrated by the higher levels of QUIN, KYN/TRP ratio and QUIN/TRP ratio in these subjects. Euthymic BD patients with PTSD also exhibited lower 5-HT levels than CLT group, though 5-HT concentrations resulted significantly higher in PTSD group with respect to DEP one. This finding confirms the role of 5-HT dysregulation in PTSD, as it plays a key modulatory role in the regulation of fear and stress loci throughout the brain, including the amygdala and hippocampus (Sari, 2004; Krystal and Neumeister., 2009). Further,

IDO enzyme and kynurenine shunt seems to be inducted by stress and proinflammatory mediator, that have been reported to be increased in PTSD (Yang and Jiang, 2020; Passos et al., 2015); however, as we will examine later, 5-HT depletion seems to have a main role in major depressive episode than PTSD, as also suggested empirically by the partial/incomplete response to serotonergic treatments that characterized more frequently PTSD than depression (Hoskins et al., 2015).

Melatonin, the hormone regulating circadian rhythmicity and sleep derived by the 5-HT metabolism, is mostly metabolized by the liver, transformed into 6-hydroxymelatonin and subsequently to 6-sulfatoxymelatonin (6-OH-Melatonin-S), which is excreted in the urine (Kondo et a., 2009). The concentration of 6-OH-Melatonin-S in urine correlates well with the total level of melatonin in the blood (Mahberg et al., 2006). Early-morning 6-OH- Melatonin-S reflects the sum of nighttime pineal melatonin secretion. Our results showed DEP group exhibiting lower 6-OH-Melatonin-S levels than CTL subjects. As aforementioned, melatonin is an indole product of tryptophan metabolism, deriving from the mood neurotransmitter 5-HT, and acts as a foremost signal of the body sleep-wake cycle, related to the daily light-dark alternation. Additionally, the 5-HT/melatonin pathway is a tryptophan metabolic branch alternative to the kynurenine shunt, which promotes the tryptophan indole-ring breakdown. The induction of the IDO enzyme and of the kynurenine shunt seems by stress and proinflammatory mediators, as happen in depression, could contribute to reduce pineal melatonin synthesis from 5-HT, leading to circadian rhythms dysregulation and insomnia, that qualify this psychopathological condition. Insomnia and sleep disorders, indeed, represents core symptoms of depression, as well as residual sign in this kind of patients after remission, exposing them to a higher risk of relapse (Nutt et al., 2008; Nierenberg et al., 2010; Paunio et al., 2015).

Conversely, euthymic BD patients with PTSD showed higher urinary nighttime cortisol than the other two groups of subjects. Cortisol is the primary effector of HPA axis, that is released in stress conditions and contribute to several functions, including immune system regulation (Palego et al., 2021). In the context of a chronically hyperarousal state, as seen in individuals suffering from PTSD

(i.e. disturbances of the sleep-wake homeostasis, leading to a perturbed activity of the arousal centers in the brainstem and mesencephalic nuclei), the dysregulation of HPA axis may contribute to a chronic low-grade inflammatory state due to alterations within the glucocorticoid (GC) receptor itself and reduced GC signalling. Changes to GC responsiveness may also contribute to allostatic load, resulting in chronic disease development. Therefore, an increase of allostatic load due to chronic stress exposure gives rise to long-term alterations, as GC receptor resistance or the loss of sensitivity of GC receptors in target cells, accompanied by diminished immunosuppressive and immunomodulatory functions of cortisol and GCs (Cohen et al., 2012). Dysregulated HPA function by enhanced glucocorticoid negative feedback (Morris et al., 2012, Yehuda, 2002) frequently lead to a lower circulating cortisol level in long-term in traumatized subjects when compared to controls (Meewisse et al., 2007, Miller et al., 2007, Morris et al., 2012; Klaassens et al., 2012). However, our sample included mainly subjects with a trauma occurred in the last year (60%), and that, together or alternatively to the GC receptor resistance or loss of sensitivity to cortisol, could explain how cortisol levels resulted higher in PTSD group with respect to DEP and CTL ones.

Further, PTSD and DEP groups showed lower levels of SOD, CAT (only PTSD group), total thiols, besides higher IL-6 concentrations than CTL subjects. Conflicting and heterogeny results have been reported in literature about the concentrations of antioxidant enzymes in patients with depression (Galeki et al., 2009; Kodydková et al., 2009; Rybka et al., 2013; Lukic et al., 2014), including SOD. The ambiguity can be caused by small study sample, heterogeneity of patients' statuses, or other variability in individual experiments (Hovatta et al., 2010, Vaváková et al., 2015). Conversely, our data showed as CAT reduction seemed to be more specifically related to PTSD, suggesting a fundamental role of oxygen radical-mediated damage (Passos et al., 2015; Karanikas et al., 2021) in this disorder. As previously reported, the reduction of plasmatic total thiols may also be a sign of oxidative status (Biswas et al., 2006; Incecik et al., 2019), as reported in several psychiatric condition as schizophrenia, neurodegenerative disorders, and bipolar disorder (Erzin et al., 2018; Topcuoglu et al., 2017; Erzin et al., 2020). Our findings suggest that there might be an increase in oxidative stress

in BD patients when compared with healthy controls. Interestingly, this is the first studying reporting data on total thiols in patients with PTSD, and their reduction in these subjects remark the increase of oxidative stress which characterize this psychopathological condition.

Depression and PTSD have been hypothesised to be characterized by an hyperinflammatory reaction and mild chronic inflammation, related to an increase in proinflammatory cytokines including TNFα, IL-1β, IL-6, IFNγ, and C-reactive protein (Tursic et al., 2014; Passos et al., 2015; Black and Miller, 2015; Koler et al., 2017). Antidepressants drugs possess anti-inflammatory effect, and the inhibition of proinflammatory cytokines or their signal pathways increases effectiveness of antidepressants and decreases depression state in patients (Hughes et al., 2016; Jeon and Kim, 2016; Schmidt et al., 2016; Vojvodic et al., 2019. Particularly, in PTSD also the impaired reaction of HPA axis to perceived stress also results in a chronic low-grade pro-inflammatory state (Morris et al., 2016; Bruenig et al., 2017; Lindqvist et al., 2017; Speer et al., 2018). This promotes the pro-inflammatory cytokines secretion in PTSD (Yang and Jang, 2020). It is noteworthy that chronic inflammation plays a decisive role in several chronic immune associated diseases, including cardiovascular disease (Welsh et al., 2017). Pro-inflammatory cytokines, indeed, contribute to endothelial function impairment (Galkina and Ley, 2009). PTSD has been associated with high risk of cardiovascular disease, as atherosclerosis, myocardial ischemia and coronary heart disease (Levine et al., 2014; Turner et al., 2013). High risk of cardiovascular disease in women with chronic PTSD has been related to increased inflammation and impaired endothelial function (Sumner et al., 2017). Further, peripheral pro-inflammatory cytokines could also cross the BBB and produce effects on the stress response system and reducing neurotrophins release (as BDNF) and neurogenesis processes (Banks et al., 2002; Kuhn and Gallinat, 2013; Monje et al., 2003).

Finally, PLT-BDNF levels resulted significantly lower in DEP group with respect to CTL one. Platelets are the main storage of BDNF secreted from all other tissues (Bus et al., 2011) and, when activated, release numerous inflammatory mediators, including BDNF (Thomas and Storey, 2015).

Up to 40%-60% of their BDNF total content could be released when platelets are activated (Fujimura et al., 2002). However, the PLT-BDNF levels seems to be more stable than PPP-BDNF, maybe because platelets either have a non-releasable pool of BDNF, or MK endogenously produce BDNF (Chacòn-Fernandez et al, 2016), and the released BDNF is sequestered by binding to a transporter or receptor on the platelet surface (Fujimura et al., 2002). PPP-BDNF levels, indeed, probably reflect also the proinflammatory status and the production by the endothelium, by the musculature smooth and by activated macrophages and lymphocytes (Karege et al., 2005). Many other studies considered peripheral BDNF levels as possible peripheral biomarker of depression or stress-related disorders (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-analyses have also shown that BDNF is more pronouncedly reduced in severe depression and increased during antidepressant 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 (Primo de Carvalho Alves and Sica da Rocha, 2018; Monteiro et al., 2017). Further, several studies found serum levels of BDNF significantly reduced during the depressive, manic and mixed phases of bipolar disorder (Yoshimura et al., 2006; Cunha et al., 2006, de Oliveira et al., 2009, Monteleone et al., 2008; Machado-Vieira et al., 2007, Piccinni et al., 2015). In addition, Fernandes et al. (2011) found a difference between BDNF levels in euthymic and non-euthymic states in bipolar disorder, suggesting peripheral BDNF levels as potential biomarkers of mood (depressive and manic) episodes. Further, serum levels of BDNF resulted significantly reduced in depressed patients and inversely correlated with severity of depressive symptoms (Karege et al., 2002, 2005; Shimizu et al. coll., 2003; Piccinni et al., 2008). Our result showing non-significant PPP-BDNF levels in patients vs. controls still requires attention. It is indeed possible that plasma BDNF is rather a state marker related to the presence of peculiar inflammation paths activated in different depressive presentations, since it has

been found even increased in depressed subjects (Serrà-Millas et al, 2011), and, as already mentioned, in mood disorder patients with resistant depression and higher IL-1β, in bipolar disorder-II vs. subthreshold bipolar patients (Bonnìn et al, 2012), in pain syndromes, and in adaptive disturbances (Bazzichi et al, 2011; Wang et al, 2016; Buselli et al, 2019; Uint et al, 2019). Moreover, subjects in a depressive phase of mood disorder with mixed features (compresence in a depressive phase of some control-polar manic symptoms) have been characterized by BDNF levels generally slightly higher than depressive ones (Piccinni et al., 2015). Ours and these results also suggest the need at further characterizing the possible various components of circulating BDNF not only in defined subgroups of patients but also in healthy subjects (Le Blanc et al, 2020).

4.3 Correlations among biochemical parameters

Results

When evaluating the Spearman correlation coefficient among biochemical parameters in the whole sample, we found that TRP levels were significantly positively correlated with 5-HT and negatively correlated to QUIN concentrations and to KYN/TRP and QUIN/TRP ratios. 5-HT levels were significantly positively correlated to 5-HT/TRP ratio, and total thiols levels, while they were negatively correlated to QUIN and IL-6 levels and QUIN/TRP ratio. KYN concentrations were positively correlated to KYN/TRP ratio, as well as QUIN levels were positively correlated to KYN/TRP and QUIN/TRP ratios. 5HT/TRP ratio was positively correlated to total thiols and negatively correlated to IL-6 levels. KYN/TRP ratio was positively correlated to QUIN/TRP ratio. 6-OH-melatonin-S concentrations were positively correlated to PLT-BDNF and negatively correlated to SOD levels. PLT-BDNF levels were also negatively correlated to CAT concentrations. SOD concentrations were positively correlated to CAT levels and negatively correlated to total thiols and cortisol ones. Finally, total thiols and IL-6 concentrations were negatively correlated. Details are reported in **Table 3**.

Table 3. Correlations (Spearman r) between biochemical parameters in the whole sample (n = 64).

	TRP, µM	5-HT, ng/mL	KYN, ng/mL	QUIN, ng/mL	5HT/TRP	KYN/TRP	QUIN/TRP	6-OH-MLT-S (urine), ng/ml	BDNF (PPP), ng/mL	BDNF (PLT), ng/mL	SOD, U/ml	CAT, nmol/min/ml	Total Thiols, µM	IL-6, pg/mL	IL-1β, pg/mL	Cortisol (urine), µg/8h
TRP, µM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-HT, ng/mL	.353**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KYN, ng/mL	-.099	.036	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QUIN, ng/mL	-.339**	-.344*	.190	-	-	-	-	-	-	-	-	-	-	-	-	-
5-HT/TRP	-.011	.915**	.036	-.249	-	-	-	-	-	-	-	-	-	-	-	-
KYN/TRP	-.696**	-.234	.740**	.406**	.012	-	-	-	-	-	-	-	-	-	-	-
QUIN/TRP	-.699**	-.439**	.192	.881**	-.196	.619**	-	-	-	-	-	-	-	-	-	-
6-OH-MLT-S (urine), ng/ml	.150	.173	-.107	-.124	.055	-.192	-.102	-	-	-	-	-	-	-	-	-
BDNF (PPP), ng/mL	-.001	.197	-.150	-.073	.215	-.068	-.070	-.203	-	-	-	-	-	-	-	-
BDNF (PLT), ng/mg proteins	.086	.083	.053	.044	.042	-.053	-.075	.357*	-.162	-	-	-	-	-	-	-
SOD, U/ml	-.043	.041	.005	.177	.111	.119	.143	-.335*	.162	-.104	-	-	-	-	-	-
CAT, nmol/min/ml	-.104	-.055	.047	.117	-.028	.066	.143	-.184	.091	-.402*	.341*	-	-	-	-	-
Total Thiols, µM	.190	.316*	.103	-.251	.280*	-.075	-.257	.086	.121	.066	-.449*	-.307	-	-	-	-
IL-6, pg/mL	-.043	-.292*	.047	.099	-.312*	.075	.031	.132	.175	-.002	-.193	-.056	-.275*	-	-	-
IL-1β, pg/mL	.012	-.044	.192	.099	-.089	.132	.093	-.012	.017	-.145	-.263	.120	.173	.101	-	-
Cortisol (urine), µg/8h	.089	.080	-.131	-.010	-.046	-.254	-.119	.206	-.245	.229	-.343*	-.238	.033	-.144	.051	-

**p<.05; **p<.01*

Discussion

The correlations between biochemical variables widely reflected the pathophysiological fundamentals of TRP metabolism, besides proinflammatory and immune response to stress. As previously discussed, 5-HT levels were positively correlated to TRP concentrations (its precursor in the indole-conservating pathway). Conversely, 5-HT and TRP levels were negatively correlated with QUIN, the metabolic product of the kynurenine shunt of TRP. 5-HT levels were also positively correlated with total thiols concentrations and negatively correlated to IL-6 levels, confirming the role of oxidative stress and proinflammatory mediators in inducing IDO, the limiting enzyme of the kynurenine pathway (Jiang et al., 2020; Tordjman et al. 2014, Savino et al. 2020; Brown et al., 2021). Significant correlations also emerged among stress-oxidative biomarkers and inflammatory profile (SOD, CAT, total thiols, IL-6), suggesting the well-known strong relationship between these two systems (Yang and Jiang, 2020; Passos et al., 2015; Erzin et al., 2020; Troubat et al., 2021). Finally, PLT-BDNF and 6-OH-melatonin-S resulted positively correlated each other, suggesting the bidirectional role of neurotrophic factor on pineal melatonin production in mood and stress-related disorders (Oglodek et al., 2016).

4.4 Correlations between biochemical parameters and psychometric instruments

Results

We also evaluated the possible correlations among biochemical parameter concentrations and the total and domain scores reported on psychometric scales. As described in the method section, for all the scales employed higher scores were associated with a higher impairment in the investigated dimension.

The HAM-D total score was significantly and positively correlated to QUIN and IL-6 concentrations and to KYN/TRP and QUIN/TRP ratios; conversely, it was negatively correlated to 5-HT, 6-OH-melatonin-S, PLT-BDNF, SOD, total thiols and cortisol levels, besides 5-HT/TRP ratio.

The YMRS total score presented a significantly positive correlation with IL-6 levels and QUIN/TRP ratio, while significantly negative correlations emerged with TRP, 5-HT, 6-OH-melatonin-S, PLT-BDNF, SOD and total thiols levels, besides 5-HT/TRP ratio.

The IES-R total and all domains' scores showed negative correlations with 5-HT, SOD, CAT and total thiols concentrations, besides 5-HT/TRP ratio. Conversely, significant positive correlations emerged among the IES-R total and all domains' scores and IL-6 and cortisol levels, besides QUIN/TRP ratio (with the exception of *Intrusion domain*). The *Hyperarousal* domain presented also positive correlations with the QUIN levels.

Considering the MOODS-SR Spectrum, significantly negative correlations were found among all domains and total scores and 5-HT levels and 5-HT/TRP ratio. Negative correlations emerged also between TRP concentrations and *Energy Depressive* and *Rhythmicity* domains besides the *Total Depressive* score. Total thiols levels negatively correlated with *Mood Manic*, *Energy Depressive and Rhythmicity* domains, besides *Total Depressive* and *Total Manic* scores. Cortisol concentrations negatively correlated to *Energy Depressive* domain, while SOD levels negatively correlated to *Total Depressive* score. Conversely, significantly positive correlations were found among all domains (with

the exception of *Cognition Maniac* domain) and total scores and QUIN/TRP ratio. Further, IL-6 levels positively correlated to *Mood Depressive*, *Mood Maniac* and *Energy Maniac* domain scores, besides to *Total depressive* and *Total Maniac* scores. Finally, IL-1β positively correlated to *Energy Maniac* and *Cognition Depressive* domains scores.

When exploring correlations between biochemical parameters and TALS-SR Spectrum, we found significantly negative correlations between all TALS-SR domains scores and 5-HT levels and 5HT/TRP ratio (with the exception of *Loss Events* domain). Negative correlations also emerged among TRP concentrations and *Loss Events*, *Maladaptive coping* and *Personal characteristics/risk factors* domains scores, besides among Total Thiols levels and *Loss Events* and *Avoidance and numbing* domains scores. Moreover, PPP-BDFN levels were found to be negatively correlated to *Re-experiencing* domain scores. Conversely, significantly positive correlations were shown among all TALS-SR domains scores and IL-6 levels (with the exception of *Loss Events, Potentially Traumatic Events* and *Re-experiencing* domains). KYN/TRP ratio positively correlated to *Loss Events* domain score, while QUIN/TRP ratio positively correlated to *Loss Events, Potentially Traumatic Events, Avoidance and numbing* and *Arousal* domains scores. Moreover, 6-OH-melatonin-S levels were found to be positively correlated to *Grief Reactions* domain scores, while IL-1β concentrations positively correlated to *Maladaptive coping* and *Personal characteristics/risk factors* domains scores.

Finally, we evaluated the correlations between biochemical parameters and the WSAS, which explores the negative impact of symptoms on different areas of functioning (the higher the score, the higher the negative impact). Significantly negative correlations emerged among all WSAS items scores and the levels of TRP, 5-HT, Total Thiols, besides 5-HT/TRP ratio. Conversely, significantly positive correlations were shown among all WSAS items scores and the concentrations of QUIN and IL-6, besides QUIN/TRP ratio.

Details are summarized in **Tables 3A** and **3B**.

Table 4A. Correlations (Spearman r) among biochemical parameters, HAM-D, YRMS, IER-R and MOOD-SR in the whole sample (n = 64).

	HAM-D	YMRS	IES-R				MOODS-SR								
	Total score	Total score	*Intrusion* domain	*Avoidance* domain	*Hyperarousal* domain	Total score	*Mood Depressive* domain	*Mood Maniac* domain	*Energy Depressive* domain	*Energy Maniac* domain	*Cognition Depressive* domain	*Cognition Maniac* domain	*Rhythmi -city* domain	*Total Depressiv -e* score	*Total Manic* score
TRP, µM	-.424**	-.335**	-.205	-.242	-.162	-.191	-.213	-.195	-.351**	-.242	-.208	-.043	-.280*	-.257*	-.185
5-HT, ng/mL	-.792**	-.634**	-.509**	-.496**	-.475**	-.505**	-.635**	-.449**	-.477**	-.496**	-.576**	-.333*	-.472**	-.617**	-.469**
KYN, ng/mL	.065	-.072	-.193	-.212	-.190	-.197	-.006	-.087	.005	-.037	-.082	.006	-.056	-.041	-.048
QUIN, ng/mL	.444**	.382**	.233	.228	.271*	.242	.256*	.217	.271*	.298*	.204	.137	.178	.248	.238
5-HT/TRP	-.678**	-.578**	-.516**	-.486**	-.494**	-.519**	-.619**	-.428**	-.390**	-.462**	-.551**	-.344*	-.405**	-.575**	-.447**
KYN/TRP	.354**	.215	.029	.019	-.019	.006	.130	.078	.261*	.130	.102	.036	.148	.151	.098
QUIN/TRP	.535**	.428**	.251	.277*	.262*	.261*	.288*	.279*	.354**	.346**	.276*	.160	.306*	.311*	.293*
6-OH-MLT-S (urine), ng/ml	-.387**	-.295*	-.067	-.055	-.021	-.045	-.096	.149	-.072	.017	.078	.206	.027	.030	.164
BDNF (PPP), ng/mL	-.230	.182	-.121	-.169	-.120	-.146	-.170	-.069	-.016	.011	-.084	-.024	-.116	-.104	-.052
BDNF (PLT), ng/mg proteins	-.398**	-.293*	-.129	-.060	-.003	-.067	-.204	-.037	-.283	-.142	-.221	.004	.046	-.250	-.046
SOD, U/ml	-.435**	-.372**	-.465**	-.476**	-.536**	-.495**	-.293	-.066	-.140	-.307	-.233	-.211	-.153	-.325*	-.166
CAT, nmol/min/ml	-.154	-.247	-.403**	-.369**	-.384**	-.400**	.174	-.056	-.026	.136	.014	.011	-.181	.054	-.013
Total Thiols, µM	-.414**	-.410**	-.411**	-.429**	-.383**	-.413**	-.251	-.358**	-.288*	-.243	-.224	-.215	-.288*	-.254*	-.316*
IL-6, pg/mL	.325*	.426**	.309*	.342*	.397**	.354**	.418**	.305*	.180	.338*	.255	.247	.232	.340*	.325*
IL-1β, pg/mL	.116	.181	.069	.065	.082	.064	.227	.213	.140	.268*	.265*	.227	.150	.235	.233
Cortisol (urine), µg/8h	-.048	-.018	.389**	.381**	.369**	.383**	-.040	-.186	-.445**	-.151	-.256	-.083	-.160	-.223	-.152

$*p<.05$; $**p<.01$

Table 4B. Correlations (Spearman r) among biochemical parameters and TALS-SR and WSAS in the whole sample (n = 64).

	TALS-SR									WSAS				
	Loss events domain	*Grief reactions* domain	*Potentially traumatic events* domain	*Reactions to losses or upsetting events* domain	*Re-experiencing* domain	*Avoidance and numbing* domain	*Malada-ptive coping* domain	*Arousal* domain	*Personal characteristics /risk factors* domain	*Work*	*Home manage-ment*	*Social leisure activities*	*Private leisure activities*	*Close relationships*
TRP, µM	-.302*	-.143	-.174	-.161	-.185	-.228	-.288*	-.147	-.304*	-.319**	-.317*	-.247*	-.276*	-.406**
5-HT, ng/mL	-.335*	-.443**	-.570**	-.436**	-.373**	-.402**	-.333*	-.468**	-.422**	-.670**	-.669**	-.674**	-.669**	-.646**
KYN, ng/mL	.043	.017	-.177	.089	.087	.048	-.040	.002	-.185	-.148	-.081	-.072	-.006	-.061
QUIN, ng/mL	.197	.193	.271*	.007	.127	.219	.091	.236	.155	.308*	.359**	.394**	.394**	.367**
5-HT/TRP	-.232	-.462**	-.560**	-.430**	-.360**	-.376**	-.292*	-.475**	-.349**	-.603**	-.595**	-.639**	-.630**	-.537**
KYN/TRP	.288*	.131	.014	.192	.220	.209	.163	.136	.061	.137	.171	.125	.191	.209
QUIN/TRP	.257*	.231	.256*	.125	.177	.284*	.210	.263*	.237	.384**	.406**	.428**	.436**	.457**
6-OH-MLT-S (urine), ng/ml	.113	.040	.098	-.038	.047	.083	.244	.237	.099	-.230	-.107	-.026	-.023	.185
BDNF (PPP), ng/mL	.027	-.147	-.033	-.166	-.270*	-.105	-.111	-.208	-.065	-.027	-.087	-.210	-.110	-.196
BDNF (PLT), ng/mg proteins	.283	.039	.061	.039	-.062	-.026	-.066	.127	-.022	-.101	-.149	-.181	-.045	.130
SOD, U/ml	.316	-.088	.052	-.054	.008	.052	-.194	-.108	-.170	.053	-.134	-.068	.030	-.260
CAT, nmol/min/ml	.004	-.129	-.013	-.115	-.192	-.071	-.108	-.284	.049	-.116	-.130	.134	.089	.057
Total Thiols, µM	-.257*	-.236	-.238	-.163	-.201	-.298*	-.169	-.216	-.226	-.291*	-.310*	-.333*	-.344**	-.277*
IL-6, pg/mL	.205	.364**	.239	.265*	.198	.343**	.348**	.287*	.339*	.342**	.364**	.387**	.379**	.422**
IL-1β, pg/mL	.224	.116	.012	.236	.172	.154	.258*	.073	.258*	.149	.066	.091	.139	.124
Cortisol (urine), µg/8h	-.022	.048	.025	-.041	.095	.118	.061	.040	-.008	-.036	.060	.012	-.041	.122

*$p<.05$; **$p<.01$

Discussion

The correlations among biochemical parameters and psychometric variables confirmed the associations of specific molecules with specific psychopathological dimensions, as previously emerged in the comparisons among groups. TRP levels negatively correlated with the actual gravity of depressive and manic symptoms, evaluated by means of the HAM-D and YMRS respectively, with some domains of the lifetime dimensional mood spectrum symptomatology assessed by MOODS-SR (particularly *Energy depressive*, *Rhythmicity* and *Total depressive score*) and with the impairment in the several areas of subject's functioning measured by means of the WSAS scale. TRP levels also correlated with some domains of the lifetime dimensional posttraumatic stress spectrum symptomatology assessed by TALS-SR (*Loss events* and *Maladaptive coping*), but not with the actual gravity of post-traumatic stress symptoms, evaluated by IES-R scale. Further, 5-HT levels negatively correlated with the actual gravity of depressive symptoms, evaluated by the HAM-D total score, as well as with the actual gravity of manic symptoms, evaluated by the YMRS total score, with the lifetime dimensional mood spectrum symptomatology assessed by MOODS-SR and with the impairment in the several areas of subject's functioning measured by means of the WSAS scale. Interestingly, 5-HT concentrations negatively correlated also with the actual gravity of post-traumatic stress symptoms, evaluated by IES-R scale, and with the lifetime dimensional posttraumatic stress spectrum symptomatology assessed by TALS-SR. However, the observed correlations of both TRP and 5-HT levels with the HAM-D total score suggest a more pronounced tryptophan and serotonin depletion in mood disorders, particularly major depressive episode, than in PTSD, as observed in the comparisons among groups previously reported. This might confirm a possible different neurobiological substrate underlying PTSD with respect to major depressive episode in BD patients, involving other pathways than 5-HT as alterations in glutamate and GABA markers and signalling (Abdallah et al., 2019).

Moreover, QUIN levels positively correlated with the global function impairment, highlighting the possible neurotoxic role of the accumulation of this metabolite. QUIN seems to promote NMDA transmission, in specific brain regions such as the striatum and the hippocampus (Lugo-Huitrón R et al. 2013; Savino et al. 2020), acting as a glutamatergic excitotoxin produced in response to inflammation (Savino et al. 2020; Brown et al., 2021). QUIN is also involved in free radicals and oxidative stress generation, including the increase of ROS, decreased glutathione levels and SOD activity (Savino et al. 2020; Brown et al., 2021). In addition, QUIN is involved in the neural apoptosis due to an intracellular calcium imbalance (Williams et al. 2017). Our data highlighted a positive correlation also between KYN/TRP and QUIN/TRP ratios and the gravity of depressive symptoms. This is in line with evidence showing as increased QUIN and KYN levels have also been associated with suicidal ideation/behaviours in depression (Erhardt et al. 2013; Sublette et al. 2011; Lim et al. 2016; Bryn et al. 2017).

6-OH-melatonin-S negatively correlated to the actual gravity of depressive and manic symptoms but not with the actual gravity of the post-traumatic stress symptoms. As aforementioned, melatonin is an indole product of tryptophan metabolism, deriving from the mood neurotransmitter 5-HT. Mood disorders phases, particularly major depressive episodes, are characterized by a reduction of pineal melatonin synthesis from 5-HT, leading to circadian rhythms dysregulation and insomnia, that qualify this psychopathological condition. Insomnia and sleep disorders, indeed, represents core symptoms of depression, as well as residual sign in this kind of patients after remission, exposing them to a higher risk of relapse (Nutt et al., 2008; Nierenberg et al., 2010; Paunio et al., 2015).

IL-6 and total-thiols concentrations correlated positively (IL-6) and negatively (total thiols) to actual and lifetime mood and post-traumatic stress spectrum symptomatology, as well as with global function impairment, pointing out the role of a low chronic inflammation status in PTSD and mood disorders. (Yang and Jiang, 2020; Passos et al., 2015; Erzin et al., 2020; Troubat et al., 2020; Cingi et al., 2018).

Conversely a correlation among CAT (negative), cortisol (positive) and the actual gravity of post-traumatic stress symptoms but not lifetime post-traumatic stress spectrum symptomatology emerged. These results might indicate different mechanisms of response in acute and chronic stress in PTSD. In the first phase after a traumatic event, stress could increase stress-oxidative leading to a CAT consumption (Dell'Oste et al., 2023). Meanwhile, increased stress could induce HPA axis response with an increased release of cortisol. Subsequently, these systems could lead to CR peripheral resistance and, ultimately, to dysregulation and exhaustion of the HPA functions. As aforementioned, our sample included mainly subjects with a trauma occurred in the last year (60%), and this could explain why cortisol levels positively correlated to the actual gravity of acute post-traumatic stress symptoms. However, other studies reported an increase in CAT concentrations in depressed with respect to controls, suggesting an overcompensation mechanism (Gałecki et al., 2009; Szuster-Ciesielska et al. 2008).

Negative correlations of PLT-BDNF with both the HAM-D and YMRS total scores have emerged herein. As mentioned above, platelets are the main storage of BDNF secreted from all other tissues (Bus et al., 2011) and, when activated, release numerous inflammatory mediators, including BDNF (Thomas and Storey, 2015). Up to 40%-60% of their BDNF total content could be released when platelets are activated (Fujimura et al., 2002). However, the PLT-BDNF levels seems to be more stable than PPP-BDNF, maybe because platelets either have a non-releasable pool of BDNF, or MK endogenously produce BDNF (Chacòn-Fernandez et al, 2016), and the released BDNF is sequestered by binding to a transporter or receptor on the platelet surface (Fujimura et al., 2002). It may be argued that there could be a depletion of the platelet content of BDNF, as a possible compensation mechanism in response to the reduction of the circulating PPP-BDNF in the active phases of mood disorders (Yoshimura et al., 2006; Cunha et al., 2006, de Oliveira et al., 2009, Monteleone et al., 2008; Machado-Vieira et al., 2007; Piccinni et al., 2015; Wang et al., 2016). Further, this may not be observed in PTSD probably because depression is a more chronic pathology that causes long-term

alterations of the neuro-metabolic pathways (Brown et al., 2021). However, no significant correlation was found between either clinical scores reported for the current depressive or post-traumatic symptoms and PPP-BDNF. Only a significant negative correlation was observed between PPP-BDNF levels and the *Re-experiencing* domain of the lifetime TALS-SR scale. These findings further suggest that PPP-BDNF may rather fluctuate, as aforementioned, in respect to specific neuroinflammatory responses and biochemical pathways not yet fully elucidated, as well as on peculiar clinical pictures.

4.5 Discriminant and multivariate analyses

Results

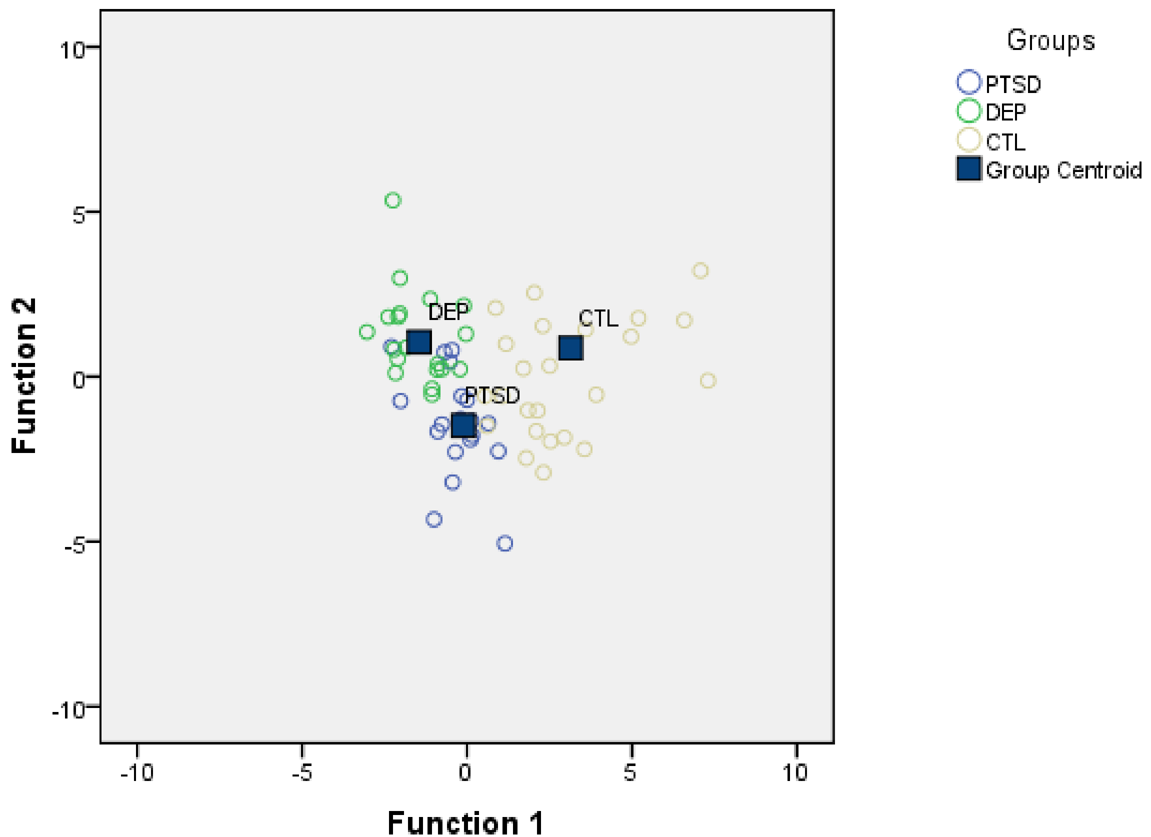
In order to evaluate which biochemical variables best differentiated each group, we performed a discriminant analysis. The analysis included the biochemical variables that presented statistically significant differences in the comparisons among groups, excluding variables more correlated to each other. The discriminant analysis revealed two discriminant functions. The first explained the 65.7% of the variance, canonical R^2=0.76 whereas the second explained the 34.3%, canonical R^2=0.62. In combination these discriminant functions significantly differentiated the groups ($p<0.001$). Structure matrix revealed that 5-HT/TRP ratio (r=0.55), Total thiols (r=0.36), SOD (r=0.32) and 6-OH-Melatonin-S (r=0.27) fell in the first function, while CAT (r=0.49), cortisol (r=-0.46), KYN/TRP ratio (r=0.39), QUIN/TRP ratio (r=0.35), PLT-BDFN (r=-0.21) and IL6 (r=-0.18) were loaded onto the second function. The discriminant function plot showed that the first function discriminated prevalently the CTL group from the other two groups (BD patients). Particularly, higher levels of 5-HT/TRP ratio, Total thiols, SOD and 6-OH-Melatonin-S characterized the CTL group with respect to the others. Further, the second function mainly differentiated PTSD and DEP groups, with the former characterized by higher cortisol, PLT-BDNF and IL-6 concentrations besides lower CAT, KYN/TRP and QUIN/TRP ratios than the latter.

Details are reported in the **Table 5** and **Figure 2**.

Table 5: Discriminant Analysis

Eigenvalues				
Function	**Eigenvalue**	**% of variance**	**Cumulative %**	**Canonical correlation**
1	3.083	65.7	65.7	.869
2	1.609	34.3	100	.785
Wilks' Lambda				
Test of Functions	**Wilks' Lambda**	χ^2	**df**	**p**
1 through 2	.094	65.057	20	<.001
2	.383	26.370	9	.002

Figure 2: Discriminant function plot



Finally, a bivariate logistic regression analysis was performed within BD patients to evaluate which biochemical parameters were most statistically predictive of being in the PTSD group with respect to the DEP one. We included as independent variables 5-HT and cortisol levels, since they were the biochemical parameters for which significant differences were found in the comparison among these two groups and that had not correlated each other. Results showed that both higher levels of 5-HT and cortisol were statistically predictive of being in the PTSD group with respect to the DEP one, with a stronger effect of 5-HT (Odds ratio=2.86) than cortisol (Odd ratio=1.08).

Details are reported in **Table 6**.

Table 6: Bivariate logistic regression

	B (S.E.)	p	Odds Ratio	C.I.
5-HT	1.05 (0.37)	0.004	2.86	1.39 - 5.88
Cortisol	0.07 (0.03)	0.026	1.08	1.01 – 1.15
K	-5.74 (1.96)	0.003	0.00	-

R^2(Cox/Smell)=.501; R^2(Nagelkerke)=.668; Overall correct classification: 85.7%

Discussion

Discriminant and logistic regression analyses contributed to identify a subset of biochemical variables more associated to both groups of BD patients with respect to CTL one, besides specific biological variables resulted more associated to the PTSD group than the DEP one. A reduction of 5-HT/TRP ratio, Total thiols, SOD and 6-OH-Melatonin-S levels seems to be a common factor in both PTSD and DEP groups, discriminating BD patients from healthy controls. Conversely, CAT, cortisol, the KYN/TRP rand the QUIN/TRP ratios, PLT-BDFN and IL-6 rather seem to discriminate the PTSD and the DEP groups, with the former characterized by higher levels of cortisol, PLT-BDNF and IL-6, while the latter by lower levels of CAT, KYN/TRP and QUIN/TRP ratios. Specifically, within both BD patients' groups, 5-HT and cortisol levels rather seem to predict the risk to be affected by PTSD instead of major depressive episode, with an Odds Ratio of 2.86 and 1.08 respectively.

The reduction of 5-HT and the induction of KYN pathway (with an increase of KYN/TRP and QUIN/TRP ratios) might be more pronounced in major depressive episode than in PTSD, probably because depression is a more chronic pathology that causes long-term alterations of the metabolic pathways (Brown et al., 2021). As previously reported, our data showed lowest levels of 5-HT in DEP group, in line with the classical link between the onset of depression and the reduction of 5-HT transmission in several brain areas, deputy to the control of mood tonus, emotion, psychomotor abilities, sleep, appetite and a variety of other stress-related functions (Charnay and Leger, 2010). The depletion of 5-HT might also determine the reduction of melatonin, since 5-HT is the precursor of this methoxy-indoleamine, which is fundamental in regulating circadian rhythmicity and sleep,

being found often reduced in depression (Nutt et al., 2008; Nierenberg et al., 2010; Paunio et al., 2015). However, also PTSD patients frequently report severe hyperarousal symptoms, including sleep problems, and the dysregulation in the production of melatonin could play a crucial role in these conditions (APA, 2022; Carmassi et al., 2020c).

Further, trauma and stress induce HPA axis and the release of cortisol. This mechanism characterized PTSD, at least in the first phase of the traumatic response. The long-term dysregulation of HPA axis may contribute to a chronic low-grade inflammatory state, with a reduced glucocorticoid signalling (Morris et al., 2016; Speer et al., 2018). As previously discussed, dysregulated HPA function frequently led to a lower circulating cortisol level in long-term traumatized subjects when compared to controls (Meewisse et al., 2007, Miller et al., 2007, Morris et al., 2012; Klaassens et al., 2012). However, our sample mainly included subjects with a trauma occurred in the last year (60%), a feature that, together or alternatively to the glucocorticoid receptor resistance or loss of sensitivity to cortisol (Morris et al., 2012, Yehuda, 2002), could explain how cortisol levels resulted higher in the here-recruited subjects with PTSD.

Finally, BD patients, both in the PTSD and in the DEP groups, showed lower levels of SOD, CAT (only PTSD group), total thiols, besides higher IL-6 concentrations than CTL subjects. As previously discussed, BD, depression and PTSD have been hypothesised to be characterized by an hyperinflammatory reaction and mild chronic inflammation, related to an increase in proinflammatory cytokines including (Tursic et al., 2014; Passos et al., 2015; Black and Miller, 2015; Koler et al., 2017). Peripheral pro-inflammatory cytokines could also produce dysregulation in the CNS neurotransmission, reduce neurotrophin release (as BDNF) and impair neurogenesis processes (Banks et al., 2002 Kuhn and Gallinat, 2013; Monje et al., 2003). Particularly, this acute inflammatory and stress-oxidative condition looked to be more marked to PTSD, also due to the impaired reaction of HPA axis to perceived stress (Morris et al., 2016; Bruenig et al., 2017; Lindqvist et al., 2017; Speer et al., 2018).

These finding highlight the key role of a chronic low-grade inflammatory state in BD patients, with specific state biomarkers of mental disorder related to the cross-sectional symptomatology (PTSD and major depressive episode). This suggest the role of peculiar neurobiological pathway in PTSD and depression, that is also empirically confirmed by the fact that existing pharmacotherapies of PTSD, based principally on SSRI drugs, are ineffective for many individuals, with an estimated 40–60% of patients not responding adequately (Bradley et al. 2005; Brady et al. 2000; Steenkamp et al. 2015). In addition, patients with non-responsive PTSD often experience problematic side effects and typically require long-term medication to maintain the therapeutic efficacy (Lee et al. 2016). In conclusion, the sizable proportion of cases of PTSD are persistent (Koenen et al. 2017) and the paucity of currently available treatments make the acknowledge for specific PTSD neurobiological biomarkers a research priority in order to develop tailored treatments.

5. Limitations and conclusion

When discussing the present results several limitations should be considered. Firstly, it was conducted in a small sample, thus limiting the impact and the extensibility of our results. Moreover, significant differences in BMI were reported among groups, and this may have led to biases also with respect to the possible changes in biochemical parameters related to these variables. In addition, several subjects, in particular within the DEP group, were affected by other disorders in comorbidity and this may also have impacted results. Therefore, we consider advisable that future studies should also include euthymic BD subjects without comorbidities. Further, PTSD and DEP groups included subjects who were under psychopharmacological treatment, that often included the same drug classes. This is, however, a common condition for studies in this area; as well, it should be pointed out that, to reduce possible interferences due to this bias, we included subjects that had a treatment stable since about a month. To assess differences depending on the presence of specific treatments, further analyses are necessary. Another limitation that should be considered is the lack of information on the nutritional state of the subjects, which may also have influenced our data. Furthermore, we used, both clinician's administered interview (SCID-5, HAM-D, YMRS), as well as self-reported psychometric instruments (IES-R, MOODS-SR, TALS-SR, WSAS). While we employed instruments considered reliable in the literature, this specific feature should be carefully considered, as subjects might have over/underestimated their own symptoms, leading to biases in the results. Lastly, the cross-sectional design of the study prevented us from hypothesizing possible temporal or causative relationships between the analysed variables.

Despite these limitations, to the best of our knowledge, this is the first study investigating and comparing a variety of possible peripheral biomarkers of PTSD and major depressive episode in patients with BD versus healthy controls. Our results seem to suggest the key role of a chronic low-grade inflammatory state in BD patients, both PTSD and depression, probably related to a

dysregulation in HPA axis and release of cortisol, with an increase in proinflammatory cytokines including IL-6 (Speer et al., 2018; Yang and Jang, 2020; Palego et al., 2021, Dell'Oste et al., 2023). This seems to be predominant in subjects with PTSD, characterized by a higher oxidative distress as measured by the decrease in circulating CAT, SOD and total thiols. Particularly, our data showed as CAT reduction seems to be more specifically related to acute symptoms of PTSD, suggesting a fundamental role of oxygen radical-mediated damage (Passos et al., 2015; Karanikas et al., 2021) in this disorder. Interestingly, this is the also the first survey reporting reduced total thiols in PTSD patients, thus remarking that an increased oxidative stress characterizes this psychopathological condition. Therefore, cortisol and antioxidant enzyme mechanisms seem to play a fundamental role in the response to stress and psychic trauma, at least in the initial stages of PTSD, leading to the involvement of immune system and neuroinflammation. Conversely, 5-HT and TRP metabolic pathway, including both KYN shunt and melatonin production, seem to be more markedly altered in depression. Our data support the hypothesis that, mostly in depression, the depletion of 5-HT could derive from the upregulation of the indoleamine 2,3-dioxygenase (IDO), the crucial enzyme in the kynurenine shunt, a main pathway of TRP degradation, resulting in the decrease of TRP concentration and the increase of several metabolites, including KYN and QUIN (Savino et al. 2020, Brown et al., 2021). This was also demonstrated by the higher levels of QUIN, KYN/TRP ratio and QUIN/TRP ratio in the DEP group. PTSD subjects also exhibited lower 5-HT levels than CLT group, though 5-HT concentrations resulted significantly higher in PTSD group with respect to DEP one. This finding also confirms the role of a 5-HT dysregulation in PTSD, as this neurotransmitter plays a key modulatory role in the regulation of fear and stress (Sari, 2004; Krystal and Neumeister, 2009), and also supports that the kynurenine shunt can be inducted by stress and proinflammatory mediators, that have been reported to be increased in PTSD (Yang and Jiang, 2020; Passos et al., 2015). However, 5-HT depletion seems to have a main role in major depressive episode than in PTSD, as also suggested both by the decrease of its metabolic derivate melatonin and by the incomplete response to serotonergic treatments that characterized more frequently PTSD than depression

(Hoskins et al., 2015). Finally, peripheral chronic pro-inflammatory status could also reflect a CNS low-grade neuroinflammation, that lead to reduced neurotrophins release (as BDNF) and neurogenesis processes (Banks et al., 2002; Kuhn and Gallinat, 2013; Monje et al., 2003). Correlations between psychometric scales and biochemical parameters also confirmed these different patterns of association, particularly among acute and lifetime symptoms.

In conclusion, our finding highlights the key role of a chronic low-grade inflammatory state in BD patients, with specific state biomarkers of mental disorder related to the cross-sectional symptomatology (PTSD and major depressive episode), suggesting the role of peculiar neurobiological pathway in PTSD and depression. Further research investigating biochemical correlates in wider samples with a longitudinal design should proceed in the framework of the possible presence of intertwined relationships between different systems and metabolic routes, as well between central and peripheral systems in shaping different psychopathological trajectories between mood and trauma-related disorders. This is fundamental to allow the development of new specific tailored psychopharmacological treatments for PTSD.

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