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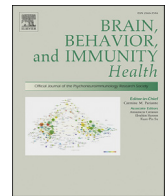
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## Full Length Article

# Blood cytokines differentiate bipolar disorder and major depressive disorder during a major depressive episode: Initial discovery and independent sample replication

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## ABSTRACT

Bipolar disorder (BD) diagnosis currently relies on assessment of clinical symptoms, mainly retrospective and subject to memory bias. BD is often misdiagnosed as Major Depressive Disorder (MDD) resulting in ineffective treatment and worsened clinical outcome. The primary purpose of this study was to identify blood biomarkers that discriminate MDD from BD patients when in a depressed state. We have used clinical data and serum samples from two independent naturalistic cohorts of patients with a Major Depressive Episode (MDE) who fulfilled the criteria of either BD or MDD at inclusion. The discovery and replication cohorts consisted of 462 and 133 patients respectively. Patients were clinically assessed using standard diagnostic interviews, and clinical variables including current treatments were recorded. Blood was collected and serum assessed for levels of 31 cytokines using a sensitive multiplex assay. A penalized logistic regression model combined with nonparametric bootstrap was subsequently used to identify cytokines associated with BD. Interleukin (IL)-6, IL-10, IL-15, IL-27 and C-X-C ligand chemokine (CXCL)-10 were positively associated with BD in the discovery cohort. Of the five cytokines identified as discriminant features in the discovery cohort, IL-10, IL-15 and IL-27 were also positively associated with BD in the replication cohort therefore providing an external validation to our finding. Should our results be validated in a prospective cohort, they could provide new insights into the pathophysiological mechanisms of mood disorders.

## 1. Introduction

Major Depressive Disorder (MDD) and Bipolar Disorder (BD) are psychiatric disorders classified under the general category of mood disorders. Both MDD and BD are characterized by severe fluctuations in mood, energy and activity. MDD is characterized by one or recurrent episodes of sadness and loss of interest, associated with sleep and appetite disorders, cognitive dysfunction and suicide ideation or

attempts. BD is also characterized by recurrent major depressive episodes (MDE), associated with episodes of drastic increase in mood, energy and activity levels defined as (hypo)manic episodes (Grande et al., 2016). In both MDD and BD, changes in mood usually alternate with periods relatively free of symptoms, of variable duration, called euthymia. Although mania and hypomania are the most recognizable characteristics of BD, depression is often its first clinical manifestation and most frequent clinical presentation across time (Judd et al., 2002). BD patients

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are much more likely to present to clinicians when they are clinically depressed, especially in outpatient settings (Hirschfeld et al., 2005). When depressed, the presentation of a patient with BD may not differ from that of an MDD patient in clinical practice.

Several clinical specifiers have been proposed to discriminate BD from MDD but their sensitivity and specificity remain low (Angst et al., 2011). This could explain why nearly 40% of BD patients are initially misdiagnosed with MDD (Hirschfeld, 2014), and why the average delay for correct BD diagnosis is 7.5 years (Ghaemi et al., 1999). Moreover, conversion from MDD to BD occurs frequently (around 7% after 24 months) (Kessing et al., 2017). Unfortunately, confusion between BD and MDD has troublesome consequences. Indeed, first-line treatments for MDD, the so-called antidepressants (Cipriani et al., 2018), aggravate the course and outcome of BD and are therefore generally contraindicated as monotherapy (Pacchiarotti et al., 2013; Vieta, 2014). When correctly diagnosed, BD patients with MDE are prescribed mood stabilizers, such as lithium, either alone or in combination with antidepressants.

Despite extensive research in this field, we still know very little about the pathophysiological mechanisms of mood disorders in general, and more specifically those that are specific to BD. Accumulating data suggest that dysregulation of immune pathways is associated with the etiology and pathophysiology of mood disorders (Goldsmith et al., 2016; Wang and Miller, 2018; Yuan et al., 2019). For example, several cytokines were reported to be present at different levels in blood and/or cerebrospinal fluids (CSF) in patients with MDD compared to healthy controls (Kohler et al., 2017; Wang and Miller, 2018). Of note, these differences were not due, at least in part, by the use of antidepressant drugs in the patient group as demonstrated in studies in which antidepressant drug-naïve patients with MDD were compared to healthy controls (Zou et al., 2018). Likewise, meta-analyses have provided evidence for significant elevation of proinflammatory, anti-inflammatory, and regulatory cytokines in BD (Modabbernia et al., 2013; Munkholm et al., 2013). On a related topic, four studies have sought to identify cytokines or soluble receptors that discriminated BD from MDD (Bai et al., 2015; Brunoni et al., 2020; Poletti et al., 2020; Wollenhaupt-Aguiar et al., 2019). In a first study comparing 130 patients with BD and 149 patients with MDD, patients with BD had higher levels of sIL-2R, sIL-6R, CRP, sTNF-R1, and CCL2 compared to those with MDD (Bai et al., 2015). In a second study, the authors measured serum levels of 10 immune or oxidative stress biomarkers in 54 MDD and 54 BD patients (Wollenhaupt-Aguiar et al., 2019). Using recursive feature elimination with a linear support vector machine kernel, these authors found that IL-4, IL-10 and thiobarbituric acid reactive substances discriminated BD from MDD. In a third study, the authors measured plasma levels of 12 immune biomarkers in 245 MDD and 59 BD patients who were in an acute depressive episode of moderate severity (Brunoni et al., 2020). Using hierarchical binary logistic regression analyses and generalized linear models, these authors found that the levels of IL-1 $\beta$ , TNF- $\alpha$ , soluble TNF receptor 1 (sTNFR1), IL-10, IL-12 and TNF- $\alpha$  were higher in MDD than in BD, while those of IL-6, IL-18, IL-33, sTNFR2ST2 and KLOTHO were lower. More recently, the plasma levels of 54 cytokines, chemokines and growth factors were measured in 308 depressed patients among whom 81 and 127 had been diagnosed with BD and MDD respectively (Poletti et al., 2020). Applying an elastic net framework to this dataset allowed for the identification of 16 cytokines, i.e. IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-16, CCL3, CCL4, CCL5, CCL11, CCL25, CCL27, CXCL11 and TNF- $\alpha$ , that discriminated BD from MDD. Although these three studies supported the hypothesis that blood immune biomarkers could discriminate BD from MDD, they have all suffered from several shortcomings. For example, Brunoni et al. did not use cross-validation techniques to evaluate model performance, and used statistical methods that did not consider multicollinearity between biomarkers (Brunoni et al., 2020). In two of these three studies, patients with relatively mild depressive symptoms, i.e. HDRS ranging from 7 to 18, were included (Poletti et al., 2020; Wollenhaupt-Aguiar et al., 2019), and results were not adjusted for important covariates such as psychiatric medication use (Wollenhaupt-Aguiar et al.,

2019) and symptom severity (Poletti et al., 2020; Wollenhaupt-Aguiar et al., 2019). Most importantly, the results of these four studies have not been replicated in independent samples.

Here, we have taken advantage of two naturalistic cohorts of MDE patients in which blood samples were collected and patients assessed at inclusion for BD and MDD. We then used a penalized logistic regression method to identify cytokines that discriminated BD from MDD, adjusting for co-variables known or suspected to impact cytokine levels or diagnosis such as gender, age, body mass index (BMI), smoking, history of alcohol or substance abuse, and current treatments.

## 2. Methods and materials

### 2.1. Study subjects

#### 2.1.1. Discovery cohort

The study sample included 462 outpatients of the Department of Emergency Psychiatry and Acute Care at the Montpellier Academic Hospital (France). Patients were included between January 17, 2015 and May 31, 2018. Patients were consecutively and systematically selected and included. Among the 462 patients included in the study, 57.1% and 42.9% were diagnosed with MDD and BD respectively. Participants underwent a standardized interview led by a psychiatrist according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (Association of Psychiatry, 1994). The diagnosis of MDE was made by experienced psychiatrists based on the DSM-IV criteria of mood disorders (Association of Psychiatry, 2000) and the MDE severity was evaluated by the 30 item Inventory of Depressive Symptomatology (IDS-C30) (Rush et al., 1986, 1996). Current psychotic symptoms were not recorded. The psychiatrists who made the diagnoses were not blind as they had access to patient charts to help give a more accurate diagnosis of BD or MDD. Current treatments, including use of selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), benzodiazepines, atypical antipsychotics, lithium, valproate salts, carbamazepine, lamotrigine and anti-inflammatory drugs, were recorded. Forty-nine patients were free of medication. Non-inclusion criteria were a diagnosis of schizophrenia, psychotic or schizoaffective disorder according to the DSM-IV, any disease or health condition that gets worse over time, resulting in a general decline in health or function, pregnancy and being under 18. Of note, including patients who are under 18 in France require a specific authorization that we did not request. All patients were evaluated by a clinician using clinical evaluation and chart review. Body temperature at the time of blood sampling was normal for all patients, and upper respiratory infection or flu/cold symptoms were clinically absent. The local Ethics Committee (CPP Sud Méditerranée IV (Montpellier, France)) approved the study protocol. All participants provided written informed consent.

#### 2.1.2. Replication cohort

A total of 148 adults diagnosed with current MDE were recruited under informed consent (Consoloni et al., 2018), together with 98 healthy subjects free of any psychiatric disorders, in several clinical sites in France from 2012 to 2015. Participants had a clinical evaluation using the DSM-IV Semi-Structured Clinical Interview (First et al., 2002) and were consecutively and systematically selected and included and followed for 30 weeks. Patients had recently been admitted to a psychiatric unit or had been recently referred to a psychiatrist. The diagnosis of MDE was made by experienced psychiatrists based on the DSM-IV criteria of mood disorders (Association of Psychiatry, 2000) and the MDE severity was evaluated by the 17-item Hamilton Depression Rating Scale (HDRS-17) (Hamilton, 1960). The psychiatrists who made the diagnosis were not blind as they had access to patient charts to have a more accurate diagnosis of BD or MDD. Out of the 148 patients who were included in the study, five exhibited delusions without hallucinations. Patients were included if they scored 19 or higher on the HDRS. Exclusion criteria were a history of substance use disorder in the past 12

months, a diagnosis of schizophrenia, psychotic or schizoaffective disorder according to DSM-IV, any disease or health condition that gets worse over time resulting in a general decline in health or function, pregnancy, vaccination within a month before the inclusion in the study and being under 18. Body temperature at the time of blood sampling was normal for all patients and upper respiratory infection of flu/cold symptoms were clinically absent. A total of 15 patients were excluded during the follow-up: 3 were diagnosed with a neurological disorder or cancer, 7 withdrew their consent and 5 patients because serum samples were not available. Overall, data from 133 MDE patients were included in the analyses. Current treatments including SSRIs, MAOIs, benzodiazepines, atypical antipsychotics, lithium, valproate salts, carbamazepine, lamotrigine and anti-inflammatory drugs were recorded. Eleven patients were free of medication. As for healthy subjects, four were excluded among whom two withdrew their consent, one was diagnosed with cancer and one because the serum sample was missing. This study was approved by the institutional ethical board of CPP Sud Méditerranée II (Marseille, France). All subjects provided written informed consent after receiving a complete description of the study. The overall design of the analyses was retrospective.

## 2.2. Blood samples

Venous blood was obtained from fasting subjects between 7:00 a.m. and 9:00 a.m. on weekdays. Five milliliters of peripheral blood were drawn by venipuncture and allowed to clot for 1 h before centrifugation (1500×g, 10 min). In the discovery cohort, serum samples were stored in 0.5 ml aliquots at  $-80^{\circ}\text{C}$  for 3–4 years and thawed on ice only once for cytokine measurement. In the replication cohort, serum samples were stored in 0.5 ml aliquots at  $-20^{\circ}\text{C}$  for 2–3 years. Serum samples were thawed on ice up to 3 times depending on the measured cytokine.

## 2.3. Measurements of serum cytokine levels

Serum samples from the discovery and replication cohorts were assessed for levels of C-C motif chemokine ligand (CCL)2, CCL3, CCL4, CCL11, CCL13, CCL17, CCL20, CCL22, CCL26, C-X-C motif chemokine ligand (CXCL)10, Interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23 p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-27, IL-31, interferon (IFN)- $\gamma$ , Tumor Necrosis Factor (TNF)- $\alpha$ , TNF- $\beta$  and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). For this purpose, the Proinflammatory Panel 1, Cytokine Panel 1, Chemokine Panel 1 and Th17 panel 1 kits were used according to manufacturer's instructions (MSD). Samples were run in batches of 80 samples together with calibrated standards. Data were acquired on the V-PLEX<sup>®</sup> Sector Imager 2400 plate reader and analyzed using the Discovery Workbench 3.0 software (MSD). Standard curves for each cytokine were generated using the premixed lyophilized standards provided with the kits. Serial 2-fold dilutions of the standards were run to generate a 13-standard concentration set, and the diluent alone was used as a blank. Cytokine concentrations were determined by extrapolation from the standard curve using a 4-parameter logistic curve fit to transform the mean light intensities into concentrations. The lower limit of detection (LLOD) was defined as the lowest concentration of each cytokine yielding a signal equal to or over 2.5 standard deviations above blank (zero calibrator). The researcher who assessed serum samples for biomarkers was blind to the diagnoses.

## 2.4. Statistical analyses

### 2.4.1. Univariate statistical analysis

Comparison analyses of variables in MDD versus BD groups of patients were performed using the Wilcoxon-Mann-Whitney *U* test for numerical variables and with the Chi-square test for categorical variables, with Benjamini & Hochberg's multiple testing correction (False Discovery Rate).

### 2.4.2. Multiple imputation (MI)

Missing data were imputed using the Multivariate Imputation by Chained Equations (MICE) procedure in R (van Buuren and Groothuis-Oudshoorn, 2011). The outcome was used in the multiple imputation process as it produces more precise estimates than standard multiple imputation. To minimize bias introduced by a mis-specified model for imputing the missing outcomes, observations with imputed outcomes were excluded from subsequent analyses (Kontopantelis et al., 2017). We generated 40 independent MI datasets as per the recommended procedure regarding power considerations (Graham et al., 2007).

### 2.4.3. Resampling (R)

Traditional methods do not provide valid confidence intervals or *p*-values for testing the significance of penalized regression coefficients. As an alternative, the non-parametric bootstrap has been used for inference in applications of penalized regression (Abram et al., 2016). The bootstrap step involved 100 resampling steps for each of the 40 MI datasets (with replacement) to create 100 different samples of each MI dataset, with the full sample size. The regression model was subsequently fitted to each of the  $40 \times 100$  samples of data.

### 2.4.4. List of variables included in logistic regressions

We have performed two logistic regressions in which the diagnosis (BD/MDD) was the dependent variable. The independent variables were 18 cytokines (CCL2, CCL3, CCL4, CCL11, CCL13, CCL17, CCL22, CXCL10, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23 p40, IL-15, IL-16, IL-27, IFN- $\gamma$  and TNF- $\alpha$ ), sex, age, BMI, tobacco smoking, history of alcohol abuse, history of substance abuse, antidepressants, benzodiazepines, antipsychotics, lithium, anticonvulsants, other psychotropic drugs, anti-inflammatory drugs and either IDS-C30 or HDRS-17 in the discovery and the replication cohort respectively.

### 2.4.5. Variable selection using the elastic net

The caret (Kuhn, 2008) and glmnet (Friedman et al., 2010) R packages were used to implement Elastic Net penalized logistic regression models with the aim of performing variable selection, leading to a sparser final model, in agreement with previous recommendations (Pavlou et al., 2016). The Elastic Net framework uses linear models with penalties to avoid extreme parameters that may cause overfitting while simultaneously performing variable selection and addressing the issue of multicollinearity (correlation between variables). Optimal alpha and lambda hyper parameters for the Elastic Net algorithm were chosen via 10-fold cross-validation, with the optimal tuning hyper parameters values chosen to maximize the Area Under the Receiving Operator Curve (AUROC). The Elastic Net is not a deterministic process since variables associated with the outcome may not be selected in every run. For this reason, the optimization steps were repeated. For MI resampling (MI-R) analyses,  $40 \times 100$  models were implemented and the hyper parameters optimization step was repeated 3 times, leading to 12,000 models. The Elastic Net was used to select a subset of variables by calculating the Variable Inclusion Probability (VIP), i.e. the percentage of times each variable was kept in the model (i.e. with an associated coefficient different from zero) out of 12,000 models. In the absence of asymptotically valid *p*-values which are (still) not available in high-dimensional regression, the VIP can be interpreted as the posterior probability of including a variable in the model and is used as a measure of the stability of the association (Bunea et al., 2011). However, determining an appropriate threshold for the VIP is challenging. In a seminal paper, the use of a conservative threshold of 50% was recommended because the goal of the authors was "not to miss any possibly relevant predictors" (Bunea et al., 2011). However, this 50% threshold increases the risk of false positives. In one of the mainstays in Bayesian Statistics, Harold Jeffreys categorized values between 50% and 75% as weak, values between 75% and 95% as positive, values between 95% and 99% as strong, and values above 99% as decisive evidence for an effect (Jeffreys, 1961). This prompted us to consider VIP above 75% to



identify variables stably associated with BD. Of note, the researcher who performed the statistical analyses was not blind to the diagnosis because the implementation of machine learning methods requires the entire dataset to be analyzed as a whole.

#### 2.4.6. Influential individuals and outliers

We estimated the influence of each individual in each model by calculating the Cook's distance using logistic regression. As a result, we obtained for each individual the percentage of the runs in which it appeared as influential. Considering a conservative threshold of 50%, there was only one influential individual in the discovery cohort and none in the replication cohorts respectively. Since we had no objective biological reason to think this individual does not represent the target population and natural variation, we decided to retain it for analysis.

#### 2.4.7. Algorithm availability

The algorithm that we have produced is available upon request subject to signing of the non-disclosure agreement between the parties.

### 3. Results

As a first step to identify cytokines associated with BD, we used a discovery cohort consisting of 462 patients experiencing an MDE among whom 42.9% and 57.1% had been previously diagnosed with BD and MDD respectively (Table 1). Serum samples from all patients were assessed for 31 cytokines using a sensitive multiplex immunoassay. Thirteen cytokines, CCL20, CCL26, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-12p70, IL-13, IL-17A, IL-31, TNF- $\beta$  and GM-CSF were below the lower limit of detection (LLOD) in more than 10% of samples. This prompted us to retain only the 18 remaining cytokines in downstream analyses (Supplementary Table 1).

The production of most cytokines involves common biochemical or partly overlapping functional pathways as supported by medium to strong correlations between cytokine pairs (Supplementary Figure 1). Further, blood cytokine levels are impacted by patient characteristics and treatments. In contrast to univariate statistical methods that assess the differential expression of individual biomarkers independently between two groups, we used a penalized logistic regression method to consider the interrelationships between cytokines by including these in the same model together with sex, age, BMI, smoking, history of alcohol and substance abuse, treatments with antidepressants, antipsychotics, benzodiazepines, anticonvulsants, lithium, anti-inflammatory drugs and the 30-item Inventory of depressive symptomatology (IDS-C30). Among several penalized logistic regression methods, we selected the Elastic Net

framework which has good performance when the number of events per variable (EPV) is low, and addresses the issue of multicollinearity while performing variable selection and computing estimates (Pavlou et al., 2016). The more frequently a variable is selected by Elastic Net, the more likely it is stably associated with the outcome. This frequency, coined Variable Inclusion Probability (VIP) (Bunea et al., 2011), is a measure of the stability of an association. Using a relatively stringent VIP threshold of 75%, we identified CXCL10, IL-6, IL-10, IL-15 and IL-27 to be associated with BD in the discovery cohort (Table 2). As for the strength of associations, the mean ORs of CXCL10, IL-6, IL-10, IL-15 and IL-27 were 1.00 (95% CI [0.99,1]), 0.95 (95% CI [0.832,0.996]), 1.18 (95% CI [0.877,1.618]), 1.20 (95% CI [0.979,1.792]) and 1.00 (95% CI [1.00, 1.01]) respectively suggesting that IL-6, IL-10 and IL-15 contributed more to the model than CXCL10 and IL-27. The mean accuracy of the model was 0.83 (95%CI [0.82–0.85]) as determined by the AUROC, and its mean sensitivity and specificity were 0.77 (95%CI [0.66–0.87]) and 0.76 (95%CI [0.65–0.86]) respectively.

As an attempt to replicate our finding in an independent cohort, we used clinical data and serum samples from a replication cohort of 133 MDE patients among whom 30.0% and 70.0% had been previously diagnosed with BD and MDD respectively (Table 3). We then assessed serum samples from these patients for the 18 cytokines which were retained for downstream analyses in the discovery cohort (Supplementary Table 2), and applied the Elastic Net framework to this dataset. Covariates in the replication and discovery cohorts were the same with the exception of history of alcohol and recreative substance abuse which were exclusion factors in the replication cohort. In addition, depressive symptom severity was assessed by the 30 item Inventory of Depressive Symptomatology (IDS-C30) (Rush et al., 1986) and the 17 item Hamilton Depression Rating Scale (HDRS-17) (Hamilton, 1960) in the discovery and replication cohort respectively. Six cytokines were associated with BD in the replication cohort (Table 2), among which IL-10, IL-15 and IL-27 were already associated with BD in the discovery cohort. As for the strength of these associations, the mean ORs of IL-10, IL-15 and IL-27 in the replication cohort were 2.07 (95%CI [0.703–7.366]), 1.80 (95%CI [1.008,4.641]) and 1.0 (95%CI [1.00,1.001]) respectively (Table 2), suggesting that IL-10 and IL-15 contributed more to the model than IL-27. The mean AUROC, sensitivity and specificity of the model were 0.83 (95%CI [0.78–0.87]), 0.75 (95%CI [0.55–0.93]) and 0.81 (95%CI [0.62–0.95]) respectively.

Because the discovery cohort lacked a control group, it did not allow us to draw any conclusions regarding whether the cytokines were increased or decreased in BD and MD patients compared to healthy subjects. However, this was possible in the replication cohort in which

**Table 1**

Characteristics of patients at baseline in the discovery cohort. For each categorical variable, the number (Nb.) and percentage (%) of patients with BD or MDD are indicated. For each continuous variable, mean and standard deviation (SD) are indicated. Statistical tests (\* Chi-square; ° Mann & Whitney) for comparing BD and MDD patients and corresponding p-values after adjustment for multiple testing are indicated. Differences between groups were considered to be statistically significant when adjusted p-values were <0.05. Non-significant (n.s.) statistical differences are indicated.

Variable	BD				MDD				Statistics p-value	Missing values	
	Nb.	%	Mean	SD	Nb.	%	Mean	SD		Nb.	%
All Patients	198	42.9			264	57.1				0	0.0
Sex (Male)	77	38.9			83	31.4			n.s.	1	0.2
Age (years)			46.4	12.5			40.1	14.6	<0.0001°	30	6.5
Body Mass Index (Kg.m-2)			24.8	4.7			23.3	5.3	<0.001°	55	11.9
Tobacco smoking	91	46.0			106	40.2			<0.01*	40	8.7
IDS-C30			25.8	15.7			34.3	13.8	<0.0001°	94	20.3
History of alcohol abuse	60	30.3			61	23.1			n.s.	5	1.1
History of substance abuse	46	23.2			47	17.8			n.s.	8	1.7
Antidepressants	88	44.4			191	72.3			<0.0001*	0	0.0
Benzodiazepines	90	45.5			156	59.1			<0.01*	0	0.0
Antipsychotics	93	47.0			72	27.3			<0.0001*	0	0.0
Lithium	39	19.7			6	2.3			<0.0001*	0	0.0
Anticonvulsants	80	40.4			30	11.4			<0.0001*	0	0.0
Other psychotropic treatments	34	17.2			48	18.2			n.s.	0	0.0
Antiinflammatory treatments	5	2.5			5	1.9			n.s.	0	0.0

**Table 2**

Associations between cytokines and BD in the discovery and replication cohorts. Odds Ratios (ORs), 95% Confidence Interval (CI) and Variable Inclusion Probability (VIP) are shown. Variables with a VIP >0.75 are bolded and labeled with a dot.

Variables	Discovery cohort			Replication cohort		
	ORs	95%CI	VIP	ORs	95%CI	VIP
Sex (Male)	1.072	[0.849,1.291]	0.66	0.800	[0.547,1.197]	<b>0.78</b>
Sex (Female)	0.933	[0.775,1.177]	0.66	1.249	[0.836,1.829]	<b>0.78</b>
Age	1.022	[1.005,1.036]	<b>1.00</b>	0.995	[0.967,1.018]	0.621
BMI (Kg.m-2)	1.027	[1.001,1.067]	<b>0.88</b>	1.049	[0.993,1.154]	<b>0.9</b>
IDS-C30	0.986	[0.972,0.998]	<b>0.98</b>			
HDRS-17				1.041	[0.932,1.149]	0.70
Tobacco smoking (no)	0.932	[0.776,1.126]	0.63	1.082	[0.695,1.706]	0.71
Tobacco smoking (yes)	1.073	[0.888,1.289]	0.63	0.924	[0.587,1.439]	0.71
History of alcohol abuse (no)	0.952	[0.738,1.169]	0.62			
History of alcohol abuse (yes)	1.051	[0.856,1.353]	0.62			
History of substance abuse (no)	0.940	[0.748,1.233]	0.66			
History of substance abuse (yes)	1.063	[0.813,1.338]	0.66			
Antidepressants (no)	1.706	[1.4,2.113]	<b>1.00</b>	1.634	[1.046,3.074]	<b>0.93</b>
Antidepressants (yes)	0.587	[0.473,0.715]	<b>1.00</b>	0.612	[0.326,0.957]	<b>0.93</b>
Benzodiazepines (no)	1.234	[1.028,1.524]	<b>0.96</b>	0.469	[0.251,0.806]	<b>0.99</b>
Benzodiazepines (yes)	0.811	[0.656,0.972]	<b>0.96</b>	2.131	[1.241,3.985]	<b>0.99</b>
Antipsychotics (no)	0.633	[0.486,0.785]	<b>1.00</b>	0.699	[0.464,0.98]	<b>0.96</b>
Antipsychotics (yes)	1.580	[1.273,2.054]	<b>1.00</b>	1.430	[1.02,2.155]	<b>0.96</b>
Lithium (no)	0.447	[0.285,0.685]	<b>1.00</b>	0.727	[0.381,1.404]	<b>0.82</b>
Lithium (yes)	2.233	[1.455,3.513]	<b>1.00</b>	1.373	[0.712,2.614]	<b>0.82</b>
Anticonvulsants (no)	0.488	[0.368,0.641]	<b>1.00</b>	0.335	[0.167,0.626]	<b>1.00</b>
Anticonvulsants (yes)	2.046	[1.553,2.717]	<b>1.00</b>	2.979	[1.592,5.98]	<b>1.00</b>
Other psychotropic drugs (no)	0.966	[0.705,1.242]	0.60	0.795	[0.534,1.237]	0.70
Other psychotropic drugs (yes)	1.034	[0.805,1.418]	0.60	1.256	[0.808,1.872]	0.70
Antiinflammatory drugs (no)	0.834	[0.372,1.819]	0.61	0.825	[0.374,1.98]	0.68
Antiinflammatory drugs (yes)	1.197	[0.551,2.689]	0.61	1.212	[0.505,2.669]	0.68
CCL2 (pg/ml)	1.001	[0.999,1.003]	0.64	0.999	[0.997,1.001]	0.65
CCL3 (pg/ml)	1.001	[0.992,1.004]	0.58	1.000	[0.976,1.023]	<b>0.78</b>
CCL4 (pg/ml)	0.999	[0.996,1.004]	0.56	0.998	[0.994,1.003]	0.66
CCL11 (pg/ml)	1.000	[0.998,1.002]	0.47	0.999	[0.997,1]	<b>0.77</b>
CCL13 (pg/ml)	1.000	[0.997,1.002]	0.53	0.999	[0.992,1.004]	0.66
CCL17 (pg/ml)	1.000	[1,1]	0.64	0.999	[0.998,1.001]	0.63
CCL22 (pg/ml)	1.000	[1,1]	0.70	1.000	[1,1.001]	0.71
CXCL10 (pg/ml)	0.999	[0.99,1]	<b>0.93</b>	1.000	[0.997,1.002]	0.65
IL-6 (pg/ml)	0.948	[0.832,0.996]	<b>0.88</b>	1.002	[0.767,1.184]	0.52
IL-7 (pg/ml)	1.000	[0.979,1.032]	0.56	1.014	[0.968,1.079]	0.60
IL-8 (pg/ml)	0.995	[0.972,1.006]	0.72	1.000	[0.999,1.001]	0.65
IL-10 (pg/ml)	1.177	[0.877,1.618]	<b>0.88</b>	2.067	[0.703,7.366]	<b>0.83</b>
IL-12/IL-23 p40 (pg/ml)	1.000	[0.997,1.004]	0.61	1.000	[0.992,1.008]	0.69
IL-15 (pg/ml)	1.198	[0.979,1.792]	<b>0.75</b>	1.800	[1.008,4.641]	<b>0.81</b>
IL-16 (pg/ml)	1.000	[0.998,1]	0.70	0.999	[0.996,1.003]	0.52
IL-27 (pg/ml)	1.000	[1,1.01]	<b>0.79</b>	1.000	[1,1.001]	<b>0.75</b>
IFN- $\gamma$ (pg/ml)	0.996	[0.977,1.015]	0.67	0.977	[0.918,1.09]	0.74
TNF- $\alpha$ (pg/ml)	1.002	[0.739,1.121]	0.69	1.258	[0.928,1.716]	<b>0.85</b>

both patients with MDE and sex-matched age-matched healthy controls were enrolled (Supplementary Table 3). Data showed that IL-10, IL-15 and IL-27 were present at higher levels in both BD patients and MDD patients compared to healthy controls (Supplementary Figure 2).

While most studies on mood disorders, including this one, have used diagnostic systems such as the DSM-IV to assign individuals to distinct nonoverlapping categories (Association of Psychiatry, 1994), dimensional approaches that characterize patients based on their most prominent symptoms have been proposed. For example, some authors have suggested that bipolarity should be considered as a continuum which can be measured using the bipolarity index (Aiken et al., 2015). Having shown that IL-10, IL-15 and IL-27 were positively associated with the BD diagnosis category, we investigated whether these cytokines were also associated with BD measured as a continuum. The bipolarity index was available in the replication cohort, but not in the discovery cohort. We therefore ran a linear regression on the replication cohort dataset using the bipolar index as the dependent variable and IL-10, IL-15 and IL-27 as independent variables. While IL-15 and IL-27 were not associated with the bipolar index, IL-10 was (coefficient = 11.5; SD = 5.8; p-value < 0.05), thereby providing a partial validation of our previous findings (Table 4).

## 4. Discussion

In this study, we have used a large naturalistic discovery cohort of 462 MDE patients among whom 57.1% and 42.9% were diagnosed with MDD and BD respectively. We analyzed serum samples for 31 cytokines and selected 18 whose levels were above the LLOD in more than 10% of the samples. We then used a penalized logistic regression method to identify cytokines associated with BD after adjustment for co-variables known or suspected to impact cytokine levels, diagnosis or both. Among the five cytokines associated with BD in the discovery cohort, IL-10, IL-15 and IL-27, were also associated with BD in the replication cohort therefore providing an external validation of our results. Of note, we did not make the hypothesis that cytokines could discriminate BD and MDD *a priori* but after data collection was finished.

### 4.1. Association of cytokines with BD

As for the strength of the association between IL-10, IL-15 and IL-27 and BD in the discovery cohort, their mean ORs were 1.18 (%95 CI [0.877,1.618]), 1.20 (%95 CI [0.979,1.792]) and 1.00 (%95 CI [1,1.01]) respectively. Because the OR for a given cytokine represents the

**Table 3**

Characteristics of patients in the replication cohort. For each categorical variable, the number (Nb.) and percentage (%) of patients with BD or MDD are indicated. For each continuous variable, mean and standard deviation (SD) are indicated. Statistical tests (\* Chi-square; ° Mann & Whitney) for comparing BD and MDD patients and corresponding p-values after adjustment for multiple testing are indicated. Differences between groups were considered to be statistically significant when adjusted p-values were < 0.05. Non-significant (n.s.) statistical differences are indicated.

Variables	BD				MDD				Statistics	Missing values	
	Nb.	%	Mean	SD	Nb.	%	Mean	SD	p-value	Nb.	%
All patients	40				93					0	0
Males	10	25.0			31	33.3			n.s.	0	0
Age (years)			44.6	10.9			44.1	15.4	n.s.	0	0
Body Mass Index (Kg.m-2)			26.9	5.28			24.3	4.75	n.s.	14	15.1
Tobacco smoking	15				39	41.9			n.s.	0	0
HDRS-17			23.7	3.57			23.0	3.15	n.s.	0	0
First MDE	5	13.9			28	32.2			<0.01	10	7.52
Number of MDE			4.41	2.76			2.63	1.92	<0.01	20	15.0
Episode duration (months)			6.53	7.57			13	30.2	<0.01	3	0.023
Disease duration (years)			16.0	11.6			11.4	11.1	n.s.	14	10.5
Current psychotic symptoms	1	2.5			2	2.2			n.s.	1	0.0075
Bipolar Type 1 disorder	19	47.5								0	0
Bipolar Type 2 disorder	21	52.5								0	0
Antidepressants	24	60.0			74	79.6			n.s.	0	0
Benzodiazepines	36	90.0			67	72.0			n.s.	0	0
Antipsychotics	24	60.0			30	32.3			n.s.	0	0
Lithium	9	22.5			6	6.5			n.s.	0	0
Anticonvulsants	15	37.5			3	3.2			<0.0001*	0	0
Other psychotropic treatments	22	55.0			22	23.7			n.s.	0	0
Antiinflammatory treatments	4	10.0			1	1.1			n.s.	0	0

**Table 4**

Association between the bipolar index and cytokine levels in the replication cohort. A linear regression was run using the bipolar index as the dependent variable and IL-10, IL-15 and IL-27 as the independent variables. Estimates, standard errors (SE), and p-values are shown. An association was considered to be statistically significant when p-value < 0.05. Significant associations were bolded.

Variable	Estimate	SE	p-value
Sex (Female)	2.56	4.13	0.54
Age (years)	-0.27	0.14	0.06
BMI (Kg.m-2)	0.24	0.40	0.56
Tobacco smoking	2.99	3.88	0.44
Antidepressors	0.30	4.89	0.95
Benzodiazepine	-0.67	-0.67	0.90
Antipsychotics	5.01	3.93	0.21
Lithium	10.77	6.03	0.08
Anticonvulsants	25.91	6.48	<b>&lt;0.001</b>
Other psychotropic drugs	0.07	4.43	0.99
Antiinflammatory drugs	3.21	8.26	0.70
HDRS-17	-0.10	0.58	0.86
IL-10 (pg/ml)	11.46	5.77	<b>&lt;0.05</b>
IL-15 (pg/ml)	4.66	6.05	0.44
IL-27 (pg/ml)	0.00	0.00	0.86

change in odds that a patient is diagnosed with BD when the concentration of this cytokine increases by one standard deviation (SD), both the distribution of each cytokine in the sample and its OR should be considered when comparing their contribution to the model. For example, while the SD of IL-27 was several orders of magnitude higher than those of IL-10 and IL-15, its computed OR was very close to 1 strongly suggesting that this cytokine contributed much less to the model compared to IL-10 and IL-15.

While BD and MDD are distinct diagnosis categories in current psychiatric nosology, some authors have proposed that BD should be considered as a continuum that could be measured using the bipolarity index (Sachs, 2004). This index being available for 111 patients of the replication cohort, it allowed us to explore the association between the bipolarity index and cytokines. Results showed that IL-10 was associated with the bipolar index but not IL-15 or IL-27, a result which was in partial agreement with our first analysis using the DSM-IV criteria for

distinguishing BD from MDD. As for IL-15 and IL-27, we could only speculate on the reasons for which they were associated with BD when we used a categorical approach, and not a dimensional approach. While the bipolarity index is a continuous variable, some authors have used this scale and a cut-off of 50 for distinguishing BD from MDD (Aiken et al., 2015). Using this cut-off, only 24 patients (out of 28) who were diagnosed with BD according to the bipolarity index fulfilled the DSM-IV criteria for BD. In contrast, only 73 patients (out of 83) who were diagnosed with MDD according to the bipolarity index fulfilled the DSM-IV criteria for MDD. This partial overlap between the two diagnosis systems may explain, at least in part, why IL-15 and IL-27 were associated with BD in one analysis and were not in the other. In addition, it is noteworthy that the bipolarity index was only available for 111 patients of the replication cohort therefore limiting the statistical power of the study.

#### 4.2. Model performance

As for the ability of blood cytokines to discriminate BD from MDD, we adjusted the model for sex, age, BMI, smoking, history of alcohol and substance abuse, treatments with antidepressants, antipsychotics, benzodiazepines, anticonvulsants, lithium, anti-inflammatory drugs and depressive symptoms measured either by IDS-C30 or HDRS-17 scales. This was necessary as cytokine levels are known or believed to be impacted by these covariates (Achur et al., 2010; Juncal-Ruiz et al., 2018; Wiedlocha et al., 2018; Yanbaeva et al., 2007). The AUROC, sensitivity and specificity of the model in the discovery cohort were 0.83 (95%CI [0.82–0.85]), 0.77 (95%CI [0.66–0.87]) and 0.76 (95%CI [0.65–0.86]) respectively. While these performances could be considered as good, we would like to emphasize that it is not possible to disentangle blood variables from clinical variables. Covariates and confounders known or believed to impact either cytokine levels or diagnosis must be included in the model, but assessing a model performance can only be done (by construction) using all included variables. Therefore, it is impossible to include confounders in a model (which should be done) and not to consider them for assessing its performances (which is the ultimate goal). This important issue has been overlooked in most studies in which model performances were reported (Poletti et al., 2020; Wollenhaupt-Aguiar et al., 2019).

#### 4.3. Discrepancies between studies

To the best of our knowledge, five studies, including this one, have aimed at identifying immune biomarkers that could discriminate patients with BD from those with MDD. However, it is difficult to compare results across studies because of differences regarding the immune biomarkers that were assessed, the sample size, the analytical methods and most importantly the statistical approaches (Supplementary Table 4). Out of the 18 cytokines that we have retained for further analysis, two, i.e. IL-27 and CCL17, have not been measured by others. Out of the remaining cytokines, we found that IL-10 was present at higher levels in BD compared to MDD, while Poletti et al. (2020) found it was present at lower levels and Brunoni et al. (2020) reported that it was present at similar levels in the two groups. Further, Wollenhaupt-Aguiar et al. found that IL-10 differentiated BD from MDD in a machine learning approach but these authors did not report whether it was more abundant in BD or MDD compared to the other group (Wollenhaupt-Aguiar et al., 2019). As for the 14 cytokines that did not discriminate BD from MDD in our study, seven were found to be expressed at different levels in BD and MDD in at least one study: CCL2, CCL3, CCL4, CCL11, IL-6, IL-7 and TNF- $\alpha$ . While these discrepancies are puzzling, it is noteworthy that in the present study both a discovery and a replication cohort were used.

#### 4.4. A possible role of IL-10, IL-15 and IL-27 in the etiology of mood disorders

The roles of IL-10, IL-15 and IL-27 as regulatory and effector molecules of the immune system are well known. However, several studies have demonstrated that these cytokines could also regulate neurodevelopment and brain function. For example, IL-10 is an important mediator of the crosstalk between microglia, astrocytes and neurons (Lobo-Silva et al., 2016). IL-10 inhibits the production of pro-inflammatory cytokines by astrocytes and microglia (Balasingam and Yong, 1996; Ledebor et al., 2002) and potentiates the production of transforming growth factor (TGF)- $\beta$  by astrocytes which, in turn, attenuates microglial activation (Norden et al., 2014). IL-10 receptor signaling promotes neuronal survival (Zhou et al., 2009a, b) and regulates adult neurogenesis (Pereira et al., 2015; Perez-Asensio et al., 2013). In addition, IL-10 treatment reduced anxiety-depressive behaviors in a mouse model of depression (Worthen et al., 2020). As for IL-15, this cytokine and its receptors are expressed throughout the brain by either glial cells or neurons, and show developmental changes, regional differences, and regulation by inflammatory challenges (Kurowska et al., 2002) (Wu et al., 2010b). In human fetal brain, IL15 and IL15R $\alpha$  mRNA levels are higher in the hippocampus and cerebellum compared to the cortex and thalamus (Kurowska et al., 2002). Other studies have shown that IL-15 regulated proliferation and self-renewal of adult neural stem cells (Gomez-Nicola et al., 2011) and that its blockade inhibited microglial activation (Gomez-Nicola et al., 2010). Most importantly, loss-of-function experiments in mice have demonstrated that IL-15 exerts anti-depressive effects by regulating the serotonin system (Wu et al., 2010a) and facilitates GABA transmission and hippocampal-dependent memory (He et al., 2010). As for IL-27, it has been reported to have an anti-inflammatory effect through regulation of T cell differentiation, resulting in reduced IL-17 and induction of IL-10. Furthermore, IL-27 can be secreted by resident cells of the central nervous system (CNS) such as astrocytes or microglia cultured *in vitro* (Li et al., 2005; Sonobe et al., 2005) as well as *in vivo* in multiple sclerosis (Senecal et al., 2016). To summarize, while there is currently no strong evidence that IL-10, IL-15 or IL-27 play a role in the etiology of mood disorders, this is an attractive possibility based on their ability to regulate neurodevelopment, brain function and behavior.

#### 4.5. Limitations

The present study has potential limitations. First, the blood samples were thawed up to three times in the replication cohort potentially introducing noise. Second, while patients in the replication cohort were followed for six months after inclusion, those in the discovery cohort were clinically assessed and sampled only once. Therefore, it is possible that at least some patients who were diagnosed as having MDD in the discovery cohort eventually converted to BD at later time points. While we acknowledge that this could be a flaw, it is noteworthy that the vast majority of studies in the field do not have a follow-up at all. In this context, we have collected all data available using patient charts, examination and, when possible, familial insight to guarantee the accuracy of the diagnosis. Diagnoses in both cohorts were made by psychiatrist experts in the field of mood disorder. Third, the penalized regression method shrinks coefficients towards 0, and therefore ORs towards 1. The computed ORs reported in the current study may therefore underestimate the strength of the association between individual cytokines and BD. Fourth, patients with mild or subthreshold depression were not included in our discovery and replication cohorts, therefore limiting the generalizability of our findings. Fifth, we did not collect data about psychotherapy. Sixth, we excluded patients who were under 18. Seventh, subjects were all white, and our data may not be generalized to other ethnic groups. Seventh, we did not include patients with severe medical conditions in both the discovery and the replication cohort. Although this is customary in such studies because other medical conditions interfere with cytokines levels (Krabbe et al., 2015), this also limits the external validity and generalizability of our findings. Last but not least, both our discovery and replication cohorts were retrospective in design and therefore subject to memory bias as opposed to prospective studies (Sedgwick, 2013).

#### 4.6. Strengths

We believe that the present study has several strengths. First, our discovery cohort consisted of a large sample of 462 patients. Second, we partially replicated our results in an independent replication cohort thereby providing an external validation to our findings. Third, patients that were included in our discovery and replication cohorts were sampled when presenting a MDE and carefully characterized for several covariates and confounders which were all included in statistical analysis. Fourth, we conducted statistical analyses using Elastic Net penalized logistic regression models which, in contrast to standard univariate and multivariate methods, consider multicollinearity between variables and perform variable selection. Fifth, the three cytokines that were associated with BD in both the discovery and replication cohorts were also more abundant in patients compared to healthy controls. Lastly, one of the three cytokines that we have identified as associated with the BD diagnosis category was also associated with BD measured as a continuum.

### 5. Conclusions and prospects

To summarize, we have shown here that IL-10, IL-15 and IL-27 could discriminate BD patients from MDD patients when in a depressed state. Because these cytokines have been previously demonstrated to regulate neurodevelopment and brain function by acting on neurons, astrocytes or glial cells, their possible role in the etiology of mood disorders is an attractive possibility which deserve to be further investigated.

#### Declaration of competing interest

NG, RB, EM, SB, PC and EI are co-inventors of a proprietary (CNRS,



Université Côte d'Azur) patent application entitled "A method for diagnosing *in vitro* a bipolar disorder or a major depressive disorder" filed in the European Patent Office on October 4, 2018 (11115 EP). EO, SG, DD and LD reported no biomedical financial interests or conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbih.2021.100232>.

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