DOTTORATO DI RICERCA IN
SCIENZE APPLICATE A BENESSERE E SOSTENIBILITÁ

CICLO XXXVII

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BORDERLINE PERSONALITY DISORDER: MOLECULAR SIGNATURES LINKED TO PATHOLOGY AND PSYCHOTHERAPY EFFECTS

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ABSTRACT

Background: Borderline Personality Disorder (BPD) is a severe mental disorder characterized by emotional dysregulation, impulsivity, and difficulties in interpersonal relationships. Despite its severity and prevalence, the underlying biological mechanisms that drive BPD pathophysiology remain poorly understood. Moreover, although it is known that psychotherapy is effective in symptom management, the biological effects of this type of treatment remain largely unexplored. This study builds upon the CLIMAMITHE project, a randomized clinical trial whose primary aim was to compare two psychotherapeutical approaches for BPD: Metacognitive Interpersonal Therapy (MIT) and Structured Clinical Management (SCM).

Methods: 50 BPD patients and 26 non affected controls were recruited and clinical assessments were conducted to evaluate core symptoms such as emotional regulation, metacognition, and symptom severity. Patients were randomly assigned to MIT or SCM, both covering a period of 1 year. Blood samples were collected at the baseline, 6 months, and 12 months for patients, and at the baseline for controls. We then quantified molecular markers associated with key features of BPD, including: Oxytocin (OXT), β-endorphin, Brain-derived Neurotrophic Factor (BDNF), and microRNAs (miRNAs). Neuroimaging data were also available for the patient group.

Results: Lower baseline OXT levels were found in BPD patients compared to controls. OXT levels increased during psychotherapy: this increment was correlated with clinical improvements in emotional regulation and BPD symptomatology. Also, OXT increase following psychotherapy negatively correlated with the decrease in the volume of the left anterior-inferior area of the hypothalamus. β-endorphin levels did not differ between patients and controls, nor any significant changes during psychotherapy were observed. Although non-significant, the T0-T12 increases of β-endorphin levels were positively correlated with the increase of the volume of the CA3 region of the hippocampus. BDNF levels were significantly higher in the group of patients, but psychotherapy was not able to modulate them. We also found a positive correlation between BDNF levels at T0 and the fractional anisotropy of the Ventral Default Mode Network and the Left Executive Control Network. Finally, eight miRNAs

were differentially expressed between BPD patients and controls, and KEGG pathway analysis showed their involvement in pathways related to neuroplasticity and stress response.

Conclusion: This study explored the role of molecular markers potentially involved in BPD, investigating their relationship with the pathology and the psychotherapies effect, highlighting alterations between patients and controls and, regarding OXT, also modulations induced by psychotherapies. Overall, these findings contribute to gain insight into the biological underpinnings of BPD, also providing potential novel markers that may be useful for the improvement of the differential diagnosis and the optimization of the therapeutic treatments for this disease.

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INTRODUCTION

Borderline Personality Disorder: Clinical and Pathophysiological Aspects

Borderline Personality Disorder (BPD) is a severe and complex mental disorder which affects about 1–2% of the general population, up to 12% of psychiatric outpatients and up to 22% of inpatients, with a higher prevalence in women than in men (Ellison et al., 2018). The onset of BPD often occurs around adolescence, and its course to adulthood is characterized by a symptomatic switch from predominant symptoms of affective instability and impulsivity to maladaptive interpersonal functioning and enduring functional impairments, with their expression depending on environmental and developmental variables. Symptoms then tend to decay and fade up to old age (Videler et al., 2019), although patients may experience relapses over time and, if not directed soon to therapy, may have a poor long-term functional status. As defined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V), BPD affects multiple domains, with clinical features that reflect: emotional dysregulation; intense unstable relationships, which include fear of abandonment and tumultuous close relationships; cognitive dysfunction, chronic depersonalization, paranoid trends, transient delusions and hallucinations; impulsivity, substance abuse, self-mutilation, and suicide attempts (Lieb et al., 2004; Paris, 2009). In relation to this last aspect, BPD is characterized by a high mortality rate due to suicide: up to 10% of patients commit suicide, a rate almost 50 times higher than in the general population (Paris, 2019). Moreover, patients with BPD often manifest comorbidities with other psychological disorders, such as Major Depressive Disorder, Post-traumatic Stress Disorder, Anxiety Disorders, and Eating Disorders (Lieb et al., 2004). BPD causes significant economic challenges, accounting for higher healthcare costs than both Major Depressive Disorder and other personality disorders (Bender et al., 2001; Stepp & Lazarus, 2017). The contributing factors to these elevated medical expenses are the great number of hospitalizations, frequent emergency room visits, and the great use of outpatient services (Bender et al., 2001).

Given the severity and the strong societal impact of the disorder, intensive research on its pathophysiological mechanisms has been conducted in last years. While there is no single cause of BPD, a complex interplay between genetic, neurobiological and environmental factors may contribute to its development, each factor interacting with the others, as discussed below.

Environmental Factors

A wide range of environmental factors may increase the risk of developing BPD, including adverse childhood experiences: exposure to maltreatment, such as neglect and abuse, are reported by 30% to 90% of BPD patients. More than any other personality disorder, BPD diagnosis is linked to child abuse and neglect (Battle et al., 2004; Yen et al., 2002). Traumatic childhood events, alongside with genetic factors, may lead to emotional dysregulation and impulsivity, which may be reinforced by consequent dysfunctional behaviors and psychosocial conflicts (Skodol et al., 2002). Among all traumas, childhood sexual abuse is the most common, severe and most associated with receiving a BPD diagnosis when adult - it is reported by 40-71% of inpatients with BPD (Dadomo et al., 2022; de Aquino Ferreira et al., 2018). However, the rates of abuse, particularly those for physical and sexual abuse, probably are underestimated because usually only repetitious abuse by parents or other full-time caregivers are reported. Moreover, underreporting of such events may result from a dissociation from that type of memory (Zanarini et al., 1989). Childhood sexual abuse plays a major role in BPD, particularly in women, predicting more severe clinical presentation and poorer prognosis(de Aquino Ferreira et al., 2018; Lieb et al., 2004). Other types of traumas, although not involving physical violence, can be equally severe and damaging. For example, emotional neglect, intended as the caregivers' failure to provide emotional support, affection, and validation, can influence the severity of BPD symptoms and lead to maladaptive attachment styles, fear of abandonment and difficulties in self-regulation and forming stable relationships (J. Lee & Choi, 2023). Emotional abuse, characterized by degrading and manipulative behaviors, has similar neurobiological effects as other forms of maltreatment and is associated with heightened risks of mental health issues, including BPD (Hoffmann & Heim, 2024). In BPD, emotional abuse manifest as unstable moods, difficulty in managing anger and higher sensitivity to rejection. Higher scores of childhood emotional abuse were found to be correlated with increased difficulties in emotion regulation, which can predict higher BPD traits (Kanj et al., 2023). Taken altogether, traumatic childhood experiences have multiple effects on patients' behavior and may predict BPD symptoms like affective and interpersonal dysfunctionalities (Dadomo et al., 2022).

Genetic Factors

The genetic architecture of BPD is likely influenced by a complex interplay of multiple genes and their interactions with environmental factors which contribute to disorder development and symptomatology, complicating the identification of specific genetic determinants (Livesley, 2008). The genetic studies on BPD are limited, primarily focusing on candidate genes and involving small samples. Lubke et al. (Lubke et al., 2014) conducted a GWAS on personality features associated with BPD using data from a total of 8'135 participants. They found seven SNPs in SERINC5, a gene involved in myelination, which role has been suggested in the development of psychiatric disorders characterized by lack of social interaction (Toritsuka et al., 2015). Notably, the strongest effects of these SNPs were observed for affect instability, one of the main features of BPD. To date, only three genome-wide association study (GWAS) were conducted on large samples of BPD patients. Witt and colleagues (Witt et al., 2017) found two genes significantly associated with BPD: Dihydropyrimidine Dehydrogenase (DPYD), an enzyme involved in the catabolism of uracil and thymidine, and Plakophilin-4 (PKP4), which plays a role in cell adhesion, organization of the cytoskeleton and regulation of cell junctions. Previous studies have already linked DPYD to Schizophrenia and Bipolar Disorder, and its connection to pyrimidine metabolism and neuronal signaling suggests it may play a role in neurodevelopmental processes relevant to psychiatric disorders, including BPD. PKP4 has also already been associated with Schizophrenia and Bipolar Disorder, and the dysregulation of adhesion and cytoskeletal organization in neurodevelopment and synaptic functioning, which often occurs in psychiatric disorders, suggesting its key role in the development of these disorders. Moreover, dysfunctions in cell adhesion mechanisms could affect brain connectivity and neural circuitry, contributing to the emotional instability and impulsivity seen in BPD. The third GWAS (Streit et al., 2023) identified two independent loci within FOXP2, a gene that is highly expressed in the brain and that plays a crucial role in proper brain development and synaptic plasticity, suggesting its potential role in neurobiological processes related to BPD. Another interesting gene that might be implicated in BPD is FKBP5, which mediates stress response by negatively regulating the hypothalamic-pituitary-adrenal (HPA) axis, crucial for stress response (Malekpour et al., 2023). Research has shown that FKBP5 variants are significantly associated with BPD, particularly in the context of childhood trauma, which can exacerbate disorder symptoms (Amad et al., 2019).

Neurobiology of BPD: Findings from Neuroimaging Studies

Alterations in brain structures and functioning are essential to contribute to the pathophysiology and, potentially, to the treatment of this disorder. Although the neural correlates of BPD clinical features are mostly unclear, a large portion of the literature concerning neuroimaging studies conducted both in adults and adolescents shows that several brain regions are potentially involved in BPD, such as the amygdala, insula, cingulate cortex, hippocampus, anterior cingulate cortex, and prefrontal regulatory regions (orbital frontal cortex, dorsal lateral prefrontal cortex, and ventral lateral prefrontal cortex) (Katherine S. Pier, 2016).

Several research findings have centered the amygdala as a core region for the neurobiology of BPD, because of its role in the generation of emotional experience. The amygdala is involved in rapid processing of stimuli, more specifically of the relevance of a stimulus with respect to the individual's ongoing motivational state. Thus, amygdala activation may be part of an affective response, but the outcomes of those responses may be vastly different depending on the context and the person itself (Cunningham & Brosch, 2012). This is consistent with the fact that functional Magnetic Resonance Imaging (fMRI) studies on emotion processing in BPD lead to discrepant findings: amygdala appears to activate more among patients with BPD than non psychiatric controls in the majority of brain-imaging studies of emotional materials (Ruocco & Carcone, 2016; Schulze et al., 2016), although there are also some conflicting results (Ruocco et al., 2013). A possible explanation for this variability could be represented by the fact that fMRI analyses compare brain activity in response to negative, positive or neutral stimuli, and that BPD patients can perceive as arousing stimuli that non psychiatric individuals interpret instead as neutral or ambiguous (faces, interactions, images...) (Daros et al., 2014; Ruocco et al., 2013). The relevance of ambiguous stimuli could fluctuate widely in individuals with BPD according to their mood and social contexts, possibly resulting in variable results regarding the activation of the amygdala, depending on relevance attributed to the emotional stimuli (Ruocco & Carcone, 2016).

Also the hippocampus has been implicated in BPD; it plays a key role in memory consolidation, emotional regulation and stress response. Dysfunctional activity of the hippocampus during memory encoding tasks has been observed in BPD subjects, who have a significantly weaker

hippocampal activation, leading to an impaired memory performance (Carcone et al., 2020). In particular, the hippocampus helps to process and retrieve traumatic memories, and dysfunctions in this area may result in intrusive thoughts, flashbacks, and difficulty in separating past trauma from the present, contributing to emotional instability and cognitive difficulties. Several neuroimaging studies showed that individuals with BPD exhibit volumetric abnormalities in the hippocampal volumes, which may contribute to their symptoms and cognitive deficits. O'Neill and colleagues observed significant reductions in the left hippocampal head, body, and tail, as well as in the right hippocampal tail in BPD patients (O'Neill et al., 2013), which were associated with stress-related mechanisms and potentially had an impact on emotional regulation and memory processes. Similarly, Luyten and colleagues observed that individuals with BPD exhibit reduced hippocampal volume, which correlated with emotional dysregulation (Luyten et al., 2023).

The cingulate cortex, particularly the anterior cingulate cortex (ACC), plays a significant role in emotional regulation, cognition, and also influences fear and pain processing. It integrates information from various brain regions, contributing to behavioral and emotional responses (Falconi-Sobrinho et al., 2024; Mahgoub et al., 2024). Individuals with BPD exhibit altered functional connectivity in ACC, together with structural changes that are associated with emotional dysregulation and impulsivity. Functional connectivity patterns involving the ACC are related with BPD features, particularly in regions related to emotion regulation and executive functions (Shafiei et al., 2024). BPD patients also show both hypometabolism and altered structural volumes of this region, indicating potential dysfunctions in emotional processing and regulation (K. Huang, 2024). Moreover, the ACC is also implicated in stress response, and studies conducted in adolescents showed an implication of ACC in self-injury, suggesting a link between ACC activity and BPD symptoms (Höper et al., 2024).

The prefrontal cortex (PFC) is essential for various cognitive functions: executive functions, which include organization and behavioral control (Zhou et al., 2024); decision-making, particularly in social contexts, as it contributes to evaluate the consequences of actions and to helps individuals making choices aligned with social norms, a vital capability for fostering social interactions and avoiding conflicts (Rolls et al., 2023); emotional responses, which involves managing responses during social interactions and processing emotional information, leading

individuals to controlled reactions, effective communication and relationship management (S. H. Lee & Williams, 2024). In a pathological context, PFC is linked to emotional and cognitive dysregulation observed in BPD. Individuals with BPD exhibit altered PFC activity, particularly under stress, which correlates with symptoms such as impulsivity and emotional instability. In particular, BPD patients show an under-activation in PFC, associated with difficulties in regulating emotions and behaviors, leading to significant interpersonal and functional challenges(Luyten et al., 2023). The involvement of PFC in emotion regulation is further highlighted by findings of reduced theta oscillatory activity (neural brain oscillations) in BPD patients during cognitive reappraisal tasks, indicating impaired emotional control mechanisms (Haaf et al., 2024). Furthermore, the heightened impulsivity typical of BPD is also linked to PFC dysfunction. This impairment contributes to behaviors such as self-harm and substance abuse (Calancie et al., 2024). PFC also works in conjunction with other brain regions, such as the amygdala, which is also involved in emotional regulation. While in BPD patients the amygdala may show an over-activation, associated with emotional dysregulation, the PFC remains poorly activated, failing to adequately downregulate these heightened responses.

Treatment Approaches for Borderline Personality Disorder

First-line treatments for BPD are psychotherapies, which enclose a broad range of treatments. Indeed, many psychotherapeutical strategies have been put into practice for BPD over the past 20 years and their efficacy in lowering symptoms and behavioral dysfunctions appears to be well-supported (Magni et al., 2019). However, how psychological therapies produce their improvement is still not fully understood.

Among the treatments, the most commonly used are: Cognitive Behavioral Therapy (CBT); Dialectical Behavior Therapy (DBT); Metacognitive Interpersonal Therapy (MIT) and Structured Clinical Management (SCM). Other approaches include also: Transference-focused Therapy (TFP); Mentalization-based Treatment (MBT); Schema-focused Therapy (SFT); Acceptance and Commitment Therapy (ACT) (Crotty et al., 2024). All these approaches have different goals and targets and are tailored with different mechanisms of action, according to their underlying specific etiology models, although the common element is the aim to ameliorate BPD pathology using verbal communication (Jm et al., 2013).

Cognitive Behavioral Therapy

Cognitive-behavioral therapy (CBT) is a pivotal psychotherapeutic intervention for BPD characterized by clear and precise treatment protocols and the largest base of empirical evidence; for this reasons CBT is used for a wide range of mental disorders (Sójta et al., n.d.). This therapy includes psychoeducation and focuses on emotional regulation and reducing maladaptive behaviors and core beliefs, effectively addressing the complex symptoms of BPD, including self-harm and emotional instability, and aiming to develop new, more adaptive beliefs about the self and others, and strategies of behavior (Stoffers-Winterling et al., 2022). In the course of the therapeutical pathway, the psychologist and the patient collaborate to fully understand the problem and to develop an effective treatment strategy. Moreover, CBT aims to lead the patients to be their own therapists, not only through the exercises carried out during the sessions, but also assigning outside "homework" in order to help developing coping skills, to help change their own thinking and problematic behaviors (*APA PsycNet*, n.d.). CBT can lead to sustained improvements in emotional and relational functioning over time, developing skills to manage intense emotions, reducing impulsive behaviors such as self-harm (Selvapandiyan, 2023; Sójta et al., n.d.). However, CBT may not address all aspects of BPD, particularly interpersonal issues, suggesting a need for integrated approaches like Dialectical Behavioral Therapy (DBT) for comprehensive care (L. Huang, 2022).

Dialectical Behavior Therapy

Dialectical behavior therapy (DBT) is a structured outpatient treatment based on cognitive-behavioral principles developed in the early 1990s for the treatment of parasuicidal behavior (defined as intentional self-injurious behavior, including suicide attempts and self-harming behaviors, with or without suicidal intent (Linehan et al., 1991) in women with BPD. To this day, DBT is the leading evidence-based psychotherapy for BPD. DBT aims to address the behavioral symptoms of BPD by replacing maladaptive behaviors with healthier coping skills, such as mindfulness, interpersonal effectiveness, emotion regulation, and distress tolerance (May et al., 2016). DBT efficacy has been proven in several clinical trial, which overall show the efficacy of DBT in numerous areas, including the reduction of self-harm episodes (Barnicot & Crawford, 2019; Chen et al., 2021) and the improvement of emotional regulation (Barnicot & Crawford, 2019). Moreover, DBT has a low overall dropout rate (Kliem et al., 2010). Regarding

the neurophysiological changes following DBT treatment, only a few studies have been conducted. Altogether, evidence shows that DBT causes a deactivation in the activity of amygdala and anterior cingulate cortex, as well as of the inferior frontal gyrus in response to arousing stimuli and increased activity in response to inhibitory control (Iskric & Barkley-Levenson, 2021).

Metacognitive Interpersonal Therapy

Metacognitive interpersonal therapy (MIT) is a cognitive behavior-based psychotherapeutic approach that has mostly been investigated in non-borderline personality disorders (Fioravanti et al., 2023). MIT aims to increase metacognitive abilities in order to improve general personality functioning and promote better interpersonal relationships. More specifically, MIT helps patients to recognize and integrate different mental states and to improve their ability to solve interpersonal problems using mentalistic knowledge of themselves and others (Magni et al., 2019; Semerari et al., 2003). To date, only two studies have assessed the clinical efficacy and the neurobiological correlates of MIT in BPD (Quattrini et al., 2022; Rossi et al., 2023). These studies will be diffusively discussed later. However, MIT is designed for personality pathology rather than for a specific disorder and it aims to promote metacognitive abilities and improve interpersonal relationships (Rossi et al., 2023) and is therefore in line with the hypothesis that consider relevant an assessment and a treatment of personality pathology, rather than a categorical approach (*APA PsycNet*, n.d.). Moreover, MIT approach is also in line with the importance of the capacity to: self-reflect, thus promoting a stable sense of self and self-directivity; and understand others' minds in order to establish and maintain empathetic and good relationships (*APA PsycNet*, n.d.). MIT therefore may represent a promising approach for BPD. The first results about the effects of MIT conducted in BPD patients on neuroimaging features (Rossi et al., 2023) will be described in one of the following paragraphs ("*The Climamithe Study*").

Structured Clinical Management

Structured Clinical Management (SCM) was developed in the late 00s using the current NICE (National Institute for Health and Care Excellence) guidelines for the management of the BPD (*Overview | Borderline Personality Disorder: Recognition and Management | Guidance | NICE*, n.d.). It was originally intended as a comparator of general psychiatric treatment against which

other specialized protocols could be tested in research studies. SCM therefore originally was not strictly a type of psychotherapy but has evolved into a distinct set of organizational psychotherapy principles (Edmonds, 2020). SCM aims to collaboratively set clear and focused goals based on a shared therapeutic hierarchy, and then to use problem-solving practices to work towards these goals, which are prioritized on the basis of their importance and urgency. These goals are generally divided into four key areas of focus: interpersonal functioning; impulsivity; emotional dysregulation; cognitive distortions. SCM reflects the "best general psychiatric treatment for BPD" and is intended to be used by practitioners and mental health clinicians with generalist expertise and skill-sets, and minimal additional training (Bateman & Fonagy, 2009). SCM has been proven to be effective for BPD in several trials, particularly within the initial 6 months of treatment (Bateman & Fonagy, 2009; Graham et al., 2019; Rossi et al., 2023), and can be faster at reducing self-harming behavior compared to other treatments such as MBT (Bateman & Fonagy, 2009).

Pharmacological Treatments

Although psychotherapy is the primary treatment for BPD, the large majority of people with BPD are prescribed psychotropic medications during the course of their illness. Pharmacological treatment may be necessary especially in case of acute crisis, when patients manifest suicidal behavior, impulsive outbreaks or any kind of aggravations (Borschmann et al., 2012). Indeed, it's common that psychotropic drugs are used as a long-term medication, even though only as an adjunctive to psychotherapy (Stoffers-Winterling et al., 2022). Antidepressants are the most frequent class of psychotropic medications used in BPD, but there is no standard medication treatment. Currently, all medications for BPD are used off-label (if not prescribed targeting the related psychopathology, such as depression or anxiety, for which there is evidence for use); however, the majority of BPD patients without co-occurring disorders still take medication to treat BPD symptoms (Stoffers-Winterling et al., 2022).

Molecular Correlates of Borderline Personality Disorder

Several studies have identified different biological systems and mechanisms that could be involved and might play a critical role in BPD. The dysregulation of these systems could manifest through specific molecular signatures observable in peripheral matrices such as blood derivatives. Besides providing insight into the underlying biological processes of the disorder, these markers could potentially offer the opportunity to improve the diagnostic accuracy, to predict and monitor disease progression, as well as response to therapy.

Oxytocin System

As previously said, emotional and interpersonal dysfunction is one of the main core components of BPD and is related to a wide range of altered basic functions such as facial emotion recognition (Daros et al., 2013) and low metacognitive functions (Fonagy & Bateman, 2016), alongside with complex functions such as trust and cooperation (Seres et al., 2009). One of the main biological regulators of social relationships is Oxytocin (OXT), a neuropeptide produced by the hypothalamus and secreted by the posterior pituitary. OXT effects are exerted in the peripheral circulation in response to various stimuli: apart from its well-known role in labor and breastfeeding (Uvnas-Moberg et al., 2020), OXT also regulates several aspects of social relationships and affiliation behavior, such as recognition of facial emotions, empathy, confidence, parent-child interactions, attachment, sexual and romantic response, and cognition (Ferreira & Osório, 2022). In general, it promotes a behaviorally appropriate social response mediated by different brain areas and circuits (Froemke & Young, 2021). Since patients with BPD present significant impairments in terms of behavior, social and emotional skills, and cognitive processing, OXT mechanism of action may be critical for the disorder. Moreover, OXT system could mediate impairments in social cognition linked to adverse early childhood experiences, which are one of the most severe environmental risk factors of BPD (Müller et al., 2019). Dysregulation of OXT in peripheral tissues of BPD patients has been shown by a handful of studies; in particular a recent meta-analysis has evidenced lower peripheral OXT levels in female patient samples (Ferreira & Osório, 2022). BPD patients also seem to show a greater aversive reaction after a paradigm of social exclusion compared to healthy subjects, which correlates with a reduction in OXT plasma levels (Reinhard et al., 2022).

In the last few decades, acute and chronic administration of intranasal OXT have been extensively used in both animal models and human preclinical and clinical studies, with the intent to increase OXT levels in the central nervous system via a direct nose-to-brain route, penetrating directly by the blood–brain barrier, and to modulate the neural mechanisms of social behavior and cognition (Yao & Kendrick, 2022). For example, BPD patients have a bias in facial emotion recognition, as they can identify more easily unpleasant emotions in others, especially rage; at this regard recent studies have shown that the more pronounced and faster initial fixation to the eyes of angry faces observed in women with BPD diminishes after administration of intranasal OXT (Bertsch et al., 2013). Moreover, Domes and colleagues showed that intranasal OXT increases affective empathy and approach motivation in BPD patients and healthy controls compared to placebo (Domes et al., 2019). Nevertheless, there is still no evidence about possible OXT modulation effects induced by psychotherapies in BPD patients and their association with symptom improvement.

Opioid System

The opioid system modulates the response to acute and chronic stressful and noxious stimuli that induce physical, emotional, or social pain. Endogenous opioids include β-endorphin, enkephalin, dynorphin, and nociceptin/orphanin FQ agonists. Opioids act on G protein-coupled receptor subtypes, including: μ-opioid receptors, which respond preferentially to morphine and β-endorphin; k-opioid receptors, which respond preferentially to ketocyclazocine; δ-receptors; and ORL-1 opioid-like receptor binding sites (Stein, 1991). The opioid system has a mediating role in separation distress, relief and pleasure of reunion, self-soothing, and pain of social exclusion and rejection, suggesting its potential contribution to the vulnerability to BPD. In particular, recent studies indicate that the experience of physical pain could be mediated by the same neural pathways involved in emotional pain, which is one of the main features of BPD, and, furthermore, that the opioid system could also mediate emotions of social exclusion, separation, abandonment (Eisenberger et al., 2003; MacDonald & Leary, 2005). Among all, the μ-opioid receptors seem to be relevant in the pathology of BPD because of their function related to social and affective regulation (Stanley & Siever, 2010). μ-opioid receptors are widely distributed throughout the human central nervous system, and

particularly high levels of binding of these receptors are found in the basolateral amygdala, nucleus accumbens, hypothalamus, thalamus, ventral tegmental area, and caudate putamen. A μ-opioid receptor agonist is β-endorphin, which is involved in stress-induced analgesia and thermal pain perception. β-endorphin, indeed, is released in response to stress and has a common precursor (pro-opiomelanocortin, POMC) with adrenocorticotropic hormone (ACTH), the pituitary hormone which mediates stress response (Pilozzi et al., 2021).

Two of the core symptoms of BPD are affective dysregulation and non suicidal self-injuries (NSSI), which appear to be associated with lower pain perception. In particular, BPD patients show lower pain sensitivity (Bohus et al., 2000) and a positive correlation between pain tolerance thresholds, aversive inner tension, and dissociation (Ludäscher et al., 2007). This alteration seems to be linked to an abnormal activation pattern of brain regions associated with the affective-motivational aspects of pain processing (Schmahl et al., 2006). Interestingly, NSSI is often followed by mood enhancement, thus this type of behavior could act as self-healing through restoration of positive and rewarding affect (Simeon et al., 1992). Some theories link these manifestations with low basal opioid levels, which cause a subsequent compensatory super-sensitivity of μ-opioid receptors: in this context, self-injuries could be a result of an increase in opioid levels after such behaviors, leading to further episodes of such behavior, which might act as a maladaptive coping mechanism. Numerous studies have already proven a relationship between altered endogenous opioid levels and self-injurious behavior, that report lower levels of β-endorphin and met-enkephalin in individuals with Cluster B personality disorder who manifest self-injury, compared to those without self-injury (Stanley et al., 2010) as well as altered pain sensitivity during that kind of episodes (Russ et al., 1992). Further evidence for such role of β-endorphin is based on the ability of opioid antagonist treatment to partially ameliorate self-injury, in particular naloxone (Symons et al., 2004) and naltrexone (Sonne et al., 1996), suggesting that they could decrease the rewarding effects of these behaviors by blocking opioid receptors.

Brain-Derived Neurotrophic Factor System

Brain-derived neurotrophic factor (BNDF) is the most abundant neurotrophin in mammals' brain: it is released from neurons both pre- and post- synaptically and can interact with two receptors: the p75 neurotrophin receptor (p75NTR) and the tropomyosin-related kinase receptor B (TrkB). The interaction with one or the other receptor depends on the proteolytical cleavage of BNDF: the uncleaved pro-form of BDNF binds preferentially to p75NTR, while mature cleaved BDNF binds to TrkB. Pro-BDNF mediates apoptosis and long-term depression; mature BDNF stimulates several effects, including neuronal differentiation, outgrowth of neurites, increased cell survival and strengthening of synapses (Mitchelmore & Gede, 2014). BDNF therefore has an important role in nervous system development and neuronal plasticity, and an involvement of BDNF have been observed in many neurological and psychiatric diseases, with different change patterns among different mental disorders (Zou et al., 2024) Furthermore, BDNF has also a role in mediating processes related to learning and memory. Alterations of BDNF expression or release have been linked to memory and cognitive impairment, which may also lead to a higher susceptibility to multiple disorders affecting nervous system functioning, including neuropsychiatric disorders such as Alzheimer's Disease, Parkinson's Disease, Affective Disorders and Schizophrenia (Balaratnasingam & Janca, 2012). Given the key role of BDNF in brain development, early modulations due to environmental effects and early stress may have long-term influence on the neurotrophic gene expression and, consequently, potentially lifelong effects on brain activity and behavior. It has been proposed that brain changes may result from epigenetic modifications due to early life adverse conditions and, since early environmental stressors contribute to the development of BPD, stress-induced changes in BDNF may contribute to increase the susceptibility to the pathology. Indeed, several animal models of early-life adversity have demonstrated the effect of environmental stressors on stable changes in both BDNF gene transcription and protein expression, with consequent behavioral outcomes, suggesting that BDNF expression could be mediated by epigenetic mechanisms, which can be influenced both by acute and chronic stress (Balaratnasingam & Janca, 2012; Boulle et al., 2012). The same epigenetic mechanisms have been observed also in humans and have been correlated to childhood trauma and a subsequent development of psychiatric disorders in adulthood (Boulle et al., 2012). Regarding BPD, the literature offers a complex and sometimes contradictory picture of its role in the

disease. Koenigsberg et al. (2012) observed decreased BDNF levels in platelets of male BPD patients compared to healthy controls, suggesting a biological deficiency in BDNF (Koenigsberg et al., 2012), while another study presented contrasting results, reporting increased BDNF levels in plasma of BPD patients, which decreased after a four-week DBT program (Perroud et al., 2013). In the same research the authors also explored BDNF methylation, showing that while there was no direct association between BDNF protein levels and methylation status, methylation patterns differed between responders and non-responders to psychotherapy: non-responders exhibited increased methylation, which typically suppresses gene expression, while responders showed a decrease in methylation (Perroud et al., 2013). Thomas and colleagues (2018) observed similar results after a 12-week DBT program, that led to a significant decrease in BDNF promoter gene methylation in saliva samples of BPD patients (Thomas et al., 2018). Interestingly, regarding methylation studies, the BDNF Val66Met polymorphism, is known to influence emotional regulation and neuroplasticity: in BPD patients, the Met-carriers exhibit prolonged and heightened amygdala activation in response to repeated exposure to unpleasant emotional stimuli, compared to non-carriers, highlighting the specific role of this polymorphism in modulating emotional responses in BPD patients (Perez-Rodriguez et al., 2017). Interestingly, mixed findings also linked BDNF to the mechanisms of addiction (initiation, maintenance or abstinence/relapse) and drug reward in rodent models. BDNF may be important in drug addiction, which is a frequently occurring issue in BPD, as it provides trophic support to midbrain dopaminergic neurons, which are critically important in mediating drug reward and relapse (Balaratnasingam & Janca, 2012).

microRNAs

microRNAs (miRNAs) are small (20-22 nucleotides) non-coding RNAs that play an important role in the post-transcriptional regulation of gene expression. In most cases, miRNAs interact with the 3’ untranslated region (3’ UTR) of target messenger RNAs (mRNAs) to induce mRNA degradation and/or translational repression. However, miRNAs can also activate translation or regulate transcription: their interaction with target genes is dynamic and varies depending on many factors (such as location of miRNAs, the abundancy of miRNAs and target mRNAs, the

affinity of the interaction). In mammals, miRNAs are predicted to control the activity of at least 50% of all the protein-coding genes (Bartel, 2009).

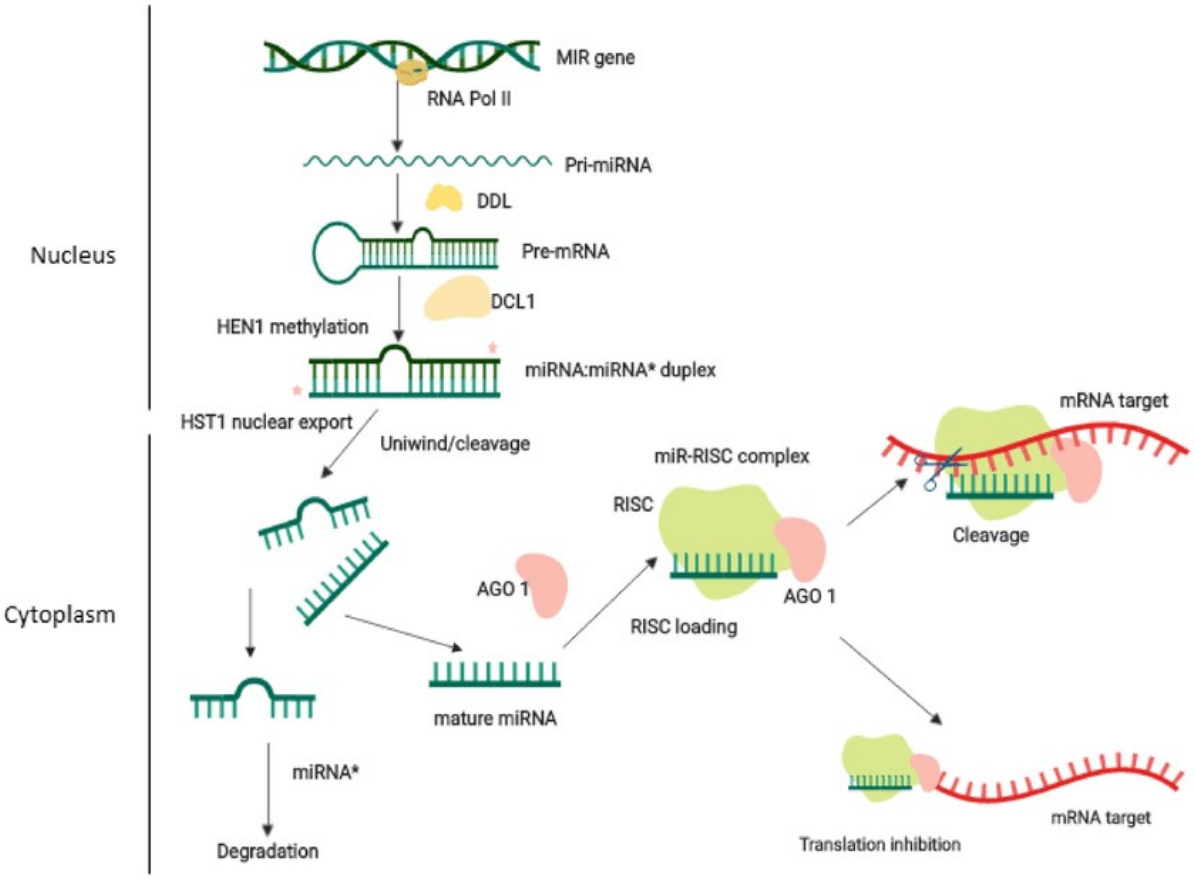


Fig. 1 : miRNA biogenesis and mode of action (Chaudhary et al., 2021)

In the field of epigenetic mechanisms, microRNAs (miRNAs) play crucial roles in regulating gene expression and are implicated in various physiological processes within the central nervous system, including neuronal development, neurogenesis, and synaptic plasticity. These molecules are not only produced within cells but are also released into body fluids such as cerebrospinal fluid, blood and its derived products, including plasma and serum: they can be transported to target cells via vesicles, such as exosomes, or bound to proteins as Argonautes (O'Brien et al., 2018). Moreover, miRNAs are able to cross the blood-brain barrier, allowing them to travel into the bloodstream. This means that circulating peripheral miRNA levels might be indicators of the biological processes occurring within the brain. Modulations in miRNA levels detected in peripheral matrix such as serum and plasma may provide insight into cerebral alterations. Alterations in circulating miRNAs have already been observed in other

psychiatric disorders such as MDD and BD (Clausen et al., 2022; Ding et al., 2023). Very few studies have shown miRNA alterations in BPD, although there is a remarkable connection between miRNAs and the intensity of childhood abuse, suggesting their potential role in the illness pathogenesis (Crowell et al., 2009). Only one research, conducted by Prados and colleagues, showed a specific CpG site, cg04927004, near the gene of miR124-3p, whose diminished methylation was strongly linked with the severity of childhood trauma and BPD symptoms, suggesting a role in the progression from early traumatic experiences to adult BPD (Prados et al., 2015). In line with the lower methylation of miR-124-3p gene, a study also evidenced higher levels of the miRNA in BPD patients (Aloi et al., 2023).

The CLIMAMITHE Study

The CLIMAMITHE study (Clinical Trials.org; NCT02370316) (Magni et al., 2019) is a longitudinal, multicenter, randomized clinical trial carried out at two centers: IRCCS Istituto Centro San Giovanni di Dio, Brescia, Italy (site 1) and Third Center of Cognitive Psychotherapy, Rome, Italy - Scuola Italiana di Cognitivismo Clinico (SICC), Rome (site 2). The primary goal was to compare two psychotherapeutical approaches for BPD, Metacognitive Interpersonal Therapy (MIT) and Structured Clinical Management (SCM), by investigating the clinical and neurobiological changes associated with these treatments.

The study included 78 BPD patients and 30 non affected volunteers, which were randomized to either MIT or SCM group using randomly generated block randomization scheme (Magni et al., 2019). Both treatments have been described above. Both MIT and SCM protocols consisted of weekly individual sessions and group sessions covering one year. Clinical evaluations and data collections included assessments conducted at three timepoints: the baseline and two follow-ups at 6 and 12 months.

The study evaluated two main clinical outcomes:

- The primary outcome was effects on emotion regulation, as measured through the Difficulties in Emotion Regulation Scale (DERS) scores. This scale is a 36-item self-report questionnaire comprising a total score and six dimensions of emotional dysregulation: Nonacceptance of emotional responses, Difficulties engaging in goal-directed behavior,

Impulse control difficulties, Lack of emotional awareness, Limited access to emotion regulation strategies, Lack of emotional clarity.

- The secondary outcomes explored broader psychological effects evaluating changes in several psychological domains: metacognitive abilities using the Zanarini rating scale for BPD symptomatology (ZAN-BPD), Metacognitive Assessment Interview (MAI); general psychopathology with the Symptoms Check-list 90 Revised (SCL-90-R); Depressive symptoms using the Beck Depression Inventory-II (BDI-II); Anger measured through the State-Trait Anger Expression Inventory (STAXI); impulsivity, measured through the Barratt Impulsiveness Scale (BIS); alexithymia (difficulty identifying and expressing emotions) through the Toronto Alexithymia Scale (TAS-20); Interpersonal functioning, assessed by Inventory of Interpersonal Problems (IIP); Childhood traumatic experiences, evaluated by the Childhood Trauma Questionnaire (CTQ); the attachment experience, measured through the Attachment Style Questionnaire (ASQ). Data on demographics, suicide attempts, self-injury and aggression episodes, hospitalizations, and pharmacotherapy was also collected.

A subset of 60 patients and all the controls also underwent functional and structural magnetic resonance imaging (MRI) before and after the year of treatment, in order to assess functional and structural changes induced by psychotherapy in brain regions associated with emotional regulation, such as the amygdala, prefrontal cortex, hippocampus, and cingulate cortex. In particular, structural MRI investigated whether therapy could lead to changes in brain volumes and connectivity, and fMRI measured brain activity in response to emotional stimuli (pleasant, unpleasant, and neutral pictures).

From a clinical point of view, both MIT and SCM showed significant improvements in emotion regulation over the course of the treatment: DERS scores decreased in both groups, with a similar large efficacy in reducing emotional dysregulation.

For secondary outcomes, both interventions positively impacted BPD core symptoms, depressive symptoms, and overall psychological functioning. Patients in both MIT and SCM showed improvements in several dimensions: Depressive symptoms (BDI-II); General psychopathology (SCL-90-R); Alexithymia (TAS-20); Interpersonal functioning (IPP scale); Metacognitive abilities (MAI); Impulsivity (BIS); BPD symptomatology (ZAN-BPD). However,

MIT showed higher effects than SCM on PD criteria, metacognitive abilities and impulsivity, while SCM showed a higher effect than MIT on BPD symptomatology (Rossi et al., 2023).

Concerning neuroimaging features, both disease-related and psychotherapy-driven differences have been observed through functional MRI experiments; in particular, a higher activation of the right amygdala in response to emotional stimuli has been described in BPD patients compared to controls, and both MIT and SCM were able to reduce this over-activation, in line with the clinically reported improved regulation of emotions (Rossi et al., 2023).

Moreover, MRI experiments evaluating structural connectivity showed altered parameters in the triple network system in BPD patients (in particular, an increased mean diffusivity in the anterior salience network, in the dorsal default mode network and in the right executive control network). This network system mediates core emotional and cognitive functions and, interestingly, the observed alterations were more pronounced in patients with higher behavioral dysregulation (Quattrini et al., 2022).

AIM OF THE STUDY

The studies here presented build on the foundations of the CLIMAMITHE project, taking a deeper dive into the biological mechanisms involved in BPD and that may drive the therapeutic effects of psychotherapy in this disorder. In particular, after the firstly reported clinical and neuroimaging data from this project demonstrated significant disease-associated and psychotherapy-mediated modifications, my attention focused on uncovering the underlying molecular processes.

The primary aim of my research was to investigate the role of specific molecular mediators involved in biological functions which have been described as altered in BPD. In particular, the objective was to study, through the evaluation of their circulating levels, key molecules such as Oxytocin (OXT), β-endorphin, Brain-Derived Neurotrophic Factor (BDNF), and microRNAs, all of which have been implicated in the regulation of core processes disrupted in BPD, such as emotional regulation, stress response, and neuroplasticity. The involvement of these molecular markers in BPD, as well as their possible modulation in response to psychotherapeutical approaches, has been investigated.

The secondary aim was to explore possible relationships between these molecular markers and available neuroimaging data gathered in the context of the Climamithe project, both evaluating their association at the baseline and during the course of psychotherapies.

METHODS

Sample cohort description

A subgroup of 50 BPD patients and 26 non affected volunteers enrolled in the framework of the CLIMAMITHE study (Clinical Trials.org as NCT02370316, Protocol number 67/2014) from whom blood samples were available were included in this study. The inclusion criteria for patients were: age 18–45; diagnosis of BPD (DSM-IV-TR). Exclusion criteria were: a lifetime diagnosis of schizophrenia; schizoaffective disorder; substance abuse or dependence in the 3 months before the enrollment; bipolar disorder; organic mental syndromes; dementia or cognitive impairment; relevant neurological signs. Furthermore, pregnant or lactating women or patients receiving concurrent psychotherapy were excluded. Non affected control volunteers were included in the study and underwent the same evaluation as BPD patients. Exclusion criteria for control subjects were: any cognitive impairment or psychiatric/neurological condition, including alcohol/substance abuse. All subjects signed an informed consent.

Clinical assessment

The Structured Clinical Interview (SCID) for DSM-IV-TR disorders I and II and the Zanarini rating scale for Borderline Personality Disorder (ZAN-BPD) were used to define the diagnosis of BPD and collect data on comorbidities. Patients and non affected controls underwent a multidimensional evaluation assessing different clinical features:

- Emotion dysregulation assessed, with the Difficulties in Emotion Regulation Scale (DERS)
- BPD symptomatology, assessed with the Zanarini rating scale for Borderline Personality Disorder (ZAN-BPD)
- Depressive symptoms, assessed with the Beck Depression Inventory-II (BDI-II)
- Impulsivity, assessed with the Barratt Impulsiveness Scale (BIS)
- Alexithymia, assessed with the Toronto Alexithymia Scale (TAS-20)
- Interpersonal functioning, assessed with the Inventory of Interpersonal Problems (IIP)

- Childhood traumatic experiences, assessed with the Childhood Trauma Questionnaire (CTQ)
- Attachment experience, assessed with the Attachment Style Questionnaire (ASQ)

Clinical evaluation, in conjunction with a blood withdrawal, were scheduled at the baseline (T0), after 6 months of treatment (T6) and after one year from the beginning of treatment (T12). Control subjects were clinically assessed and underwent the blood withdrawal only at T0.

Psychotherapy

After the diagnosis, all the 50 patients were randomly allocated to one of the two different psychotherapeutical protocols groups: Metacognitive Interpersonal Therapy (MIT) or Structured Clinical Management (SCM). MIT was divided into five phases, each focusing on different metacognitive functions: the first phase aimed to enhance the patient's ability to monitor problematic mental states; the second phase focused on helping the patient to develop an integrated understanding of his current mental state; during the third phase, the focus shifted to the patient's ability to consider the representational nature of thoughts; the fourth phase sought to raise awareness of dysfunctional interpersonal patterns; the fifth phase aimed to develop a sense of self-agency. SCM targeted BPD symptomatology using a supportive approach with case management and advocacy. Key components included psychoeducation, problem-solving, explicit safety planning, medication reviews, and assertive follow-up if appointments were missed. Both treatments consisted in individual and group sessions (manualized metacognitive skill training in MIT and a problem-solving group in SCM) covering a period of about six months during one year of treatment. Among BPD patients, 26 were randomly allocated to MIT, whereas 24 were allocated to SCM. Treatment retention was measured by the total number of weeks with at least one session and the number of weeks from the first to the last session attended. Patients were classified as having completed treatment if the time between the first and the last sessions was at least 12 months between the first and the last session. Dropouts were defined as those missing four consecutive sessions with no ascertained reason. When needed, BPD patients received pharmacological treatment.

14 patients were drug-free at the baseline, while 36 were in treatment with psychotropic drugs.

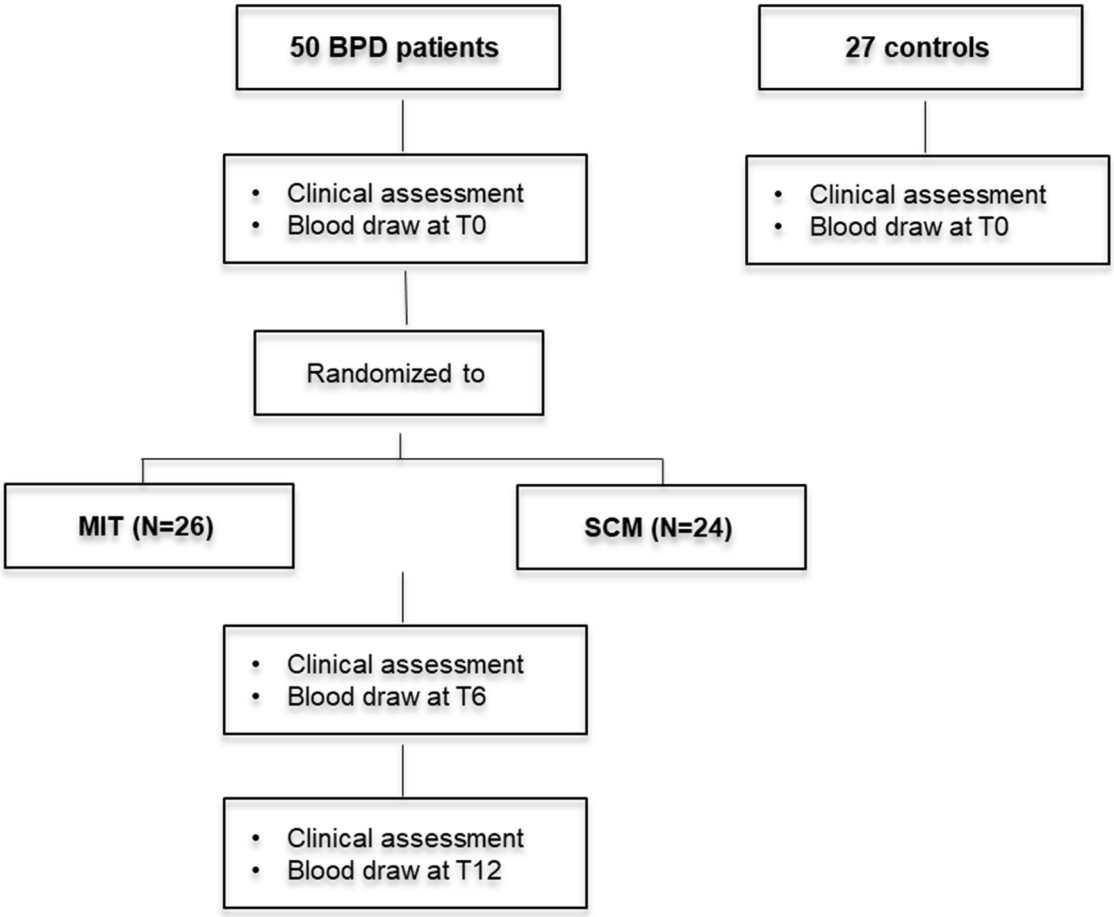


Fig 2 : *Project flow chart*

Neuroimaging

For this study, neuroimaging data collected at the baseline (T0) and at the end of treatment (T12), were available from a subgroup of 35 patients who underwent a 3T MRI exam (Siemens Magnetom Skyra), equipped with a 64 Channels RF HEAD COIL, at the Neuroradiology Unit of the Spedali Civili Hospital (Brescia, Italy). The multimodal protocol included functional (fMRI), diffusion-weighted (DWI), and structural (3D T1-weighted) sequences, acquired according to the following parameters: (i) fMRI (EPI sequence), TR=2000 ms, TE=30 ms, voxel size of 2.2 × 2.2 × 3.5 mm; (ii) DWI (axial spin-echo EPI sequence), TR=8300 ms, TE=75 ms, voxel

size=2 mm isotropic, 64 non-collinear gradient directions (b=1000 s/mm^2), 5 non-weighted directions (b=0 s/mm^2), 5 non-weighted scans with reversed phase-encoding blips for distortion correction; (iii)3D T1-weighted (MPRAGE sequence), TR = 2300 ms; TE=2 ms; flip angle=9; spatial resolution=1 mm isotropic, 176 sagittal slices.

For the purpose of the present study, we selected all patients for whom baseline (T0) and follow-up (T12) scans were available. We considered for the analyses the variables referring to the Fractional Anisotropy and the Mean Diffusivity of four networks: the default mode network (dorsal and ventral), the salience network (anterior and posterior), the left executive control network and the right executive control network. Moreover, we considered the volumetric data of the hippocampal subfields, of the nuclei of the amygdala and of the subunits of the hypothalamus. Finally, to highlight connectivity patterns, we considered large-scale distributed networks in the human cerebral cortex.

Functional magnetic resonance (fMRI) procedure

All participants received training prior to the fMRI procedure. During the acquisition, an experimental task was administered to participants using a screen through a mirror mounted onto the head coil (Magni et al., 2019): (i) a total of 96 intermixed unpleasant, neutral, and pleasant photographic images from the International Affective Picture System (IAPS) (Bradley & Lang, 2017) were presented twice in a random order, for a total of 192 trials; (ii) a dark image with cross was shown during the rest periods. After the stimulus presentation, the participant was asked to indicate the emotional valence between unpleasant, neutral, or pleasant, using a joypad.

Imaging Data Processing

The fMRI image processing was carried out using SPM12 (http://www.fil.ion.ucl.ac.uk/spm). Each image was adjusted for variations in the slice acquisition time and realigned to the field map. Then, a mean functional image volume was computed, aligned with the high-resolution structural image, and then normalized using affine and nonlinear transformations. A Gaussian filter (6-mm full-width half maximum [FWHM]) was used to spatially smooth the images.

ArtRepair (http://cibsr.stanford.edu/tools/human-brain-project/artrepair-software) was used on individual subjects to further correct head movement differences across consecutive volumes.

The DWI data processing was performed using FMRIB's Software Library (FSL) (http://www.fmrib.ox.ac.uk/fsl/, version 5.0.9; RRID:SCR_002823) (Smith et al., 2004). First, EPI images acquired with opposite directions were combined to estimate the susceptibility-induced distortions, as implemented in the *topup* tool (Andersson et al., 2003). Then, the DTI sequences were corrected for eddy current-induced distortions and subject movements using *eddy* (Andersson & Sotiropoulos, 2016). For each subject, the diffusion tensor was estimated with *DTIfit* from FDT tool (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FDT), and the diffusion maps (fractional anisotropy [FA], mean diffusivity [MD]) were created. The longitudinal diffusion processing pipeline was used following a previously described optimized protocol (Engvig et al., 2012) for the Tract-Based Spatial Statistics (TBSS; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/TBSS) (Smith et al., 2006). For each subject, the first brain-extracted b=0 volume at T00 and T12 were aligned and resampled to a common in-between halfway space using SIENA (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/SIENA) (Smith et al., 2002, 2004). SIENA automatically extracted the brain and the skull, which were then aligned each other using the skull images to constrain the registration scaling. Brain images of each timepoint were resampled to the halfway space, creating the halfway map and calculating the related deformation matrix. The deformation matrix was then applied to FA and M maps, to co-register each timepoint to the halfway map using FLIRT (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FLIRT) (Jenkinson et al., 2002; Jenkinson & Smith, 2001). The halfway FLIRT-registered FA images were averaged to create a subject-wise mid-space template. Each template was then processed using the standard TBSS pipeline(Smith et al., 2006). Briefly, TBSS identifies the most representative image to be used as target, computes the affine- and non-linear transformations warping each subject to the target image and then to the MNI space, and generates a mean FA map from all subjects. The mean FA map was thresholded (FA>0.2) to create a binary skeleton mask for the statistical analysis. Finally, the halfway FLIRT-registered FA and MD images were smoothed (sigma=2) and projected to the previously generated skeleton mask using TBSS.

For the triple network system analyses, the WM pathways connecting hubs of the SN (anterior [aSN] and posterior [pSN]), the DMN (dorsal [dDMN] and ventral [vDMN]), and the ECN (left [LECN] and right [RECN]), were identified using a probabilistic fMRI-guided normative atlas (Boettiger et al., 2015). For each network subcomponent, the corresponding WM probability map was thresholded at 5% to exclude low probability voxels and then binarized. These masks were then overlaid to the spatially normalized individual DTI maps normalized to MNI space with the TBSS procedure, and the mean values were finally extracted.

Finally, structural MPRAGE were processed using the FreeSurfer v6.0 longitudinal pipeline (https://surfer.nmr.mgh.harvard.edu/). First, images were processed using the cross-sectional procedure; then, for each subject, an intermediate template was computed across timepoints and used to initialize the longitudinal stream. In addition, the longitudinal procedure was also implemented for the segmentation of the hippocampal subfields and nuclei of the amygdala (https://surfer.nmr.mgh.harvard.edu/fswiki/HippocampalSubfieldsAndNucleiOfAmygdala), while the procedure for the hypothalamic subunits segmentation (https://surfer.nmr.mgh.harvard.edu/fswiki/HypothalamicSubunits) was applied to the output of the standard longitudinal procedure.

Blood Sample Processing

Peripheral venous blood samples were collected in the morning using Vacutainer™ collection tubes. For plasma collection, blood samples were collected in the morning, after an overnight fast in EDTA tubes; the tubes were kept on ice for 30 minutes, subsequently plasma was separated by centrifugation at 1680 g for 15 minutes at 4 °C. Plasma samples were aliquoted and stored at -80 °C until the time of analysis. For serum collection, blood samples were collected in the morning, after an overnight fast, in tubes without EDTA; the tubes were left at room temperature for 1 hour until serum separation, which occurred by centrifugation at 1680 g for 15 minutes at room temperature. Serum samples were aliquoted and stored at -80 °C until the time of analysis.

Oxytocin dosage

Plasma OXT concentrations were quantified by Radioimmunoassay (RIA) (RIAgnosis, Munich, Germany). 300 µl of each plasma sample was extracted using LiChroprep® Si60 (Merck) heat-activated at 690 °C for 3 hours. 20 mg of LiChroprep® Si60 in 1mL distilled water were added to each sample, mixed for 30 min, washed twice with distilled water and 0.01 mol/L HCl and eluded with 60% acetone. 50 µl of assay buffer was then added to the extract followed by 50 µl antibody against OXT (raised in rabbits). After a 60-min preincubation interval, 10 µl 125I-labeled tracer (PerkinElmer, USA) was added, and samples were allowed to incubate for 3 days at 4◦C. Unbound radioactivity was precipitated by activated charcoal (Sigma–Aldrich, St Louis, MO, USA). Under these conditions, an average of 50% of total counts are bound with <5% non-specific binding. The detection limit is in the 0.1-0.5 pg/sample range, depending on the age of the tracer, with typical displacements of 20–25% at 2 pg, 60–70% at 8 pg and 90% at 32 pg of standard neuropeptide. Cross-reactivity with arginine vasopressin (AVP), ring moieties and terminal tripeptides of both OXT and AVP and a wide variety of peptides comprising 3 (α-melanocyte-stimulating hormone) up to 41 (corticotropin-releasing factor) amino acids are <0.7% throughout.

β -Endorphin dosage

Serum β-endorphin levels were determined using the enzyme-linked immunosorbent assay (ELISA) kit E90806Hu obtained from Cloud-Clone Corp. (USA). The kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of β-endorphin in human serum, plasma, cerebrospinal fluid and other biological fluids. Serum samples were thawed to room temperature and 50µL were added to each well of a 96-well plate pre-coated with a β-endorphin specific monoclonal antibody. 50µL/well of a biotin-labeled competitor were added and the plates were set to incubate for 1 hour at 37 °C. After incubation, the plate was washed off and 100µL of avidin conjugated to horseradish peroxidase (HRP) were added to each well. After 30 min of incubation at 37 °C, the wells were washed again and 90µL of HRP-substrate solution was added. From this point, the plate was kept protected from light. After 15 min of incubation at 37 °C, 50µL of Stop solution were added and mixed uniformly to each well. Finally, absorbance was measured immediately at

450 nm. The intensity of color developed (OD) was inversely proportional to the concentration of β-endorphin in the sample. Standard curve was constructed by plotting the OD values (X-axis) against the log of concentration of each standard (Y-axis) and the concentration of β-endorphin (pg/mL) was determined through the points of intersection of each sample on the graph. All samples were dosed in duplicate.

Brain-Derived Neurotrophic Factor dosage

Serum BDNF levels were determined using the enzyme-linked immunosorbent assay (ELISA) kit DuoSet human BDNF, DY248 obtained from R&D Systems (USA). The kit is a direct sandwich enzyme immunoassay technique for the in vitro quantitative measurement of BDNF in both human and mouse serum, plasma, cerebrospinal fluid, other biological fluids and most cell cultures supernatant. The day before the assay, a 96-well plate was coated overnight at room temperature with 100µL of a BDNF specific monoclonal antibody. The day of the assay, the unbound antibody was washed off and the wells were blocked by adding 300 µL of Reagent Diluent. Serum samples were thawed to room temperature and diluted 1:100. 100µL of each diluted samples were then added to each well and the plate was set to incubate at room temperature. After incubation, the plate was washed off and 100 µL of a biotin-labeled detection antibody were added. The plate was incubated again at room temperature. After incubation, the unbound antibody was washed off and 100 µL of the streptavidin-HRP solution was added to each well. From this point, the plate was kept protected from light. After 20 minutes of incubation at room temperature, the plate was washed again and then 100 µL of HRP-Substrate Solution was added to each well. After 20 minutes of incubation at room temperature, 50 µL of Stop Solution was added and mixed uniformly to each well. Finally, absorbance was measured immediately at 450 nm with a wavelength correction at 540 nm. The intensity of color developed (OD) was directly proportional to the concentration of BDNF in the sample. Standard curve was constructed by plotting the OD values (Y-axis) against the concentration of each standard (X-axis) and the concentration of BDNF (pg/mL) was determined through the points of intersection of each sample on the graph. All samples were dosed in duplicate.

Quantitative Real-Time PCR for microRNA analysis

A subgroup of 12 BPD patients and 12 controls, matched for age and sex, were selected to assess potential differences in miRNA expression profile between the two groups. For this kind of study, we decided to use a -omic approach without a priori hypotheses about the involvement of specific miRNAs in the disorder. Instead, we decided to explore a wide set of miRNAs to identify potential modulations. Total miRNA isolation from serum samples at T0 was performed using the NucleoSpin miRNA Mini kit for circulating miRNA (Macherey-Nagel, DEU), following the manufacturer's instructions. RNA was then subjected to reverse transcription using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (ThermoFisher, USA), according to the manufacturer's instructions. Serum miRNA levels were assessed using TaqMan™ Advanced miRNA Human Serum/Plasma Card (ThermoFisher, USA), a panel allowing to profile 188 target miRNAs, including endogenous and exogenous miRNA controls for normalization of data results. Quantitative real-time PCR (qRT-PCR) assays, carried out on a QuantStudio 12K Flex (Applied Biosystems, USA), were performed in duplicate for each sample. miRNAs showing Ct values > 32 in more than 3 samples were excluded from the analysis. Additionally, as a quality control (QC) procedure on each miRNA we excluded values with excessive variance (Ct SD = 1.00) between the duplicates and/or showing an unconventional exponential trend in the amplification curves (double-peaked curves, linear slopes, jagged curves…). Data passing the quality control were then normalized on the geometric mean of the Ct values of the more stably expressed miRNAs, thus assumed as endogenous controls (miR-126-3p, miR-93-5p, miR-144-3p). The stability of the endogenous controls was evaluated taking into account the variation of their Ct between the patients and the control group, their variance between subjects and their mean Ct. miRNA levels were calculated by the 2^-ΔCt method. Fold changes between BPD patients and controls for each miRNA were calculated as the ratios (or as negative reciprocal values of the ratios, for ratios < 1) between the means of miRNA levels, expressed as 2^-ΔCt, in the two groups.

In order to identify the biological processes possibly regulated by the differentially expressed miRNAs, we conducted a target gene prediction and pathway analysis using the freely available online software mirPath v.4 (http://62.217.122.229:3838/app/miRPathv4).The identification of miRNA-regulated pathways was obtained through the combination of predicted interactions

of miRNAs with their target genes (whether miRNA target sites locates in CoDing Sequence CDS regions or untranslated 3'-UTR regions), provided by the DIANA-microT-CDS algorithm, and a pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

Statistical analysis

Data were analyzed using the latest version of SPSS software for Windows. Descriptive statistics were presented in terms of mean and standard deviation (SD) for continuous variables or in terms of frequency and percentage for the categorical ones. Group comparisons between BPD patients and controls at the baseline were performed by t-test, or Mann–Whitney test when appropriate. Correlations among continuous variables were tested by Pearson's correlation analysis, evaluating the r coefficient. Pearson's correlation analysis was used also for assessing the correlations between changes of different variables across time, and in particular from T0 to T12. This variation (also defined "T0-T12" from here on) was calculated as the delta obtained by subtracting T0 from T12 values.

For the longitudinal evaluation of changes in the continuous variables and for the analysis of possible differences in their modulation between the two intervention groups (MIT and SCM), generalized linear mixed models were applied by setting time (3 time points: T0, T6, T12), group (MIT vs SCM) and time x group interaction as fixed effects.

RESULTS

Demographical and clinical data

Demographical data of BPD patients and control groups were compared at the baseline. The mean age of BPD patients was 30.1 years (SD = ±8.1) and the mean age of controls was 28.6 (SD = ±6.8). The majority of both BPD patients and controls were females, respectively 82% and 84.6%. No statistically significant differences were observed between the two groups for age and sex. We also compared clinical data at the baseline between the patients and the control group. About 48% and 58% of the patients showed a lifetime history of alcohol and substance abuse (but not in the three months before the enrollment), respectively. 48% of the patients manifested Non Suicidal Self Injuries (NSSI). Scores in interpersonal functioning (IIP) and attachment style (ASQ) scales were significantly higher in patients (Table 1).

Table 1*: demographical and clinical characteristics of patients and control group.*

	Patients	**Controls**	**p-value**
	N=50	**N=26**	
Sex (females)	41 (82%)	22 (84.6%)	0.778
Age (mean (SD))	30.1 (8.1)	28.0 (6.28)	0.255
Alcohol abuse	24 (48%)	0 (0%)	#
Substance abuse	29 (58%)	0 (0%)	#
Non Suicidal Self-injury (NSSI)	24 (48%)	0 (0%)	#
Psychotropic Drugs	36 (72%)	0 (0%)	#
Inventory of Interpersonal Problems (IIP) (mean (SD))	2.2 (0.7)	0.7 (0.4)	<0.001
Attachment Style Questionnaire subscales:			
Confidence (mean (SD))	26.3 (6.4)	32.7 (3,8)	<0.001
Discomfort with Closeness (mean (SD))	40.1 (6.0)	32.8 (5.1)	<0.001
Relationships as Secondary (mean (SD))	17.7 (6.4)	14.2 (6,1)	0.024
Need for approval (mean (SD))	29.4 (6.6)	19,0 (4,9)	<0.001
Preoccupation with Relationships (mean (SD))	36.2 (6.9)	25.8 (5.6)	<0.001
Difficulties in Emotion Regulation Scale (mean (SD))	121.7 (25.6)	62.4 (12,1)	<0.001
Zanarini Rating Scale for BPD	15.6 (4.6)	1.8 (1.2)	<0.001
Childhood Trauma Questionnaire (mean (SD))	57.2 (16.7)	31.9 (4,8)	<0.001
Barratt Impulsiveness Scale (mean (SD))	74.2 (12.5)	51.7 (7.7)	<0.001
Toronto Alexithymia Scale(mean (SD))	60.9 (12.7)	38.7 (7.0)	<0.001
Beck Depression Inventory-II (mean (SD))	33.1 (11.9)	3.3 (2.9)	<0.001

Out of the 50 patients enrolled in this study, 41 completed the 6 months follow-up, whereas 9 patients dropped out. Out of the 41 patients which reached the T6 step, 35 also completed the 12 months of psychotherapy treatment and all the relative clinical evaluations, whereas 6 dropped out in this phase. Full response to psychotherapy was defined as a percentage decrease from T0 to T12 (calculated as T12-T0/T0) of at least 25% of DERS score, partial response as a decrease of at least 12.5%, stable condition as any changes between ±12.5% and worsening as an increase of at least 12.5%. Out of the 35 patients that completed the treatment, 9 were full responders, 8 were partial responders and 18 were non responders. Considering the two different psychotherapies, among those who followed MIT 6 patients were full responders, 2 were partial responders and 8 were non responders, while among those who followed SCM 3 patients were full responders, 6 were partial responders and 10 were non responders.

Considering BPD symptomatology, ZAN scores were reduced by 42.12% in the subgroup of 35 patients during treatment with psychotherapies ($p < 0.001$). Considering the two different psychotherapies, those who followed MIT showed a ZAN reduction of 28.45%, while those who followed SCM showed a reduction of 54.27%. The decreases in other clinical scale scores were not statistically significant in this subsample.

Oxytocin

Baseline evaluation

At T0, mean plasma OXT concentration in the patient group was 2.34 pg/mL ± 0.48, while mean plasma T0 concentration in the control group was 3.16 pg/mL ± 1.41. OXT concentration levels were compared at the baseline between BPD and control groups using a Mann-Whitney test (as data did not show a normal distribution). Results showed significantly lower plasma OXT levels in BPD patients compared to controls ($p = 0.002$) (fig. 3).

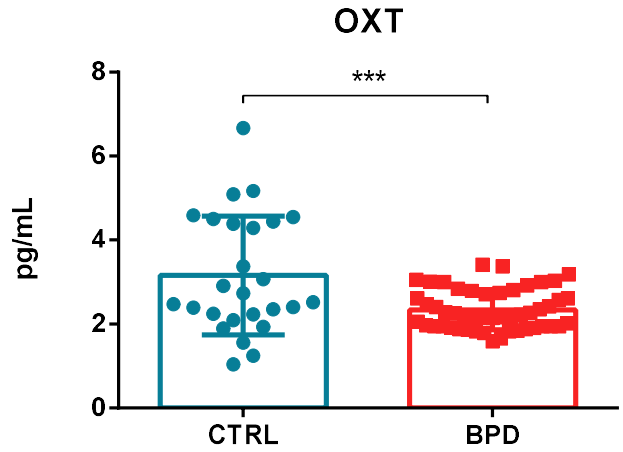


Fig. 3 : OXT plasma levels in BPD patients at T0 compared to CTRL group. OXT plasma levels are expressed as pg/mL

No statistical difference in OXT concentrations at the baseline was evidenced between patients who followed MIT and those who followed SCM (MIT: 2.33 pg/mL ± 0.46, SCM: 2.36 pg/mL ± 0.50) (fig. 4).

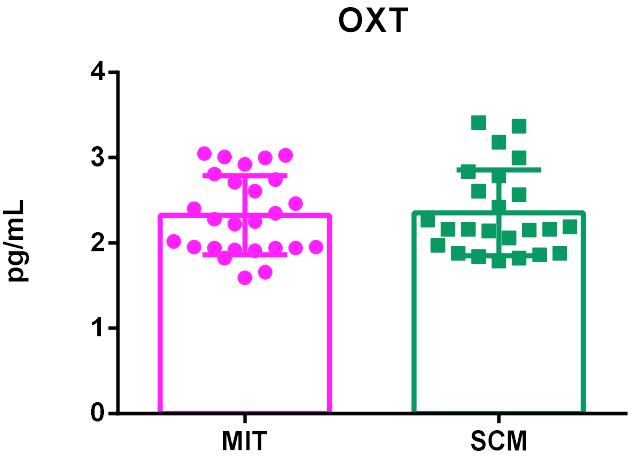


Fig. 4 : OXT plasma levels in patients at T0 between MIT and SCM group. OXT plasma levels are expressed as pg/mL

Also, no difference in baseline plasma OXT levels was evidenced between drug-free patients and those under pharmacological treatment. No difference in baseline plasma OXT levels was evidenced either considering alcohol abuse and substance abuse.

When testing correlations between OXT levels and clinical measures, a negative correlation was evidenced in BPD patients only between baseline OXT concentrations and scores in the subscale ASQ-PR ("Preoccupation with Relationships") ($r = -0.36$, $p = 0.017$) (fig. 5).

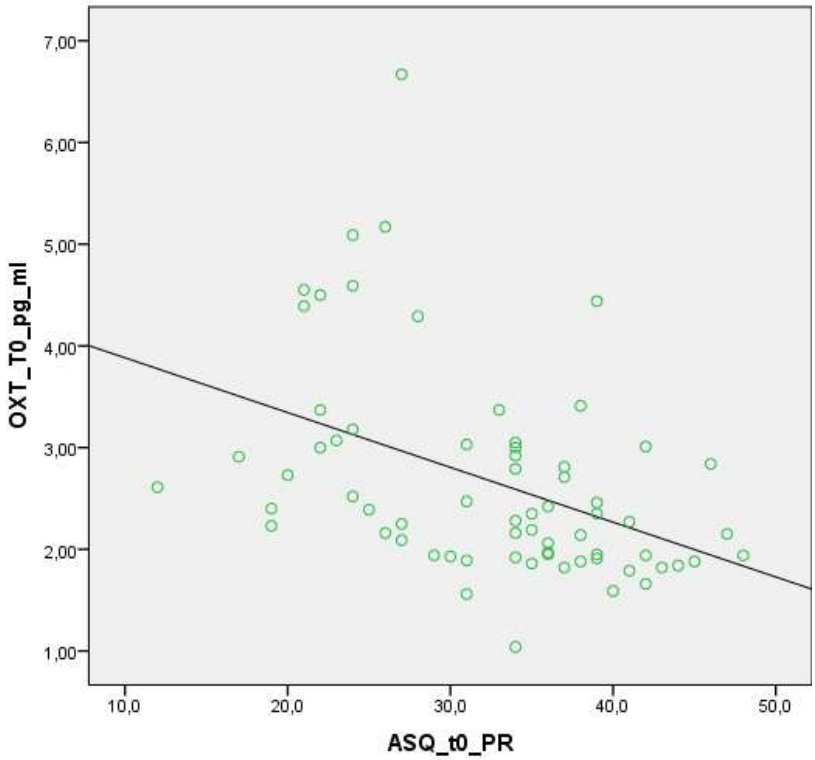


Fig. 5 : correlation, in BPD patients, between OXT levels at T0 and ASQ-PR score. OXT levels are expressed as pg/mL

No correlation was evidenced between OXT baseline levels and T0-T12 changes in clinical scale scores, and no difference in baseline OXT levels was observed when comparing responders vs. non responders to treatment interventions.

Longitudinal evaluation during psychotherapy

As previously described, mean plasma OXT concentration in the patient group at T0 was 2.34 pg/mL ± 0.48. After the first six months of psychotherapy (T6) OXT levels in BPD patients were 2.59 pg/mL ± 0.54 and at the end of the treatment (T12), OXT concentrations were 2.54 pg/mL ± 0.58. We used a generalized linear mixed model for evaluating longitudinal variations at the two follow-up. In the whole patients group, OXT levels increased significantly after the 12 months of treatment (T12) ($p = 0.049$), with a more relevant increment between the baseline and T6 ($p = 0.022$) (fig. 6). No difference in OXT variations across time was evidenced between the two intervention groups (MIT and SCM), both between T0-T6 and between T0-T12 (time x group interaction). However, patients in the MIT group, in contrast to patients in the SCM group, showed maintenance of OXT increased values also at T12 (fig. 7) even though difference between treatment groups were not significant.

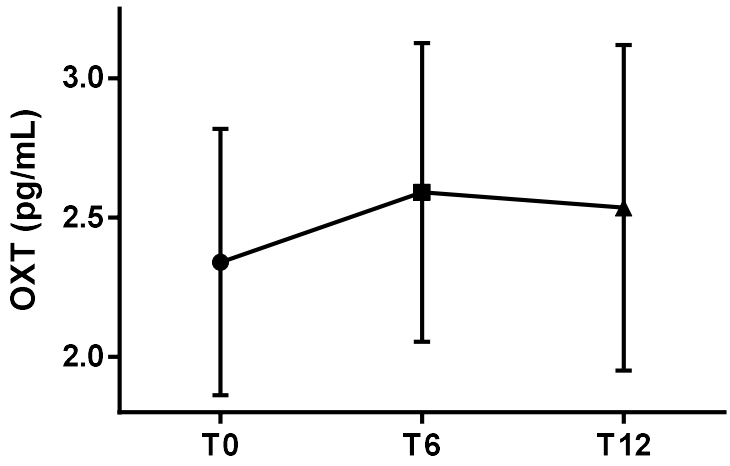


Fig. 6 : OXT plasma changes in patients during psychotherapies. OXT levels are expressed as pg/mL

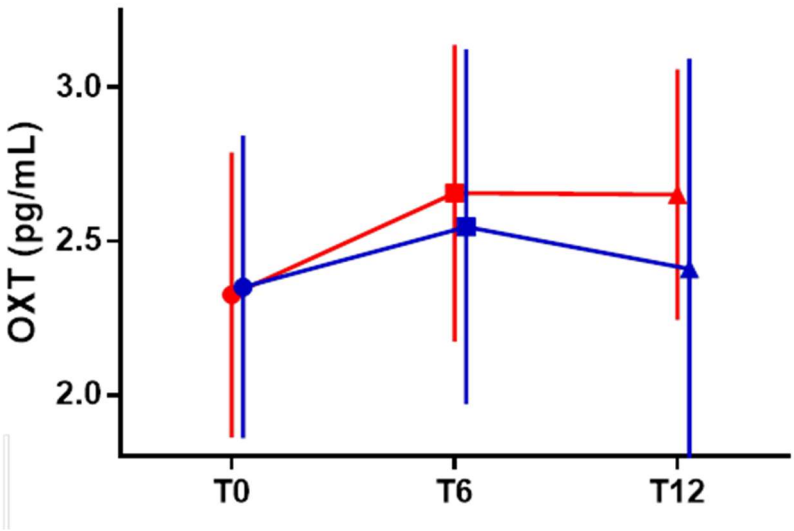


Fig. 7 : OXT plasma changes in patients during psychotherapies in the two intervention groups. The red line corresponds to the MIT group; the blue line corresponds to the SCM group.

Moreover, increase of plasma OXT (T0-T12) was positively correlated with the decrease in DERS scores (r = 0.387 p = 0.005) (fig. 8) and in ZAN scores (r = 0.387 p = 0.006) (fig. 9).

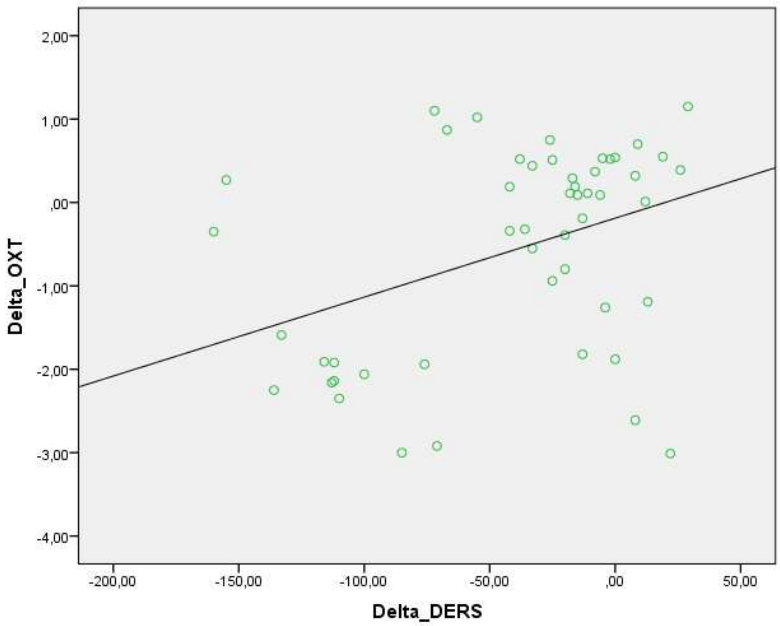


Fig. 8 : Correlation between OXT variation between T0 and T12 and DERS scores variation between T0 and T12

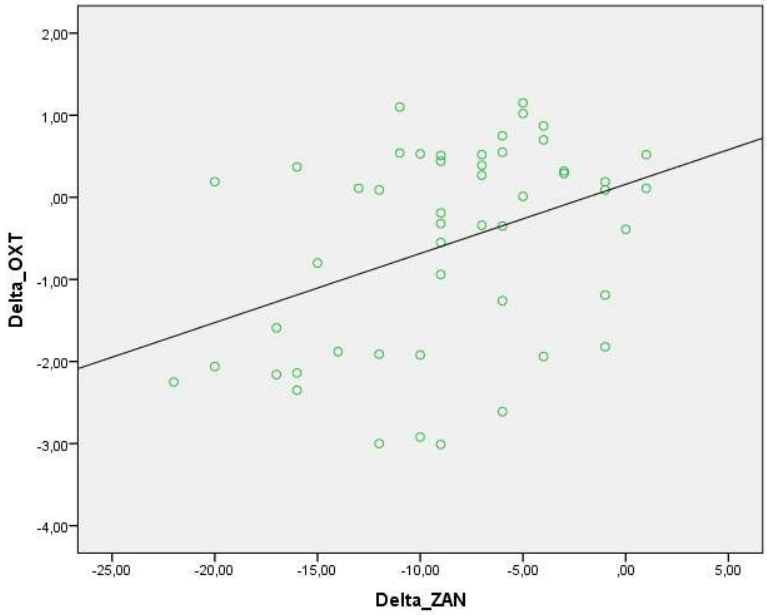


Fig. 9 : Correlation between OXT variation between T0 and T12 and ZAN scores variation between T0 and T12

β -Endorphin

Baseline evaluation

Mean serum T0 β-endorphin concentration in the patient group was 146.41 pg/mL ± 64.99, while in the control group was 135.84 ng/mL ± 46.74, no difference was evidenced between the two groups (p = 0.533) (fig. 10).

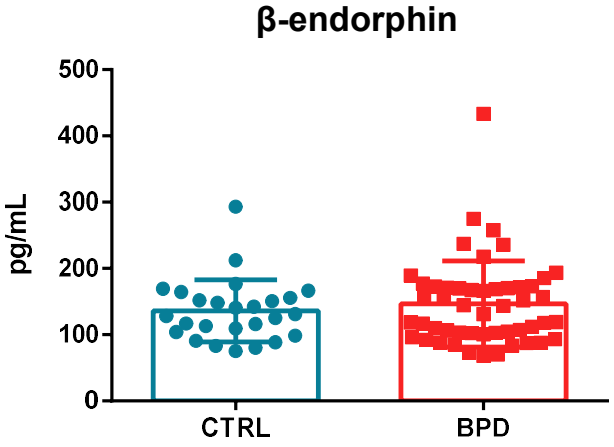


Fig. 10 : β-endorphin serum levels in BPD patients at T0 compared to CTRL group. B-endorphin serum levels are expressed as pg/mL

No statistical difference in β-endorphin concentrations at the baseline was evidenced between patients who followed MIT and those who followed SCM (MIT: 138.89 ± 42.05 pg/mL, SCM: 154.58 ± 83.32 pg/mL) (fig. 11).

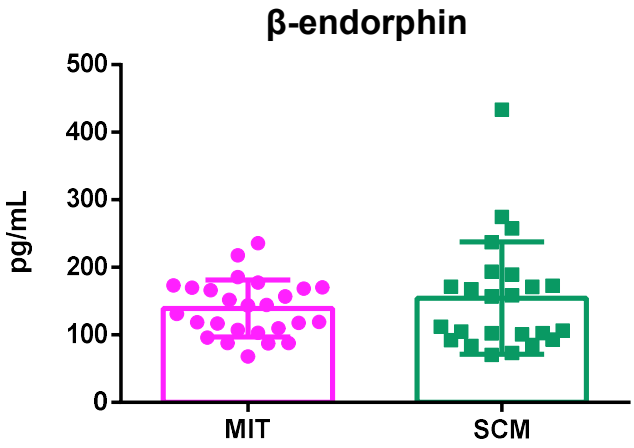


Fig. 11 : β-endorphin serum levels in patients at T0 between MIT and SCM group. B-endorphin serum levels are expressed as pg/mL

We then conducted correlation analyses, between β-endorphin levels at T0 and clinical measures, evidencing no significant correlation.

No statistical difference in β-endorphin concentrations at the baseline was evidenced between the patients who were following a pharmacological treatment and those who were drug-free. No difference in baseline plasma β-endorphin levels was evidenced either considering alcohol abuse and substance abuse

Considering the biological role of β-endorphin in pain perception and also evidence collected in literature, we were particularly interested in evaluating whether β-endorphin concentrations at T0 were different in BPD patients who manifested Non Suicidal Self Injuries (NSSI). Mean T0 β-endorphin concentration in NSSI patients was 141.59 pg/mL ± 52.08, while in patients who didn't manifest NSSI was 152.57 ± 77.29. revealing no statistical difference (fig. 12).

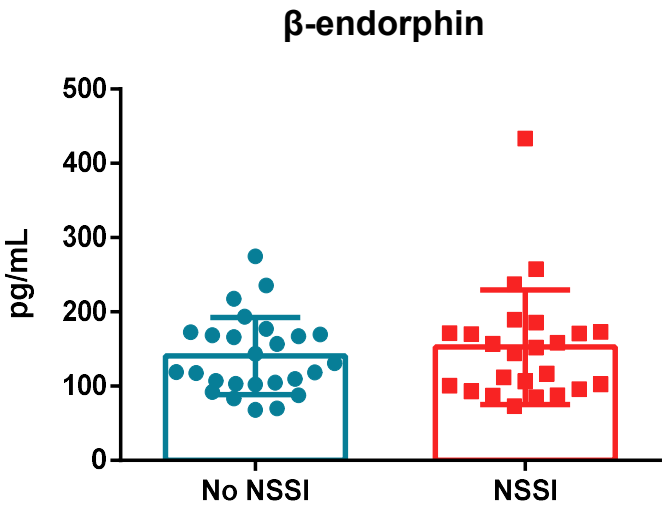


Fig. 12 : β-endorphin serum levels at T0 in patients who manifested NSSI and patients who didn't manifest NSSI. B-endorphin serum levels are expressed as pg/mL

No correlation was evidenced between β-endorphin baseline levels and T0-T12 changes in clinical scale scores and no difference was observed between responders and non responders to treatment interventions.

Longitudinal evaluation during psychotherapy

Mean serum β-endorphin concentration in the patient group at T0 was 146.41 pg/mL ± 64.99, 152.28 pg/mL ± 70.67 at T6 and 152.59 pg/mL ± 68.25 at T12 showing no significant changes in β-endorphin concentrations, suggesting that this neuropeptide was not modulated by psychotherapies (fig. 13). Modifications across time were not significantly different also considering the MIT and the SCM treatment group (time x group interaction) (fig. 14).

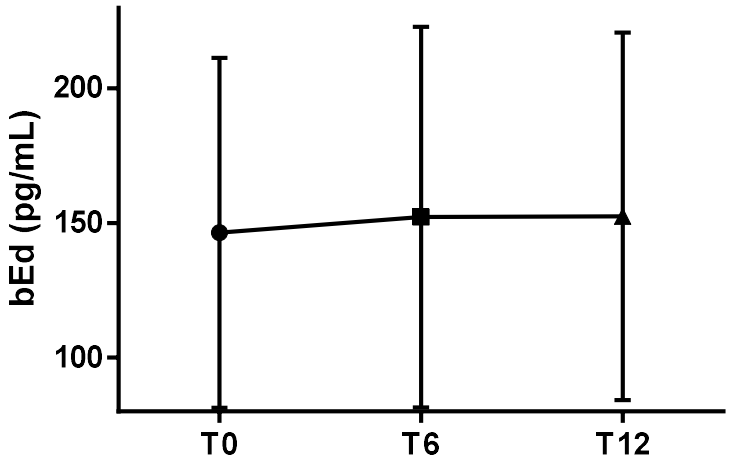


Fig. 13 : β-endorphin serum changes in patients during psychotherapies. B-endorphin serum levels are expressed as pg/mL

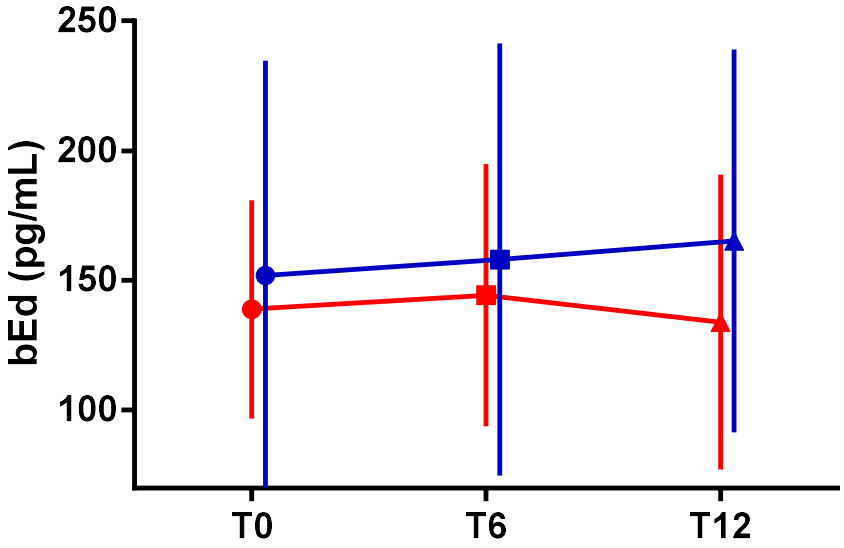


Fig. 14 : β-endorphin serum changes in patients during psychotherapies. The red line corresponds to the MIT group; the blue line corresponds to the SCM group.

No correlation was evidenced between β-endorphin T0-T12 variation and changes in clinical scale scores.

Brain-Derived Neurotrophic Factor

Baseline evaluation

Mean T0 BDNF concentration in the patient group was 42.44 ng/mL ± 12.78, while in the control group was 32.30 ng/mL ± 13.17 evidencing higher serum BDNF levels in BPD patients (p = 0.001) (fig.15).

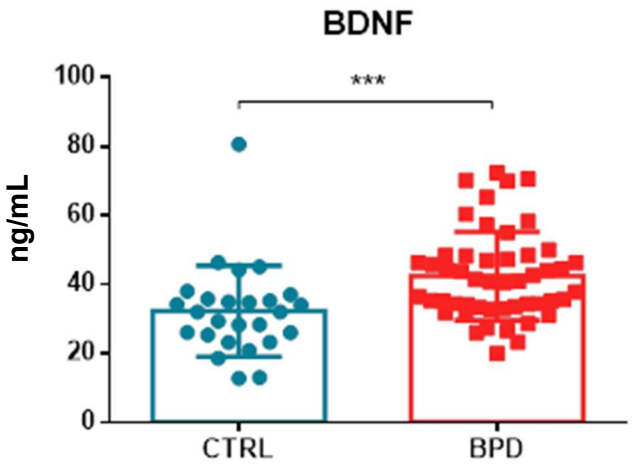


Fig. 15 : BDNF serum levels in BPD patients at T0 compared to CTRL group. BDNF serum levels are expressed as ng/mL

At the baseline, no statistical difference in BDNF concentrations was evidenced between patients who followed MIT and those who followed SCM, (MIT: 40.64 ± 12.07 ng/mL, SCM: 44.39 ± 13.48) (fig. 16).

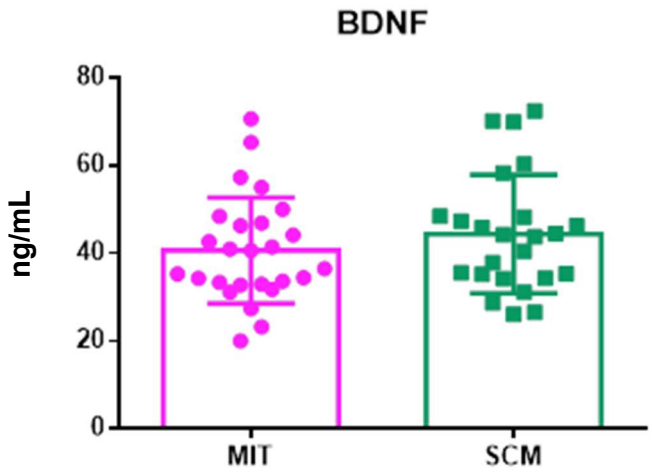


Fig. 16 : BDNF serum levels in patients at T0 between MIT and SCM group. BDNF serum levels are expressed as ng/mL

No statistical difference in T0 BDNF concentrations was evidenced between the patients who were following a pharmacological treatment and those who were drug-free. Since antidepressant treatment is known to affect BDNF levels, we also tested possible differences between patients who were under treatment with these drugs and those who were not, evidencing no significant result. No difference in baseline plasma BDNF levels was evidenced either considering alcohol abuse or substance abuse.

We then conducted correlation analyses between BDNF levels at T0 and clinical measures: negative correlations were evidenced between BDNF serum concentration at T0 and ZAN-cog (cognitive symptoms) scores at T0 ($r = -0.343$; $p = 0.015$) or IPP-Interpersonal Ambivalence (the simultaneous desire to develop and avoid close interpersonal relationships) scores at T0 ($r = -0.286$; $p = 0.047$). Considering the literature evidence that links the biological role of BDNF to traumatic events, especially in early life, we analyzed possible correlations between BDNF concentrations at T0 in BPD patients and CTQ scores reporting negative findings (data not shown). No correlation was found between these variables.

No correlation was evidenced between BDNF baseline levels and T0-T12 changes in clinical scale scores and no difference was observed between responders and non responders to treatment interventions.

Longitudinal evaluation during psychotherapy

Mean serum BDNF concentration in the patients group at T0 was 41.34 ng/mL ± 13.11, 41.29 ng/mL ± 12.21 at T6 and 39.57 ng/mL ± 11.36 at T12 showing no significant changes during the treatment, suggesting that the neurotrophin was not modulated by psychotherapies (fig. 17). Modification across the entire time course were not significantly different between the MIT and SCM treatment froup: however, a significant time x group interaction was evidenced between T6 and T12 (p= 0.017) (fig. 18).

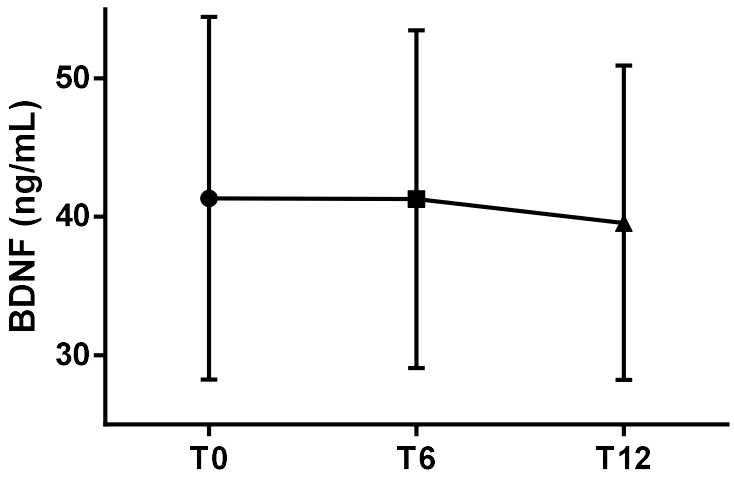


Fig. 17 : BDNF serum changes in patients during psychotherapies. BDNF levels are expressed as ng/mL

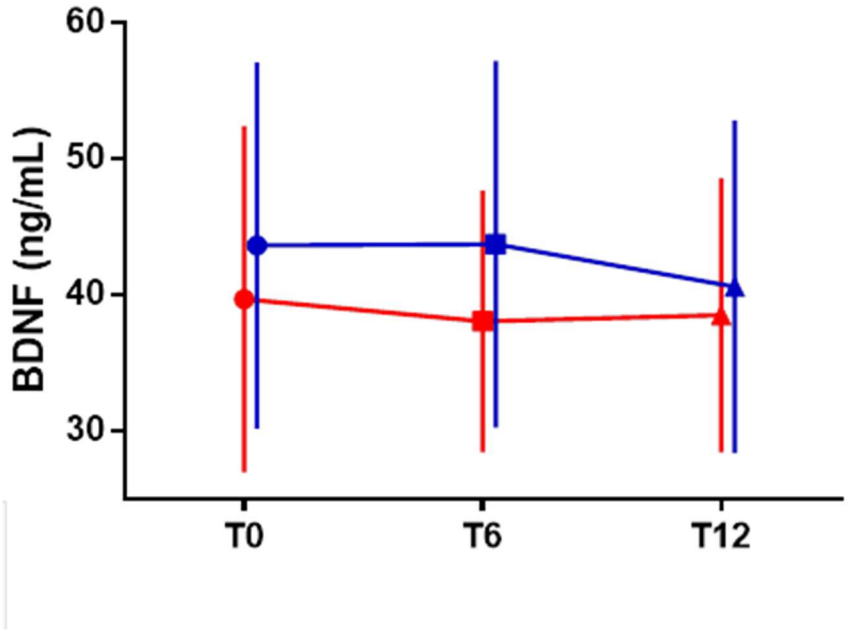


Fig. 18 : BDNF serum changes in patients during psychotherapies. BDNF serum levels are expressed as ng/mL. The red line corresponds to the MIT group; the blue line corresponds to the SCM group

No correlation was evidenced between BDNF T0-T12 variation and changes in clinical scale scores.

Correlations between molecular markers and neuroimaging features

First, we tested possible correlations between baseline levels of molecular markers (OXT, β-endorphin and BNDF) and all the available baseline neuroimaging measures in the whole group of BPD patients.

The most interesting correlations were observed for BDNF. In particular, positive correlations were observed between T0 BDNF levels and the fractional anisotropy (FA) of the ventral default mode network (DMN) ($r = 0.369$; $p = 0.029$) (fig. 19) and of the left executive control network (LECN) ($r = 0.416$; $p = 0.013$) (fig. 20) (fractional anisotropy is a measure referring to the integrity of the axonal fibers connecting brain regions through the white matter).

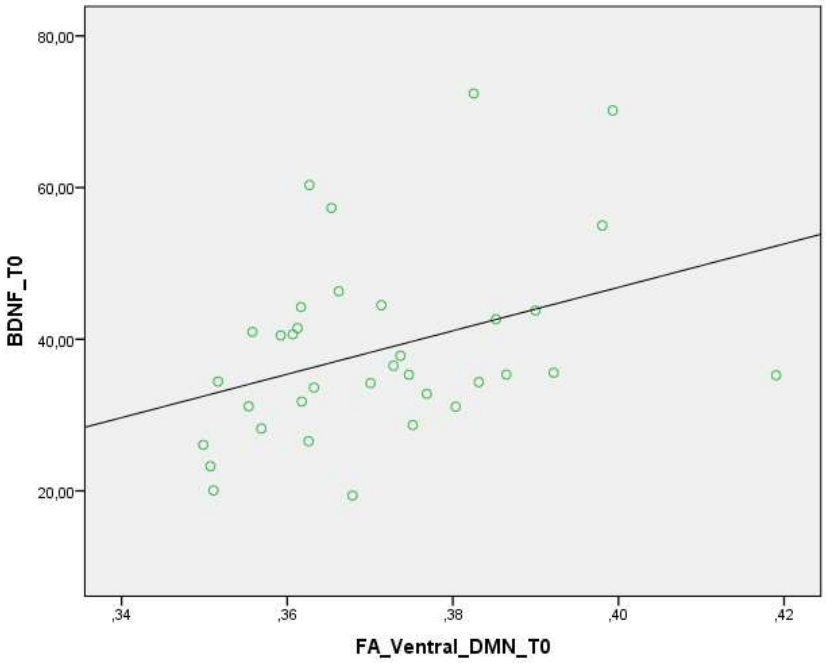


Fig. 19 : Correlation between BDNF concentration at T0 and the fractional anisotropy of the ventral default mode network at the same timepoint. BDNF serum levels are expressed as ng/mL

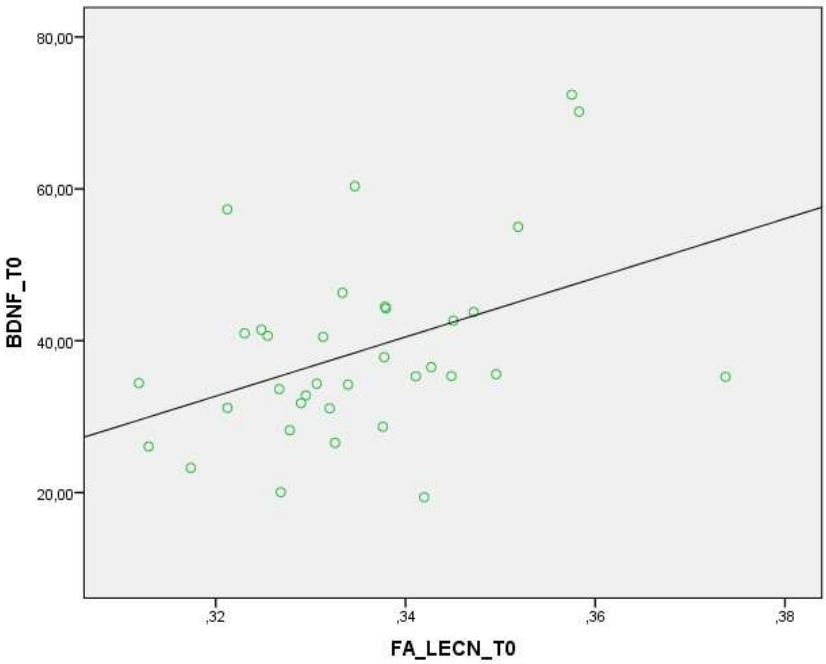


Fig. 20 : Correlation between BDNF concentration at T0 and the fractional anisotropy of the left executive control network at the same timepoint. BDNF serum levels are expressed as ng/mL

Moreover, we focused on the neuroimaging variables that showed a significant correlation with the molecular markers at the baseline (T0) and also on the ones that showed changes following treatment (T0-T12) in our study sample (data not shown). We explored possible correlations between T0-T12 changes in these variables and the T0-T12 variations in the levels of OXT, BDNF, and β-endorphin, with the aim to evaluate if cerebral changes were linked to peripheral molecular variations over time.

We evidenced a negative correlation between OXT increase during treatment and the reduction, although non-significant, of the volume of the left anterior-inferior portion of the hypothalamus (r -0.532, p = 0.003) (fig. 21), as measured with structural MRI. Interestingly, the cerebral nuclei belonging to this area are the suprachiasmatic nucleus and the supraoptic nucleus, which primary function is the release of Vasopressin and OXT.

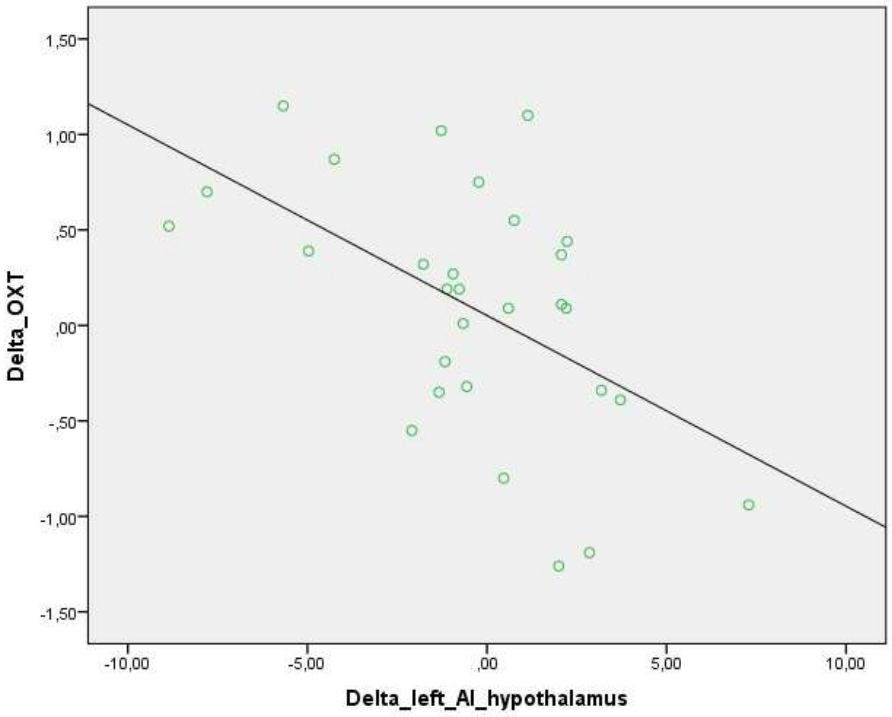


Fig. 21 : correlation between T0-T12 OXT variations and T0-T12 variations of the volume of anterior-inferior portion of the hypothalamus

Moreover, we evidenced a positive correlation between β-endorphin levels increase during treatment, (although non significant) and the significant increase in the hippocampus CA3 region volume (r = 0.413 p = 0.026), a subfield that plays a specific role in rapid memory recall and encoding (fig. 22).

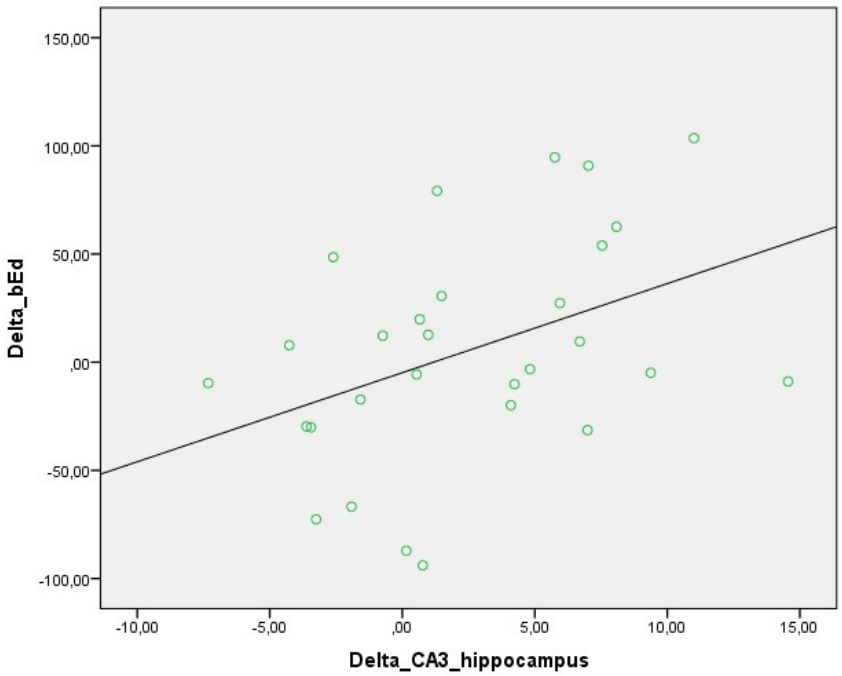
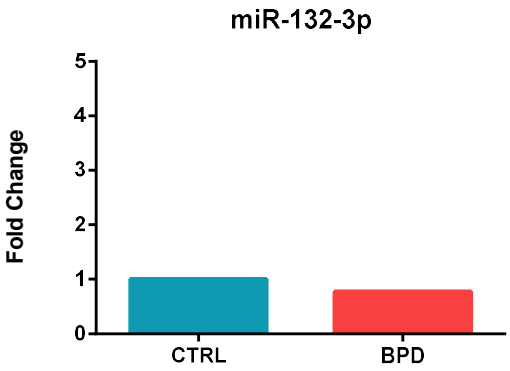
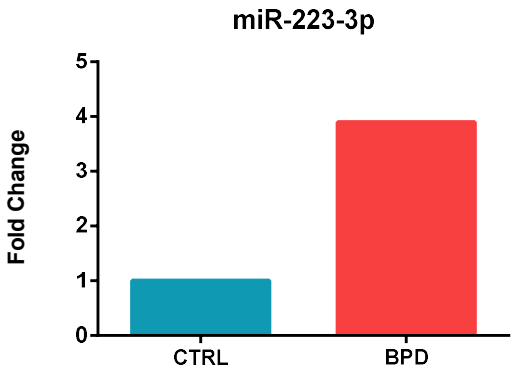


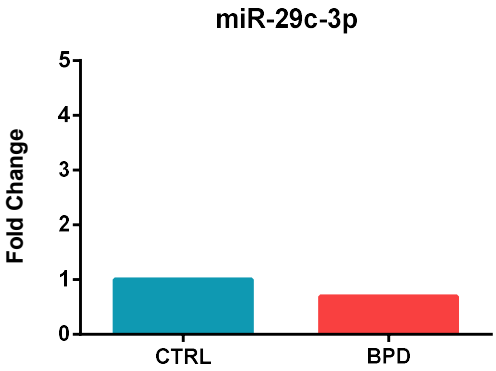
Fig. 22 : correlation between T0-T12 β-endorphin variations and T0-T12 variations of the volume of the CA3 region of the hippocampus

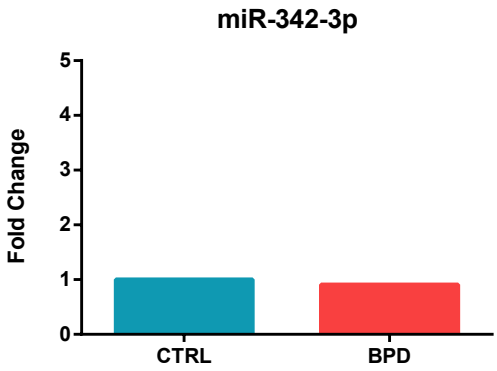
Evaluation of serum miRNA levels

Of the 188 miRNAs evaluated in the TaqMan™ Advanced miRNA Human Serum/Plasma Card panel, 164 were expressed in the analyzed samples and passed the applied QC; thus, they were then considered in the subsequent analyses. miRNA expression was compared at the baseline between the two subgroups of 12 BPD patients and 12 controls, showing eight miRNAs differentially expressed at a nominal level (Mann-Whitney test). In particular three miRNAs were down-regulated: hsa-miR-132-3p, hsa-miR-342-3p and hsa-miR-29c-3p; and five miRNAs were up-regulated: hsa-miR-409-3p, hsa-miR-543, hsa-miR-223-3p, hsa-miR-382-5p and hsa-miR-451a (fig. 23). All the Fold Changes (FC) and p-values are reported in Table 2.


miR-132-3p
Fold Change
5
4
3
2
1
0
CTRL
BPD


miR-223-3p
Fold Change
5
4
3
2
1
0
CTRL
BPD


miR-29c-3p
Fold Change
5
4
3
2
1
0
CTRL
BPD


miR-342-3p
Fold Change
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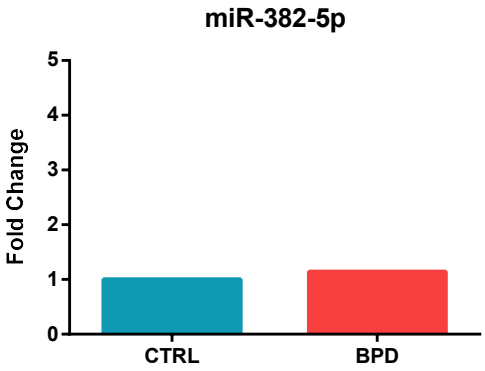
4
3
2
1
0
CTRL
BPD


miR-382-5p
Fold Change
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4
3
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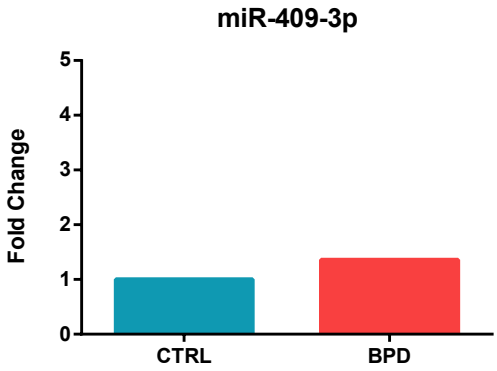
CTRL
BPD


miR-409-3p
Fold Change
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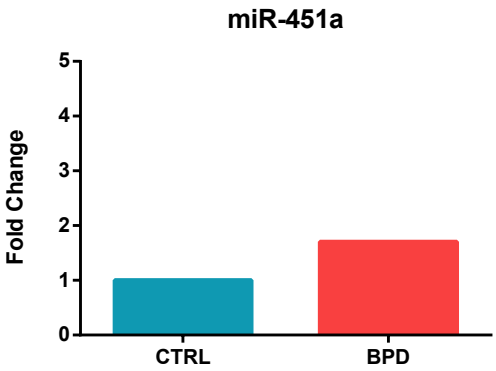
BPD


miR-451a
Fold Change
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4
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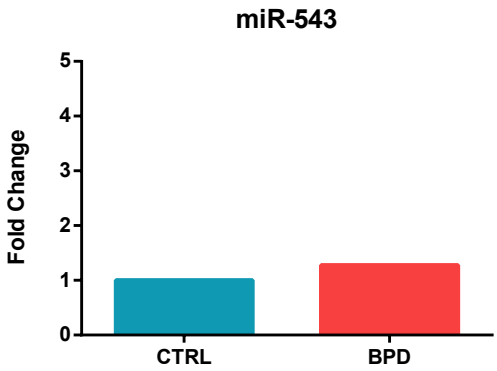
2
1
0
CTRL
BPD


miR-543
Fold Change
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4
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2
1
0
CTRL
BPD

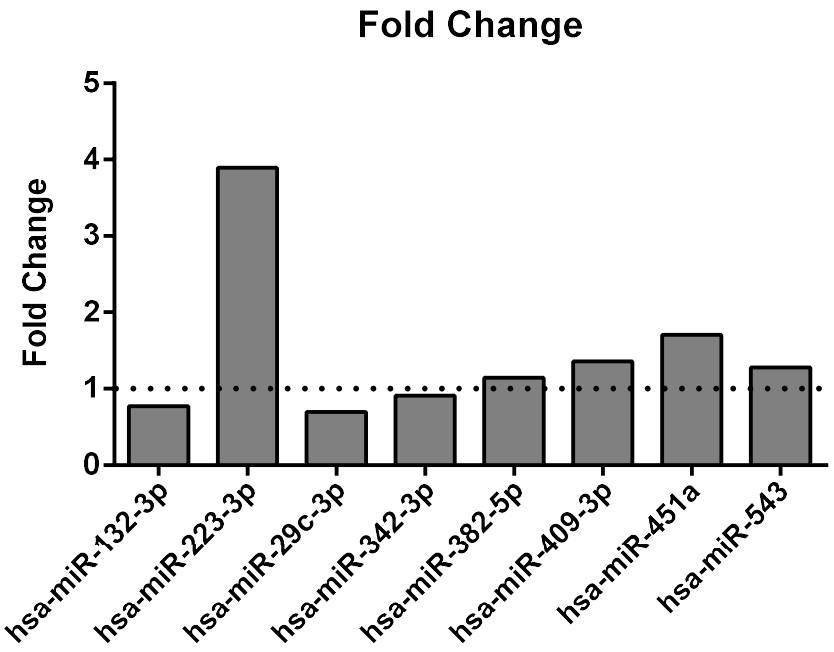

Fig 23 : Fold Change of miRNA expression levels between patients and controls

***Table 2**: Fold Changes and p values of modulated miRNAs*

miRNA ID	miRBase accession	Fold Change	p value (not adjusted)
hsa-miR-132-3p	MIMAT0000426	0,77	0,01
hsa-miR-409-3p	MIMAT0001639	1,36	0,012
hsa-miR-29c-3p	MIMAT0000681	0,69	0,020
hsa-miR-543	MIMAT0004954	1,28	0,024
hsa-miR-223-3p	MIMAT0000280	3,89	0,033
hsa-miR-382-5p	MIMAT0000737	1,15	0,033
hsa-miR-451a	MIMAT0001631	1,71	0,033
hsa-miR-342-3p	MIMAT0000753	0,91	0,045

However, when adjusting all the p-values for multiple analyses using the Benjamini-Hochberg correction, no statistically significant changes were observed.

We then performed a target gene prediction and KEGG analysis for the eight modulated miRNAs using the microT-CDS algorithm (https://dianalab.e-ce.uth.gr/microt_webserver/#/) that predicted interactions of miRNAs with both CDS or in 3'-UTR regions of potential target genes. A total of about 6000 possible target genes were identified.

The KEGG pathway analysis identified 81 enriched pathways (p<0.05, FDR-corrected). Among them, we found several biological pathways involved in BPD pathophysiology, such as *Oxytocin signaling pathway* and *Neurotrophin signaling pathway*; as well as other brain-related pathways implicated in important cerebral functions like *Colinergic synapse*, *Glutamatergic synapse*, *Dopaminergic synapse*, *Long-term depression*, *Long-term potentiation*, *Axon guidance, PI3K-Akt signaling pathway, cAMP signaling pathway, Wnt signaling pathway, Circadian rhythm* (table 3).

Among the genes belonging to these pathways, we highlighted many genes previously associated with BPD, as:

- BDNF (Brain-Derived Neurotrophic Factor, targeted by hsa-miR-409-3p, hsa-miR-382-5p, hsa-miR- 543)
- HTR1A (Serotonin 1A Receptor, targeted by hsa-miR-223-3p)
- MAOA (Monoamine Oxidase A, targeted by hsa-miR-132-3p, hsa-miR-543)
- OXTR (Oxytocin Receptor, targeted by hsa-miR-409-3p, hsa-miR-29c-3p)

Table 3: *KEGG pathway enrichment analysis (p-value < 0.05, FDR-Corrected)*

Term Name	N. of target Genes	N. of miRNAs	P (FDR)
Axon guidance	86	7	1,5556E-07
TGF-beta signaling pathway	53	8	1,9499E-06
Hippo signaling pathway	74	7	3,0966E-06
Pathways in cancer	193	8	2,2503E-05
Ubiquitin mediated proteolysis	62	8	9,9268E-05
PI3K-Akt signaling pathway	134	8	0,00013602
Autophagy - animal	62	8	0,00019018
Dopaminergic synapse	61	8	0,00019018
Glutamatergic synapse	52	7	0,00020382
Long-term depression	33	7	0,00021203

Spinocerebellar ataxia	61	8	0,00023643
Colorectal cancer	41	8	0,00029053
EGFR tyrosine kinase inhibitor resistance	39	7	0,00029053
Focal adhesion	82	8	0,00029053
Signaling pathways regulating pluripotency of stem cells	64	7	0,00029053
Adherens junction	38	7	0,00029053
Protein processing in endoplasmic reticulum	76	8	0,00029053
Rap1 signaling pathway	82	7	0,00031819
AGE-RAGE signaling pathway in diabetic complications	50	7	0,00031819
Transcriptional misregulation in cancer	79	7	0,0004136
Melanoma	36	7	0,00044963
Circadian entrainment	44	7	0,00058354
ErbB signaling pathway	39	8	0,000669
Phosphatidylinositol signaling system	44	7	0,00069266
Cholinergic synapse	51	7	0,00069266
Renal cell carcinoma	33	7	0,0008738
FoxO signaling pathway	56	8	0,0009683
Gastric cancer	63	8	0,0011491
Long-term potentiation	33	7	0,0011491
Oocyte meiosis	54	7	0,00115217
Ras signaling pathway	87	7	0,00135452
Relaxin signaling pathway	55	8	0,00135452
Proteoglycans in cancer	80	8	0,00184315
Small cell lung cancer	42	8	0,00201071
cAMP signaling pathway	81	7	0,00269295
Hepatocellular carcinoma	66	7	0,00269295
AMPK signaling pathway	51	8	0,00294364
Pancreatic cancer	34	7	0,00294364
Regulation of actin cytoskeleton	80	7	0,00294364
Longevity regulating pathway	43	8	0,00294364

Apelin signaling pathway	54	7	0,00315493
Hepatitis C	64	7	0,00347188
Wnt signaling pathway	64	8	0,00347188
Circadian rhythm	17	6	0,00347188
Chronic myeloid leukemia	34	8	0,00348135
Prostate cancer	41	7	0,00403997
Morphine addiction	41	7	0,00403997
Bacterial invasion of epithelial cells	34	8	0,0043091
mRNA surveillance pathway	43	7	0,00466672
Neurotrophin signaling pathway	48	7	0,00485655
Breast cancer	60	7	0,00514801
Choline metabolism in cancer	42	8	0,00566999
Oxytocin signaling pathway	59	7	0,00619503
Growth hormone synthesis, secretion and action	49	8	0,00650365
Prolactin signaling pathway	31	7	0,00654006
Sphingolipid signaling pathway	50	8	0,00731063
Protein digestion and absorption	44	7	0,00746384
MAPK signaling pathway	108	8	0,007767
Protein export	13	6	0,00836678
Nicotine addiction	20	7	0,00865909
Platinum drug resistance	31	7	0,00999843
cGMP-PKG signaling pathway	62	8	0,01048413

We then checked the expression abundance in the cerebral tissue of identified 8 miRNAs using the miRNA Tissue Atlas database (https://www.ccb.uni-saarland.de/tissueatlas2): hsa-miR-29c-3p, hsa-miR-451a and hsa-miR-342-3p were among the 100 miRNAs more abundant in the brain, indicating their potential involvement in key neural processes.

This miRNomic study was conducted as a preliminary analysis in order to identify a panel of candidate miRNAs, potentially involved in the molecular underpinnings of BPD pathophysiology, for further extended replication studies. Their expression will be evaluated

in the whole group of 50 BPD subjects and 26 controls to confirm their modulation and to further understand their implication in BPD mechanisms; indeed, the wider sample size will allow to evaluate the possible relationships between these miRNAs and specific BPD symptomatological features. Moreover, we plan to investigate the levels of these miRNAs also during psychotherapy, with the aim to evaluate their possible involvement in the molecular mechanisms underlying the efficacy of this therapeutical approach.

DISCUSSION

The studies here presented aimed to investigate the role of different molecular mediators, mainly selected on the basis of their involvement in key biological processes altered in BPD, both in relation to the presence of the disease, as well as to its clinical and neurobiological features, and in regard to the effects of psychotherapeutical interventions.

The obtained results indicated that, for some of these markers, peripheral concentrations were altered in BPD patients compared to non affected controls and also correlated to some specific clinical characteristics and neuroimaging hallmarks; moreover, some longitudinal modulations in their levels have been observed during the course of psychotherapy and in some cases are in correlation with changes in clinical scores and neuroimaging features. All the relevant results are discussed in the following paragraphs.

Oxytocin

Results obtained from OXT plasma dosages indicate that OXT concentrations are decreased in patients with BPD compared to controls, corroborating previous findings (Carrasco et al., 2020; Ferreira & Osório, 2022; Mielke et al., 2023). At the baseline, we did not evidence any correlation between OXT plasma levels and BPD symptomatology or emotional dysregulation as measured by the ZAN-BPD scale and the DERS scale, respectively. However, a negative correlation was found between baseline OXT levels and ASQ - Preoccupation with Relationships (ASQ-PR) scores suggesting that this attachment pattern may be somehow linked to the neuropeptide alterations. Research consistently demonstrates that OXT plays a crucial role in the formation and maintenance of attachment bonds, both in early developmental stages and throughout life (Buchheim & Diamond, 2018; Sharma et al., 2020). Previous evidence has shown that plasma OXT shows significantly lower levels in BPD patients, and especially in those who have a disorganized attachment style (Bertsch et al., 2013; Carrasco et al., 2020; Diaz-Marsá et al., 2024; Ferreira & Osório, 2022; Jobst et al., 2016; Mielke et al., 2023). Recent studies have shown that exogenous administration of OXT (for example delivered through methods such as intranasal sprays) can significantly ameliorate attachment-related behaviors (Bernaerts et al., 2017; K. Zhang et al., 2021).

In this study, no significant correlation was found between childhood adversity and plasma OXT levels, a finding that contrasts with the results obtained by Mielke et al. (Mielke et al., 2023), who reported an association between low OXT concentration and adverse childhood experiences in a larger sample of women exhibiting BPD traits, across a large age span (12-50 years), suggesting that early-life trauma influences the oxytocinergic system. Another study conducted in this field found a negative correlation between OXT levels and the extent of a childhood history of trauma – lower levels of OXT corresponded to higher scores of self-reported experience of childhood trauma as measured with the Childhood Trauma Questionnaire (Bertsch et al., 2013). A possible explanation for this discrepancy could be the difference in age and clinical characteristics between the different study populations.

Moreover, no significant difference was observed in baseline OXT levels between drug-free patients and those undergoing pharmacological treatment, suggesting that the BPD-related reduced OXT levels found in the study are more likely linked to the underlying pathology of the disorder itself rather than to an effect of medication. However, the possibility of confounding factors affecting these results cannot be entirely ruled out, and further controlled studies are needed to clarify these potential influences.

These findings are consistent with previous studies conducted on plasma samples, suggesting that dysregulation in the oxytocinergic system may play a central role in the pathogenesis of BPD. A reduction in OXT receptor (OXTR) expression in blood mononuclear cells has been documented in BPD patients (Carrasco et al., 2020; Diaz-Marsá et al., 2024), further supporting this hypothesis, and genetic studies suggested that the OXTR gene may be a potential contributor to BPD susceptibility. Variations in the OXTR gene can interact with family dynamics (Hammen et al., 2015) and childhood maltreatment (Flasbeck et al., 2018; M. Zhang et al., 2020).

Remarkably, this study is the first to report an increase in plasma OXT levels during psychotherapeutic treatments, particularly those specifically focused on BPD, that correlated with symptom improvement: indeed, a positive correlation was observed between the increase in OXT levels and the decrease in DERS and ZAN scores. This finding suggests that these therapies may exert their effects also modulating the oxytocinergic system, helping to normalize its functioning. In the context of the CLIMAMITHE clinical trial (Rossi et al., 2023) a

near-significant difference in amygdala activation between BPD patients and controls was observed in response to emotional stimuli. Moreover, the trial demonstrated that both MIT and SCM therapies reduced this right amygdala activation, which is noteworthy considering the involvement of the amygdala in emotion processing and its implication in emotional dysregulation in BPD (Schmahl et al., 2014). Based on these results, it can be hypothesized that the observed changes in amygdala function and the therapeutic effects of psychotherapy may be mediated by changes in OXT expression. Studies conducted in rodent models have shown that oxytocinergic projections to the amygdala are critical for emotional state discrimination (Ferretti et al., 2019). In humans, higher endogenous OXT levels have been linked to reduced amygdala volume and decreased activation in response to negative and threatening stimuli (Lancaster et al., 2018). Similarly, intranasal OXT treatment has been shown to attenuate amygdala hyperactivation in BPD patients (Lischke et al., 2017), further underscoring the potential role of OXT in modulating amygdala responses.

The biological mechanism that underpins the efficacy of psychotherapy remain limitedly explored, yet emerging evidence suggests the role of hormones like OXT in successful treatment outcomes (Fischer & Zilcha-Mano, 2022). While most of the research on neuroendocrine markers in psychotherapy has focused on major depression, important findings have begun to shed light on how OXT may influence therapeutic success across other mental health disorders. For instance, studies have shown that low OXT levels are correlated with reduced symptom improvement following cognitive behavioral therapy in depressed patients (Jobst et al., 2018). This suggests that lower OXT levels may hinder the effectiveness of therapy. Moreover, OXT reactivity in response to interventions has been associated with greater treatment improvement in patients with depression (Atzil-Slonim et al., 2022). Notably, the therapist's OXT response may also mediate treatment outcomes (Fisher et al., 2023), suggesting that the hormonal dynamics between therapist and patient, in this case the endogenous release of OXT, may enhance synchronization during sessions and therapeutic alliance, thereby increasing treatment effectiveness (Palmieri et al., 2021). Other studies have highlighted the importance of OXT synchrony between patients and therapists in the context of psychodynamic therapy. Specifically, interpersonal difficulties and lower levels of OXT synchrony have been linked to poorer treatment outcomes, suggesting that OXT may play a crucial role in fostering empathic connections and therapeutic alliance, both of which are

central to psychotherapy effectiveness (Zilcha-Mano et al., 2021). In the context of BPD, preliminary research indicates that human and animal-assisted skills training may increase salivary OXT levels, although these findings are based on a small sample and did not reach statistical significance (Plett et al., 2023).

Our data confirm a dysregulation of OXT in BPD patients, as well as the positive regulatory effect of long-term psychotherapy on the oxytocinergic system, supporting the hypothesis that OXT is implicated in both the pathology and treatment of BPD. The fact that psychotherapy stimulates OXT release suggests that these interventions may enhance social interactions and emotional regulation by modulating the activity of brain regions such as the amygdala, which is heavily involved in these functions. Moreover, the increase in OXT levels following psychotherapy could play a crucial role in managing core symptoms of BPD, including impulsivity and self-destructive behaviors. Given the role of OXT in promoting prosocial behaviors and inhibiting impulsive actions, its increase during the treatment could help patients to improve overall interpersonal functioning and reducing impulsive behaviors. Finally, plasma OXT increase following psychotherapy negatively correlated with the decrease in the volume of the left anterior-inferior area of the hypothalamus. This region encompasses the suprachiasmatic nucleus (SCN) and the supraoptic nucleus (SON). The SCN controls the endogenous circadian rhythm, and emerging evidence shows that dysfunctions of the SCN and the consequent sleep disruptions may play an important role in clinical manifestations of BPD. The SON is involved in the regulation and release of OXT and Vasopressin (VP), which once again supports the hypothesis that the therapeutic effects of psychotherapy may be mediated by changes in the OXT system.

The relationships between the oxytocinergic system, the pathogenesis of BPD and the outcomes of psychotherapy are complex, and future studies addressing these aspects are needed to clarify the underlying causal mechanisms.

β-endorphin

Based on the fact that several studies have indicated that some of the core symptoms of BPD may be related to a dysregulation of the endogenous opioid system, and that β-endorphin is implicated in biological processes dysregulated BPD, like emotional regulation and stress response (Pilozzi et al., 2020), we investigated serum β-endorphin levels in BPD patients compared to controls. However, we found no statistically significant difference between the two groups, and no relationships of β-endorphin concentrations with clinical features of the disease.

To date, this is the only study that evaluated serum β-endorphin concentrations in BPD patients. However, several studies have focused on peripheral β-endorphin levels in individuals exhibiting Non Suicidal Self Injury (NSSI), a behavior frequently observed in BPD patients. These investigations, some of which conducted also in the context of other psychiatric disorders, have reported lower concentrations of this opioid, both in plasma and serum, in subjects showing NSSI (Cakin Memik et al., 2023; Stanley et al., 2010; van der Venne et al., 2021). Moreover, Van der Venne and colleagues linked lower levels of β-endorphin to increased pain thresholds and lower pain intensity in NSSI subjects; this greater pain threshold correlated positively with borderline personality disorder (BPD) symptoms (van der Venne et al., 2021). When comparing BPD patients with and without NSSI in our sample cohort, we did not observe any differences in β-endorphin levels. This is also the first study that has investigated possible β-endorphin modulation in response to psychotherapies, evidencing no significant result. Although β-endorphin concentrations in this study did not significantly vary during the treatment course, a positive correlation was evidenced between increases in β-endorphin levels and increases in the volume of the CA3 hippocampal region, which was significantly enhanced by psychotherapy. CA3 is a subfield of the hippocampus that plays a specific role in rapid memory recall and encoding (Chiang et al., 2018) and that previously has been described as decreased in volume in patients with BPD (Bøen et al., 2014) and altered by environmental stressors (McEwen, 2002; Teicher et al., 2012; Wang et al., 2010). The observed correlation between variations in this brain region and β-endorphin levels could thus be linked to the role played by this endogenous opioid in memory modulation (Netto, 2022)

The heterogeneity of BPD symptoms and the complexity of self-injurious behaviors are problematic aspects to consider while investigating the role of β-endorphin. The dysregulation in this neuropeptide (and more generally in the opioid system) might occur in close time proximity to acute stress events, rather than being detectable in resting states. This is suggested, for example, by studies conducted in subjects showing repetitive NSSI, in which the levels of β-endorphin were lower immediately before NSSI acts and higher immediately after (Störkel et al., 2021). Therefore, the fluctuations of β-endorphin might be dynamic and complex to observe. Moreover, serum levels of β-endorphin might not reflect the activity within the brain, failing to detect possible brain-specific alterations. Opioids dysregulation also might be related to other mechanisms, including modulation of μ-opioid receptor, rather than β-endorphin, as indicated for example by a study in which altered levels of μ-opioid receptor binding potential were observed in different brain areas in BPD patients compared to controls (Prossin et al., 2010).

Brain-Derived Neurotrophic Factor

The results of our study evidenced higher serum BDNF levels at the baseline in BPD patients compared to controls. As reported in a recent meta-analysis, for the majority of psychiatric disorders, including major depressive disorder, schizophrenia, bipolar disorder, generalized anxiety disorder, obsessive-compulsive disorder and panic disorder, lower peripheral BDNF levels have been observed, whereas for post-traumatic stress disorder the concentration of this neurotrophin was increased (Zou et al., 2024). Concerning BPD, to date only two studies have been conducted. Perroud and colleagues, in line with our results, described higher concentrations of BDNF in BPD patient plasma samples accompanied by higher methylation status of the BDNF promoter. This relationship was unexpected, since an increased methylation of the BDNF promoter has been repeatedly associated with reduced expression levels of BDNF, coherently with the fact that methylation typically suppresses gene expression. Moreover, in this study a brief (4 weeks) course of psychotherapy, consisting in particular in intensive dialectical behavioral therapy, induced a decrease in BDNF protein levels over time which was inversely associated with treatment response (Perroud et al., 2013). Although we did not observe a significant modulation of BDNF during the course of psychotherapy, this

finding suggests that psychotherapeutical interventions, at least at particular time intervals, could exert modulatory effects on the heightened levels of BDNF observed in BPD patients. Another study was conducted by Koenigsberg and colleagues evaluating BDNF levels in platelets and evidencing, on the contrary, a reduced content of this neurotrophin in BPD patients compared to controls (Koenigsberg et al., 2012). Although we did not observe a difference in BDNF levels when comparing patients under treatment with antidepressants versus those who were not, it cannot be excluded that the heightened levels of BDNF observed in our study and in the one by Perroud (Perroud et al., 2013) could depend on the effects of antidepressants, which are known to heighten BDNF concentrations (Madsen et al., 2024), also in combination with possible effects of other kinds of psychotropic drugs concomitantly administered; further studies in larger sample cohorts are necessary to clarify this aspect.

If confirmed, increased BDNF levels in BPD patients could be explained by its role in overconsolidation of traumatic memories. As already mentioned, patients with PTSD show increased levels of BDNF (Zou et al., 2024); Hauck and colleagues showed that PTSD patients who recently experienced trauma had significantly higher serum BDNF levels compared to healthy controls, regardless psychotropic medication, or past psychiatric history. BDNF levels decreased as time passes since the traumatic event: after a year, patients' BDNF levels were comparable to those of the control group, suggesting that BDNF overexpression could be more likely a short-term response to trauma, rather than a chronic response (Hauck et al., 2010). However, another study conducted on patients with chronic PTSD highlighted higher serum BDNF levels that persisted over time, as seen in a follow-up evaluation of approximately three years (Wu et al., 2021). Taking into account these results, and also considering the role of BDNF in memory processes, and particularly in the strengthening of synaptic connections, BDNF might play a role in the excessive consolidation (immediate and long-term) of adverse memories linked to trauma; higher levels of this neurotrophin, in this pathological condition, could exacerbate the symptoms linked to trauma by reinforcing the traumatic memory associations, rather than alleviating them. Early life traumas (emotional or physical trauma, abuse, neglect, abandonment…) are one of the main environmental factors that increase the risk of developing BPD. A similar mechanism of heightened memory consolidation mediated by BDNF might be involved in BPD biological underpinnings. Instead of promoting "healthy" neuroplasticity, BDNF might be involved in maladaptive plasticity, reinforcing the traumatic

memories and contributing to the development of BPD symptoms. The overexpression of BDNF could also be explained as a compensatory attempt to counteract the neurobiological disruptions caused by the disorder, trying to promote neural repair and improve plasticity. As previously reported, BDNF plays a key role in stress response. BDNF upregulation might suggest the development of a compensatory neuroplastic response to chronic stress and emotional trauma as the brain attempts to reverse or block the effects of stress, maintaining neural plasticity and resilience, and might therefore play a role in helping with the consolidation of traumatic memories, extinction learning (learning to dissociate trauma from triggers), and reconsolidation (modifying the emotional weight of the memories). BPD-related BDNF modulations suggest the existence of complex mechanisms, involving neuroplasticity changes, underlying the pathophysiology of the disorder, leading to the possibility of developing through further research a deeper comprehension of the biological processes that may contribute to the development and symptoms of BPD. Considering its relationship with neuroimaging features, BDNF baseline levels positively correlated with the baseline fractional anisotropy of the ventral default mode network (FA-vDMN). The DMN is a network of interacting brain areas which are generally activated during moments of rest by specific activities. The distribution of the cerebral areas belonging to this network may vary among different individuals, but can be generally traced back to some major areas such as the hippocampus, the hippocampal gyrus, the medial prefrontal cortex, the lateral and parietal temporal regions, and the medial posterior cortex (Immordino-Yang et al., 2012). The activation of the DMN seems to be associated with mind wandering, remembering past experiences, thinking about other people's mental states, imagining the future and processing language. Menon et al. hypothesized that these functions could be all connected and useful to help individuals reflect on who they are in relation to others and to recall past experiences, synthesizing these aspects into a coherent self-narration (Menon, 2023). At this regard BPD patients show lower DMN functional connectivity, which is also positively correlated with the anger-state intensity and expression (Quattrini et al., 2019). The same group also found an impairment in the same network, specifically concerning the mean diffusivity (a marker of microstructural axonal organization damage) of the dorsal DMN. Increased diffusivity, corresponding to increased axonal damage, were more pronounced in individuals with higher

behavioral dysregulation (suicidal attempting, self-harm, and aggressiveness) (Quattrini et al., 2022).

BDNF at the baseline was also positively correlated with baseline fractional anisotropy of the left executive control network (L-ECN). The L-ECN is a network that includes areas like the dorsolateral prefrontal cortex (DLPFC) and the lateral posterior parietal cortex (PPC) which are generally involved in executive functions like emotion regulation, paying attention and working memory. More specifically, the L-ECN is primarily involved in cognitive and language paradigms (Edinoff et al., 2022). Impairments in the ECN are hypothesized to play a significant role in BPD, affecting both cognitive functions and behavioral regulation. Previous studies showed a decreased functional connectivity in ECN of BPD patients (Doll et al., 2013; Wolf et al., 2011). Quattrini et al. also found ECN abnormalities in BPD patients: specifically, they found that increased mean diffusivity in ECN was linked to more pronounced behavioral dysregulation, suicidal attempt, self-harm, and aggressiveness.

Concerning both its relationship with the DMN and the SCN, BDNF is closely linked to preserved white matter integrity, and therefore to FA: research show that higher serum BDNF levels correlate with increased FA in several white matter regions, whose integrity was associated with preserved and better executive functions, suggesting a protective role against cognitive decline vascular brain injury (Maillard et al., 2016). This interplay highlights the importance of BDNF in maintaining white matter integrity and its implications for neuropsychiatric health and recovery processes. These studies are coherent with the correlation that we observed in our cohort of patients, as higher levels of BDNF correlated with higher FA, supporting the protective role of BDNF on white matter integrity, and further suggesting the possible development of a compensatory response to the disruption and stress-related damages involved in BPD, in the attempt to promote neuroplasticity and neural repair.

miRNAs

Regarding the results about miRNA expression levels in serum samples, the comparison between BPD patients and controls revealed a down-regulation of three miRNAs (hsa-miR-132-3p, hsa-miR-342-3p and hsa-miR-29c-3p) and an up-regulation of five miRNAs (hsa-miR-

409-3p, hsa-miR-543, hsa-miR-223-3p, hsa-miR-382-5p, and hsa-miR-451a). These findings suggest a potential biological relevance of these miRNAs in the pathophysiology of BPD. Three of the miRNAs differentially expressed (hsa-miR-29c-3p, hsa-miR-451a, and hsa-miR-342-3p) were among the top 100 most abundant in the brain, suggesting that they might be involved in critical neurobiological processes within the central nervous system, including cerebral functions linked to BPD neuropathology.

miRNA target gene prediction and KEGG pathway enrichment analysis suggest their effect on biological mechanisms possibly implicated in BPD, like the Oxytocin and the neurotrophic signaling or other pathways involved in neurotransmission (cholinergic, glutamatergic, and dopaminergic synapses) and influencing synaptic transmission and connectivity (axon guidance, long-term depression and long-term potentiation). Moreover, many target genes may play a role in the pathophysiology of BPD and are known to regulate processes relevant to mood regulation, stress response, emotional dysregulation, and cognitive functions such as BDNF, OXTR, FKBP5 (FKBP Prolyl Isomerase 5), HTR1A (5-hydroxytryptamine receptor 1A), MAOA (monoamine oxidase A), SLC6A4 (solute carrier family 6 member 4), and TPH1 (tryptophan hydroxylase 1). More in detail, BDNF is targeted by three miRNAs (hsa-miR-409-3p, hsa-miR-382-5p, hsa-miR-543), all of which are up-regulated in BPD patients, and two miRNAs (hsa-miR-409-3p and hsa-miR-29c-3p) potentially target OXTR; based on the previously discussed evidence, this finding further supports an involvement of BDNF and oxytocinergic system in BPD.

Another interesting gene is FKBP5, that mediates stress through the negative regulation of the HPA axis(Malekpour et al., 2023). Genetic variants in this gene have already been associated with BPD, particularly in the context of childhood trauma (Amad et al., 2019), and studies in animal models have shown that stress exposure induces a dramatic increase of FKBP5 expression in many brain regions (Scharf et al., 2011). Our target gene analysis indicated that FKBP5 is predicted to be targeted by one miRNA, miR-223-3p, which was up-regulated in our BPD patient sample (this was the highest up-regulation among the eight identified miRNAs). An up-regulation of miR-223-3p could therefore induce a down-regulation of FKBP5, altering stress sensitivity.

The serotonergic system also emerged as a potentially modulated process, with several miRNAs targeting genes related to serotonin regulation: HTR1A (Serotonin 1A receptor), targeted by the up-regulated hsa-miR-223-3p, and SLC6A4 (Serotonin transporter gene), targeted by the down-regulated hsa-miR-342-3p. Serotonergic dysfunctions have been associated with BPD: in particular, reduced 5-HT1A receptor sensitivity has been linked to impulsivity and suicidal behavior in this disorder (Joyce et al., 2013), and the HTR1A G allele variant has been linked with an increased risk of BPD, highlighting its role in the disorder etiology (Hansenne et al., 2002). Furthermore TPH1 (Tryptophan hydroxylase 1), targeted by the up-regulated hsa-miR-382-5p, has also been linked to serotonergic dysfunction in BPD, reinforcing the impairment in decision-making observed in this disorder and also possibly being linked to suicidal behaviors (Maurex et al., 2009). Polymorphisms in TPH1 have been associated with the diagnosis of BPD, also in interaction with a childhood abuse history (Wilson et al., 2009, 2012).

Finally, genetic variations in Monoamine oxidase A (MAOA), targeted by hsa-miR-132-3p (down-regulated) and hsa-miR-543 (up-regulated), are implicated in BPD, particularly concerning impulsivity and aggressivity (Kolla & Vinette, 2017).

Overall, these results support the choice of these miRNAs as promising candidate for further studies in BPD. Notably, the regulation of miRNA in BPD is quite unexplored and this is the first study that focuses on serum miRNA: this biological matrix offers unique insight about several processes happening throughout the body, often distinct from what is observable in whole blood, which contains a large amount of cellular RNA. Serum is also an ideal matrix due to its easy collection. Altered expression of miRNAs in serum could serve as an indicator of pathological brain changes involved in psychiatric disorders (Roy et al., 2023). As previously said, circulating miRNAs are either enclosed in microvesicles (exosomes, microparticles, apoptotic bodies) or exist as free miRNAs bound to proteins and lipoproteins. Furthermore, serum miRNAs are highly stable and resistant to degradation by endogenous RNase activity (Weber et al., 2010).

CONCLUSION

This study explored the role of molecular markers as Oxytocin (OXT), β-endorphin, and Brain-Derived Neurotrophic Factor (BDNF), hypothesized to have a key role in BPD psychopathology, investigating their relationship within the disease, its clinical features and the effects during treatment with psychotherapies. We evidenced in particular an involvement of Oxytocinergic system, as patients had lower plasma OXT levels compared to controls. Notably, psychotherapies had a positive modulatory effect on OXT levels, which decreased after a year and moreover correlated with improvements in emotional regulation and BPD symptoms reduction, suggesting that psychotherapies effects may exert their positive effect by modulating OXT. The study also reported higher baseline serum BDNF levels in BPD patients, suggesting that increased BDNF might contribute to the disorder acting perhaps on the maladaptive consolidation of traumatic memories and influencing white matter integrity in brain networks known to be involved in BPD. However, psychotherapy did not induce modulations in BDNF levels. No alteration in β-endorphin levels was found in BPD patients, nor modulation during psychotherapies, although we observed a correlation between changes in this neuropeptide and volumetric changes in the hippocampal CA3 region volumes, which plays a role in memory processes. We also conducted a first exploratory miRNomic investigation about the involvement of these small regulatory RNAs in BPD, identifying promising candidates for future studies.

Overall, these findings contribute to gain insight into the biological underpinnings of BPD, providing potential novel molecular markers that may be useful for the improvement of the differential diagnosis and the optimization of therapeutic treatments for BPD.

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