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Regione Lombardia
ASST Fatebenefratelli Sacco

UNIVERSITÀ DEGLI STUDI DI MILANO

BipOlAR Disorder Integrative stagiNG: incorporating the role of biomarkers into Progression AcrosS Stages (BOARDING-PASS)

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Rationale

Bipolar Disorder (BD) is a highly prevalent (approximately 1% in the general population; American Psychiatric Association, 2013) and disabling condition, characterized by a chronic and progressive course (McIntyre et al., 2020). The natural history of BD typically includes an initial asymptomatic phase, followed by a prodromal stage, and subsequently the onset of a first syndromic episode (depressive or manic), which is usually followed by recurrent episodes, often in the absence of full inter-episodic recovery.

A growing body of evidence supports the notion that the longitudinal course of BD is associated with an active process of neuroprogression, characterized by progressive brain alterations and functional impairment (Berk et al., 2007; Berk et al., 2014; Grewal et al., 2022). Several clinical factors may influence the trajectory of the disorder, including the number of affective episodes, the presence of psychiatric and medical comorbidities, exposure to stressful life events, and a family history of psychiatric disorders (Post, 2020).

In order to better define this progressive trajectory, over recent decades several authors have conceptualized clinical staging models for BD, with the primary aim of predicting illness evolution over time and identifying stage-specific therapeutic interventions (Fernandes et al., 2017). In 2012, Kupka and Hillegers proposed a staging model integrating clinical phenomenology with predictors of unfavorable illness course (Kupka & Hillegers, 2012). This model was subsequently tested retrospectively in a sample of 99 patients with BD to evaluate illness progression over time. Results showed a transition toward increasingly advanced stages of illness within five years from onset, with specific variables—namely biphasic onset episodes (mania–depression or depression–mania) and male sex—significantly influencing the rate of stage transition (van der Markt et al., 2019).

Neuroimaging studies have consistently supported the neuroprogressive hypothesis in BD (Serafini et al., 2021), demonstrating structural and functional brain alterations that progressively emerge as the illness advances. Abnormalities have also been described at the level of large-scale neural networks (Gong et al., 2021). In particular, the Default Mode Network appears to exhibit altered patterns of hyper- and hypo-connectivity with affective, fronto-parietal, and attentional systems, involving key hubs responsible for efficient large-scale brain communication. Moreover, structural and functional alterations have also been identified in unaffected relatives of patients with BD (Cattarinussi et al., 2019).

Studies investigating additional biomarkers in BD have further provided evidence supporting a stage-related progression of the disorder, particularly with respect to Brain-Derived Neurotrophic Factor (BDNF) and inflammatory cytokines. Regarding BDNF, several studies have reported reduced levels during manic and depressive episodes (Kauer-Sant'Anna et al., 2008; Fernandes et al., 2015). Other investigations have suggested that such reductions may correlate with downregulation of BDNF gene expression associated with hypermethylation of the BDNF promoter region (D'Addario et al., 2012; D'Addario et al., 2013). Higher levels of DNA methylation have been observed in depressed compared with manic/mixed patients (Dell'Osso et al., 2014) and, notably, in individuals with early-onset BD compared with those with later onset (Nassan et al., 2020), supporting the hypothesis that BDNF levels and/or downregulation of BDNF gene expression may be associated with illness progression across different stages over time.

With respect to pro-inflammatory cytokines, available data suggest the presence of a chronic low-grade inflammatory state in patients with BD, which may worsen in later stages of the disorder. The association between inflammatory biomarkers and BD appears to be strongly dependent on both illness stage and

phase (Rosenblat & McIntyre, 2016; Tatay-Manteiga et al., 2017). Notably, increases in pro-inflammatory cytokines (e.g., IL-1β, IL-2, IL-4, IL-6, TNF-α, soluble TNF-α receptor type 1, C-reactive protein) during depressive phases seem to increase the likelihood of subsequent transition to mania. In addition, some studies have reported an association between alterations in IL-6 and TNF-α and reduced BDNF expression, suggesting the co-occurrence of broader neuroplastic changes during these phases (Lima Giacobbo et al., 2019).

Overall, recent advances in the field of clinical staging of BD, together with progress in the identification of biological markers (e.g., gene transcription modulation, neurotrophic signaling, immuno-inflammatory pathways, microbiota) and neuroimaging (structure and function of large-scale brain systems), provide a strong rationale for the development of a multidimensional staging model**.** Such a model would integrate clinical and neurobiological data, allowing for greater diagnostic, prognostic, and therapeutic precision.

To this end, machine learning (ML) algorithms have already been applied to develop diagnostic classification models and to predict illness trajectories (Jan et al., 2021; Colombo et al., 2022). Specifically, ML approaches enable the integration of multi-level patient data to generate multivariate and individualized staging models, thereby supporting a personalized approach to the management of patients with BD.

Study Objectives

The study pursues the following three objectives:

1. To longitudinally evaluate the clinical progression of Bipolar Disorder (BD) in a sample of subjects according to the staging model proposed by Kupka and Hillegers, as detailed in the subsequent sections;
2. To analyze the role of gene transcription regulation, inflammation, microbiota-related features, and neuroimaging in the progression across stages. With regard to gene transcription regulation, inflammation, and microbiota-related characteristics, genomic DNA or exosomal miRNA derived from blood and saliva samples will be analyzed. For neuroimaging, participants will undergo 3 Tesla (3T) structural and functional brain magnetic resonance imaging (MRI) in order to construct a structural connectome based on the gyrification index and a resting-state functional connectome;
3. To implement a machine learning (ML) model based on all acquired variables to predict progression across stages of BD. The integration of biological and neuroimaging contributions into the model may help overcome a traditional phenotype-based clinical approach and allow for a more refined characterization of patients with BD, with a significant impact on prognosis and treatment strategies.

Materials and Methods

Study Sample

The study involves the consecutive recruitment of 120 subjects enrolled at three of the four participating centers: UO1 (ASST Fatebenefratelli-Sacco, Milan), UO2 (ASST Papa Giovanni XXIII, Bergamo), and UO3 (ASL 2 Abruzzo, Lanciano–Vasto–Chieti). Inclusion and exclusion criteria are detailed below.

Inclusion Criteria

- Subjects affected and unaffected by BD whose clinical stage falls within those defined by the Kupka and Hillegers model, namely:

stage 0 (increased risk: having a first-degree relative with BD, in the absence of psychiatric symptoms);

stage 1 (having a first-degree relative with BD, in the presence of non-specific psychiatric symptoms or depressive episode(s));

stage 2 (first hypo/manic episode allowing a diagnosis of BD type I or II according to DSM-5; APA, 2013);

stage 3 (recurrent episode(s): depressive, hypo/manic, or mixed);

stage 4 (persistent non-remitting disorder: chronic depressive, manic, or mixed episodes, including rapid cycling);

- Individuals of both sexes;
- Age ≥ 18 years and ≤ 70 years;
- Ability to provide valid written informed consent.

Exclusion Criteria

- Inability to provide valid written informed consent;
- Presence of intellectual disability;
- Presence of a severe concomitant medical condition;
- Presence of a current substance use disorder.

Study Design and Assessment

Of the four centers participating in the study, three (UO1, UO2, and UO3) will contribute to subject recruitment. Of the total 120 participants, n = 40 will be consecutively recruited at the outpatient clinics of the Psychiatry Unit 2 of Luigi Sacco Hospital in Milan (Coordinating Center, UO1). The remaining participants will be consecutively recruited at the outpatient clinics of ASST Papa Giovanni XXIII in Bergamo (UO2, n = 40) and ASL 2 Abruzzo in Lanciano–Vasto–Chieti (UO3, n = 40), following approval by the respective local Ethics Committees.

At enrollment, all participants will be administered the Structured Clinical Interview for DSM-5 Disorders (SCID-5) to assess psychiatric diagnoses according to DSM-5 criteria (American Psychiatric Association, 2013). All subjects meeting inclusion criteria will be considered eligible for the study.

After providing written informed consent to participate, enrolled subjects will undergo baseline assessment (T0) and will then enter an 18-month follow-up period consisting of three subsequent time points: T1 (6 months after T0), T2 (12 months after T0), and T3 (18 months after T0). At T0 and at each subsequent time point, participant assessment will include: (i) clinical and psychometric evaluation; exclusively at T0, T2, and T3, (ii) biological marker assessment; and (iii) brain magnetic resonance imaging for acquisition of structural and functional MRI data.

Clinical and Psychometric Assessment

At baseline (T0), for each study participant, the main sociodemographic and clinical variables will be collected and entered into an anonymized database. These include:

(a) sociodemographic data: age, sex, ethnicity, educational level, marital status, and occupational status;

(b) clinical characteristics: family history of psychiatric disorders, BD subtype, age at onset of BD and associated stressful life events, illness duration, duration of untreated illness, age at first depressive and hypo/manic episode, polarity of the first and most recent affective episode, predominant polarity, total lifetime number of affective episodes, presence of mixed or rapid-cycling features, current and previous psychopharmacological treatments, medical and psychiatric comorbidities, history of substance use disorder, lifetime number of hospitalizations, and suicide attempts.

These variables will be used to assign the clinical stage at T0 according to the Kupka and Hillegers model (Kupka & Hillegers, 2012). At each subsequent time point, sociodemographic and clinical data will be updated in order to reassign the corresponding stage and to evaluate potential associations between clinical variables and the probability of progression to more advanced stages of illness.

Clinical assessment will further include the administration of the following psychometric scales and questionnaires: the Test di Intelligenza Breve (TIB; Sartori, 1997; Colombo, 2002), administration time approximately 5 minutes; the Hamilton Depression Rating Scale (HDRS-21; Hamilton, 1960; Cassano et al., 1991), administration time approximately 20 minutes; the Hamilton Anxiety Rating Scale (HARS; Hamilton, 1959; Cassano et al., 1991), administration time approximately 15 minutes; the Montgomery–Åsberg Depression Rating Scale (MADRS; Montgomery & Åsberg, 1979; Palma et al., 1999), administration time approximately 15 minutes; the Young Mania Rating Scale (YMRS; Young et al., 1978; Palma et al., 1999), administration time approximately 15 minutes; and the Global Assessment of Functioning (GAF; Hall, 1995; APA, 1996), administration time approximately 2 minutes; Family Interview for Genetic Studies (FIGS); Drugs Abuse Screening Test (DAST-10), administration time has been estimated in nearly 5 minutes.TWEAK test with a completion time of roughly 2 minutes. Childhood Trauma Questionnaire (CTQ), it requires 5–10 minutes and will be administered at baseline. Paykel Scale for Recent Life Events, its administration takes approximately 15 minutes and will be conducted at baseline; Clinician Rating Scale (CRS), recorded in nearly 2 minutes at each time point and aimed at evaluating patients' adherence to pharmacological treatment.

Biological marker assessment: Gene transcription regulation, inflammation, microbiome data

Biological samples for gene expression, inflammation, and microbiome analyses will be collected at baseline, T2 and T3. Specifically:

a. unstimulated saliva samples —i.e., whole saliva collected under resting conditions without gustatory, masticatory, or pharmacological stimulation— will be obtained using cotton buccal swabs (Salivette, Sarstedt, Nümbrecht, Germany) and stored at -20 °C until genomic DNA (gDNA) extraction. Exosomes wil be also isolated from saliva and miRNAs purified using an exosome RNA isolation kit.

b. peripheral venous blood samples will be collected in two 5 ml vacutainer tubes containing sodium citrate. Serum and cellular components will be separated and total RNA as well as gDNA will be extracted from PBMCs.

All biological samples collected at the three recruiting sites (Units 1, 2 and 3) will be transferred to the central laboratory (Unit 4) for standardized processing and analyses, including:

- LIPIDOMICS to analyze short chain fatty acids (SCFAs) extracted from saliva derivatization for LC–MS/MS analysis will be carried out.

Molecular biology studies:

- gene expression analysis. Relative abundance of mRNA species in PBMCs will be assessed by real-time RT-PCR and Digital PCR.

- DNA methylation in both blood and saliva cells a. general DNA methylation status will be analyzed using the Reduced representation bisulfite sequencing (RRBS) method (SBS sequencing of the SURFseq5000 platform); b. gene-specific DNA methylation study will be performed on amplified bisulfite (BS) treated DNA and methylation levels analyzed using PyroMark Q48 (64).

- salivary exosomal miRNAs: miRNOme analysis and selected miRNAs after networking analysis by RealTime PCR and Digital PCR.

- Transcriptional factors DNA-binding. ALPHAScreenTM assay technique to verify if identified recognition elements at genes promoter bind to different transcriptional factors and if this binding is directly modulated by the methylation degree of CpG motifs.

- Salivary MICROBIOTA COMPOSITION by 16S rRNA Microbiome sequencing.

Neuroimaging assessment

MRI assessments will be performed using 3T scanners at T0, T2, and T3, and comprised:

- Structural MRI (sMRI): 3D T1-weighted images will be acquired using a SPGR sequence (TE = minimum (full); flip angle, 6°; FOV, 250 mm; bandwidth, 31.25; matrix, 256 x 256) with 124 axial slices of 1.3 mm thickness. Following cortical surface reconstruction, local gyrification indices will be computed for 68 parcellated cortical regions based on the Desikan Atlas using FreeSurfer v7.1.0. A jackknife bias estimation procedure will then be applied to determine each individual's contribution to group-level covariance structure, generating a 68×68 individual-wise distance matrix. The topological organization of the resulting structural covariance networks will subsequently be analyzed using the Graph Analysis Toolbox.

- Resting-state functional MRI: rs-fMRI images will be acquired using a gradient-echo EPI sequence with 36 axial slices (TE = 30 ms; TR = 2000 ms; voxel size: 3×3×4 mm3; matrix size: 64× 64; FOV: 192×192 mm2);, acquired in interleaved order. Each resting-state session will consist of 400 volumes. Pre-processing will be conducted using a combination of FMRIB's Software Library (FSL) and custom MATLAB scripts. The pipeline will include the following steps: (1) reorientation to standard space; (2) detection of outlier volumes, followed by spline-based interpolation of outlier timepoints; (3) spatial and temporal preprocessing, including motion correction (MCFLIRT), temporal high-pass filtering, and spatial smoothing (FWHM = 5 mm); (4) brain extraction of the structural image; (5) nonlinear registration to the MNI152 standard space using FSL-FNIRT. Static and dynamic functional connectomes will be estimated by calculating z-transformed Pearson correlation coefficients between all pairs of brain regions in the adopted parcellation scheme. Dynamic connectivity will be computed using a sliding-window approach with a window length of 30 TRs and a step size of 2 TRs. These steps will be implemented through in-house software developed in MATLAB. Graph-theoretical measures will be computed through the Brain Connectivity Toolbox (MATLAB).

To minimize inter-site variability in neuroimaging data, both structural and functional MRI acquisitions will performed using harmonized protocols across the two imaging centers, each equipped with a 3 T scanner.

ML algorithms

A ML framework will be developed to predict clinical stage transitions in BD by integrating collected clinical, biological, and neuroimaging data. To manage the integration of multimodal data, we will adopt robust pre-processing pipelines including data normalization, outlier detection, and imputation methods for handling missing values (e.g., k-nearest neighbor or multiple imputation). ML analyses will start at month 6 of the study and will be conducted using MATLAB's Statistics and Machine Learning Toolbox, initially supported by NeuroMiner software (http://proniapredictors.eu/neurominer/index.html), a validated software platform designed to manage heterogeneous datasets. NeuroMiner provides a broad range of cross-validation frameworks, preprocessing strategies, supervised learning algorithms, feature selection tools, and external validation methods. The ML approach will be based on supervised classifiers, primarily Support Vector Machine (SVM) and Bayesian models. In the preliminary phase, classifiers will be trained and tested on preliminary datasets to compare alternative predictive models in a controlled setting and to identify the best-performing algorithmic configuration. Subsequently, feature selection procedures will be employed to identify the most discriminative variables from the pool of candidate features. This step is crucial to enhance model interpretability and to prevent overfitting, especially in small-sample, high-dimensional datasets typical of multimodal studies. In fact, by reducing the number of input variables, feature selection minimizes noise, lowers model complexity, and improves generalizability of the ML predictions. If needed (i.e. if the dimensionality of the feature space is still too high), to further reduce overfitting and the computational burden, Principal Component Analysis might be applied. SVM classifiers will be prioritized due to their robustness in handling high-dimensional, small-sample data. In addition, class weighting will be applied so that errors on minority stages are penalized more heavily, reducing the bias introduced by uneven group sizes. Model performance will be assessed using cross-validation techniques (e.g. leave-one-out or stratified k-fold, depending on data structure and class distribution) and evaluated in terms of sensitivity, specificity, F1 score and overall accuracy, in order to account for potential class imbalance. A predefined minimum target accuracy of 90% is required for model deployment on the full dataset. For external validation, ML-derived stage predictions will be compared against those assigned by expert clinicians, considered the diagnostic gold standard. An interim evaluation of model accuracy has been planned after acquisition of 50% of the total dataset, serving as a critical checkpoint to assess the predictive performance of the model and to estimate its expected accuracy upon completion of data collection. This intermediate analysis is particularly relevant should the timeframe between data acquisition completion and the project conclusion proves insufficient for final model training and validation.

Data analysis

For stage progression analysis, data from repeated assessments of clinical stages across follow-up visits will be analyzed using generalized estimating equations implemented via the PROC GENMOD procedure in SAS®, applying a multinomial distribution with a logit link function. This approach allows evaluating associations and interactions between stage transitions and relevant sociodemographic and clinical variables over time. Additionally, stage transition probabilities will be modeled using a multi-state Markov approach provided by the 'mstate' package in R. Statistical significance was set at $p \leq .05$. Biological and neuroimaging data analyses will be performed by linear mixed-effects models to evaluate the associations between biomarkers and neuroimaging outcomes across BD stages at different time points. Omnibus one-way ANOVA will be used for molecular measures, complemented by post-hoc analyses for multiple comparisons when significant effects are identified. ML analyses will be conducted using MATLAB's Statistics and Machine Learning Toolbox, supported by NeuroMiner software.

Sample size estimation was guided by multiple methodological considerations. Given the multidimensional nature of the study, several independent measures - clinical, biological, and neuroimaging - will be collected longitudinally and subsequently integrated into a unified ML predictive model. Therefore, the sample size was determined to ensure sufficient statistical power for traditional analyses, while also supporting the training and validation of robust ML algorithms. Specifically, the required sample size has been determined based on two complementary criteria. First, among the multiple domains investigated, a power analysis

conducted using G*Power 3.1.9.7 indicated that a total of N=120 participants was sufficient to detect significant differences across five BD stages (0, 1, 2, 3, 4), assuming a statistical power of 0.80, an alpha=0.05, and a medium effect size (f=0.32). Second, from a computational perspective, this sample size was considered adequate for training supervised ML models with acceptable performance. Previous ML-based studies in BD have shown that models trained on ~50 subjects reached an accuracy of ~64%, while those trained on samples approaching 90 participants achieved accuracy up to 99%.

Study sites and organizational structure

BOARDING-PASS study will be conducted across four operational units (UOs), each with defined roles to ensure high-quality data acquisition and standardized clinical procedures.

- UO1 (ASST Fatebenefratelli-Sacco, Milan) is the coordinating center, responsible for patient recruitment and baseline diagnostic assessments, standardized collection of clinical and biological data. UO1 investigators will provide coordination and oversight within the multicenter research network, maintaining effective communication among clinicians, researchers, and technical staff.

- UO2 (ASST Papa Giovanni XXIII, Bergamo) is responsible for participant recruitment, diagnostic assessment, collection of biological samples, structural and resting-state MRI data acquisition also on behalf of UO1, and structural neuroimaging analysis.

- UO3 (ASL 2 Lanciano-Vasto-Chieti) will conduct participant recruitment and assessment, data collection and management, biological sample collection, as well as structural and functional neuroimaging data acquisition.

- UO4 (University of Teramo) serves as the centralized facility responsible for biological analyses, specifically gene transcription regulation and microbiota analyses. UO4 is also responsible for the analysis of functional neuroimaging data and for the implementation of ML algorithms.

Screening and Informed Consent

Screening of potential study participants will be conducted at the clinical facilities of UO1, UO2, and UO3. For UO1, screening activities will take place at the outpatient clinics of Psychiatry Unit 2 of Luigi Sacco Hospital in Milan. Investigators will select subjects based on the inclusion and exclusion criteria outlined above. For identified candidates, the presence of diagnostic criteria for Bipolar Disorder will be verified through administration of the Structured Clinical Interview for DSM-5 Disorders (SCID-5; American Psychiatric Association, 2013).

Enrollment of eligible subjects will occur only after the acquisition of valid written informed consent. Each participant will be adequately informed about the potential risks and benefits associated with study participation, as well as about the possibility of withdrawing consent at any time during the course of the study. The studies involving humans were approved by Research Ethics Committee of Milan, Area 1 (N.0008265/2023 on 24/02/2023)

Privacy

All data and information collected regarding study participants will be kept confidential. Should data derived from this study be published, this will occur in a manner that does not allow identification of individual subjects. The Ethics Committee will be permitted to review any documentation related to participants' involvement in the study. All data will be securely stored in compliance with Regulation (EU) 2016/679 (General Data Protection Regulation, GDPR), and access will be restricted to medical personnel directly involved in the study. Reference is made to Articles 15 to 22 of the GDPR regarding the rights of data subjects.

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