

# The predictive role of proinflammatory and neuroendocrine factors in the acute phases of bipolar disorder: study protocol of a longitudinal controlled trial

MATTEO DI VINCENZO<sup>1</sup>, MARIO LUCIANO<sup>1</sup>, BARBARA COLLACCHI<sup>2</sup>, GIANMARCO DE FELICE<sup>1</sup>, LETIZIA GIONA<sup>2</sup>, ELENA PIEGARI<sup>1</sup>, IRENE RUSPANTINI<sup>3</sup>, ANTONIO VOLPICELLI<sup>1</sup>, FRANCESCA CIRULLI<sup>2</sup>, ANDREA FIORILLO<sup>1</sup>, ALESSANDRA BERRY<sup>2</sup>

<sup>1</sup>Department of Psychiatry, University of Campania Luigi Vanviteli, Naples, Italy; <sup>2</sup>Center for Behavioural Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy; <sup>3</sup>Core Facilities, Istituto Superiore di Sanità, Rome, Italy.

**Summary. Introduction and aims.** Bipolar disorder (BD) is a severe and recurring mental illness associated with a significant personal and social burden. It has been recently hypothesized that increased levels of pro-inflammatory cytokines and cortisol, which is also associated with a reduced expression of the brain-derived neurotrophic factor (BDNF), may influence affective recurrences in BD. Our study aims to: 1) assess changes in the levels of peripheral cytokines, BDNF and salivary cortisol during acute and euthymic phases of bipolar disorder, compared to that of a sample of healthy controls; 2) evaluate whether these changes represent a biosignature for the different phases of the illness. **Materials and methods.** Patients aged 18-65 years old, with a diagnosis of BD I or II types, will be enrolled during an acute episode, according to DSM-5 criteria, together with age- and gender-matched healthy controls. Blood and salivary samples will be collected at baseline and after 3 and 6 months. Validated assessment instruments will be administered to all participants for the evaluation of symptom severity, global functioning, suicidal risk, stress levels and physical comorbidities. **Expected Results.** We expect changes in inflammatory and neuroendocrine indices to be predictive of the onset of an acute phase of bipolar disorder and that overall levels of cytokines, cortisol and BDNF are overall significantly different between BD patients and healthy controls. **Conclusions.** The longitudinal design of the study will allow to assess whether the presence of acute affective symptoms in BD patients correlates with significantly higher levels of cytokines and salivary cortisol and with reduced BDNF levels compared to euthymic phases. Moreover, the comparison with healthy control subjects will allow to understand if inflammatory mediators as well as the hypothalamic-pituitary-adrenal (HPA) axis are chronically elevated in BD patients and are independent from mood swings.

**Key words.** BDNF, bipolar disorder, cortisol, cytokines, inflammation, longitudinal.

*Il ruolo predittivo dei fattori pro-infiammatori e neuroendocrini nelle fasi acute del disturbo bipolare: protocollo di uno studio longitudinale.*

**Riassunto.** Introduzione e scopo. Il disturbo bipolare (DB) è un disturbo mentale grave e ricorrente, associato a elevati costi personali e sociali. Numerosi studi recenti hanno suggerito un possibile ruolo dell'infiammazione nell'influenzare il decorso a lungo termine del disturbo. Questo studio si propone di valutare se i livelli di citochine, cortisolo e BDNF si modificano nelle diverse fasi di malattia (eutimia vs. fasi acute) rispetto a un gruppo di controlli sani e se una loro alterata espressione possa costituire un biomarcatore complesso del DB. **Metodi.** Verranno reclutati pazienti di età compresa tra 18 e 65 anni con diagnosi di DB di tipo I o II, arruolati durante un episodio acuto di malattia secondo i criteri del DSM-5, e controlli sani appaiati per età e sesso. Al reclutamento, dopo 3 e 6 mesi, verranno raccolti campioni di sangue per la valutazione dell'emocromo, delle principali citochine e chemochine, e campioni di saliva, per la valutazione della variazione del cortisolo al risveglio. Inoltre, verranno somministrati numerosi questionari per la valutazione dei livelli di gravità dei sintomi psichiatrici, del funzionamento psicosociale, del rischio suicidario, dei livelli di stress e delle comorbidità fisiche. **Risultati attesi.** Ci aspettiamo che le variazioni degli indici infiammatori possano avere un ruolo predittivo rispetto all'insorgenza degli episodi attivi di malattia e che i livelli di citochine, cortisolo e BDNF siano significativamente diversi tra i pazienti affetti da DB e i controlli sani. **Conclusioni.** Il disegno longitudinale dello studio consentirà di valutare se il pattern infiammatorio e immuno-neuroendocrino si modifica nei pazienti nelle differenti fasi del disturbo bipolare. Inoltre, il reclutamento di controlli sani consentirà di valutare se l'aumento degli indici infiammatori nei pazienti con DB sia dovuto alle fasi acute di malattia o sia presente anche nelle fasi di eutimia.

**Parole chiave.** BDNF, citochine, cortisolo, disturbo bipolare, infiammazione, studio longitudinale.

## Introduction

Bipolar Disorder (BD) is a chronic, recurring, heterogeneous and severe mental illness, affecting more than 1% of the population worldwide<sup>1</sup>, with a lifetime prevalence of 2.4%<sup>2</sup>. BD is associated with cognitive<sup>3,4</sup> and functional<sup>5</sup> disability and with a significant personal and social burden<sup>6,7</sup>. Despite the availability of effective treatment strategies, most patients do not achieve a full functional recovery<sup>8</sup>, mainly because of persistency of subthreshold symptoms<sup>1,9,10</sup>, high rates of treatment resistance<sup>11</sup> and of suicide<sup>12,13</sup>, delays in appropriate diagnosis<sup>14</sup>. The identification of reliable biomarkers might allow for an early and timely diagnosis of BD and for the development of interventions aimed at improving the personalized management of affective episodes as early as possible<sup>15</sup>.

In recent years, there has been a growing interest towards the role played by the immune system and inflammatory pathways in the pathophysiology of mental disorders<sup>16</sup> and of BD<sup>17</sup>. Apart from the high levels of comorbidity between several inflammatory diseases and BD<sup>18-21</sup>, which significantly reduces patients' life expectancy and quality of life<sup>22</sup>, immune system dysfunction is likely to be involved in the pathophysiology of BD with a multidirectional pattern of interaction<sup>23</sup>. In fact, when compared with healthy subjects, patients suffering from BD show increased serum levels of proinflammatory cytokines (in particular, IL-4, TNF- $\alpha$ , soluble IL-2 receptor, IL-1 $\beta$ , IL-6, soluble receptor of TNF- $\alpha$  type 1)<sup>24</sup> and C-reactive protein (CRP)<sup>25</sup> specifically varying according to the type of acute mood episode<sup>26</sup>. Moreover, increased levels of proinflammatory cytokines, in particular IL-6, during a depressive phase, are associated with a more frequent transition to mania<sup>27</sup>. On the other hand, during the euthymic phases of the disorder PET studies have detected an increased activation of hippocampal microglia, which represents the first and the main form of active immune defense in the central nervous system, as an index of neuroinflammation<sup>28</sup>, whilst a higher expression of pro-inflammatory signals has been found in the peripheral blood monocytes<sup>29</sup>. An association between levels of proinflammatory cytokines and cognitive impairment has been also reported in BD patients<sup>30</sup>. It is worth noticing that pro-inflammatory cytokines may modulate the biosynthesis of monoamines such as dopamine, noradrenaline, adrenaline and serotonin, strengthening the hypothesis of a link between inflammatory state and the mood state<sup>31</sup>.

Immune function is strictly and bidirectionally related to that of the hypothalamic-pituitary-adrenal (HPA) axis, which is involved in the etiopathogenesis of many psychiatric disorders, including BD<sup>32</sup>. Numerous cytokines may increase the secretory activity of the

HPA axis and are in turn inhibited by glucocorticoids (GCs)<sup>33</sup>. Secretion of GCs is increased by the HPA axis as a result of stressful environmental conditions: in the short-term this increase is essential for the maintenance of homeostasis, while in case of prolonged stress conditions, hypercortisolism might redirect metabolic, inflammatory/immune, neuroendocrine and behavioral responses<sup>34</sup>. A recent meta-analysis has revealed that the levels of morning cortisol are increased in BD patients compared to healthy controls<sup>35-37</sup>, with important clinical implications in terms of relapses, treatment resistance, functional impairment and cognitive deficits<sup>38,39</sup>. There is evidence that hypercortisolism may be central to the pathogenesis of depressive symptoms and cognitive deficits<sup>40</sup>. Manic episodes may be preceded by increased cortisol levels, leading to cognitive problems and functional impairment<sup>38</sup>. Moreover, hypercortisolism could increase treatment resistance and relapses, worsening the outcome and determining cognitive deficits in BD patients<sup>38,41</sup>. Chronically elevated levels of glucocorticoids can greatly interfere with the production of Brain-Derived Neurotrophic Factor (BDNF)<sup>42,43</sup>, which plays a pivotal role in neurodevelopment and brain plasticity (including the glutamatergic and GABAergic pathways), in the modulation of serotonergic and dopaminergic neurotransmission as well as of cognitive, behavioral, emotional and metabolic processes<sup>44,45</sup>. Interestingly, by freely crossing the blood-brain barrier, BDNF presents a blood concentration which reflects its levels in cerebrospinal fluid<sup>46</sup>. According to the "neurotrophic hypothesis" - which postulates that stress would lead to reduced expression of this neurotrophin and to hippocampal atrophy<sup>47,48</sup> - it has been suggested that lower levels of peripheral BDNF expression could represent a potential biosignature in BD patients<sup>49</sup>. In addition, a meta-analysis involving 52 studies and more than 6,000 participants has documented the fluctuating course of peripheral BDNF levels during BD phases (i.e., lower levels during the acute phases and increased levels during euthymic phases of the disorder), further suggesting that BDNF could represent a potential "biomarker of disease activity" rather than being "trait-related"<sup>50</sup>.

Although available evidence highlights a strong association between inflammation, HPA axis activation, low BDNF levels and BD, most studies in this field have important methodological limitations, including small sample sizes, low statistical power and absence of comparisons with healthy subjects.

Furthermore, available evidence does not allow to clearly establish whether the inflammatory state and the activation of HPA axis are consistently elevated in subjects suffering from BD. Moreover, currently available data do not allow for a full elucidation of the complex relationship between course of BD and changes in peripheral inflammatory markers, HPA axis, BDNF, and peripheral blood cells. Available data on the dif-

ferences in biological concomitants among depressive, manic and hypomanic episodes in BD are still scattered, making difficult to draw firm conclusions. We hypothesize that similar alterations in the biosignature can be observed in either phases of the disease namely, depressive, manic or hypomanic episodes. To this regard, according to one of the most recent systematic reviews and meta-analyses<sup>51</sup>, the most relevant differences across the bipolar spectrum can be detected between acute and euthymic phases (rather than between depressive and manic/hypomanic phases).

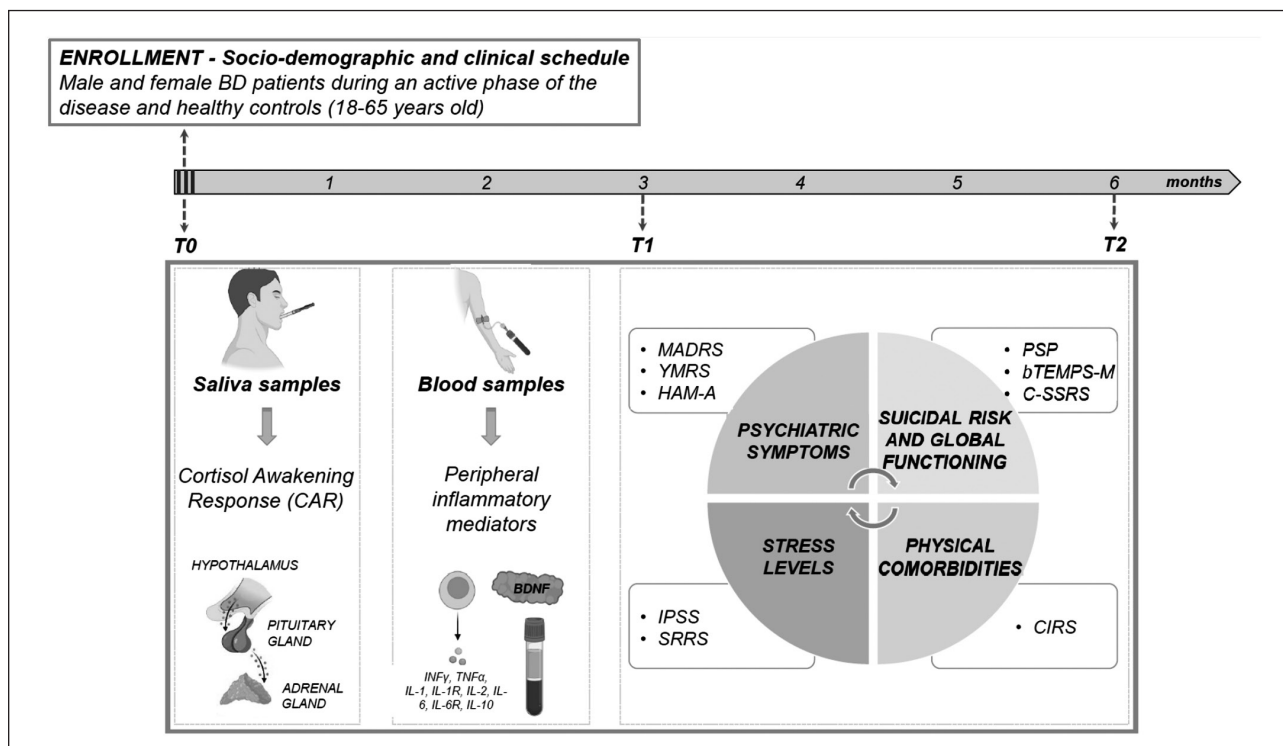
Aim of this study is to characterize a range of reliable peripheral biomarkers in order to identify a biosignature that might allow for future timely diagnosis and early intervention in BD patients during the acute phase of the disease. Moreover, this biosignature might be used to predict the occurrence of an acute phase of the disease leading to a more efficient management of the affective episodes. Taking advantage of a longitudinal approach, we will assess whether the presence of affective symptoms in BD patients is positively correlated with significantly higher levels of cytokines and salivary cortisol and reduced BDNF levels than during euthymia. In addition, the enrolment of control subjects will allow assessing whether the inflammatory state and the activation of the HPA axis in BD patients are constantly elevated.

More specifically, the primary aim of our study will be the assessment of differences between patients and healthy controls in the maximum variation of cytokines, salivary cortisol and BDNF levels across three time points (T0, T1 and T2). In patients, the variation would reflect the maximal change from the acute and the euthymic phase. In healthy controls, it would represent the maximal variation due to seasonality. Secondary aims will include the comparison of the differences between mean values (across the three time points) of cytokines, salivary cortisol and BDNF levels of healthy controls and patients during acute (T0) and euthymic phases (T2). Mean values in primary outcomes across three time-point in healthy subjects are calculated in order to minimize their physiological variation due to seasonality.

## Materials and methods

### DESIGN

This is a longitudinal controlled study, funded by the Istituto Superiore di Sanità (Rome, Italy), including both subjects with BD during active phases of the disease and healthy controls, enrolled at the Department of Psychiatry of University of Campania “Luigi Vanvitelli” in Naples (figure 1).



**Figure 1.** Study design.

**Legend:** BD= Bipolar Disorder; BDNF= Brain-Derived Neurotrophic Factor; INF $\gamma$ = Interferon  $\gamma$ ; TNF $\alpha$ = Tumor Necrosis Factor  $\alpha$ ; IL= Interleukin; MADRS= Montgomery-Asberg Depression Rating Scale; YMRS= Young Mania Rating Scale; HAM-A= Hamilton Anxiety Rating Scale; PSP= Personal and Social Performance Scale; b-TEMPS-M= Brief Temperament Evaluation in Memphis, Pisa and San Diego; C-SSRS= Columbia-Suicide Severity Rating Scale; IPSS= Italian Perceived Stress Scale; SRRS= Social Readjustment Rating Scale; CIRS= Cumulative Illness Rating Scale.

## DATA COLLECTION

### *Recruitment procedure*

Patients aged 18-65 years old, with a diagnosis of bipolar I or II disorder and experiencing an acute phase of the disease (i.e., depressive or manic/hypomanic episode) according to Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)<sup>52</sup>, will be recruited. The diagnosis will be formulated by expert psychiatrists, according to the and will be confirmed by the administration of the Structured Clinical Interview for DSM-5 (SCID-5)<sup>53</sup>. All patients will receive treatment as usual at the time of recruitment. At the same time, healthy controls, matched for age and sex with BD patients, will be recruited according to the following inclusion criteria: 1) absence of past and/or current diagnosis of any psychiatric disorder according to the DSM-5<sup>52</sup>; 2) absence of chronic inflammatory or other immune-mediated diseases that could significantly influence blood levels of biomarkers. Both patients and healthy subjects will be excluded in case of 1) pregnancy or breastfeeding; 2) presence of mood disorders due to a general medical condition or mood disorders not otherwise specified<sup>52</sup>; 3) inability to provide a written consent. Moreover, subjects suffering from acute or chronic systemic inflammatory diseases under prolonged treatment with steroidal and non-steroidal anti-inflammatory drugs or immunosuppressive, immunomodulatory or cytotoxic medications and those suffering from hypercortisolism, such as Cushing Syndrome, will be excluded as well. Patients with DSM-5 substance abuse disorders will be excluded from the study.

On the contrary, patients with occasional use of alcohol or substances will be recruited in order to have a better and more real characterization of enrolled patients, since acute use of alcohol or substances has a limited impact on the basal levels of inflammation.

### *Assessment*

According to the longitudinal design of the trial, assessments will be carried out both in patients and healthy controls at baseline (T0), and after 3 (T1) and 6 (T2) months from enrollment.

Blood and salivary samples at each time point of assessment will be collected both in BD patients and in healthy subjects. Blood sample will include: 1) complete blood count to collect total and percentages of white cells, lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR); 2) cytokines (IL-1, IL-2, soluble IL-2 receptor, IL-6, soluble IL-6 receptor, IL-10, TNF- $\alpha$ , IFN- $\gamma$ ); 3) BDNF. Blood specimens for the evaluation of cytokines and BDNF will be collected in serum clot activator tubes (VACUETTE). Samples will be centrifuged and stored at -80°C.

Salivary samples will be used to assess HPA axis function by measuring the cortisol awakening response (CAR). Participants will be instructed to refrain from eating, drinking (except water), smoking and brushing teeth until the sampling is completed. They will be asked to collect saliva samples at home on a working day, immediately upon awakening and after 30 and 60 min by holding a cotton swab in their mouth, slightly chewing it until it became soaked. At the end of the procedure the cotton swab will be sealed into an ad-hoc plastic tube (Salivette). At the end of the procedure, all subjects will store their saliva samples in home freezers (-20°C) before returning them to the lab, where saliva will be extracted from the swab by centrifugation and stored at -80°C. Cortisol concentrations will be measured by an enzyme immunoassay method, using a commercially available ELISA kit.

All recruited subjects will be assessed with the validated Italian versions of the following scales: 1) Montgomery-Asberg Depression Rating Scale (MADRS) to assess depressive symptoms<sup>54</sup>; 2) Young Mania Rating Scale (YMRS) to assess manic symptoms<sup>55</sup>; 3) Hamilton Anxiety Rating Scale (HAM-A) to assess anxious symptoms<sup>56</sup>; 4) Personal and Social Performance Scale (PSP) to assess global functioning<sup>57</sup>; 5) Brief Temperament Evaluation in Memphis, Pisa and San Diego (bTEMPS-M) to assess affective temperaments<sup>58,59</sup>; 6) Columbia-Suicide Severity Rating Scale (C-SSRS) to assess suicidal risk<sup>60</sup>; 7) Italian Perceived Stress Scale (IPSS)<sup>61</sup> and the Social Readjustment Rating Scale (SRRS)<sup>62</sup>, to assess global levels of stress and stressful life events during last year respectively; 8) Cumulative Illness Rating Scale (CIRS) to assess physical comorbidities<sup>63</sup>.

Moreover, at baseline, the following information will be collected: 1) sociodemographic characteristics (age, nationality, educational level, marital and employment status); 2) psychiatric history (comorbidity with other mental health problems, number of previous hospitalizations, presence of suicide attempts, number and type of lifetime relapses, duration of illness, use of psychotropic drugs); 3) anthropometric parameters (weight, height, waist and hip circumference).

All assessment instruments will be administered at each time point. Remission will be determined on the basis of the clinical evaluation, a MADRS score  $\leq 12$  for depressive symptoms or a YMRS score  $\leq 12$  for hypomanic/manic symptoms. In the case of an affective episode with mixed features, both requisites will have to be met.

### SAMPLE SIZE CALCULATION

Sample size has been calculated using G\*Power software<sup>64</sup>, considering pro-inflammatory cytokines, cortisol and BDNF levels as outcome measures of the study. After that, the measure which resulted in



the largest sample size (BDNF levels) was taken as the reference. The calculation was based on a non-parametric test of the main outcome (Mann-Whitney with min ARE parent distribution), establishing error level  $\alpha$  at 0.05, power at 0.95 and Cohen's effect size  $d=1.0$ . Based on this setting, a sample size of 64 subjects (32 patients with BD and 32 healthy controls) was obtained. However, since a dropout rate of 20% is expected, additional 8 participants per each group will be recruited, leading to a total number of 80 subjects as the final sample size (40 patients with BD and 40 healthy controls).

With respect to secondary objectives, setting alpha level at 0.025 (with Bonferroni's correction to account for the two comparisons) and power at 0.95, the above sample size will allow to identify  $d = 1.07$  for the comparisons among mean values of proinflammatory cytokines, cortisol and BDNF of healthy subjects and the acute and euthymic phase of the disorder using Mann-Whitney test with min ARE parent distribution.

### STATISTICAL ANALYSIS

Statistical analysis will be performed by the Center for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy. Non-parametric tests will be used to analyze the levels of biological markers and psychiatric assessment scores. In particular, the Mann-Whitney test will be applied for assessing the variation ( $\delta$ ) described above. In addition, a multiple linear regression model will be performed to quantify the effect of the variation in inflammatory markers on patients' clinical scores.

We will include patients' mood polarity at baseline as a covariate in multivariable analyses in order to account for possible effects in the biosignature related to the specific type of BD phase.

### ETHIC STATEMENT

This study will be carried out in accordance with globally accepted standards of good clinical practice, in agreement with the Declaration of Helsinki<sup>65</sup>. Ethical approval has been obtained by the Ethical Review Board of the University of Campania "Luigi Vanvitelli" in Naples, which is responsible for enrolling human subjects (Protocol number: 0003238/i/2023). At the time of recruitment, all subjects willing to participate in the study will receive individual explanations about the aims, the course and the implications of the study. Thereafter, they will be asked to sign a written consent. All data collected in this trial will be held confidential and made anonymous during the first step of the collection. Patients' data and samples will be uniquely coded and stored in compliance with the GDPR UE/697/2016.

## Discussion

Major depressive and bipolar disorders (BD) are the most common affective disorders. Etiologically related but clinically distinct, they share several clinical features, resulting in high rates of misdiagnosis due to frequent occurrence of depressive episodes and later onset of manic episodes in BD subjects, who can await years for a proper diagnosis, resulting into a greater severity of symptoms, impaired psychosocial functioning, treatment resistance and higher suicidality<sup>66</sup>. The delay in establishing a correct diagnosis is associated to higher number of lifetime relapses and hospitalizations, with consequent increased direct and indirect costs associated to the treatment and the management of MDD and BD<sup>67</sup>.

Increased immunological/inflammatory processes have been consistently reported to play a pivotal role in the pathophysiology of affective disorders<sup>68</sup>. Extreme fluctuations in mood, such as those characterizing BD, associate with main energy unbalance and neuroendocrine-immune dysfunction. In the last decades a number of studies have been carried out with the aim to identify new treatment strategies to improve long-term outcome of BD<sup>69</sup> reporting contrasting results<sup>70</sup>, mainly related to methodological weaknesses<sup>71-73</sup>.

One of the main strengths of the present protocol is the attempt to identify a biosignature to predict the onset of acute phases of BD. This is of particular relevance at clinical and research levels, considering that BD is one of the most pleiotropic and heterogeneous psychiatric disorders, with a fluctuating course among phases of opposite mood polarity and impairments in several areas, such as a low global functioning and a high suicidal risk<sup>74,75</sup>. The identification of a predictive biosignature could improve the early identification of an acute phase of the disorder and, therefore, significantly improve its management. Previous literature corroborates our hypotheses since during acute phases of BD higher levels of proinflammatory cytokines, salivary cortisol and lower BDNF levels, compared to euthymic state and to healthy controls have been detected<sup>35-37</sup>. Moreover, from a methodological viewpoint, our study will include the assessment of a series of explanatory variables, which may result useful to link the severity of the clinical picture of the recruited patients and the analyzed biosignature. Explanatory variables included in the study are the number of voluntary and compulsory hospitalizations, number and polarity of relapses, polarity index, days lost at work, duration of illness, age at onset of the disorder, seasonality and suicidality. All these "hard clinical indicators" are considered a proxy of the severity of BD<sup>76</sup>. Moreover, we will include the assessment of global functioning and suicidal risk,

in order to assess whether more severe clinical presentations of BD (i.e., those with a significant impairment in functioning, with history of repeated suicide attempts or with a frequent presentation of clinically relevant suicide ideation) are associated to specific alteration in inflammatory pathways. As explanatory variables, a series of physical anthropometric parameters will be assessed, including BMI, physical comorbidities, waist circumference and hip circumference. Some studies have highlighted that variations in BMI and in the waist-to-hip ratio are strongly related to the inflammatory state in BD<sup>77-81</sup>. In fact, it has been reported that obesity is a chronic, low-grade inflammatory state, which is a key mechanism through which obesity causes diseases such as cancer, atherosclerosis, and type II diabetes<sup>82,83</sup>. Adipose tissue produces and secretes chronic high concentrations of proinflammatory cytokines, including interleukins-6 and 8 (IL-6, IL-8), and tumor necrosis factor-alpha (TNF- $\alpha$ ). Therefore, we believe that the inclusion of BMI and waist-to-hip ratio as explanatory variables will add significant knowledge in the study of the relationship between BD and neuroinflammation.

The choice to include a questionnaire aimed at assessing affective temperament<sup>58,59</sup> deserves a special mention. According to Akiskal and Akiskal<sup>84</sup> affective temperaments, defined as genetically determined ways to behave in response to environment<sup>85</sup>, play a significant role in mood disorders, conditioning clinical presentation, course, severity, and treatment response<sup>86</sup>. The term "temperaments" refers to the emotional domain of personality<sup>86</sup>, and can be considered the interface between biological and psychological features of affective disorders<sup>87</sup>. Temperaments are defined as a stable trait of personality and refer to patients' activity levels, social and biological rhythms, mood disposition and daily variability<sup>88,89</sup>. Hence, it will be relevant to measure the association between clinical course, inflammatory parameters and the "biological scaffolds" of personality, assessed at baseline through a validated Italian patient-reported questionnaire<sup>90</sup>.

It is worthy noticing that available studies have largely focused on these different aspects singularly, providing incomplete or sometimes contrasting results. The strength of the present study is the complete assessment of different aspects of inflammatory and neuroendocrine pathways, in order to provide a wider and more comprehensive oversight. Another important implication of the present study is the potential identification of new pharmacological targets for BD.

Lastly, both patients with bipolar I and II disorder will be included in the studied sample. If from one hand this could limit the specificity of our findings, it has to be said that, to our knowledge, a few data are available on the differences in biological signature

between type bipolar disorders type I and II. Therefore, we decided to include both subtypes of bipolar disorders in order to identify possible variations in biosignature according to the main diagnosis.

Our study has some limitations. We will not differentiate among depressive and hypomanic/manic phases when patients will be recruited. However, our aim is not to identify a biosignature for a specific phase of the disorder, but to discriminate among acute and euthymic phases. Moreover, we will not apply restrictions in recruiting patients under pharmacological treatment, although antidepressants, mood stabilizers and II- and III-generation antipsychotics may differently impact on the inflammatory outcomes<sup>91-94</sup>. However, our multivariable analyses will be corrected for pharmacological treatments in order to partially counterbalance this bias. Furthermore, the panel of inflammatory indices we selected is not exhaustive, since we will not make the complete dosage of what is nowadays available to assess inflammation: cytokines, chemokines, growth factors and specific T-cells populations<sup>95</sup>. Another possible limitation of this study relies on the procedures of the CAR assessment, which will encompass the collection of saliva samples at home and not under rigorous monitoring in the research unit. In particular, we will not be able to monitor objectively the delay between awakening and the collection of the first saliva sample. However, as suggested by Stalder et al.<sup>96</sup>, we will try to obtain the participants' adherence to the sampling rules by motivating them, by providing them with take-home written instructions and, whenever possible, by asking a relative to supervise the whole sampling procedure.

Another possible limitation is the exclusion of patients with actual comorbid alcohol and/or substance abuse disorders, which could possibly limit the generalizability of our results. Chronic alcohol and substance abuse are highly frequent in BD patients; however, such comorbidity holds a significant impact on the levels of basal inflammation and shall be considered as a potential confounding factor in many chronic inflammatory or other immune-mediated diseases<sup>97,98</sup>. Thus, we have decided to remove from our analyses all possible confounders in order to obtain a specific biosignature in BD patients, although we are aware that this choice does not reflect real-world clinical practice. If our hypotheses will be confirmed, larger studies taking into account the impact of all possible confounders (including the comorbidity with substance abuse disorders) on BD biosignature will be carried out.

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## Conclusions

Bipolar disorder is a chronic, severe, remitting and disabling illness, whose difficult clinical management is due to unpredictable staged course and important

complications, including suicidal risk. Many needs still remain unmet since most patients do not achieve a full recovery. To our knowledge, this is the first study aimed at assessing biological and affective indices in BD according to a longitudinal design, so as to detect changes across different stages (acute periods vs. euthymia), in comparison with not affected subjects. Despite some operational limitations, we expect the levels of cytokines, awakening cortisol and BDNF to undergo meaningful changes and will allow us to describe a biosignature of BD. Predicting the onset of an impending acute phase through peripheral indices, such as blood and saliva samples, will allow for a more accurate and appropriate therapeutic management.

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Corresponding author:  
 Mario Luciano, MD, PhD  
 Department of Psychiatry  
 University of Campania "L. Vanvitelli"  
 Largo Madonna delle Grazie  
 80039, Naples, Italy  
 E-mail: mario.luciano@unicampania.it