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Neurotrophic and inflammatory markers in bipolar disorder: A prospective study

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ABSTRACT

Altered neurotrophic signaling is thought to impair neuroplasticity in bipolar disorder (BD). Brain-derived neurotrophic factor (BDNF) is proposed as a neurotrophic marker in BD. However, the current evidence for its use in monitoring disease activity and illness progression is conflicting and an exploration of additional neurotrophic markers is needed. This prospective case-control study investigated mood-specific changes in potential neurotrophic markers and their association to inflammatory activity. Patients with BD were included during an acute mood episode, either depressive (n = 35) or (hypo)manic (n = 32). Fifty-nine patients (88%) and 29 healthy controls (97%) completed the study. Peripheral blood levels of BDNF, vascular endothelial growth factor A (VEGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and tumor necrosis factor alpha (TNF-α) were measured at baseline and after 2 months. Biomarker levels in patients were compared to controls and correlated to HDRS-17 and YMRS total scores and the PANSS positive subscale scores. Linear mixed model analysis revealed no significant differences in neurotrophic markers between patients and controls. We found significantly increased TNF-α levels in patients and a subsequent normalization during euthymia. None of the biomarkers strongly correlated to mood symptom severity. Despite standardized methodological practices, BDNF and VEGF levels had a wide distribution range. We need a better understanding of methodological aspects influencing the analysis of neurotrophic factors to improve future research on markers for mood state monitoring and illness progression in BD.

1. Introduction

Changes in neurotrophic signaling leading to impaired neuroplasticity are thought to play a role in the pathophysiology of bipolar disorder (BD) (Berk et al., 2011; Grande et al., 2012). Several neurotrophins and trophic factors are thought to be involved, with most evidence for changes in brain-derived neurotrophic factor (BDNF) expression in patients with BD (Scola and Andreazza, 2015). Decreased BDNF levels were shown during acute mood episodes and correlated negatively to the severity of mood symptoms (Cunha et al., 2006; Machado-Vieira et al., 2007). In line with the latter, drug responders had increasing BDNF levels while non-responders had stable lower levels (de Sousa et al., 2011; Tramontina et al., 2009). On the long term, a negative correlation between BDNF levels and duration of illness was found (Kauer-Sant'Anna et al., 2009). These findings put BDNF forward as a promising biomarker for both acute mood state monitoring and documenting illness progression in BD (Grande et al., 2010). However, the evidence on BDNF as a biomarker in BD is increasingly conflicting. While decreased BDNF levels in patients with BD compared to healthy controls (HCs) are confirmed in recent meta-analyses, state-related BDNF alterations or a link to illness progression were not consistent (Fernandes et al., 2015; Munkholm et al., 2016; Polyakova et al., 2015). Furthermore, the meta-analyses point out methodological problems such as between-study heterogeneity, insufficient control for

Abbreviations: BD, bipolar disorder; BDNF, brain-derived neurotrophic factor; CV, coefficient of variation; ECT, electroconvulsive therapy; HC, healthy control; HDRS, Hamilton depression rating scale; MDD, major depressive disorder; PANSS, Positive and negative syndrome scale; sFlt-1, soluble fms-like tyrosine kinase-1; TNF-α, tumor necrosis factor-alfa; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; YMRS, Young mania rating scale

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confounding factors, publication bias and a lack of longitudinal designs (Munkholm et al., 2016). Consequently, the initial enthusiasm on BDNF has tempered and efforts shifted towards an exploration of other potential biomarkers in BD (Scola and Andreazza, 2015). Vascular growth factor A (VEGF) is an angiogenic protein with neurotrophic capacities induced by hypoxia and pro-inflammatory cytokines (Ruiz de Almodovar et al., 2009). VEGF signals through the VEGF receptor (VEGFR)-1 and VEGFR-2, which are both involved in neuroprotection and neuroregeneration. The soluble form of VEGFR-1 (soluble fms-like tyrosine kinase-1, sFlt-1) is induced by hypoxia and acts as an anti-angiogenic factor by scavenging free VEGF and attenuating its trophic effects (Ruiz de Almodovar et al., 2009). Soluble Flt-1 also sensitizes endothelial cells for pro-inflammatory cytokines making them more susceptible to damage (Lizano et al., 2016). Disturbances in VEGF signaling were reported in schizophrenia (Lee et al., 2015; Lopes et al., 2015), but studies in BD are scarce (Scola and Andreazza, 2015). A single study showed a mood state-related increase in VEGF levels in major depressive disorder (MDD) and BD (Lee and Kim, 2012). Lithium treatment downregulated *VEGF* transcription in leukocytes of patients with BD (Kikuchi et al., 2011). These findings may represent mood state-related VEGF changes, but without prospective studies, the evidence remains weak. Increased sFlt-1 levels were found in a young population at high risk for psychosis, but again there are no studies in BD (Lizano et al., 2016).

Immune system alterations in patients with BD have been demonstrated extensively (Haarman et al., 2014; Munkholm et al., 2013; Rosenblat et al., 2014). An increased pro-inflammatory state during mood episodes as well as a role of inflammation in illness progression have been described (Berk et al., 2010; van den Ameele et al., 2016). Although both neurotrophic factors and inflammatory cytokines are proposed as biomarkers in BD, study designs exploring the interaction between alterations in neurotrophic signaling and immune system activity are scarce (Jacoby et al., 2016; Kauer-Sant'Anna et al., 2009). A better exploration of possible interactions between neurotrophic and inflammatory pathways could contribute to better insight in the underlying pathophysiology of acute mood episodes and illness progression.

We aimed to prospectively investigate mood state-specific changes in neurotrophic markers (BDNF, VEGF and sFlt-1) in patients with BD compared to HCs. Since there is great evidence on increased tumor necrosis factor-alfa (TNF-α) levels in BD (Modabbernia et al., 2013; Munkholm et al., 2013) with even indications of a mood state-related increase (Fiedorowicz et al., 2015; Modabbernia et al., 2013; van den Ameele et al., 2016), TNF-α was included as a marker of inflammation to study the interaction between inflammatory changes and neurotrophic markers in BD. We hypothesized TNF-α, VEGF and sFlt-1 levels to be increased in patients with BD in all affective states compared to HCs and that these levels would positively correlate to the severity of mood symptoms. The inverse was hypothesized for BDNF.

2. Methods

2.1. Participants

Inpatients were recruited in 3 psychiatric centers in the region of Antwerp, Belgium. Outpatients were recruited *via* the Flemish patient association. The inclusion criteria were age 18–65 years, DSM-IV diagnosis of BD type I, type II or schizoaffective disorder and suffering from a depressive or (hypo)manic episode. Healthy controls were recruited mainly among staff members. Patients and HCs were age and gender matched because of the impact of these factors on inflammatory activity and neurotrophic factors (Bus et al., 2011; Michaud et al., 2013). We aimed for an equal distribution of inclusions of HCs and patients throughout the year to account for seasonality in immune system activity and BDNF levels (Molendijk et al., 2012; Nelson, 2004). Exclusion criteria for both patient and control group were: substance abuse, use of anti-inflammatory drugs within 2 weeks before screening or test days, acute infection, autoimmune diseases, chronic inflammatory or neurological diseases, pregnancy or breastfeeding, electroconvulsive therapy (ECT) within 6 months before screening or during follow-up, mental retardation, significant disturbances on a screening blood test evaluating a complete blood count, blood chemistry, fasting glucose, lipid profile, liver, kidney and thyroid function, and serology (human immunodeficiency virus, hepatitis B and C). Urine drug testing was routinely done at screening and repeated on subsequent test days when drug abuse was suspected (e.g. history of substance abuse, unreliable anamnesis). In the control group, additional exclusion criteria were applied: current or past diagnosis of MDD, BD or psychotic syndrome as defined by DSM-IV criteria and BD or psychotic syndrome in a first-degree family member, use of psychopharmacological drugs, benzodiazepines or benzodiazepine-like products. There were no other restrictions regarding medication use.

Participants were recruited between March 2015 and May 2016. The study was approved by the Committee for Medical Ethics of the University Hospital Antwerp and the Antwerp University with protocol number B300201421645. The local ethical committees of the participating centers approved the protocol. All participants agreed to participate in the study and signed informed consent. The study complied with the Declaration of Helsinki.

2.2. Study design

Patients were recruited during an acute mood episode, either depressed (BD-D) or (hypo)manic (BD-M). Patients with mixed features were allocated to the mood state of which the symptoms were most pronounced, according to clinical assessments at screening. All participants were tested for the first time within 1–5 days after screening and a second time at 2 months follow-up. Clinical assessments and blood draws were always done on the same day. During the study period, patients received treatment as usual without intervention of the investigators.

2.3. Clinical assessments

The M.I.N.I.-plus, International Neuropsychiatric Interview, version 5.0.0 was chosen as diagnostic instrument in patients and HCs because of its accurate structured DSM-IV diagnosis and convenience to administer (Sheehan et al., 1998). In patients, mood symptom severity was assessed by the 17-item Hamilton Depression Rating Scaling (HDRS-17) (Hamilton, 1960) and the Young Mania Rating Scale (YMRS) (Young et al., 1978) at screening and on both test days. At screening, threshold scores for inclusion were set at $\geq$17 for the HDRS-17 or $\geq$13 for the YMRS, corresponding to moderate depression and hypomania respectively (Vieta, 2009; Zimmerman et al., 2013). Positive psychotic symptoms were evaluated on test days with the positive subscale of the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987). In the control group, the occurrence of mood episodes during follow-up was evaluated based on a short screening questionnaire on the 2nd test day. For all participants, we assessed medication use and the occurrence of any exclusion criteria on both test days. All clinical assessments were done by a psychiatrist in training (SvdA) and supervised by a specialist in psychiatry (MM).

2.4. Laboratory assessments

Blood samples were obtained between 8.00 AM. and 10.30 AM. Blood was drawn by venipuncture into an EDTA-coated vacuum tube (5 ml). Tubes were immediately stored at 4 °C and centrifuged at 2*g* and 4 °C for 10 min within 2 h after the blood draw. Plasma was aliquoted and stored at −70 °C until assayed.

TNF-α, BDNF, VEGF and sFlt-1 were measured in duplicate by an electrochemiluminescence immunoassay technique (Mesoscale

Table 1
Baseline demographic and metabolic characteristics. Data presented as mean ± SD (range) or n (%).

	BD-D	BD-M	BD-total	HC	*p*-value[a]	
					BD-total ↔ HC	*BD-D ↔ BD-M ↔ HC*
N	35	32	67	30		
Female (%)	24 (68.6)	15 (46.9)	39 (58.2)	16 (53.3)	0.654	0.182
Age, years	43.7 ± 9.7 (28–61)	42.9 ± 12.7 (23–62)	43.3 ± 11.1 (23–62)	43.0 ± 11.4 (22–61)	0.982	0.903
Caucasian (%)	33 (94.3)	30 (93.8)	63 (94.0)	29 (96.7)	0.587	0.859
Education, total years	13.34 ± 2.2 (9–17)	14.2 ± 1.8 (10–17)	13.7 ± 2.1 (9–17)	15.1 ± 1.7 (11–18)	**0.001**	**0.002**[b]
Smokers (%)	18 (51.4)	14 (43.8)	32 (47.8)	6 (20.0)	**0.010**	**0.029**
BMI, kg/cm^2	24.3 ± 3.5 (18–33)	26.4 ± 4.7 (20–39)	25.3 ± 4.2 (18–39)	23.7 ± 2.5 (20–28)	**0.027**	**0.017**[b]
Waist, cm	87.1 ± 11.7 (66–111)	91.2 ± 12.4 (71–122)	89.1 ± 12.1 (66–122)	85.4 ± 9.8 (67–104)	0.163	0.144
Fasting glucose, mg/dL	88.9 ± 8.8 (69–107)	91.1 ± 9.8 (74–116)	90.0 ± 9.3 (69–116)	87.9 ± 7.1 (73–102)	0.281	0.337
Cholesterol, mg/dL						
Total	189.1 ± 42.7 (128–341)	183.7 ± 47.3 (101–290)	186.5 ± 44.7 (101–341)	195.5 ± 43.4 (132–283)	0.360	0.584
HDL	61.0 ± 15.6 (34–96)	56.0 ± 19.9 (24–102)	58.6 ± 17.8 (24–102)	61.2 ± 19.8 (28–118)	0.522	0.439
LDL	108.4 ± 41.5 (48–264)	102.4 ± 40.6 (44–202)	105.6 ± 40.9 (44–264)	113.1 ± 33.3 (67–104)	0.386	0.565

BD-D: patient group with depressive episode at baseline; BD-M: patient group with (hypo)manic episode at baseline; BD-total: total patient group; HC: healthy controls; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein.

[a] *p*-values of *t*-test, Chi-squared test or oneway ANOVA, *p*-values < 0.05 are presented in bold.

[b] *Post-hoc* correction for multiple testing by Tukey HSD shows a significant higher education in HCs compared to BD-D ($p = 0.001$); a significant higher BMI in the BD-M group compared to HCs ($p = 0.019$).

Discovery, Rockville, USA) according to the manufacturer's instructions. Kits used for detection were V-plex human TNF-α kit; Multi-Spot Special Order Human BDNF & VEGF; V-plex human Flt-1 kit. The lower limits of detection were respectively 0.18, 4.31, 0.92 and 4.73 pg/mL. Sample signals were fitted on a 4-parametric logistic calibration curve to calculate concentrations. One sample was excluded from the BDNF analyses due to a signal below the detection range. For the other assays, all signals were within the detection range.

Patient and HC samples were analyzed in randomized sequence with both samples of a single subject on the same plate and an equal distribution of patients and HCs per plate. Samples with a coefficient of variation (CV) > 15% were excluded for statistical analysis. For the included samples, the mean CV and standard deviation of TNF-α, BDNF, VEGF and sFlt-1 were 3.01 (2.52), 5.42 (3.71), 3.58 (2.96) and 2.81 (2.56) respectively. We visually evaluated sample quality and corrected for overt hemolysis in the statistical analysis.

2.5. Statistical analysis

Normality of outcome variables, and homoscedasticity of residuals were evaluated by visual inspection. For the regression modelling, VEGF and BDNF concentrations were log-transformed to obtain a normally distribution variable. Homogeneity of variances was assessed by Levene's test and further analyses were adapted accordingly.

Baseline differences in clinical and demographic characteristics between the total patient group (BD-total) and HCs were examined by two-tailed independent *t*-tests for continuous variables and Pearson chi-square test for categorical variables. Differences between BD-D, BD-M and HCs were analyzed by one-way ANOVA and *post-hoc* analysis with Tukey correction for multiple testing.

Longitudinal data were examined using linear mixed model analysis with the biological parameters as outcome variable. Based on the *LogLikelihood* value, we fitted a model that included the subjects as random intercept and the subject*moment interaction as random slope. We included following factors as fixed effects: group (BD-D *vs*. BD-M *vs*. HC or BD-total *vs*. HC), moment (1st *vs*. 2nd test day), and a group by moment interaction (group*moment). In the adjusted model, we included BMI, baseline smoking status, hemolysis, sex, age and season of sampling (spring/summer *vs*. autumn/winter) as covariates. The output from the mixed model analysis, is reported as "F-ratio (DF); *p*-value; *b*".

The relation between mood state and concentration of biological parameters, is studied by comparing levels of biological markers in patients with an acute mood episode with levels in euthymic patients and by correlating HDRS and YMRS total scores and PANSS positive subscale scores to the levels of biological markers.

For the mixed model analyses, both duplicate measurements were included. By adding the subject ID as a random intercept to the model, we accounted for the non-independence between observations within the same individual. For the other analyses, means of duplicate measurements were calculated.

All statistical analyses were performed in JMP, 2015 in collaboration with StatUA, core facility for statistical data analysis at the University of Antwerp.

2.6. Sample size and power analysis

We performed a power analysis based on data of earlier research for TNF-α, BDNF and VEGF (Lee and Kim, 2012; Munkholm et al., 2014; Ortiz-Dominguez et al., 2007). For sFlt-1, no earlier research in BD was available, so this study was considered as a pilot study. We aimed to reach sufficient power to assess differences between the BD-total and HCs and between 3 groups: BD-D, BD-M and HCs. For BDNF, we established a minimal sample size of 35 by group, for VEGF 30. Since the longitudinal design with 2 test moments for every subject, we estimated that 30 subjects by group would be sufficient to obtain 80% power at an α-level of 0.05. Power analysis was performed in JMP, 2015.

3. Results

3.1. Demographic and clinical characteristics

Sixty-seven patients with BD and 30 HCs were included. At baseline 32 patients were in a (hypo)manic and 35 in a depressive episode. Demographic and clinical characteristics and details on statistical tests are shown in Tables 1 and 2. In both patient groups the percentage of smokers was high compared to HCs. BMI was higher in the BD-M group compared to the HCs. No other significant differences in metabolic parameters were seen between patients and HCs.

Fifty-nine patients (88%) and 29 HCs (97%) completed the study. Drop-out in the patient group was due to: chronic use of low-dose acetylsalicylic acid (2), substance abuse (2), ECT (2) and loss of contact (2). In the control group, there was 1 drop-out due to difficult blood draws. In 56% of the patients, the prescribed psychotropic drug changed during follow-up.

For the evolution of clinical scores, see Table 3 and Fig. S1. The mean HDRS total scores in the BD-D group decreased significantly

Table 2
Clinical characteristics and baseline data of patients. Data presented as mean ± SD (range) or n (%).

	BD-D	BD-M	BD-total
N	35	32	67
Diagnosis, n (%)			
BD type I	16 (45.7)	26 (81.3)	42 (62.7)
BD type II	19 (54.3)	4 (12.50)	23 (34.3)
Schizoaffective disorder	0 (0)	2 (6.2)	2 (3)
Age at onset, years	22.5 ± 10.9 (8–55)	27.5 ± 11.8 (10–50)	24.9 ± 11.5 (8–55)
Duration of illness, years[a]	20.6 ± 10.2 (3–49)	14.3 ± 11.7 (0–41)	17.6 ± 11.3 (0–49)
Episode at onset: depression, n (%)	23 (67.7)	17 (53.1)	40 (60.6)
Age first depression, years	24.7 ± 12.3 (8–55)	27.5 ± 12.0 (10–50)	25.9 ± 12.1 (8–55)
Age first mania/hypomania, years	27.2 ± 11.4 (8–55)	30.7 ± 12.4 (13–59)	28.9 ± 11.9 (8–59)
Lifetime psychotic features, n (%)	13 (37.1)	24 (75.0)	37 (55.2)
Total number of hospitalizations, n (%)			
0	6 (17.1)	3 (9.4)	9 (13.4)
1–5	23 (65.7)	22 (68.8)	45 (67.2)
6–10	6 (17.1)	7 (21.9)	13 (19.4)
Lifetime substance abuse, n (%)	16 (45.7)	14 (43.8)	30 (44.8)
Alcohol	11 (31.4)	7 (21.9)	18 (26.9)
THC	6 (17.1)	7 (21.9)	13 (19.4)
Hard drugs	4 (11.4)	1 (3.1)	5 (7.5)
Baseline clinical scales, total score			
HDRS	21.2 ± 4.0 (15–31)	8.1 ± 5.7 (0–25)	
YMRS	5.5 ± 4.8 (0–16)	21.0 ± 5.1 (15–36)	
PANSS positive subscale	9.8 ± 3.4 (0–19)	15.0 ± 4.7 (8–25)	
Duration of index episode, n (%)			
< 1 w	0 (0)	3 (9.4)	3 (4.5)
1–4 w	7 (20.0)	18 (56.3)	25 (37.3)
1–6 m	19 (54.3)	9 (28.1)	28 (41.8)
6–12 m	8 (22.9)	2 (6.2)	10 (14.9)
Baseline medication use, n (%)			
Medication-free	3 (8.6)	3 (9.4)	6 (9.0)
Lithium	13 (37.1)	11 (34.4)	24 (35.8)
Valproate	6 (17.1)	3 (9.4)	9 (13.4)
Carbamazepine	1 (2.9)	2 (6.3)	3 (4.5)
Lamotrigine	7 (20.0)	1 (3.1)	8 (11.9)
Antipsychotic	19 (54.3)	23 (71.9)	42 (62.7)
Antidepressant	25 (71.4)	6 (18.8)	31 (46.3)
Benzodiazepine	8 (22.9)	16 (50.0)	24 (35.8)

BD-D: patient group with depressive episode at baseline; BD-M: patient group with (hypo)manic episode at baseline; BD-total: total patient group; HC: healthy controls; THC: tetrahydrocannabinol; HDRS: Hamilton depression rating scale; YMRS: Young mania rating scale; PANSS: positive and negative symptom scale.
[a] Defined as number of years since first mood episode.

between both test days from moderate towards mild depressive scores, similar to the YMRS total scores in the BD-M group. Both patient groups had a significant decrease in PANSS positive subscale scores.

3.2. Biological parameters in patients compared to HCs

For concentrations of biological parameters, see Table 3. The linear mixed models with TNF-α, BDNF, VEGF and sFlt-1 as dependent variables, revealed no significant effect of the factors group (BD-D *vs*. BD-M *vs*. HC), moment, or the group by moment interaction. See Table S1 for more details. By adding the covariate 'sex' to the model, we saw significant increased TNF-α levels in the BD-D group compared to HCs ($F(94.0) = 3.7$; $p = 0.028$; $b = 0.45$ pg/mL; Tukey HSD for multiple comparison). Adjustment with the other covariates (BMI, smoking status, hemolysis, age, season of sampling) did not influence the significance of the model. By combining the BD-D and BD-M into a single patient group (BD-total, see Table S2), we observed a significant group effect for TNF-α indicating higher TNF-α levels in the BD-total group ($F(96.02) = 4.75$; $p = 0.032$; $b = 0.32$ pg/mL). A significant group by moment interaction effect was found for VEGF ($F(85.32) = 4.89$;

Table 3
Clinical scales and concentrations (pg/mL) at baseline and follow-up. Presented as means (SD).

	BD-D		BD-M		HC	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
N	35	31	32	28	30	29
HDRS	21.2 (3.8)	14.1 (6.4)	8.1 (5.7)	9.7 (7.8)		
YMRS	5.5 (4.8)	3.9 (3.6)	20.9 (5.1)	7.7 (9.0)		
PANSS positive subscale	9.8 (3.4)	8.6 (2.3)	15.0 (4.7)	10.1 (4.8)		
TNF-α	2.6 (0.7)	2.7 (0.8)	2.6 (0.7)	2.4 (0.7)	2.2 (0.7)	2.3 (0.7)
BDNF	212.3 (273.2)	197.9 (250.7)	277.0 (371.4)	241.2 (168.9)	275.5 (308.1)	161.0 (119.6)
VEGF	23.8 (10.2)	26.6 (12.3)	25.5 (11.6)	25.8 (8.3)	25.6 (14.9)	20.4 (5.3)
sFlt-1	73.0 (15.0)	74.5 (17.0)	72.5 (14.6)	69.8 (15.0)	74.0 (21.1)	71.0 (24.6)

BD-D: patient group with depressive episode at baseline; BD-M: patient group with (hypo)manic episode at baseline; HC: healthy controls; HDRS: Hamilton depression rating scale; YMRS: Young mania rating scale; PANSS: positive and negative symptom scale; TNF-α: tumor necrosis factor-alfa; BDNF: brain derived neurotrophic factor; VEGF: vascular endothelial growth factor; sFlt-1: soluble Fms-like tyrosine kinase-1.

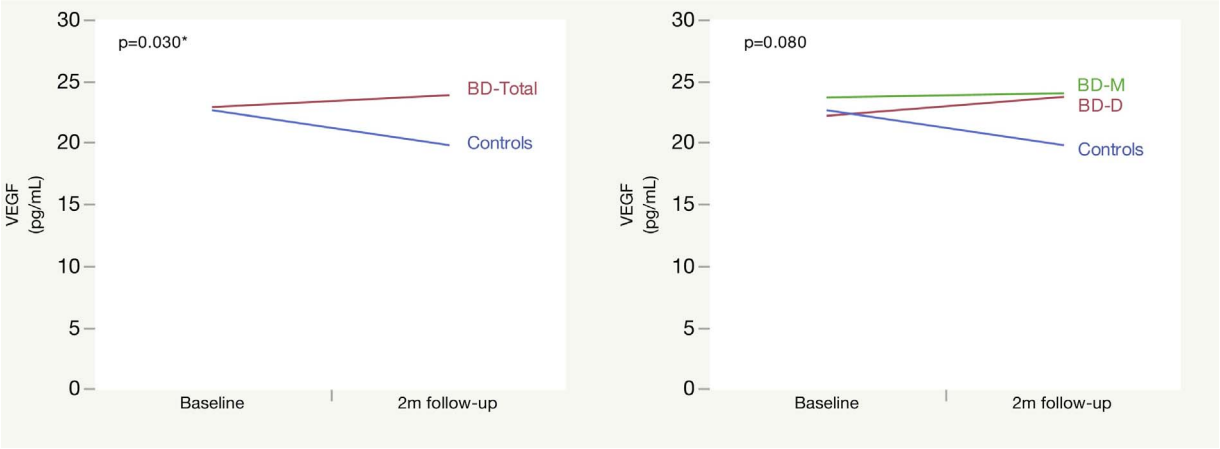


Fig. 1. Group by moment interaction VEGF.
Legend: BD-total: total patient group; BD-D: patient group with depressive episode at baseline; BD-M: patient group with (hypo)manic episode at baseline; VEGF: vascular endothelial growth factor.

$p = 0.030$) indicating that between the 1st and 2nd test day, VEGF decreases in HCs whereas it increases in patients. The increase was most pronounced in the BD-D group (see Fig. 1). However, no significant within-group changes in VEGF levels between the 1st and 2nd test day were observed in patients or HCs ($p > 0.05$). In the adjusted model, the interaction effect on VEGF remains significant ($p = 0.015$), whereas the increased TNF-α levels in patients lost statistical significance ($p = 0.080$). This was mainly due to addition of smoking status, as in a stepwise backward regression with all covariates and group, only the factor smoking status remained in the model ($p = 0.021$). No significant effects were found for BDNF and sFlt-1. See Table S2 for further details.

3.3. Relation between clinical and biological parameters in patients

Total clinical scores (HDRS, YMRS, PANSS positive subscale) did not show any strong correlation with any of the biological parameters. Changes in clinical scores between both test days did not correlate with either baseline concentrations of biological parameters or changes in concentrations. See Supplementary Tables S3–5 for further details. Comparing biological parameters in strictly euthymic patients (HDRS < 8 and YMRS < 8; $n = 14$) to patients with current mood symptoms, we found a mild, but significant increase in TNF-α levels in patients with mood symptoms ($F(198.2) = 4.08$; $p = 0.045$; $b = 0.16$). TNF-α levels of euthymic patients at the 2nd test day did not differ significantly to levels of HCs ($F(40) = 2.08$; $p = 0.157$; $b = 0.35$), whereas these patients had increased TNF-α levels at baseline compared to HCs ($F(41) = 5.85$; $p = 0.020$; $b = 0.56$). We found no significant differences for BDNF, VEGF and sFlt-1 between euthymic patients and patients with mood symptoms ($p > 0.05$).

Post-hoc analyses with clinical characteristics as BD type I or II, mixed features, current psychotic features, lifetime psychosis, age of onset, duration of illness and number of hospitalizations did not reveal significant associations with any of the biological parameters ($p > 0.05$). All biological parameters showed higher concentrations when the duration of current mood episode was shorter. This effect was significant for TNF-α ($p = 0.002$), VEGF ($p < 0.0001$) and sFlt-1 ($p < 0.0001$), even in the adjusted model (see supplementary Fig. S2).

4. Discussion

In this prospective study, we investigated mood-specific changes in neurotrophic and inflammatory markers in patients with BD. Levels of TNF-α, BDNF, VEGF and sFlt-1 were measured in patients with BD during a manic or depressive mood state and after a follow-up of 2 months. Levels in patients were compared to HCs and correlated to mood symptoms and clinical characteristics.

Impaired neurotrophic signaling accompanying both acute mood episodes and the underlying illness progression, is thought to play a role in the pathophysiology of BD (Berk et al., 2011; Grande et al., 2012). Since we found no differences in the levels of neurotrophic markers (BDNF, VEGF and sFlt-1) between patients and HCs, our data cannot support this hypothesis. Correlations between symptom severity scales and neurotrophic markers were weak and not significant. Although we observed no significant evolution in VEGF levels between the 1st and 2nd test day in either patients or HCs, VEGF levels revealed a significant group by moment interaction. Patients showed an increasing trend towards the 2nd test day, while HCs showed a decreasing trend. This finding seemingly contradicts previous reports of decreased VEGF levels in both depression and schizophrenia following psychopharmacological treatment (Lee et al., 2015; Sharma et al., 2016), but it is in line with increased VEGF levels as reported in responders to antidepressant treatment (Fornaro et al., 2013). The decreasing trend of VEGF levels we observed in HCs, however makes interpretation of the data ambiguous and is suggestive for a confounding methodological factor. We could not readily identify such methodological confounder. Blood sampling and handling procedures were highly standardized and the interaction effect was not affected by statistical adjustment for hemolysis. Samples of both test days were analyzed in batch and all laboratory procedures were uniform and meticulously followed. However, the susceptibility of VEGF immunoassays to methodological variation in both the pre-analytical and analytical phase has been reported repeatedly (Dittadi et al., 2001; Hormbrey et al., 2002).

A pro-inflammatory state in patients with BD has been documented extensively (Munkholm et al., 2013; Rosenblat et al., 2014). We confirmed increased TNF-α in patients, however after adjusting for smoking status, patients and HCs no longer significantly differed. The high proportion of smokers in the patient group is not in balance with the low proportion in the control group. Interpretation of these findings is therefore difficult. Smoking is known for its systemic pro-inflammatory effects (Arnson et al., 2010; O'Connor et al., 2009; Tanni et al., 2010), however similar cytokine levels between smokers and non-smokers have been found (Lee et al., 2017). Our data do not allow us to differentiate between increased TNF-α levels due to an inherent pro-inflammatory state in BD or due to smoking habits. Cautious interpretation of increased cytokine levels found in patients with BD compared to HCs is thus warranted, as has been stressed by others (Modabbernia et al., 2013; Munkholm et al., 2013).

While a state-related increase in TNF-α has been reported (Fiedorowicz et al., 2015; Modabbernia et al., 2013; van den Ameele

et al., 2016), this has not been confirmed in meta-analyses (Munkholm et al., 2013). In our data, TNF-α levels did not differ between patients in different mood states (depression *vs.* mania) and did not correlate to the severity of mood symptoms. However, TNF-α levels normalized at follow-up in the subgroup of patients reaching euthymia. The number of patients in this subgroup is small and possible confounding factors such as psychopharmacological treatment, baseline symptom severity or lifestyle factors have not been accounted for. Firm conclusions are therefore impossible, but since baseline TNF-α levels are similarly increased in patients achieving euthymia or not, we hypothesize that a normalization of TNF-α levels accompanies the waning of mood symptoms. This hypothesis is supported by earlier findings of decreasing inflammatory parameters in patients with a good response to pharmacological treatment (Guloksuz et al., 2012; Hornig et al., 1998). Future research should include a longer follow-up period to have more patients reaching euthymia.

Overall, our findings do not suggest mood state-related alterations in neurotrophic markers and show weak evidence for mood state-related TNF-α levels. Additionally, the few significant findings should be interpreted in view of a possible type I error. Due to the exploratory character of this study regarding VEGF and sFlt-1, we did not perform a formal Bonferroni correction to prevent an overconservative interpretation of the data. However, the *p*-values of both the interaction effect for VEGF and the increased TNF-α levels in patients with normalization during euthymia would not stand a Bonferroni correction which would lower the significance-threshold to at least $p = 0.0125$.

Our main findings are in line with a recently published study with similar design and stringent methodological procedures (Jacoby et al., 2016). Even with a longer follow-up period and a higher occurrence of euthymia, the authors found no mood state-related alterations in BDNF or inflammatory markers, except a decreased high sensitivity C-reactive protein during depression. Previously, the same group reported that they found no state-related differences in BDNF levels in patients with BD type rapid cycling, although BDNF was significantly increased in patients with BD compared to HCs (Munkholm et al., 2014). Interestingly, Munkholm and colleagues indicated in a meta-analysis on BDNF levels in patients with BD *vs*. HCs to observe smaller differences in BDNF levels in more recent studies with higher methodological quality (Munkholm et al., 2016). Many promising and valuable results have been reported on the role of neurotrophic and inflammatory markers in the understanding of disease expression, treatment response and illness progression (Frey et al., 2013). However, our results could not validate a role for these markers in monitoring disease activity.

5. Strengths and limitations

We included patients in both manic and depressive episodes, enabling comparison between both mood states and HCs. The longitudinal design allows assessment of within-group state-related differences. We minimized the impact of methodological bias by a standardized blood sampling procedure and uniform, meticulous laboratory procedures. All statistical analyses were adjusted for potential influence of BMI, smoking status, age, sex, hemolytic samples and seasonal variance. All clinical assessments were done by the same clinician-researcher, reducing the interrater bias. We carefully collected data on illness course and medication use. We used robust, transparent statistical methods and corrected for repeated measurements and missed moments. Drop-out rate was low, therefore bias due to drop-out is unlikely.

The naturalistic design has several inherent limitations. Despite strict in- and exclusion criteria, the patient sample remained heterogeneous regarding characteristics as illness severity, duration of illness, treatment history, diagnosis and history of substance abuse. Sample heterogeneity may hide relevant information that could have been discerned in a more homogenous patient group. We carefully collected data regarding course of illness and patient characteristics and integrated these data in the statistical analysis. *Post-hoc* analyses on the relation between illness course characteristics and biological markers revealed no significant effects, except for a decrease in TNF-α, VEGF and sFlt-1 with a longer duration of episode. Since patients and HCs did not differ at baseline, we are reluctant to draw conclusions from these data.

Apart from the use of anti-inflammatory medication and ECT, there were no treatment restrictions during follow-up. More than half of the patients had changes in psychopharmacological treatment. We evaluated the effect of the specific psychotropic drugs on the biological parameters *post-hoc* (data not shown) and found small effects for valproate, antipsychotics and antidepressants. Lithium use was not related to levels of any of the biological parameters. Biological parameters in medication-free patients were similar to medicated patients, which suggests that medication-use did not affect the results significantly. However, subgroups are small making the design inappropriate to evaluate the possible confounding effect of psychopharmacological drug use.

Twenty-four percent ($n = 14$) of the patients reached the criteria of strict euthymia (HDRS & YMRS < 8) at 2 months follow-up. This low rate of full recovery at 2 months is expected in view of the natural course of a bipolar mood episode (Pallaskorpi et al., 2015). Sixty-nine percent of the patients achieved significant symptom reduction and did present with mild mood symptoms or euthymia (HDRS < 17 and YMRS < 14) at 2 months follow-up. The sample size may be too small to find significant differences in biological markers between categories as strict euthymia and acute mood state. However, the continuous character of levels of biological markers and mood scales scores, enables to correlate these variables and detect more subtle associations between mood symptom severity and levels of biological markers. We did not find any significant correlation between biological markers and mood symptom severity, so cannot support the use of BDNF, VEGF or sFlt-1 as appropriate markers of mood state alterations in BD. A longer follow-up and longer duration of euthymia could have changed the profile of biological markers. However, this scenario is unlikely as we did not see differences between acute mood states and HCs.

The M.I.N.I. plus 5.0.0. was used as a structured instrument to confirm the diagnosis and screen for exclusion criteria in both patients and HCs. We did not document comorbid psychiatric disorders and cannot exclude their potential confounding effect. Comorbid anxiety disorders are especially frequent in BD and have been associated with decreased BDNF levels (McElroy et al., 2001; Suliman et al., 2013). As we did not detect significant differences between patients and HCs in biological markers, we assume a low impact of psychiatric comorbidities in our sample.

Since our results rejected our main research hypotheses, a critical assessment for the risk of a type II error is appropriate. In view of the recent meta-analyses on BDNF, our study may suffer from insufficient power due to limited sample size. Two recently published meta-analyses on BDNF levels in BD recommend a minimal sample size based on the effect sizes found in their meta-analyses (Fernandes et al., 2015; Munkholm et al., 2016). Since both meta-analyses were not published at time of our study design, we calculated our sample sizes based on earlier research. Because of the longitudinal design, we reached a considerable number of blood samples in patients ($n = 127$) and HCs ($n = 59$). The wide difference between the proposed samples sizes in the meta-analyses (from 11 to 159) however, makes the recommendations of limited use and possibly indicates that the adequate sample size remains to be determined. In line with earlier research, BDNF and VEGF levels in both patients and HCs are characterized by a wide distribution range (Hormbrey et al., 2002; Lee et al., 2015; Munkholm et al., 2016). To reach sufficient power, a strong effort to reduce variation in sample distribution should accompany the formulation of adequate sample sizes. Methodological studies on biomarker stability are needed. Strict consideration of blood sampling procedures, storage conditions, used blood fraction, type of assay and data analysis is a prerequisite for qualitative recommendations on biomarker research in psychiatric

disorders. Only with a solid methodological basis of both clinical and laboratory procedures, external validity of clinically relevant biomarkers can be guaranteed.

6. Conclusion

This prospective study investigated mood-specific changes in neurotrophic and inflammatory markers in patients with BD compared to HCs. Neurotrophic factors (BDNF, VEGF, sFlt-1) did not differ between patients with BD and HCs. We found significantly increased TNF-α levels in patients compared to HCs and a subsequent normalization in euthymic patients. None of the studied biomarkers correlated to mood symptom severity. Despite standardized methodological practices, levels of neurotrophic markers were characterized by a wide distribution range. Better understanding of methodological aspects influencing the analysis of neurotrophic factors is necessary for future research on markers for mood state monitoring and illness progression in BD.

Conflict of interest

SvdA was supported by a grant from Janssen Research and Development, a division of Janssen Pharmaceutica N.V. PDB and MT are employees of Janssen Pharmaceutica N.V. MM received grants and personal honoraria from Janssen Pharmaceutica N.V., AstraZeneca, Lundbeck, Bristol-Myer Squibb and Eli Lilly.

All authors declare they have no conflict of interests associated with this manuscript.

Role of the funding source

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Authors' contributions

MM, VC and SvdA developed the study protocol. SvdA did the patient recruitment, screening and clinical assessments and first drafted the manuscript. SvdA and JS did the laboratory analyses. Statistical analyses were done by SvdA supervised by EF. All authors contributed to the development of the manuscript and have approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2017.07.003.

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