Psychoneuroendocrinology
Manuscript Draft

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Title: Peripheral blood mRNA expressions of stress biomarkers in manic episode and subsequent remission

Article Type: Research paper

Section/Category: Clinical science

Keywords: bipolar disorder; brain-derived neurotrophic factor; tissue plasminogen activator; glucocorticoid receptor; heat shock protein 70; tumour necrosis factor-alpha

Abstract: Theoretical models of the neuroprogressive nature of bipolar disorder (BD) are based on the hypothesis that it's an accelerated aging disease, with the allostatic load playing a major role. Glucocorticoids, oxidative stress markers, inflammatory cytokines and neurotrophins play important roles in BD. The messenger ribonucleic acid (mRNA) expressions of brain-derived neurotrophic factor (BDNF), tissue plasminogen activator (tPA), glucocorticoid receptor (GR), heat shock protein 70 (HSP70), tumour necrosis factor-alpha (TNF-α) were examined in the peripheral blood of 20 adult male, drug-free BD patients during manic and remission periods and in 20 adult male, healthy controls. mRNA expression was measured using the quantitative real-time polymerase chain reaction (qRT-PCR). Compared to the controls, the expressions of BDNF and tPA mRNA were down-regulated in mania. In remission, BNDF and tPA mRNA levels increased, but they were still lower than those of the controls. Between mania and remission periods, only the change in mRNA levels of BDNF reached statistical significance. The results suggest that BDNF and tPA may be biomarkers of BD and that proteolytic conversion of BDNF may be important in the pathophysiology of BD. The change in BDNF levels between mania and remission could be adaptive and used to follow the progression of BD.

***Response to Reviewers**

Reviewers' comments:

Reviewer #2: In this study, the authors compared the blood gene expression levels of BDNF, tPA, GR, HSP70 and TNF-<alpha> between manic patients (during manic state and remission state) and normal controls. The findings are interesting. In general, the manuscript is not well organized and too long. The report needs much re-writing and shortening. I have several comments for the authors.

1. Title: Please add "blood" to the title. Title is “Peripheral blood mRNA expressions of stress biomarkers in manic episode and subsequent remission”.
2. Abstract: The authors stated "allostatic load plays the major role in the pathogenesis of bipolar disorder (BD)". I cannot find the connection between "allostatic load" and the molecules tested in this study. I tried to explain the connection. Kapczinski et al. had proposed allostatic load in BD and they also described dimensions composed of suggested molecules. Page 2: “In BD, allostasis was proposed to explain the link between disease progression and medical comorbidity (Kapczinski et al., 2008). ” Page 4: “Kapczinski et al. (2011) proposed a ‘systemic toxicity index’ composed by these dimensions: neurotrophins, oxidative stress markers and inflammatory markers.”
3. Method (Statistical analysis), Page 6. In this study, the authors made multiple comparisons. How did the authors perform the corrections for multiple comparisons? Page 7, 8: “Correlations were assessed by Pearson’s or Spearman’s correlation coefficients.”
4. Discussion, Page 9. It is not clear why the authors made this conclusion from their findings "By measuring gene expressions we eliminated these kind of posttranscriptional interaction consequences." The sentence has been erased, but meant that in the protein synthesis process many additional factors are added to the process that have effects on the measured possible biomarkers.
5. Discussion, Page 10. "Conversion of pro-BDNF to mature BDNF by tPA/plasmin system has been proposed as an adaptive mechanism against acute stress". Please add the reference for this statement. I cannot find the connection between this sentence and previous/next sentences. Part of the Discussions are not well-organized or coherent. Page 10: “In an animal study, Revest et al. (2014) found that stress-induced GR activation resulted in glucocorticoid secretion, leading to increases in the expression of both pro-BDNF and tPA. They concluded that the conversion of pro-BDNF to mature BDNF by the tPA/plasmin system could facilitate adaptation to acute stress.”
6. Table 2 "BDNF mRNA: mania 8.29 ± 0.96; remission 5.23 ±1.07; normal 5.09 ± 0.98". This is not compatible with the statements in Result that "BDNF and tPA mRNA expressions were down-regulated in manic patients." What's the unit for the mRNA expression in Table 2? Page 7: This difference is because after substraction the values ara reversed due to 2-ΔΔCt formula. This tablo has been changed a little for to be more clear. mRNA has no unit.
7. Highlight, "Their interactions seem to be reciprocal and nonlinear." From the study, I cannot find any finding to support this statement. Highlights are also changed.
8. This manuscript needs to be edited by a native English-speaking editor. The manuscript is edited by a native English-speaking editor.

Reviewer #3: The authors purpose to evaluate a few biomarkers involved in the stress response, inflammation and neuroplasticity (GR, HSP70, TNF-a, BDNF and tPA during the mania and remission of bipolar patients.

The method used to do it are good, and the patients conditions are very well characterized - this beeing one of the strongest point of the article - manic, markedly ill and drug-free bipolar patients. The "n" is a little small but I believe it is due the very homogeneous subset they decide to investigate. The way the results are shown and calculated are suitable for this sample and design; the discussion covers all the point and questions bring up by the results, and the conclusions were interpreted in the light of the study limitations.

Despite a very simple and low impact of the results on their own, I recommend the publication of the study with two minor recommendations:

- In the abstract section: where it says: "... expressions were examined in peripheral blood mononuclear cells ...", take off the mononuclear cells part as in the methods section it says that the mRNA was extracted from the whole blood. Page 1: “Brain derived neurotrophic factor (BDNF), tissue plasminogen activator (tPA), glucocorticoid receptor (GR), heat shock protein 70 (HSP70), tumour necrosis factor-alpha (TNFα) messenger ribonucleic acid (mRNA) expressions were examined in peripheral blood of 20 adult male, drug-free manic patients, their remission period, and 20 adult male, healthy controls.”
- In table 1: to do a proper table of patients and controls characteristics with all the information (age, comorbidities, BMI, ...). In table 1 only the patient characteristics are given.

Reviewer #4: Robert Dantzer, DVM, PhD
Editor-in-Chief
Psychoneuroendocrinology

Background: the authors compare brain derived neurotrophic factor (BDNF), tissue plasminogen activator (tPA), glucocorticoid receptor (GR), heat shock protein 70 (HSP70), tumour necrosis factor-alpha (TNF-alpha>) messenger ribonucleic acid (mRNA) of 20 male, drug-free manic patients, their remission period, and 20 male, healthy controls. Their results show that compared to controls BDNF and tPA mRNA expressions were down-regulated, and GR, HSP70, TNF alpha mRNA expressions were up-regulated in mania. In remission BNDF, tPA, HSP70 mRNA levels increased, GR and TNF-alpha mRNA levels decreased compared to mania. BDNF and tPA changes were statistically significant between mania and controls. Between mania and remission only BDNF reached statistical significance. The authors concluded that current results suggest that tPA and BDNF may be a biomarker of BD.

Overall feedback: I appreciate the opportunity to review this interesting paper focusing on mRNA levels of stress and inflammation related markers in BD patients before and after remission and HC as it targets an intriguing research area with currently no real empirical evidence. I commend the authors for a number of strengths of their work including the inclusion of both glucorticoid, BDNF, and inflammatory markers and the comparisons of patients before and after remission. This is a neat study design. Considering these strengths, though, as I read the manuscript I found some areas in which I would have appreciated greater clarity. I believe the paper could be further strengthened by addressing the following points:

1. Abstract
a. Please provide participants' demographics such as age, gender and IQ (if available). Page 2: “Adult” is added to the second sentence. “…20 adult male, drug-free manic patients, their remission period, and 20 adult male, healthy controls.” Under the methods section incluision and exclusion criteria are described.
Under methods please provide a general list of the clinical measures used (e.g. YMRS and please provide full name of each questionnaire before mentioning the acronym). Page 5: SCID-I, YMRS, and HDRS are used and their full names are given under the methods section.
Please add reference to the repeated measure nature of this study (blood tests taken before and after, mean number of months in between blood draws). Page 5: From the patients two blood samples were taken; first one in the first morning after hospitalization, the second when the remission criteria is fullfilled (approximately 8 weeks after hospitalization). The patients were hospitalized during the entire manic episode period. Only one sample of blood was drawn from the healthy controls, because it was hard to optimize their living conditions.
When the authors mention that "tPA may be a biomarker of BD like BDNF" it is unclear what they mean. I assume they mean that in the literature BDNF is a well-established marker of the disease but this should be explicitly stated beforehand. Page 1: “Our results suggests that BDNF and tPA may be biomarkers of BD.”

2. Title: the authors could consider reformulating the title by either referring to remitted manic patients and stress/inflammation as these are relevant keywords and points of novelty of the study. Title is "Peripheral blood mRNA expressions of stress biomarkers in manic episode and subsequent remission".

3. Introduction

a. Please provide references for the statement "brain responds to acute and chronic stress by changing chemistry, even morphology". Page number 2: "In response to acute and chronic stress, the chemistry of the brain changes; its morphology may also change (Jeanneteau and Chao, 2013; McEwen, 2008)."

b. Please explain why you want to study gene expression vs levels of BDNF and other markers. A brief references would provide a more robust rationale. Page number 2 and 3: I simplified the introduction part, so the proposed mechanism can be more clear. "One of the first responses of the brain against stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis to secrete glucocorticoids. The effects of glucocorticoids are mediated by binding to the intracellular glucocorticoid receptor (GR). In the absence of glucocorticoids, GRs and heat shock proteins (HSPs) remain as a complex in the cytoplasm. In the presence of high levels of glucocorticoids, they bind to GRs, which then dissociate from the HSPs (Bei et al., 2013). HSPs perform a chaperone function by facilitating protein refolding and maintaining normal cellular homeostasis in response to stress. They are also referred to as stress proteins and up-regulated as part of the stress response (Santoro, 2000). Increased Hsp70 expression is a universal response to cell damage caused by increased oxidative stress (Martinez de Toda and De la Fuente, 2015). Increased expression of the HSP70-GR complex was shown in BD patients as compared to controls. The authors interpreted the increase as due to changes in the affinity of HSP70-GR interactions or alterations in the relative amounts of total cellular GR and HSP70 (Bei et al., 2013). HSP70 is also thought to protect the brain, and up-regulation of HSP70 was shown to exert anti-inflammatory effects on the brain by inhibiting the release of pro-inflammatory cytokines (Yu et al., 2015). In the rat hippocampus, acute immobilisation stress led to a decrease in mRNA levels of the brain-derived neurotrophic factor (BDNF) and an increase in the levels of HSP70 (Kim et al., 2014). A recent meta-analysis of the HPA axis in BD concluded that the disorder was associated with state and trait hyperactivity of the HPA axis, resulting from abnormalities in glucocorticoid signalling. In that meta-analysis, the cortisol levels of the BD patients, especially those with mania, were higher than those of the controls (Belvederi Murri et al., 2015). Cortisol-bound GR functions as a transcription factor to modulate the expression of a variety of genes, including the transcription of many genes involved in inflammatory signalling . Interestingly, high levels of cytokines have been shown to trigger increased secretion of cortisol, which in turn cyclically repressed immune gene expression. A disruption in any part of this pathway led to increased inflammation in the periphery system or central nervous system and consequent cellular damage. According to an earlier study, dysregulation of cytokine mRNA expression in psychiatric disorders may be associated with GR mRNA abnormalities (Fillman et al., 2014). Dysregulation of GR was observed in the brains of both schizophrenia and BD patients (Sinclair et al., 2011). Stress-induced activation of GR stimulates pro-BDNF and tissue plasminogen activator (tPA) proteins, which induce an increase in mature BDNF, because tPA mediates the conversion of pro-BDNF (apoptotic) to mature BDNF (neuroprotective) (Pang et al., 2004; Revest et al., 2014)."

c. How long does it take for mRNA levels of these markers to change? What does previous literature suggest? I could not find any prospective study on the subject. Gene expression studies are usually cross-sectional. I think life span of the circulating blood cells is the answer. Since we detect mRNA expressions from these cells, the levels will vary with the cells' life spans. Their life cycles are from a few hours to a few days, and the mobilization of blood cells from tissues to circulation also changes with stress duration (depends on whether the stress is acute or chronic)(Epel, 2012). I did not include this information in the text.

d. The fact that neuroprogression in BD leads to BD is still speculative and no solid evidence exists at this stage. I would recommend that the authors use more cautious language "may lead/is associated to BD". Page 2: "Neuroprogressive decay within years, together with increased morbidity and mortality may put the disorder in the category of accelerated aging diseases (acceleration of cellular/biological aging). The regeneration ability of the brain and body diminishes with age and with chronic diseases

(Rizzo et al., 2014)."
e. Explain why you decided to pick the reported stress/inflammatory markers and whether some of them (e.g. BDNF) have been reported in previous literature in brain function and cognition in the literature (e.g. Peruzzolo et al. 2015, Bauer et al. 2014, Aas et al. 2013, Rosenblat et al. 2015). Hint: This would help the reader explain why the authors compare tPA to BDNF in their conclusions for instance. Page 5: "The hypothesis of the present study was that BDNF and tPA gene expression would be altered in manic patients. To shed light on the neurogenesis process, we selected the studied possible biomarkers, focusing on evidence based on findings in peripheral blood (Fernandes et al., 2015; Frey et al., 2013; Goldstein and Young, 2013; Kapczinski et al., 2011; Leboyer et al., 2012; Maletic and Raison, 2014; Rizzo et al., 2014)."

4. Methods
a. Overall provide full name of all markers/questionnaires prior to providing acronyms (e.g. YMRS). Page 5: Full names of acronyms are provided.
b. Please explain why only males were selected and whether age was an inclusion/exclusion criteria. Page 5: "Males were selected as the study group to eliminate effects of the menstrual cycle on the studied parameters and to avoid confounding of the results by gender bias (Oertelt-Prigione, 2012). To exclude potential age-related differences, all the participants were aged 19-45 years."
c. Why only bipolar I? Page 5: "As mania is a clinical hallmark of BD (Frey et al., 2013), the present study included only BD patients with mania."
d. Were comorbidities such as substance use allowed? Please explain why yes/no. Page 13: "Although subjects with substance abuse and dependence were excluded in both the BD group and control group, those with nicotine abuse and dependence were not. Bipolar patients are known to have higher rates of smoking than the general population (Jackson et al., 2015). "
e. Did the authors consider other sources of stress during the "treatment period"? e.g. divorce, marriage, employment status change, in both groups? if they did not this could be mentioned in the discussion. Page 13: "The patient group remained hospitalised throughout the study period, and they followed the same daily routines. However, major life events in the patient and the control group were not assessed."
f. Which medical illnesses were excluded? e.g. TBI, epilepsy, neurological disorders, cardiovascular diseases…Page 5 and 6: "Exclusion criteria for the patient and the control groups were major medical diseases, including cardiovascular and endocrine diseases, especially diabetes, obesity and metabolic syndrome. Patients with neurological diseases, including epilepsy and a history of stroke, were also excluded. For both medical and neurological diseases, exclusion was based on the results of general medical and neurological examinations."
g. Was the current health state of participants assessed at the time of the blood draws? I am wondering whether viruses/cold/flu/sepsis would have been exclusion criteria for instance. Presence of active infection was an exclusion criteria (page 6) and was verified by total blood counts and CRP levels.
h. The authors mention the participants' BMI in their results and their conclusions but not in their methods. Could the authors explain if cardiovascular disorders, obesity, diabetes were exclusion criteria? Was BMI a source of concern given the nature of the study and being obesity considered an inflammatory condition (Bond et al. 2015). Page 5: "To exclude potential age- and weight-related differences, inclusion criteria included being 19–45 years of age and having normal weight. The body mass index (BMI) was defined as the body mass divided by the square of the body height, with normal weight considered 18.5–25 kg/m^2." Page 13: "All the participants had a BMI within the normal range to exclude the effects of obesity on the stress biomarkers (Sies, 2007). To exclude the presence of other metabolic comorbid diseases (Esser et al., 2015; Rani et al., 2016) that could affect the studied parameters, all the participants underwent general medical and neurological examinations."
i. Did the authors assess the severity of symptoms using scales other than YMRS? If not why? Only YMRS and HDRS are used. These are our standard follow-up scales in BD.
j. Why were blood draws conducted between 8am and 10am? Page 6: "To take account of GR-regulated transcription of the biomarkers (Wiley et al., 2016), blood samples were taken from the patients and controls between 08:00 and 10:00 a.m."
k. The abstract mentions that the authors used peripheral blood monuclear cells but the methods section does not appear to mention this. I was wondering if the authors could clarify what kind of cells

they used,e.g. lymphocytes? Also have other studies used the same types of cells (if so please provide reference) or rather serum? We used whole blood. Muncholm reviewed 17 studies performed on gene expressions of BD patients, the investigators used whole blood or lymphocytes (Muncholm et al. 2012). Today whole genome studies can be performed and I think that measuring gene expression levels is more appropriate. There are only a mumber of studies on BD. I did not want to go in details about the gene expression results.
l. Were blood draws conducted at time 1 done when BDs were still inpatients or outpatients? Page 6: "The samples from the patients were taken the day after their admittance to the inpatient clinic. A second set of blood samples was taken when the patients fulfilled remission criteria, as defined by a total score on the YMRS of < 4 (Berk et al., 2008)."
m. Were there exclusion/inclusion criteria regarding the "waiting time" between pre and post-remission blood draws? When did the authors conduct the second blood draw in HC? Were BDs and HCs matched in terms of time between blood draw 1 and 2? Waiting time is described in measures section. Only one blood sample was obtained from the HC because it was difficult to optimise their living conditions (page 6 and 7).
n. Did HCs take medication/supplements at the time of the study? If so which ones? Page 6: "All the patients and controls stated that they were not on a diet and that they did not take part in strenuous exercise activity. They stated that they were not taking any kind of medication, including antibiotics, anti-inflammatory drugs, vitamins, dietary supplements or energy drinks."
o. Did the authors plan to match HC and BDs in terms of demographics for instance? Please address this in methods or discussion. Page 13: "We could not match the patient and control groups in terms of demographic other than gender, age and weight."

5. Analyses
a. Please mention what kind of statistical software you used. Page 8: "All statistical analyses were conducted with SPSS, version 20.0 for Windows (SPSS, Chicago, IL, USA)."
b. Were results Bonferroni corrected? No. Bonferroni corrected p value would be 0.01
c. Did the authors consider including covariates e.g. age, gender, BMI or even time between blood draw 1 and 2 if HC and BD differed in those terms? If not why? Please discuss in methods/discussion. Page 8: "Correlations were assessed by Pearson's or Spearman's correlation coefficients. The covariates were age in years, BMI, illness duration, total number of manic episodes, total number of depressive episodes, psychotic symptom presence, YMRS at admission, YMRS at the end of the study, time to reach remission, mood stabilisers used (lithium, valproate) and antipsychotics used (risperidone, ketiapine, olanzapine and haloperidol)." The results are given and discussed.

6. Results
a. I would recommend that the authors structure the results section using 3 headers: e.g. demographics/clinical, BD vs HC, and pre- and post/remission vs HC. This would improve readability and understanding of the results. Page 8 and 9: Section is divided into 4 headers. Demographics, Mania comparison with the controls, Remission comparison with the controls, Mania versus remission comparison within the patient group
b. When mentioning that "BDNF levels were statistically significant" please indicate if they mean they were significantly higher/lower. The authors often reported results without indicating whether the levels were higher/lower, and whether the comparison group (remission/HC). Please make this clearer. Page 8 and 9: "The BDNF and tPA levels of the manic patients were significantly lower than those of the controls ($p = 0.03$ and $p = 0.01$, respectively)" "The BDNF levels were significantly lower in mania compared to the remission ($p = 0.01$)"
c. Please indicate where you would place table 1 and figure 1 in the manuscript. Page 8 and 9
d. In table 1 please add mean/SDs for age, education, IQ (if available), clinical measures (YMRS), GAF etc. in both BD and HC. Also please provide F/X2 or T and corresponding p values/post-hoc p values.
e. In table 2 please add a column providing p values for mania vs remission. Please indicate if these p values are corrected for multiple comparisons.
f. Figure 1: please provide results for HCs too. In the caption please add 1 sentence summarizing the primary finding.

7. Discussion

a. Could the authors explain how they corrected for differences in medication in the BD during treatment? How do primary BD medications affect inflammation/stress and mRNA levels of these markers? Please provide an overall take-home message. Page 9: "When we assessed the correlations, a significant positive relationship existed between the remission levels of GR (patient group) and olanzapine use" Page 12: "Although atypical antipsychotics were shown to have neuroprotective effects (He et al., 2009), the claims of neuroprotective properties of antipsychotics seem premature (Lepping et al., 2011)." "In the present study, there was no correlation between the mood stabiliser used and the studied parameters. According to previous research, mood stabilizers, especially lithium, can prevent or even reverse the harmful central and peripheral biological effects associated with BD (Machado-Vieira et al., 2014)."

b. Overall discuss further how you interpret the fact that only BDNF and tPA varied between pre and post-remission. Could this be due to the time to remission? And are BDNF mediators/catalyzers for changes in the other markers such as GR, HSP70 etc. In other words do the authors think that the other markers will eventually change too? I tried to focus on that in the discussion section. Page 14: "The results showed that BDNF and tPA may be biomarkers of BD and that the proteolytic conversion of BDNF by the tPA/plasmin system may be important in the pathophysiology of BD. The change of BDNF expression levels between manic and remission periods could be adaptive, and BDNF expression levels could be used to follow the progression of BD. Based on the findings of the present study, we can speculate that neuroprotection against stress may be limited in mania but restored in remission to a degree."

c. Overall I elaborate a bit further in terms of limitations, strengths, future directions and clinical implications. The authors wrote a very short paragraph about strengths and limitations and did not provide an overall conclusion. I would suggest that they shorten and tighten their discussion up. They could then have sufficient space/words available to discuss further what an ideal time to remission would be and why. Further, would BDI differ from BD II? Were remission time and ageing considered in the study design? Why would cortisol have been beneficial to the study? What about gender related differences? These are just examples of how the authors could improve their discussion

8. Highlights

Authors could discard the first highlight and rather mention that 1. They compared BD patients before and after remission; they used mRNA levels of stress/inflammatory markers. Highlights: Peripheral blood mRNA expression levels of brain-derived neurotrophic factor (BDNF) and tissue plasminogen activator (tPA) are down-regulated in mania compared to controls. Expression level of BDNF is changed between mania and subsequent remission. The proteolytic conversion of BDNF by tPA is important in pathophsiology of Bipolar Disorder.

9. Overall: I would highly recommend that the authors get editing help from someone with full professional proficiency in English (a few typos and ambiguous sentences). The manuscript had been evaluated by a professional editing service.

Highlights

- Peripheral blood mRNA expression levels of brain-derived neurotrophic factor (BDNF) and tissue plasminogen activator (tPA) are down-regulated in mania compared to controls.
- Expression level of BDNF is changed between mania and subsequent remission.
- The proteolytic conversion of BDNF by tPA is important in pathophsiology of Bipolar Disorder.

Abstract

Theoretical models of the neuroprogressive nature of bipolar disorder (BD) are based on the hypothesis that it's an accelerated aging disease, with the allostatic load playing a major role. Glucocorticoids, oxidative stress markers, inflammatory cytokines and neurotrophins play important roles in BD. The messenger ribonucleic acid (mRNA) expressions of brain-derived neurotrophic factor (BDNF), tissue plasminogen activator (tPA), glucocorticoid receptor (GR), heat shock protein 70 (HSP70), tumour necrosis factor-alpha (TNF-α) were examined in the peripheral blood of 20 adult male, drug-free BD patients during manic and remission periods and in 20 adult male, healthy controls. mRNA expression was measured using the quantitative real-time polymerase chain reaction (qRT-PCR). Compared to the controls, the expressions of BDNF and tPA mRNA were down-regulated in mania. In remission, BNDF and tPA mRNA levels increased, but they were still lower than those of the controls. Between mania and remission periods, only the change in mRNA levels of BDNF reached statistical significance. The results suggest that BDNF and tPA may be biomarkers of BD and that proteolytic conversion of BDNF may be important in the pathophysiology of BD. The change in BDNF levels between mania and remission could be adaptive and used to follow the progression of BD.

Comment [r1]: Insignificant results are deleted.

Keywords: Bipolar disorder; brain-derived neurotrophic factor; tissue plasminogen activator; glucocorticoid receptor; heat shock protein 70; tumour necrosis factor-alpha

1. Introduction

Bipolar disorder (BD) is a cyclic and progressive disorder (Belmaker, 2004). In patients with BD, there are symptomatologic differences between manic and depressive periods. The identification of biomarkers of these periods would be useful in the management of the disease.

Neuroprogressive decay within years, together with increased morbidity and mortality may put the disorder in the category of accelerated aging diseases (acceleration of cellular/biological aging). The regeneration ability of the brain and body diminishes with age and with chronic diseases (Rizzo et al., 2014). Progressive exposure to cellular stress also leads to allostatic load (homeostatic derangements). Multiple mediators of adaptation interact with each other and by time over/under production of primary biomarkers (stress hormones and inflammatory cytokines) affect systemic parameters (Juster et al., 2010). In BD, allostasis was proposed to explain the link between disease progression and medical comorbidity (Kapczinski et al., 2008).

Comment [r2]: Added to meka clear the connection between AL and BD.

In response to acute and chronic stress, the chemistry of the brain changes; its morphology may also change (Jeanneteau and Chao, 2013; McEwen, 2008). These changes are largely reversible if the stress is short term (i.e. weeks) but may be irreversible if the stress is long term (months or years) (Juster et al., 2010; McEwen, 2008). Glucocorticoids, oxidative stress markers, inflammatory cytokines and neurotrophins are thought to play significant roles in the homeostatic regulation (Maletic and Raison, 2014). One of the first responses of the brain against stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis to secrete glucocorticoids. The effects of glucocorticoids are mediated by binding to the intracellular glucocorticoid receptor (GR). In the absence of glucocorticoids, GRs and heat shock proteins (HSPs) remain as a complex in the cytoplasm. In the presence of high levels of glucocorticoids, they bind to GRs, which then dissociate from the HSPs (Bei et al., 2013).

Comment [r3]: Reference added

HSPs perform a chaperone function by facilitating protein refolding and maintaining normal cellular homeostasis in response to stress. They are also referred to as stress proteins and up-regulated as part of the stress response (Santoro, 2000). Increased Hsp70 expression is a universal response to cell

damage caused by increased oxidative stress (Martinez de Toda and De la Fuente, 2015). Increased expression of the HSP70-GR complex was shown in BD patients as compared to controls. The authors interpreted the increase as due to changes in the affinity of HSP70-GR interactions or alterations in the relative amounts of total cellular GR and HSP70 (Bei et al., 2013). HSP70 is also thought to protect the brain, and up-regulation of HSP70 was shown to exert anti-inflammatory effects on the brain by inhibiting the release of pro-inflammatory cytokines (Yu et al., 2015). In the rat hippocampus, acute immobilisation stress led to a decrease in mRNA levels of the brain-derived neurotrophic factor (BDNF) and an increase in the levels of HSP70 (Kim et al., 2014).

Comment [r4]: Literature added.

A recent meta-analysis of the HPA axis in BD concluded that the disorder was associated with state and trait hyperactivity of the HPA axis, resulting from abnormalities in glucocorticoid signalling. In that meta-analysis, the cortisol levels of the BD patients, especially those with mania, were higher than those of the controls (Belvederi Murri et al., 2015). Cortisol-bound GR functions as a transcription factor to modulate the expression of a variety of genes, including the transcription of many genes involved in inflammatory signalling . Interestingly, high levels of cytokines have been shown to trigger increased secretion of cortisol, which in turn cyclically repressed immune gene expression. A disruption in any part of this pathway led to increased inflammation in the periphery system or central nervous system and consequent cellular damage. According to an earlier study, dysregulation of cytokine mRNA expression in psychiatric disorders may be associated with GR mRNA abnormalities (Fillman et al., 2014). Dysregulation of GR was observed in the brains of both schizophrenia and BD patients (Sinclair et al., 2011).

Comment [r5]: Literature added.

Stress-induced activation of GR stimulates pro-BDNF and tissue plasminogen activator (tPA) proteins, which induce an increase in mature BDNF, because tPA mediates the conversion of pro-BDNF (apoptotic) to mature BDNF (neuroprotective) (Pang et al., 2004; Revest et al., 2014). tPA is a serine protease produced by the endothelial cells of blood vessels. It converts plasminogen to plasmin, which is responsible for the removal of fibrin deposits, and is used as a thrombolytic agent for myocardial infarction and stroke. tPA is also found in neurons and functions independently of plasminogen-plasmin conversion (Yepes, 2015). Both BDNF and tPA contribute to synaptic plasticity

and have neuroprotective effects (Rothman and Mattson, 2013; Yepes, 2015). In animal models, tPA was associated with both acute and chronic stress responses (Melchor et al., 2003; Pawlak et al., 2005). The ratio of mature and pro-BDNF and the levels of mature BDNF were higher in patients with BD than in controls. However, in the same study, serum pro-BDNF levels were lower in the BD patients compared to the controls, which suggests that the alteration in the conversion of the pro to the mature form of BDNF may be associated with the pathophysiology of BD (Sodersten et al., 2014).

Comment [r6]: Removed from discussion to introduction and some literature has been added.

A recent meta-analysis showed that compared to healthy controls, peripheral blood BDNF levels were decreased in bipolar episodes and increased after treatment of acute manic but not depressive episodes. BDNF levels were negatively correlated with the severity of either episode, but they were not associated with the duration of the illness. The authors suggested that peripheral BDNF may be used in the future as part of a blood protein composite measure to assess disease activity in BD (Fernandes et al., 2015). In pre-clinical models, BDNF modulated the coupling of neurogenesis and vasculogenesis, and vascular levels of BDNF mRNA expression were comparable to those in the brain (Li et al., 2006). According to a previous study, BDNF and endothelial function may share a bidirectional association, as vascular endothelial cells produce BDNF (Nakahashi et al., 2000). Recent findings from the Collaborative Depression Study cohort indicated that after controlling for age, gender and smoking, manic symptoms were associated with poorer endothelial function, whereas this association was not observed for depressive symptoms (Fiedorowicz et al., 2012).

Comment [r7]: Literature addition to make clear the connection between tPA and BDNF.

Kapczinski et al. (Kapczinski et al., 2011) proposed a 'systemic toxicity index' composed of the following dimensions: neurotrophins, oxidative stress markers and inflammatory markers. They concluded that peripheral markers of allostatic adaptations in BD may aid understanding of the pathophysiology and progression of this disorder. Further, they suggested that state markers could help in differentiating between different episodes of BD or that they may only be useful when measured during a specific mood episode.

Comment [r8]: Added. Showing why we selected the studied molecules.

Most previous studies consisted of mixed groups of BD patients (mania, hypomania, depression or euthymia) and controls (Fernandes et al., 2015; Frey et al., 2013; Goldstein and Young, 2013; Kapczinski et al., 2011). According to a literature search, no previous human studies have examined

the relationship between BD and tPA. As mania is a clinical hallmark of BD (Frey et al., 2013), the present study included only BD patients with mania. The hypothesis of the present study was that BDNF and tPA gene expression would be altered in manic patients. To shed light on the neurogenesis process, we selected the studied possible biomarkers, focusing on evidence based on findings in peripheral blood (Fernandes et al., 2015; Frey et al., 2013; Goldstein and Young, 2013; Kapczinski et al., 2011; Leboyer et al., 2012; Maletic and Raison, 2014; Rizzo et al., 2014). In the present study, the mRNA expressions of BDNF, tPA, the GR, HSP70 and tumour necrosis factor-alpha (TNF-α) were examined in the peripheral blood of adult male, drug-free BD patients during manic and remission periods and a healthy male control group.

Comment [r9]: Added.

Comment [r10]: Previous literature pointing the importance of the studied biomarkers.

2. Methods

Comment [r11]: Section revised and made clearer.

2.1. Study design and participants

This was a case-control study with a prospective design. The study consisted of 20 male, drug-free manic patients and 20 age- and gender-matched healthy controls. Males were selected as the study group to eliminate effects of the menstrual cycle on the studied parameters and to avoid confounding of the results by gender bias. To exclude potential age- and weight-related differences, inclusion criteria included being 19–45 years of age and having normal weight. The body mass index (BMI) was defined as the body mass divided by the square of the body height, with normal weight considered 18.5–25 kg/m^2. The diagnosis of BD I and lack of psychiatric diagnoses in the controls were confirmed through use of the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, fourth edition (SCID-I). The patients were assessed additionally by Young Mania Rating Scale (YMRS) and Hamilton Depression Rating Scale (HDRS). BD patients with a total score of ≥ 25 on the YMRS (defining markedly ill) and a score of ≤ 7 on the HDRS were recruited from our inpatient clinic.

Exclusion criteria for the patient and the control groups were major medical diseases, including cardiovascular and endocrine diseases, especially diabetes, obesity and metabolic syndrome. Patients

with neurological diseases, including epilepsy and a history of stroke, were also excluded. For both medical and neurological diseases, exclusion was based on the results of general medical and neurological examinations. Patients and controls with mental retardation, according to a psychiatric examination, current or past substance abuse and drug dependence, except nicotine were excluded, as well as those with active infection (verified by total cell counts and C-reactive protein levels) and a history of head injury, with cognitive sequelae.

Psychotrophic drug-using patients were also excluded. Purification periods for psychotrophic drug were 2 weeks for oral drugs, 1 month for parenteral drugs and blood levels of 0 for mood stabilisers. Control subjects having first-degree relatives with BD, schizophrenia or other psychotic disorders were excluded. All the patients and controls stated that they were not on a diet and that they did not take part in strenuous exercise activity. They stated that they were not taking any kind of medication, including antibiotics, anti-inflammatory drugs, vitamins, dietary supplements or energy drinks.

The study was approved by the local ethical committee (TÜTF-BAEK 2014/198) and Trakya University Scientific Research Project Committee (TÜBAP 2015/08). The study conformed to the provisions of the Declaration of Helsinki, and only subjects who gave informed consent participated in the study. The patients were followed by psychiatrists other than the writers, and none of the drug treatment regimens of the patients were changed.

2.2. Measures

All 40 participants completed the study. To take account of GR-regulated transcription of the biomarkers (Wiley et al., 2016), blood samples were taken from the patients and controls between 08:00 and 10:00 a.m. In all cases, trained nurses obtained the samples. The samples from the patients were taken the day after their admittance to the inpatient clinic. Only lorazepam was allowed before the blood collection. A second set of blood samples was taken when the patients fulfilled remission criteria, as defined by a total score on the YMRS of < 4 (Berk et al., 2008). None of the patients had mixed episodes or episodic shifts during the study, and none of the patients withdrew consent. Only

one sample of blood was drawn from the healthy controls because it was difficult to optimise their living conditions.

2.3 Gene expression analysis

The gene expression analysis measurements were done at the laboratories of the Technology Research and Development Centre of Trakya University (TÜTAGEM). For gene expression analysis, blood samples were collected in tubes with EDTA and stored at -80° C until the assay. Total RNA was isolated from whole blood using the PureLink RNA Mini Kit (Life Technologies, USA), according to the manufacturer's instructions. The extracted RNA concentrations were measured using a Qubit Fluorometer (Life Technologies, USA). The first strand of cDNA was synthesised using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). The cDNA synthesis was performed using a thermal cycler (Veriti, Applied Biosystems, USA). The cDNA was stored at -20° C for subsequent steps of the analysis.

The gene expression levels of BDNF, tPA, GR, HSP70 and TNF-α were analysed by the quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Select Master Mix (Life Technologies, USA) on an ABI Step One Plus Real-Time PCR system.

2.4. Statistical analysis

The comparative cycle threshold (2-ΔΔCt) method was used to analyse the expression levels of mRNAs, where Ct (cycle threshold) indicated the fractional cycle number at which the amount of amplified target reached a fixed threshold and ΔCt corresponded to normalised Ct values and was equal to the difference in cycle thresholds between the target (gene expressions of predetermined molecules) and reference genes (housekeeping genes). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the housekeeping gene. ΔΔCt corresponded to the difference between the expression level of the average ΔCt of the patient group and that of the control group. 2-ΔΔCt denoted the fold change (FC) in the expression level change of the following measures of the patient group compared to the control group (Livak and Schmittgen, 2001).

The Mann–Whitney test was used to compare the levels of the biomarkers in the mania and remission groups compared to those of the controls. The Wilcoxon signed-rank test was used to compare the levels of the biomarkers in subjects during manic and remission states. Comparisons within subjects for manic and remission states were made using the Wilcoxon signed-rank test. Correlations were assessed by Pearson's or Spearman's correlation coefficients. The covariates were age in years, BMI, illness duration, total number of manic episodes, total number of depressive episodes, psychotic symptom presence, YMRS at admission, YMRS at the end of the study, time to reach remission, mood stabilisers used (lithium, valproate) and antipsychotics used (risperidone, ketiapine, olanzapine and haloperidol). All statistical analyses were conducted with SPSS, version 20.0 for Windows (SPSS, Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

Comment [r12]: Revised with a statistician from the university.

3. Results

Comment [r13]: Divided into subsections.

3.1. Demographics

None of the subjects in either group had comorbid diseases. The mean ages (in years) of the patient and control groups were similar (31.8 ± 8.2 and 32.3 ± 7.5, respectively; $p = 0.858$). The BMI of the patients and controls was within the normal range (24.4 kg/m^2 ± 4.0 kg/m^2 and 24.8 kg/m^2 ± 2.1 kg/m^{2,} respectively; $p = 0.714$). In the patient group, the time to reach remission was 8.2 ± 1.2 weeks. Table 1 presents the characteristics of the patients.

Table 1

3.2. Mania comparison with the controls

The expression levels of BDNF and tPA mRNA were down-regulated in manic patients, both with the levels of expression 90% lower than those of the controls. The mRNA expressions of the other studied genes were up-regulated in manic patients. As compared to the controls, the GR, Hsp70 and TNF-α expression levels were 70%, 50% and 70% higher, respectively, in the manic patients. The BDNF and

tPA levels of the manic patients were significantly lower than those of the controls (for BDNF Z = -2.16 and $p = 0.03$; for tPA Z = -2.58 and $p = 0.01$) (Table 2).

Table 2

3.3. Remission comparison with the controls

The expression levels of BDNF and tPA mRNA remained down-regulated in the remission period of the same patients compared to the controls. The expression levels of BDNF and tPA were lower than those of the controls (10% and 60% lower, respectively). The expression levels of GR and HSP70 remained up-regulated compared to the controls (40% and 120% higher, respectively). Compared to the controls TNF-α was up-regulated in mania, but became down-regulated in remission (40% lower). Figure 1 shows the FCs in the gene expression levels of the patients.

Figure 1

3.4. Mania versus remission comparison within the patient group

During the remission period, the BDNF, tPA and HSP70 levels increased compared to the levels in the manic state. The expression levels of GR and TNF-α mRNA decreased during the remission period compared to the levels in the manic state. The BDNF levels were significantly lower in mania compared to the remission (Z = -2.41 and $p = 0.01$) (Table 2).

When we assessed the correlations, a significant positive relationship existed between the remission levels of GR (patient group) and olanzapine use ($\rho = 0.65$, $p = 0.01$). Significant relationships were also found between the illness duration (patient group) and age ($\rho = 0.47$, $p = 0.05$) and the total number of manic episodes ($\rho = 0.47$, $p = 0.05$).

4. Discussion

The present study assessed promising biomarkers of stress pathways in BD. Based on the findings, BDNF seems to be a promising BD biomarker, as proposed earlier (Goldstein and Young, 2013).

Glucocorticoids, inflammatory cytokines, oxidative stress markers and neurotrophic factors constitute the main steps of the stress response (Kapczinski et al., 2008; McEwen, 2008). The aim of the present study was to determine whether differences existed in the levels of various candidate biomarkers in BD patients during manic and remission episodes, as compared to a control group. The statistically significant findings of the study were the decreased mRNA expression of BDNF and tPA in mania compared to controls and the increased BDNF expression in remission compared to mania. The expression levels of GR, HSP70 and TNF-α also differed between the patient group with mania and the controls, but the results were not statistically significant. Based on our findings, we suggest that BDNF and tPA may be biomarkers of BD and that proteolytic conversion of BDNF may be important in the pathophysiology of BD. Furthermore, the change of BDNF levels between mania and remission could be adaptive.

Comment [r14]: Added and conclusions of nonsignificant results deleted.

In the present study, we hypothesised that the gene expression of tPA and BDNF might be changed in BD and that these changes could be detected in peripheral blood. The results confirmed such changes in the peripheral blood of the patient group. Abnormalities of neurotrophic factors, especially BDNF, orchestrate important alterations, which may be implicated in the aetiology of BD (Fernandes et al., 2015; Scola and Andreazza, 2015). Both tPA and BDNF are found in neurons and vascular endothelium (Li et al., 2006; Yepes, 2015), and tPA plays a role in the conversion of BDNF to its neuroprotective form (Pang et al., 2004).

Comment [r15]: Focused on BDNF and tPA.

Changes in BDNF levels in the hippocampus after acute and chronic stress showed that the timing of the stress was important (Gray et al., 2013). Previous research reported decreased BDNF mRNA levels in the hippocampus of subjects with BD, suggesting that BD may cause chronic changes in BDNF levels (Ray et al., 2014). Although the response to acute stress is adaptive, the response to chronic stress can lead to dysregulation of the stress mediators and exacerbate pathophysiology of the diseases. Certain types of acute stress and many chronic stressors suppress neurogenesis (McEwen, 2008). It is possible that some of the changes seen in manic episodes of BD could be adaptive and reversible. Page: 10

Based on the results of the present study, we can speculate that the change in BDNF levels between mania and remission periods is an adaptive response.

Chronic glucocorticoid exposure causes neuronal cell damage, reduces hippocampal neurogenesis and leads to alterations of BDNF and tPA mRNA expression (Suri and Vaidya, 2013; Yepes, 2015). In an animal study, Revest et al. (2014) found that stress-induced GR activation resulted in glucocorticoid secretion, leading to increases in the expression of both pro-BDNF and tPA. They concluded that the conversion of pro-BDNF to mature BDNF by the tPA/plasmin system could facilitate adaptation to acute stress. Alterations in the conversion of pro to mature BDNF have been proposed to be associated with the pathophysiology of BD (Sodersten et al., 2014).

In an animal study, tPA administration induced an increase in mature BDNF expression in the hippocampus, and tPA contributed to the control of brain BDNF synthesis through a plasmin-independent mechanism (Rodier et al., 2014). tPA knockdown in the hippocampus increased anxiety- and depression- like behaviours in adult mice, and these effects were reversed when tPA-overexpressing vectors were injected in the hippocampus. BDNF protein levels were elevated in the hippocampus of mice administered tPA-expressing vectors (Bahi and Dreyer, 2012). Studies also reported that BDNF and TNF-α modified tPA gene expression in cultured human cells, depending on the cell type (Castorina et al., 2015; Kruithof and Dunoyer-Geindre, 2014).

According to previous research, chronic over-activation of the HPA axis, which is seen in BD, may lead to chronic hypercortisolemia and impaired neuroplasticity, with resultant negative cognitive and mood effects (Rosenblat and McIntyre, 2015). Inflammation, obesity, insulin resistance, increased rates of smoking and impaired sleep in BD subjects were also reported to potentially increase the risk of vascular complications and cognitive impairment (Goldstein and Young, 2013). BD patients have increased risks of venous thromboembolism, cerebrovascular diseases, cardiovascular complications, all-cause diabetes and vascular pathologies (Domingueti et al., 2015; Hoirisch-Clapauch et al., 2013; Prieto et al., 2016). According to previous work, these may be related to dysregulation of the tPA/plasmin system (Domingueti et al., 2015; Hoirisch-Clapauch et al., 2013; Prieto et al., 2016). The

identification of the underlying mechanisms linking BD and associated comorbidities can help to guide aetiological research and treatment planning.

BDNF has been associated with changes in mood states in BD, the most dramatic change being in mania, but changes have also been observed in euthymia (Scola and Andreazza, 2015). In patients with acute mania, BDNF levels were shown to increase in response to treatment (Fernandez-Funez and Rincon-Limas). In an animal study, BDNF levels returned to normal after the cessation of stressors (Lakshminarasimhan and Chattarji, 2012). In the present study, the BDNF levels of the patients were increased but still lower than those of the controls. However, whether these changes were due to the treatment or the cessation of the episode is not known.

Although drug-free manic patients were included in the study, in the remission stage, all the patients were using one mood stabiliser (lithium or valproate), as well as one antipsychotic drug (risperidone, ketiapine, olanzapine or haloperidol). Among these drugs, in the present study, olanzapine was positively related to GR expression levels. Olanzapine-induced weight gain can be reversed by a GR antagonist, mifepristone (Gross et al., 2009). Although atypical antipsychotics were shown to have neuroprotective effects (He et al., 2009), the claims of neuroprotective properties of antipsychotics seem premature (Lepping et al., 2011).

In the present study, there was no correlation between the mood stabiliser used and the studied parameters. According to previous research, mood stabilizers, especially lithium, can prevent or even reverse the harmful central and peripheral biological effects associated with BD (Machado-Vieira et al., 2014). Lithium exerts mood-stabilising effects mainly by inhibiting glycogen synthase kinase-3 and valproate, thereby inhibiting histone deacetylases. Mood stabilisers, such as lithium, were hypothesised to regulate the transcription and gene expression of factors critically involved in neuroprotection effects; anti-inflammatory effects; neurogenic-, angiogenic- and antidepressant-like effects; and anxiolytic effects (Chiu et al., 2013). The neuroprotective effects of lithium could be mediated by its ability to regulate various phosphatases and kinases at the post-translational level and subsequently protein activities (Nciri et al., 2014). Lithium and valproate have been shown to involve induction of neurotrophic/neuroprotective proteins, including BDNF and HSP70 (Chuang, 2005).

Lithium and valproate were reported to increase vascular endothelial growth factor (VEGF) levels in endothelial cells (Chiu et al., 2013). VEGF, a prominent angiogenic factor, was shown to modulate neurogenesis (Jin et al., 2002). Recombinant tPA treatment was reported to increase VEGF (Suzuki et al., 2015). Valproic acid induced the expression of tPAin cultured endothelial cells, and this was associated with increased histone acetylation at the tPA promoter (Larsson et al., 2012).

Comment [r16]: Added.

A strong point of the present study is the study population, which consisted of BD patients with severe mania (Lukasiewicz et al., 2013) and no psychotrophic drug use or medical comorbidities. Although the recruitment of only men allowed us to control for potentially confounding sex differences, such as those caused by the effect of the menstrual cycle on inflammation (Oertelt-Prigione, 2012), it also limits the generalizability of the results. All the participants had a BMI within the normal range to exclude the effects of obesity on the stress biomarkers (Sies, 2007). To exclude the presence of other metabolic comorbid diseases (Esser et al., 2015; Rani et al., 2016) that could affect the studied parameters, all the participants underwent general medical and neurological examinations. Having first-degree relatives diagnosed with BD or any psychotic disorder is a risk determinant. For the control group, high-risk individuals were excluded, depending on the data on high-risk groups showing dysregulation of the studied parameters (Brietzke et al., 2012; Duffy et al., 2015).

Although subjects with substance abuse and dependence were excluded in both the BD group and control group, those with nicotine abuse and dependence were not. Bipolar patients are known to have higher rates of smoking than the general population (Jackson et al., 2015). In the patient group, those with axis I disorders, except for substance-related ones, were not excluded, and patients with mental retardation were excluded based on a psychiatric evaluation. The patient group remained hospitalised throughout the study period, and they followed the same daily routines. However, major life events in the patient and the control group were not assessed.

Comment [r17]: Revised.

We could not match the patient and control groups in terms of demographic other than gender, age and weight. The small sample size may explain the detection of a difference in the neurotrophic factors only during manic episodes. Between mania and remission, only the expression levels of

BDNF changed, and this could be due to the length of time between the measures. The time to remission (8.2 ± 1.2 weeks) found in the present study was similar to that reported in an earlier study (Leelahanaj et al., 2013).

In conclusion, the GR and inflammatory system interact with each other, with cortisol being one of the most potent suppressors of immune function. Oxidative stress and neurotrophic factors affect and are affected by cortisol and inflammation. Abnormalities in stress-related biomarkers may contribute to a common pathophysiology in psychiatric disorders. It is not known whether alterations in stress-related biomarkers co-occur in BD and whether these change in response to treatment (Belvederi Murri et al., 2015). The results showed that BDNF and tPA may be biomarkers of BD and that the proteolytic conversion of BDNF by the tPA/plasmin system may be important in the pathophysiology of BD. The change of BDNF expression levels between manic and remission periods could be adaptive, and BDNF expression levels could be used to follow the progression of BD. Based on the findings of the present study, we can speculate that neuroprotection against stress may be limited in mania but restored in remission to a degree.

Comment [r18]: Revised.

To the best of our knowledge, this is the first study in the field of psychiatry to show gene expression changes in stress-pathway biomarkers of BD patients and treatment-related changes in the levels of these biomarkers. Follow-up of the same patient group will be needed to verify these findings.

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Table(s)

Table 1. Clinical patient characteristics and drugs use during remission

Clinical characteristics	mean ± SD or n (%)
Illness duration	7.9 ± 7.1
The number of manic episodes	3.0 ± 2.4
The number of depressive episodes	1.2 ± 2.2
YMRS at admission	27.8 ± 8.8
YMRS at the end of the study	2.0 ± 1.2
Psychotic symptom presence	17 (85)
Drug use at the end of the study	
Lithium	13 (65)
Valproate	7 (35)
Risperidone	7 (35)
Ketiapine	6 (30)
Olanzapine	4 (20)
Haloperidol	3 (15)

n=20
SD, standard deviation.

Table 2. qRT-PCR results of patients and controls

Gene	Patients (n=20) mean±SEM		Controls (n=20) mean±SEM	Mania vs. controls*	Remission vs. controls*	Mania vs. remission**
	Mania	Remission		*p*	*p*	*p*
BDNF	8.29 ± 0.96	5.23 ±1.07	5.09 ± 0.98	0.03	ns	0.01
tPA	12.54 ± 0.82	11.29 ± 0.71	9.78 ± 0.62	0.01	ns	ns
GR	3.86 ± 0.49	4.14 ± 0.45	4.63 ± 0.37	ns	ns	ns
HSP70	6.66 ± 0.89	7.28 ± 0.78	7.81 ± 1.07	ns	ns	ns
TNF-α	5.69 ± 0.66	7.14 ± 0.51	6.53 ± 0.81	ns	ns	ns

BDNF, brain derived neurotrophic factor; tPA, tissue plasminogen activator; GR, glucocorticoid receptor; HSP70, heat shock protein 70; TNF-α, tumour necrosis factor-α; SEM, standard error of mean.
Means are average ΔCt (cycle treshold) values.
**p* values are calculated by using Mann-Whitney test.
***p* values are calculated by Wilcoxon signed-rank test.
$p < 0.05$, significance level; NS, non significant.

Figure(s)

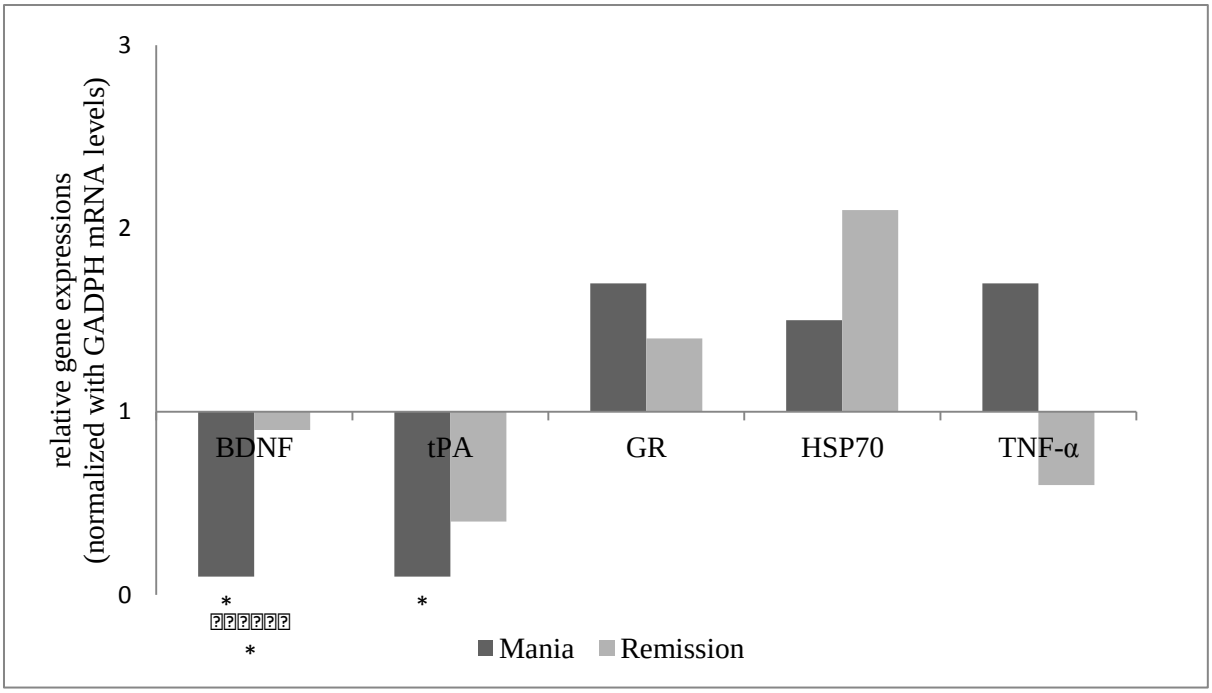

Figure 1. Fold changes relative to controls between the manic and remission periods. Controls are 1 and not represented in the figure.
GADPH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; BDNF, brain derived neurotrophic factor; tPA, tissue plasminogen activator; GR, glucocorticoid receptor; HSP70, heat shock protein 70; TNF-α, tumour necrosis factor-α.
*, $p < 0.05$.

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Conflict of interest

None.

Contributors

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