Toxicity and neuroprotection in bipolar disorder

The acute mood episode and beyond

Seline van den Ameele

Promotoren: Prof. Dr. Manuel Morrens en Prof. Dr. Bernard Sabbe

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Medische Wetenschappen
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Promotoren:
Prof. Dr. Manuel Morrens & Prof. Dr. Bernard Sabbe
Leden van de interne doctoraatscommissie:
Prof. Dr. John-Paul Bogers & Prof. Dr. Sebastiaan Engelborghs
Leden van de externe doctoraatscommissie:
Prof. Dr. Stephan Claes & Dr. Bartholomeus Haarman

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CHAPTER 1

General background

General background

Bipolar disorder

Mood fluctuations are an inherent part of life and a normal reaction when facing exceptional life events. When these mood fluctuations become very intense and of long duration, involve great suffering and disturb daily functioning, they are considered as pathological. Persons with a unipolar mood disorder, present themselves with depressive symptoms only. Those with bipolar disorder (BD), previously known as manic-depressive disorder, show episodes of mood elevation (Grande et al., 2016; Souery and Martens, 2015). BD is a lifelong illness with a highly variable course among affected individuals. The Diagnostic and Statistical Manual of Psychiatric Disorders (DSM-5) (2013) discerns 2 types of BD: bipolar I disorder (BD-I) and bipolar II disorder (BD-II). BD-I is characterized by the occurrence of manic episodes and often also depressive episodes, while BD-II is defined by the presence of hypomanic and depressive episodes. A manic episode is marked by elevated or irritable mood, increased energy and activities, decreased need for sleep, feelings of grandiosity, being more talkative, increased distractibility, flight of ideas, psychomotor agitation and increased risk-taking behavior. Hypomania presents with similar symptoms, but less pronounced and not affecting daily functioning to the same extent as mania. During a depressive episode, individuals typically suffer from sad or flattened mood, loss of interest or pleasure, low energy, sleep disturbances, psychomotor changes, intrusive thoughts of guilt and worthlessness, concentration difficulties and recurrent thoughts of death. When both manic and depressive symptoms co-occur, DSM-IV classified such a mood episode as ‘mixed’. The latest DSM edition (DSM-5) labels it however as a ‘depressive or manic episode with mixed features’. Mood episodes can be accompanied by psychotic symptoms, as grandiose delusions during mania, or poverty and nihilistic delusions during depression. Episodes of altered mood are interspersed by periods of normal, stable mood, called ‘euthymia’. Patients with BD often have cognitive dysfunctions which involve impairments in attention, working memory, verbal learning and executive functioning (Bora and Pantelis, 2015; Kurtz and Gerraty, 2009). Cognitive impairment may persist during euthymia and is related to poorer quality of life (Bourne et al., 2013; Brissos et al., 2008; Toyoshima et al., 2018).

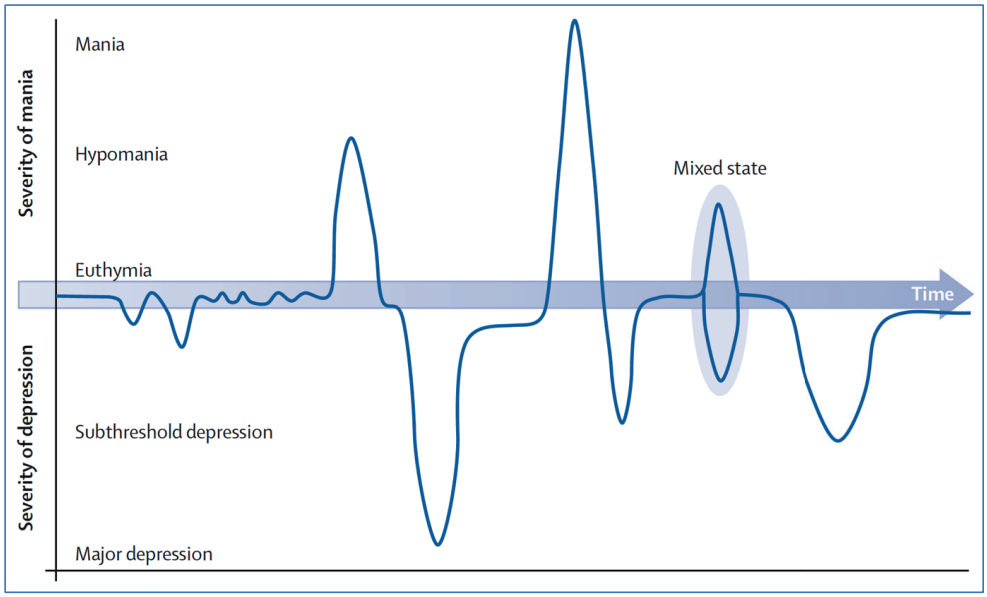

Figure 1. Mood states appearing in patients with bipolar disorder. Manic and hypomanic episodes are represented above the euthymic zone (normal mood state), whereas depressive episodes are depicted below. Mixed states are characterized by symptoms of both the depressive and manic episode. Reproduced from Grande et al., 2016.

BD has a worldwide lifetime prevalence of 0.6% for BD-I and 0.4% for BD-II (Merikangas et al., 2011). When including subthreshold BD, thus broadening the bipolar spectrum, prevalence rates rise above 2% (Merikangas et al., 2011). The disease is accompanied by high disability with an underestimated economic burden (Fajutrao et al., 2009). According to the WHO world health survey (2001), BD is ranked as the ninth among all health conditions in terms of causing disability (defined as years lived with disability). In addition to the BD-related symptoms, patients also suffer from a high psychiatric (e.g. anxiety disorders, substance abuse) and somatic comorbidity (e.g. cardiovascular disorders, metabolic syndrome) (Kilbourne et al., 2004; McElroy et al., 2001; Merikangas et al., 2011), important functional impairment (Bonnin et al., 2010) and increased risk for suicide (Plans et al., 2019; Tondo et al., 2003).

Our understanding of the pathophysiology of BD has advanced in the last decades. Evidence shows that diverse pathological processes underlie highly heterogeneous symptoms that we somewhat artificially cluster under the umbrella diagnosis of BD (Maletic and Raison, 2014). Family and twin studies indicate a strong heritability and genome-wide-association studies have identified several risk alleles that modestly increase the odds of developing BD (a

polygenic risk) (Craddock and Sklar, 2013). Historically, disturbances in monoaminergic neurotransmitters were thought to underlie the pathogenesis of BD. Such imbalances in neurotransmitters have been described (van Enkhuizen et al., 2015), but recent studies suggest that these are merely the consequence of disturbed signal transduction pathways and impaired neuronal and synaptic plasticity in brain areas essential for mood regulation and cognitive function (Martinowich et al., 2009). Current areas of study are the immune system and hypothalamic-pituitary-adrenal (HPA) axis dysregulation, alterations in glial-neuronal interactions and neurotrophic factors, oxidative stress and mitochondrial dysfunction (Berk et al., 2011; Maletic and Raison, 2014). Recent insights in these pathological processes have often been interpreted within the conceptual framework of 'neuroprogression'.

Neuroprogression in BD

Clinical evidence shows that a substantial proportion of patients with BD present with a progressive illness course (Angst and Sellaro, 2000; Passos et al., 2016). Several unfavorable clinical outcomes appear along the course of illness: a shortening of the interepisodic euthymic intervals, decreasing responsiveness to treatment with incomplete remission and development of chronic mood dysregulation (Magalhaes et al., 2012; Perlis et al., 2009), high rates of somatic and psychiatric comorbidity (Kilbourne et al., 2004; McElroy et al., 2001), increasing cognitive and functional impairment (Lewandowski et al., 2011; Rosa et al., 2012) and augmented risk of suicide (Plans et al., 2019). In search for a biological validation of these clinical observations, Berk (2011) introduced the concept of 'neuroprogression'.

Neuroprogression is used to describe the pathological process occurring along the longitudinal illness trajectory that underlies the cumulative clinical and cognitive burden (Berk et al., 2011; Fries et al., 2012). Several theoretical models have been proposed to understand neuroprogression in BD. Post (1992) laid the foundation with his 'kindling hypothesis', according to which major stressors are required to trigger initial mood episodes, but due to evolving neurobiochemical processes and altered gene expression, successive episodes become progressively less dependent on such stressors and may eventually occur autonomously (Post, 1992). Kapczinski et al. (2008b) extended this model with the allostatic load theory to account for the cumulative damage and impaired resilience associated with BD. See figure 2 for a schematic representation of the allostatic load theory.

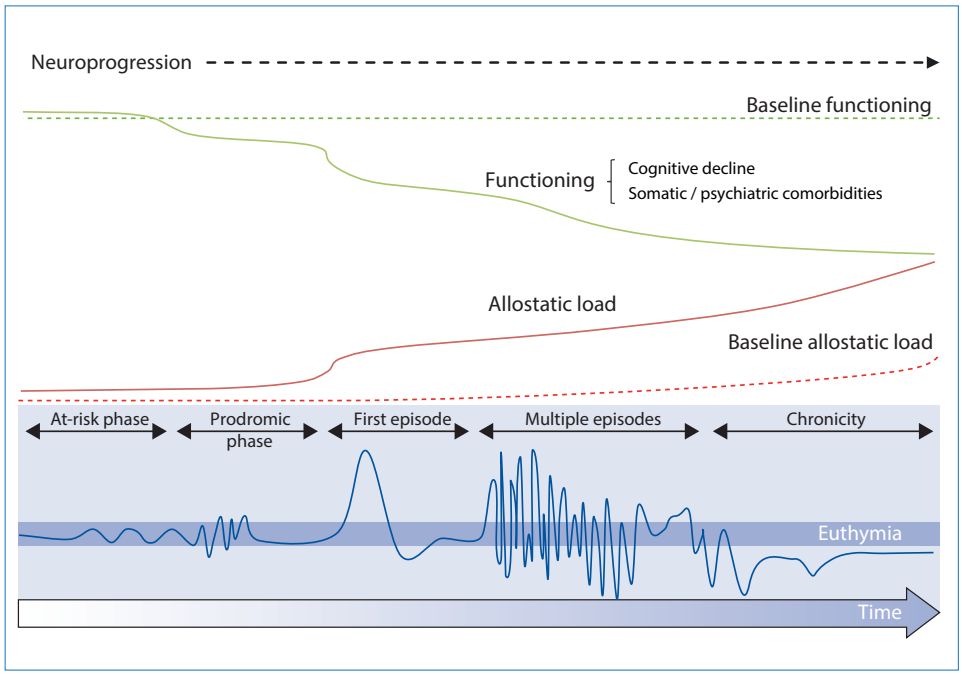

Figure 2. Neuroprogression and allostatic load in bipolar disorder. During the course of bipolar disorder, functioning begins to decrease in the prodromic phase with further decrease with cumulative episodes. Meanwhile, allostatic load increases leading to neuroprogression. Adapted from Grande et al., 2016.

The term 'allostasis' or 'stability through change' was first used by Sterling and Eyer (1981) and further elaborated by McEwen (1998) in the context of stress and its systemic consequences. Allostasis is defined as the capacity to achieve stability through change produced by adaptive mechanisms. It is essential for maintaining homeostasis when dealing with daily life events. The term to define the cumulative cost for the system of these allostatic adaptive mechanisms is 'allostatic load', which steadily increases throughout life. In BD, multiple mood episodes repeatedly affect allostatic mechanisms, leading to an allostatic overload in a non-adaptive way resulting in increasing vulnerability to stress, disease, and accelerated aging (Kapczinski et al., 2008b). Proposed allostatic mechanisms are the immune system, the HPA-axis, the autonomic nervous system, several metabolic systems and neuroplasticity (Kapczinski et al., 2008b; McEwen, 1998). Evidence on their specific contribution to neuroprogression in BD is still limited and relies mainly on cross-sectional or retrospective study designs (Berk et al., 2011; Passos et al., 2016). Because of their relevance for this thesis, below, we first describe the contribution of the immune system on the

pathogenesis and neuroprogression in BD. Second, we discuss the role of the kynurenine pathway and BH_4-dependent monoamine synthesis in the pathogenesis of BD. Both pathways are thought to impact neurotransmission, cause neurotoxicity, and are hypothesized to be strongly influenced by immune system activity. Finally, we discuss the role of neurotrophic factors and their hypothesized impact on decreased neuroplasticity in BD.

The inflammatory hypothesis of BD

Evolutionarily, inflammation is a mechanism of great benefit for maintaining homeostasis in the body. The inflammatory response may be appropriate and necessary in the presence of infection, cellular damage, physical or psychological stress. Conversely, it may be inappropriate and damaging when it is out of proportion to the stimulus (Rosenblat et al., 2014). Aberrant activation of the immune system has been described repeatedly in patients with mood disorders with findings pointing towards an underlying abnormally activated mononuclear phagocyte system (Beumer et al., 2012). Chronic activation of macrophages and microglia leads to excessive production of cytokines which may affect neurotransmission, neuroendocrine function and neural plasticity (Beumer et al., 2012; Miller et al., 2009). Increased inflammatory markers such as cytokines and acute phase proteins have repeatedly been demonstrated in peripheral blood (Dargel et al., 2015; Munkholm et al., 2013) which is in line with findings of a pro-inflammatory gene expression pattern in monocytes of patient with BD (Padmos et al., 2008). Positron emission tomography (PET) allows to visualize inflammation in the brain by using PET-tracers that mark activated microglia. Through such an approach, Haarman et al. (2014) demonstrated microglial hyperactivity in the right hippocampus of patients with BD (Haarman et al., 2014). Given the high prevalence in patients with BD of autoimmune disorders and other physical conditions with inflammation as a contributing pathological factor (e.g. metabolic syndrome, cardiovascular disorders), BD has even been proposed as a multi-system inflammatory disease (Leboyer et al., 2012; Rosenblat and McIntyre, 2016) .

The immune response impacts neurotransmission and neuroplasticity in several ways. Below we describe the effects of inflammation on the kynurenine pathway and BH_4-dependent monoamine synthesis. Although most current evidence comes from pre-clinical studies, pro-inflammatory cytokines are thought to influence both pathways leading to altered

monoamine synthesis and neurotransmission, and increased neurotoxicity (Rosenblat and McIntyre, 2017).

Inflammation and the kynurenine pathway

The initial step in the kynurenine pathway (see figure 3) is the conversion of tryptophan (TRP), a precursor of serotonin (5-HT), into kynurenine (KYN) by indoleamine 2, 3 deoxygenase (IDO-1). IDO-1 is specifically activated by cytokines as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). Several neuroactive metabolites are generated along 2 branches downstream of KYN: a 'neurotoxic' branch mainly seen in microglia and a 'neuroprotective' branch in astrocytes (Savitz, 2017; Schwarcz et al., 2012). The neurotoxic branch results in the formation of three-hydroxykynurenine (3-HK) and quinolinic acid (QA). Both 3-HK and QA result in an increase of free radicals and thus increase oxidative stress. Additionally, QA has a strong excitotoxic effect by its N-methyl-D-aspartate (NMDA) receptor agonism. The neuroprotective branch results in the formation of kynurenic acid (KYNA). KYNA has a possible neuroprotective role by competitive NMDA-receptor antagonism and anti-oxidant properties (Schwarcz et al., 2012). Increased KYNA levels or an increased KYNA/3-HK ratio are however thought to contribute to psychosis by NMDA receptor antagonism and dysregulation of glutaminergic and dopaminergic signaling (Erhardt et al., 2016). KYNA was initially thought to contribute to the development of cognitive deficits due to an alpha7 nicotinic (α7nACh)-receptor antagonism, though this has been questioned more recently because of poor replication results (Albuquerque and Schwarcz, 2013; Dobelis et al., 2012; Hilmas et al., 2001).

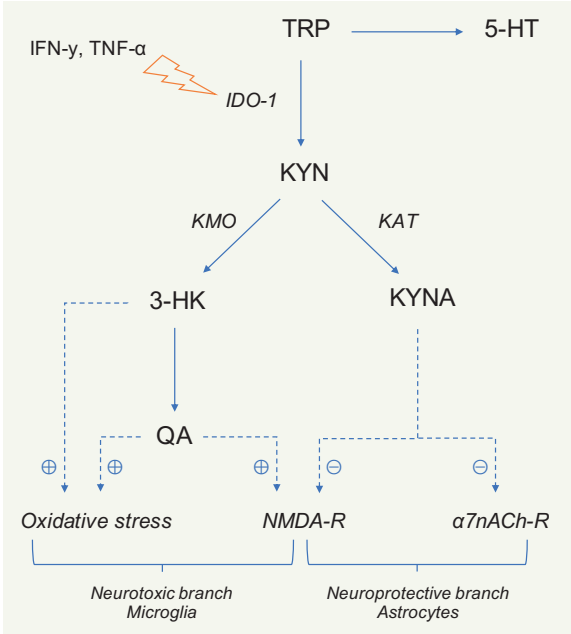

Figure 3. The neurotoxic and neuroprotective branch of kynurenine metabolism. Cytokines as interferon-γ (IFN-γ) and tumor necrosis factor-alfa (TNF-α) activate indoleamine 2,3 deoxygenase (IDO-1) to metabolize tryptophan (TRP) into kynurenine (KYN) at the expense of serotonin (5-HT) formation. Further downstream the pathway, KYN is metabolized along two branches. Three-hydroxykynurenine (3-HK) and quinolinic acid (QA) are preferentially formed in microglia and have neurotoxic properties by increasing oxidative stress and QA's N-methyl-D-aspartate receptor (NMDA-R) agonism. Kynurenic acid (KYNA) is formed in astrocytes and thought to be neuroprotective by its NMDA-R antagonism. KYNA is thought to contribute to the development of cognitive deficits due to an alpha7 nicotinic (α7nACh)-receptor antagonism. The schematic representation is not comprehensive.

The kynurenine pathway has mainly been studied in schizophrenia and major depressive disorder (MDD) (Erhardt et al., 2016; Savitz, 2017). In schizophrenia increased KYNA levels have been documented in cerebrospinal fluid (CSF) and postmortem brain, but findings on KYNA levels and neurotoxic kynurenine metabolites in peripheral blood are inconsistent (Erhardt et al., 2016). MDD seems to be associated with increased neurotoxic metabolites and decreased neuroprotective ratios (Myint et al., 2007b; Savitz, 2017).

In BD, the kynurenine pathway has been less studied. As in schizophrenia, elevated KYNA levels have been found in CSF, postmortem brain and fibroblast cultures of patients with BD, and are mainly associated with a history of mania or psychosis (Erhardt et al., 2016; Lavebratt et al., 2014; Olsson et al., 2010; Olsson et al., 2012; Sellgren et al., 2019; Sellgren et al., 2015). Studies in peripheral blood could not find differences in KYNA levels between patients with mania or history of psychosis and healthy controls (Myint et al., 2007a; Sellgren et al., 2019)

or showed decreased KYNA levels in affective psychosis (Wurfel et al., 2017). Findings in bipolar depression are similar to those in MDD. Savitz (2015a) found a decreased neuroprotective ratio KYNA/QA in patients with bipolar depression, but no differences in absolute kynurenine metabolite levels. Decreased KYNA and increased neurotoxic ratios also have been reported in euthymic to mild depressive patients with BD (Birner et al., 2017; Poletti et al., 2018; Sellgren et al., 2019). A recent meta-analysis on kynurenine metabolites in mood disorders found no significant differences between patients with BD and controls (Arnone et al., 2018), but included a limited number of studies with high heterogeneity.

Although pro-inflammatory cytokines are hypothesized to be the main trigger for kynurenine pathway activation leading to psychiatric symptoms, the interaction between inflammation, kynurenine metabolism and clinical symptoms is scarcely investigated in psychiatric populations (Chiappelli et al., 2018; Dahl et al., 2015; Quak et al., 2014; Schwieler et al., 2015).

Inflammation, neopterin and BH_4-dependent monoamine synthesis

In chronic inflammation, activation of guanosine triphosphate cyclohydroxylase 1 (GTP-CH1) by the pro-inflammatory cytokines interferon gamma (IFN-γ) and tumor necrosis factor-alfa (TNF-α) results in increased neopterin production at the expense of tetrahydrobiopterin (BH_4). Neopterin is a marker of activated cell-mediated immunity and increased oxidative stress (Fuchs et al., 1988; Murr et al., 2002), while BH_4 is an essential cofactor in the synthesis of monoamine neurotransmitters dopamine, noradrenaline, adrenaline and serotonin (Capuron et al., 2011b; Neurauter et al., 2008; Werner et al., 2011). Inflammatory markers have already been correlated with neopterin production and monoamine synthesis in chronic inflammatory disorders as human immunodeficiency virus (HIV), cancer or autoimmune diseases (Murr et al., 2002; Neurauter et al., 2008). In a study on healthy aging, low-grade inflammation correlated to increased neopterin levels, and increased GTP-CH1 activity was associated with depressive symptoms (Capuron et al., 2011b). Decreased levels of BH_4 are found in patients with MDD and schizophrenia (Hashimoto et al., 1994; Richardson et al., 2007). Few studies report neopterin levels in BD and the results are inconsistent (Hoekstra et al., 2006; Mukherjee et al., 2017; Reininghaus et al., 2013). The relation between inflammatory changes, neopterin and BH_4-dependent monoamine synthesis in BD has not yet been studied.

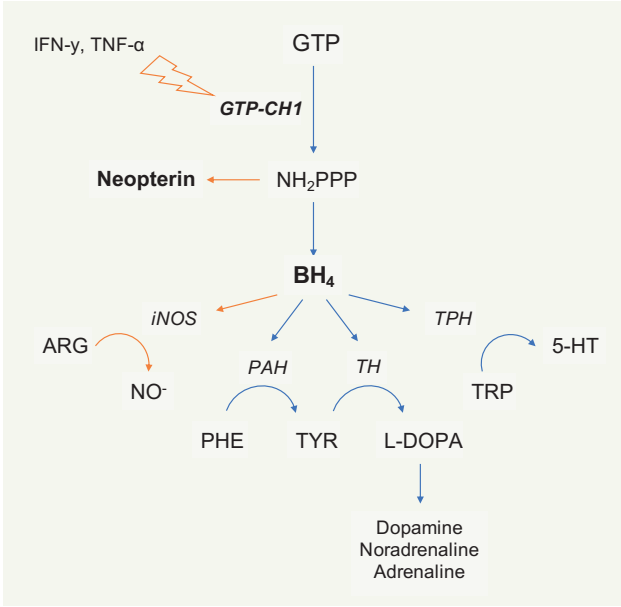

Figure 4. Neopterin and BH_4-dependent monoamine synthesis. IFN-y and TNF-α stimulate GTP-CH1, resulting in the synthesis of BH_4 and neopterin. Neopterin is almost exclusively synthesized in and released by activated cells of the monocyte/macrophage system and used as a marker for activated cell-mediated immunity. Increased neopterin levels also indicate increased oxidative stress induced by immune-mediated processes. In chronic inflammation, mainly neopterin is released at the expense of BH_4 production. BH_4 is an essential cofactor in the synthesis of dopamine, noradrenaline, adrenaline and serotonin. In inflammatory conditions, BH_4 is preferentially used as cofactor of iNOS activity for synthesis of NO^-, leading to lower availability for PAH, TH and TPH. Preferential pathways in inflammatory conditions are marked in orange. The schematic representation is not comprehensive. 5-HT: serotonin; ARG: L-Arginine; BH_4: tetrahydrobiopterin; GTP: guanosine triphosphate; GTP-CH1: guanosine triphosphate cyclohydroxylase 1; IFN-y: interferon gamma; iNOS: inducible nitric oxide synthase; L-DOPA: L-3,4-dihydroxyphenylalanine; NH_2PPP: dihydroneopterin triphosphate; NO^-: nitric oxide; PAH: phenylalanine hydroxylase; PHE: phenylalanine; TH: tyrosine hydroxylase; TNF-α: tumor necrosis factor alpha; TPH: tryptophan hydroxylase; TRP: tryptophan; TYR: tyrosine.

Neurotrophic factors

Recent studies in BD show changes in neurotrophic signaling leading to impaired neuroplasticity, especially regarding neuronal connectivity and cell resilience (Berk et al., 2011; Grande et al., 2012). Several trophic factors, especially brain-derived neurotrophic factor (BDNF), are thought to be involved (Scola and Andreazza, 2015). Decreased BDNF levels were found 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 increased 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). Recent meta-analyses confirmed decreased BDNF levels in patients with BD compared to healthy controls, but state-related BDNF alterations or a link to illness progression were not consistently reported (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 confounding factors, publication bias and a lack of longitudinal designs (Munkholm et al., 2016). Consequently, the initial enthusiasm for BDNF has tempered and efforts have shifted towards an exploration of other biomarkers in BD (Scola and Andreazza, 2015).

CHAPTER 2

Aim and outline

Aim and outline

The concept of neuroprogression describes the pathological process underlying the cumulative clinical and cognitive burden in BD (Berk et al., 2011; Fries et al., 2012). Many individual mechanisms are hypothesized to contribute to neuroprogression, but we lack a longitudinal biological validation (Berk et al., 2011). Regarding the role of inflammation, nowadays there is a consensus on a chronic mild pro-inflammatory state accompanying the disease process (Rosenblat and McIntyre, 2017). Some studies even indicate a further increase in pro-inflammatory markers during acute mood episodes which would be in line with the concept of neuroprogression (Fernandes et al., 2016; Munkholm et al., 2013). Though, current evidence on inflammation in psychiatric disorders and BD in particular, is still characterized by many limitations. The often naturalistic design of clinical studies implicates the presence of many confounding factors with known, but also unknown, effects on inflammation. The extent to which factors as medication use or unhealthy lifestyle contribute to the co-occurrence of inflammation and mood disorders has not been determined yet. Furthermore, most studies on biological markers are cross-sectional or have short follow-up periods, making conclusions on state-related dynamics of biological markers very difficult (Munkholm et al., 2013; Munkholm et al., 2016).

Preclinical studies have documented a link between inflammation, kynurenine metabolism, monoamine synthesis and the development of mood symptoms (O'Connor, J.C. et al., 2009; Savitz, 2017). These data have theoretically been extrapolated to human pathophysiology and are nominated as very promising (Myint and Kim, 2014; Schwarcz and Stone, 2017), but in fact data from clinical studies are lacking. An integrated evaluation of these pathways and their mutual interaction in patients with psychiatric disorders is rare (Vancassel et al., 2018). Especially in BD, a better understanding of the interaction between different pathological processes and their relation to mood fluctuations is essential to determine their contribution to the pathogenesis and neuroprogression of the disease.

Finally, according to the concept of neuroprogression, neurotrophic capacities in BD would decrease along with increases in neurotoxic metabolites and disturbances in neurotransmission. BDNF has often been proposed as a neurotrophic marker of illness

progression (Kapczinski et al., 2008a; Kauer-Sant'Anna et al., 2009). While there is consensus on decreased BDNF levels in patients with BD, findings on its kinetics throughout the course of illness are more controversial (Fernandes et al., 2015; Munkholm et al., 2016). Exploration of other neurotrophic markers and their relation to illness course is warranted (Scola and Andreazza, 2015). Furthermore, research on neurotrophic markers is poorly integrated with research on more toxic processes as inflammation.

The objective of this thesis was to contribute to our understanding of the mechanisms underlying neuroprogression in BD. We investigated inflammation and its relation to kynurenine metabolism, BH_4-dependent monoamine metabolism and neurotrophic markers during an acute mood episode and several months beyond. We aimed to better understand the pathophysiological, potentially toxic, processes accompanying the acute mood episode and the subsequent capacities of recovery. A schematic presentation of the theoretical background and specific aims is presented in figure 5.

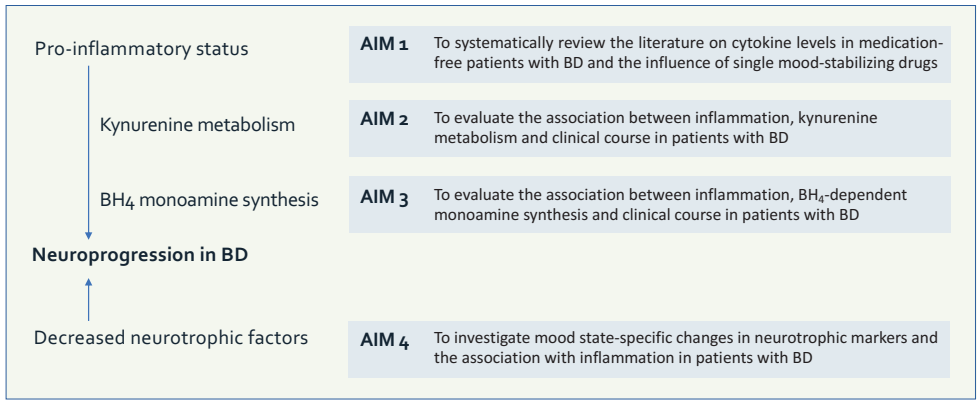

Figure 5. Theoretical background and specific aims. Neuroprogression describes the pathological process underlying the cumulative burden in BD. Several mechanisms are hypothesized to contribute to neuroprogression. Research shows a chronic pro-inflammatory state with an impact on kynurenine metabolism and BH_4 monoamine synthesis. Furthermore, a decrease in neurotrophic markers has been demonstrated. We formulated four specific aims regarding each of these aspects.

Overview of the study design

The original studies that aimed to address the above aims 2, 3 and 4 were realized within the same research project. The design is presented in figure 6.

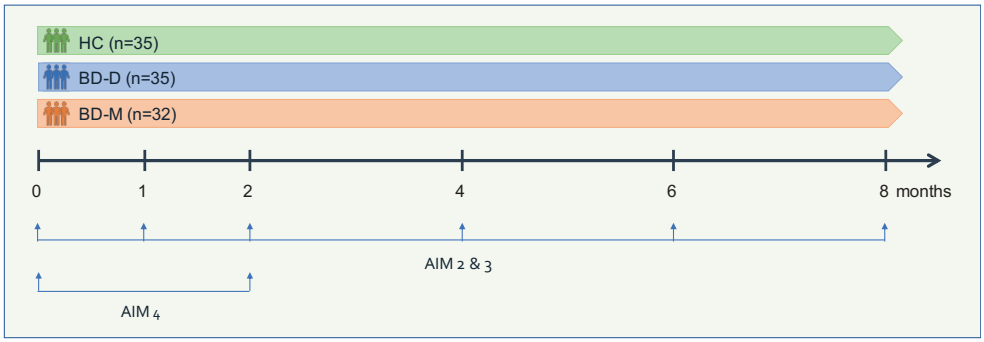

Figure 6. Study design. Patients were recruited during an acute mood episode and assigned to either the depressed (BD-D) or (hypo)manic (BD-M) subgroup. Patients and healthy controls had test days at baseline and after 1, 2, 4, 6 and 8 months follow-up. All six test moments were included for the analysis for aim 2 and 3. For aim 4, only the baseline and 2 months test days were included. HC: healthy controls; BD-D: patients with bipolar disorder and depressive mood state at baseline; BD-M: patients with bipolar disorder and (hypo)manic mood state at baseline.

Patients were recruited during an acute mood episode and assigned to either the depressed (BD-D) or (hypo)manic (BD-M) subgroup. In both patients and controls, screening was followed by a 1st test day after 1-5 days. Subsequent test days were planned after respectively 1, 2, 4, 6 and 8 months of follow-up, resulting in 6 test days per participant over the course of 8 months. Every test day included clinical and laboratory assessments as described in the chapters below. For aim 2 and 3 (Chapter 3 and 4), all 6 test moments were incorporated in the analyses. For aim 4, analyses were performed only at baseline and at 2 months.

CHAPTER 3

Inflammation and mood-stabilizing drugs

van den Ameele S, van Diermen L, Staels W, Coppens V, Dumont G, Sabbe B, Morrens M. The effect of mood-stabilizing drugs on cytokine levels in bipolar disorder: A systematic review. Journal of Affective Disorders. 2016; 203: 364-73. (IF 3.79)

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Review article

The effect of mood-stabilizing drugs on cytokine levels in bipolar disorder: A systematic review

Seline van den Ameele [a,b,*], Linda van Diermen [a,b], Willem Staels [c,d,e], Violette Coppens [a,b], Glenn Dumont [f], Bernard Sabbe [a,b], Manuel Morrens [a,g]

[a] Collaborative Antwerp Psychiatric Research Institute (CAPRI), Department of Biomedical Sciences, University of Antwerp, Belgium
[b] University Psychiatric Hospital Duffel, VZW Emmaüs, Duffel, Belgium
[c] Diabetes Research Center, Vrije Universiteit Brussel, Belgium
[d] Department of Pediatrics, Division of Pediatric Endocrinology, Ghent University Hospital, Ghent, Belgium
[e] Department of Pediatrics and Genetics, Ghent University, Ghent, Belgium
[f] Department of Pharmacy, Academic Medical Center, University of Amsterdam, The Netherlands
[g] Psychiatric Hospital Broeders Alexianen, Boechout, Belgium

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ABSTRACT

Objectives: Cytokine level alterations suggest a role for the immune system in the pathophysiology of bipolar disorder (BD). Pharmacotherapy is an important confounding factor in clinical research on cytokine levels. In this systematic review we collate the evidence on blood cytokine levels in medication-free BD and the effects of single mood-stabilizing drugs on these levels.
Methods: A systematic review was conducted according to the PRISMA statement. We searched the Pubmed and Embase databases for clinical studies reporting either on cytokine levels in medication-free BD or on the effects of single mood-stabilizing drugs on cytokine levels in BD.
Results: Of the 564 articles screened, 17 were included. Fourteen articles report on medication-free patients with BD and indicate state-related cytokine alterations. Six articles discuss the effect of lithium. Whereas no data on short-term effects of lithium were found, ≥ 2 months lithium use in euthymic populations is associated with normal cytokine levels. Two studies report no effect of valproate and no studies were found on carbamazepine, lamotrigine or antipsychotics.
Limitations: The available studies are characterized by a broad methodological heterogeneity and limited replication between studies.
Conclusions: This systematic review suggests the presence of state-related cytokine level alterations in medication-free BD with most evidence pointing to a proinflammatory cytokine response in mania. Euthymia and long-term lithium use are associated with normal cytokine levels. To improve our understanding of the impact of mood-stabilizing drugs on cytokine levels, longitudinal studies with medication-free baseline, randomized controlled single-drug treatment protocols and close mood state monitoring are needed.

Contents

Abbreviations: BD, Bipolar disorder; CRP, C-reactive protein; HCs, Healthy controls; IFN, Interferon; IL, Interleukin; LPS, Lipopolysaccharide; PHA, Phytohemagglutinin; PRISMA-P, Preferred reporting items for systematic review and meta-analysis protocols; sIL-R, Soluble interleukin receptor; sTNF-R, Soluble tumor necrosis factor receptor; TGF, Transforming growth factor; TNF, Tumor necrosis factor

* Correspondence to: University Psychiatric Hospital Duffel - CAPRI, Stationsstraat 22c, 2570 Duffel, Belgium.
E-mail address: seline.vandenameele@uantwerpen.be (S. van den Ameele).

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0165-0327/

1. Introduction

Bipolar disorder (BD) is a group of affective disorders characterized by episodes of mania or hypomania, often alternating with depressive episodes and interspersed with euthymic intervals (Phillips and Kupfer, 2013). The disease is associated with a progressive decline in cognitive and psychosocial functioning, the latter aspects accounting for a significant reduced quality of life (Levy and Manove, 2012). In search of the neurobiological underpinnings of BD, abnormalities in brain structure, brain function, neurodevelopment, neurotransmission, inter- and intracellular signaling and (epi)genetics have been identified (Maletic and Raison, 2014). Recently gained insights emphasize the role of the immune system in the pathophysiology of BD (Goldstein et al., 2009; Maletic and Raison, 2014; Stertz et al., 2013). Several studies show raised levels of inflammatory markers, notably cytokines (Hornig et al., 1998; Modabbernia et al., 2013; Munkholm et al., 2013).

Cytokines are small soluble proteins crucial for immune response regulation. They can have pro- or anti-inflammatory characteristics and some have a broader regulatory role in the immune response. Typical proinflammatory cytokines are tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6. They are mainly secreted by macrophages in response to noxious stimuli and contribute to a rapid local inflammatory response. Other important cytokines are interferon gamma (IFN-γ), IL-2, IL-4 and IL-10. These have a diverse set of functions. Basically, IFN-γ and IL-2 enhance the cellular immune response, while IL-4 and IL-10 activate the humoral immune response and have an anti-inflammatory effect through negative feedback on immune cell activation (Drexhage et al., 2010; Liao et al., 2011; Shabgah et al., 2014). Cytokines exert their function through binding to cytokine receptors. Both membrane-bound and soluble forms of cytokine receptors have been identified. Soluble cytokine receptors are readily detectable in peripheral blood samples and have been proposed as suitable markers of disease activity in various inflammatory diseases such as cancer, infections and autoimmune diseases (Barbosa et al., 2011; Cetin et al., 2012; Diez-Ruiz et al., 1995; Kronfol and Remick, 2000). Binding of the soluble receptors with their respective cytokines can result in both agonistic (soluble TNF receptor 1 and 2 (sTNFR1&2), sIL-6R) and antagonistic (sIL-2R) effects on cytokine signaling (Breunis et al., 2003; Wolf et al., 2014).

The repeated observation of elevated cytokine levels in both BD and major depressive disorder has put forward the 'cytokine-hypothesis of mood disorders'. According to this hypothesis, excessive production of cytokines due to chronic activation of macrophages, microglia, and T-cells is suggested to cause psychiatric signs and symptoms through effects on neurotransmission, neuroendocrine function and neural plasticity (Beumer et al., 2012; Haarman et al., 2014; Maes et al., 1995b; Miller et al., 2009; Rosenblat et al., 2014).

While several clinical studies and 2 recent meta-analyses (Modabbernia et al., 2013; Munkholm et al., 2013) show significant cytokine changes in BD, cautious interpretation of these results should be emphasized. The design of the individual studies is highly heterogeneous, generally small in sample size and often lacks control for important confounding factors. Indeed, age, gender, smoking habits, metabolic syndrome, somatic comorbidities and use of mood-stabilizing drugs are known to influence peripheral cytokine levels (Drexhage et al., 2010; Haack et al., 1999), but are often not accounted for. These confounders impede a conclusive interpretation of the current data on cytokine level alterations in BD. The severity and phasic course of BD often imposes complex psychotropic drug regimens on patients (Bauer et al., 2013). Consequently, the use of mood-stabilizing drugs at time of inclusion in clinical studies is often inevitable and unconfounded medication-free cytokine levels in patients with BD are therefore hard to obtain. Some *in vitro* studies on the influence of mood-stabilizing drugs (mainly lithium) on cytokine production are available. However, results are conflicting and translation to clinical populations is difficult (Himmerich et al., 2013, 2014; Kleinerman et al., 1989; Knijff et al., 2007; Petersein et al., 2015). Clinical studies on the other hand, rarely have patients on standardized treatment regimens and are therefore limited to a comparison of cytokine levels in 'medication-free and 'medicated' patients. However, as the individual mechanisms of action differ greatly between various mood-stabilizing agents, we can equally hypothesize different effects on the immune system. A separate evaluation of single drugs is therefore crucial.

This systematic review will focus on cytokine levels in medication-free patients with BD and the influence of single mood-stabilizing drugs on cytokine levels. We discuss limitations in the current body of literature and formulate suggestions for future research.

2. Methods

This systematic review was conducted and reported according to the PRISMA-P (preferred reporting items for systematic review and meta-analysis protocols) guideline (Moher et al., 2015). Objectives and eligibility criteria were specified in advance and documented in a protocol. For protocol details and PRISMA checklist, see Supplementary material.

2.1. Eligibility criteria

In order to obtain original studies on cytokine levels in medication-free patients with BD as well as original studies reporting on the effect of single mood-stabilizing drugs on blood cytokine levels in BD, we applied the following eligibility criteria:

- Patients with BD type I or II as confirmed by DSM-III-R, DSM-IV, DSM-IV-TR, DSM-5, ICD-9 or ICD-10 criteria.
- Studies assessing levels of cytokines or soluble cytokine receptors in peripheral blood.
- Studies evaluating cytokine levels in medication-free BD with a cross-sectional design comparing cytokine levels in patients with BD to those in healthy controls (HCs), or;
- Studies evaluating the effect of mood-stabilizing drugs on cytokine levels with a longitudinal design reporting cytokine levels pre- and post-treatment; or with a cross-sectional design reporting cytokine levels in medication-free compared to medicated patients in similar mood state or to HCs; and,
- Results concerning the effect of a single mood-stabilizing drug on cytokine levels are provided or could be obtained by contacting the authors, and;
- The evaluated mood-stabilizing drug is recommended by the current international standards on the pharmacological treatment of BD (*i.e.* lithium, valproate, carbamazepine, lamotrigine or atypical antipsychotics) (Cipriani et al., 2011; Geddes and Miklowitz, 2013; Soares-Weiser et al., 2007; Yatham et al., 2013).

The use of the above eligibility criteria implies that we did not exclude studies on the basis of concomitant drug use for purposes other than mood stabilization. Several studies mentioned the use of antipsychotics or benzodiazepines as concomitant drugs in a subgroup of patients and were included for qualitative review. However, results of patient groups using more than one drug administered for mood-stabilizing reasons without a separate evaluation of the effect of a single mood-stabilizing drug, were excluded. Several studies show cytokine levels analyzed in mitogen-stimulated blood cells. The interpretation of the results of these *ex vivo* studies remains controversial. Stimulating immunocompetent cells with activators such as phytohemagglutinin (PHA) or lipopolysaccharide (LPS) and thereafter analyzing the pattern of cytokine production is defended as a reliable, practical and dynamic approach to study disturbances in cytokine synthesis (Himmerich et al., 2011; Knijff et al., 2007; Xia et al., 1996). However, others argue that these studies on mitogen-stimulated cells do not necessarily reflect the endogenous immune response but only model an immune response to specific immune challenges (Munkholm et al., 2013). The inclusion of these studies increases the heterogeneity in our systematic review, however we choose for this comprehensive approach combined with a careful indication of the specific methodological characteristics of these experiments.

2.2. Information sources

A search was conducted in the Pubmed database (January 1946 – January 2016) and Embase database (1947 – January 2016) for English language articles using the following search terms: (bipolar OR mania OR manic) AND (cytokine OR interferon OR interleukin OR "tumor necrosis factor" OR TNF OR "transforming growth factor" OR TGF) AND ("medication-free" OR unmedicated OR lithium OR valproate OR carbamazepine OR lamotrigine OR antipsychotic). Specifying the product names of antipsychotic drugs in the search string did not change the number of hits. Reference lists of retrieved articles were searched by hand.

2.3. Study selection

The titles and abstracts were screened independently by SvdA and LvD to eliminate obvious irrelevant publications. Full-texts of the potential relevant articles were further screened for eligibility. Disagreements were resolved by discussion between SvdA and LvD.

2.4. Data extraction

We developed a data extraction sheet with following data: mood state, number of subjects, age, sex, study design, severity of mood symptoms, mood-stabilizing drug-use, duration of medication-free period, duration of treatment with mood-stabilizing drug, concomitant drug use, duration of illness, age of onset, body mass index (BMI), smoking status, sample type, type of cytokine assay, specific cytokines and cytokine receptors analyzed, correlations between cytokine levels and clinical characteristics. If cytokines were measured after stimulation of blood cells with mitogens, the following data were additionally extracted: type and concentration of mitogen, duration of exposure to mitogen, cell type and culture medium.

SvdA initially extracted these predefined data, LvD checked the data extraction sheet. Disagreements were resolved by discussion between SvdA, LvD and a third independent researcher WS. If no agreement was obtained, we further discussed with two senior researchers, VC and MM. If needed, authors were contacted for further information or clarification.

We assessed risk of bias in individual studies by carefully reviewing study designs, methodology and interpretation of data. Heterogeneity between studies was evaluated in detail. Risk of bias and limitations were extensively discussed with all authors and are described in the discussion and limitations sections below. For a detailed data extraction sheet, see Supplementary Tables 1–3.

3. Results

3.1. Results of systematic literature review

The search of the Pubmed and Embase databases resulted in an initial 591 records. Adjusting for duplicates resulted in 511 remaining records. Based on a preliminary screening of titles and abstracts, 465 records were excluded. Eligibility of the remaining 46 records was assessed by a detailed evaluation of the full-texts. Thirty-two records did not to meet the eligibility criteria because of the following reasons: no separate data on patients with BD ($n=3$); no analysis of cytokines or cytokine-receptors ($n=2$); no separate evaluation of a single mood-stabilizing drug effect on cytokine levels ($n=13$); no HC group or medication-free baseline measurement ($n=13$); conference abstract on the same data as an original research article ($n=1$). For 4 records, we contacted the study authors in order to obtain more information on the characteristics of the study population or for clarification of the results. One of these could provide the necessary information. Three additional eligible articles were identified by hand search of reference lists. A total of 17 studies was found eligible for the systematic review. Fourteen studies provided information on cytokine levels in medication-free patients with BD, 8 studies on the effect of mood-stabilizing drugs on cytokine levels. For a schematic representation of the study selection process, see Fig. 1.

In the following paragraphs, we subsequently describe cytokine profiles in medication-free and medicated patients with BD.

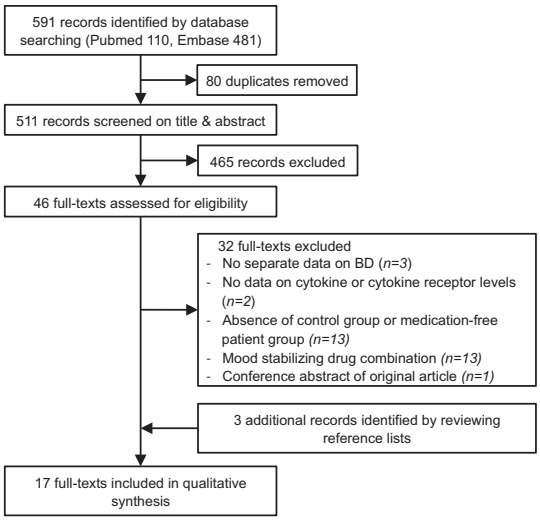

Fig. 1. Study selection process.

3.2. Cytokine alterations in medication-free patients with BD

Fourteen studies reported on cytokine levels in medication-free patients with BD. The medication-free period varied from 1 week or more without any mood-stabilizing drug use to medication-naïve patients. Details on individual studies are presented in Table 1 and Supplementary Table 1. Methodological details on *ex vivo* studies can be found in Table 2. Below we summarize the results for each mood state separately.

The majority of the studies ($n=10$) reported on cytokine levels in manic medication-free patients. For TNF-α, IL-6, sIL-2R and sIL-6R, both increased (Kim et al., 2007; Maes et al., 1995a; Ortiz-Dominguez et al., 2007; Uyanik et al., 2015) or similar (Li et al., 2015; Maes et al., 1995a; Tsai et al., 2003, 2001) cytokine levels have been found in patients compared to HCs. Particularly for the studies analyzing sIL-2R and sIL-6R, the number of patients is small and the medication-free period short (> 1–2w) (Maes et al., 1995a; Tsai et al., 2003, 2001). The other studies investigating TNF-α and IL-6 are characterized by larger patient groups, a longer medication-free period (> 3w-4m) and a more transparent description of methodology (Kim et al., 2007; Li et al., 2015; Ortiz-Dominguez et al., 2007; Uyanik et al., 2015). The *ex vivo* study of Kim et al. (2007) was performed with PHA and LPS stimulated whole blood.

For TGF-β, both increased (Li et al., 2015) and decreased (Kim et al., 2004) levels have been found in patients with mania. Interleukin-2 has been studied in 3 articles. Under stimulated conditions, similar levels were found in patients with mania compared to HCs (Kim et al., 2007; Liu et al., 2004), however *in vivo* decreased IL-2 levels were found (Ortiz-Dominguez et al., 2007). For IL-10, no differences were found in 3 studies on IL-10 levels in both stimulated (Liu et al., 2004) and non-stimulated (Li et al., 2015; Uyanik et al., 2015) conditions. While IFN-γ levels in 2 studies in stimulated conditions were found to be similar (Kim et al., 2007) or decreased (Liu et al., 2004), 2 *in vivo* studies show increased (Kim et al., 2004; Uyanik et al., 2015) levels in patients with mania compared to HCs. The same variability was found for IL-4 (similar (Liu et al., 2004; Uyanik et al., 2015) vs. decreased (Kim et al., 2007) vs. increased (Kim et al., 2004; Ortiz-Dominguez et al., 2007)). Single studies reported increased IL-23 levels (Li et al., 2015), similar IL-12 (Kim et al., 2002) and IL-17 levels (Li et al., 2015) and decreased IL-1β levels (Ortiz-Dominguez et al., 2007) in manic medication-free patients compared to HCs.

Two studies reported on cytokine and cytokine receptor levels in depressive medication-free patients with BD. A first study in a small patient group showed increased levels of TNF-α and IL-6, decreased IL-2 levels and no difference in IL-1β and IL-4 compared to HCs (Ortiz-Dominguez et al., 2007). A second study reported increased levels of sTNFR1&2 (Teixeira et al., 2015). Although methodology is clearly presented and both studies are properly designed, the number of patients is rather small and none of the performed cytokine analyses been replicated in other samples of medication-free patients with bipolar depression.

A few studies assessed the relationship between mood symptom severity and cytokine levels in both manic and depressive medication-free patients, but no correlations were found (Kim et al., 2007, 2004, 2002; Rapaport et al., 1999; Teixeira et al., 2015; Tsai et al., 2001; Uyanik et al., 2015).

Two studies in euthymic patients with BD found no differences in TNF-α, INF-γ, IL-4, IL-10 (Guloksuz et al., 2010), IL-2 (Guloksuz et al., 2010; Rapaport, 1994) and sIL-2R (Rapaport, 1994) levels compared to HCs. For measurement of IL-2 levels, Rapaport (1994) used PHA-stimulated lymphocytes. The number of patients in both studies is limited, but methodology is specifically developed to investigate cytokines in euthymic patients. Finally, 1 study in medication-free patients with rapid cycling BD in different mood states reported a trend towards increased sIL-2R and sIL-6R levels (Rapaport et al., 1999). Six studies evaluated correlations between cytokine levels and severity scores on the mood symptom scales in medication-free patients with BD, but no correlation could be reported in either the manic (Kim et al., 2007, 2004, 2002; Maes et al., 1995a; Uyanik et al., 2015) or the depressive (Teixeira et al., 2015) patient samples. More details on correlations between cytokine and cytokine receptor levels and clinical characteristics can be found in Supplementary Table 1.

In summary, most studies on cytokine levels in medication-free patients with BD have been conducted in samples of patients with mania. These results suggest an increase of proinflammatory cytokines like TNF-α and IL-6 during mania. We found conflicting results for IL-4 and IFN-γ. Levels of IL-10 do not seem to be altered in medication-free patients with mania. Other cytokines are insufficiently studied to make conclusive statements. Interestingly, in 2 samples of euthymic medication-free patients with BD, no significant differences in cytokine levels compared to HCs have been found.

3.3. Cytokine concentrations in medicated patients with BD

Six studies report on the effect of lithium on cytokine levels in patients with BD (Table 3). Two studies report on the effect of valproate (Table 4). We found no eligible studies for carbamazepine, lamotrigine or antipsychotics. Methodological details on *ex vivo* studies are indicated in Table 2. For more details on the individual studies, see Supplementary Tables 3 and 4.

3.3.1. Lithium

We selected 3 studies in euthymic patients with BD, all of them with a cross-sectional design, *i.e.* cytokine levels in lithium users vs. HCs and/or medication-free euthymic patients with BD (Guloksuz et al., 2010; Rapaport, 1994; Remlinger-Molenda et al., 2012). One study, with a design specifically developed to investigate the effect of lithium monotherapy on cytokine levels, reported elevated TNF-α and IL-4 levels in a euthymic BD group on ≥ 8 weeks lithium monotherapy compared to both a medication-free euthymic BD group and HCs (Guloksuz et al., 2010). In contrast, no differences in TNF-α levels between euthymic chronically

Table 1
Cytokine levels in MF-BD compared to HCs.

Mood state	MF-BD (*n*)	HCs (*n*)	Mean age BD/ HCs	Duration MF period	PHA/LPS	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IL-17	IL-23	sIL-2R	sIL-6R	sTNFR1&2	TGF-β	TNF-α	Reference
Mania	25	85	29/33	> 16w	n								↔								(Kim et al., 2002)
	70	96	33/30	> 16w	n	↑			↑										↓		(Kim et al., 2004)
	37	74	38/38	> 16w	y	↔		↔	↓		↑									↑	(Kim et al., 2007)
	41	36	38/37	> 4w	n							↔							↑	↔	(Li et al., 2015)
	18–25	29–45	33/27	> 2w	y	↓		↔	↔			↔									(Liu et al., 2004)
	10	21	33/41	> 1w	n						↔					↑	↑				(Maes et al., 1995a)
	10	33	29/32	> 3w	n		↓	↓	↑											↑	(Ortiz-Dominguez et al., 2007)
	13	31	32/33	> 2w	n											↔	↔				(Tsai et al., 2001)
	7	36	34/?	> 2w	n											↔	↔				(Tsai et al., 2003)
	30	28	33/33	> 4w	n	↑			↔		↑	↔								↑	(Uyanik et al., 2015)
Depression	10	33	40/32	> 3w	n		↔	↓	↔		↑									↑	(Ortiz-Dominguez et al., 2007)
	29[b]	27	28/28	> 6w	n													↑			(Teixeira et al., 2015)
Euthymia	16	16	32/32	> 4w	n	↔		↔	↔	↔		↔								↔	(Guloksuz et al., 2010)
	12	34	41/39	> 8w	y/n[a]			↔								↔					(Rapaport, 1994)
Various	17[c]	18	39/36	> 2w	n			[d]								↔[e]	↔[e]				(Rapaport et al., 1999)

MF: medication-free; BD: bipolar disorder; HCs: healthy controls; PHA: phytohemagglutinin (mitogen-induced cytokine production); LPS: lipopolysaccharide (mitogen-induced cytokine production); IFN: interferon; IL: interleukin; sIL-R: soluble interleukin receptor; TGF: transforming growth factor; TNF: tumor necrosis factor.

[a] IL-2 levels produced by PHA-stimulated lymphocytes, sIL-2R determined in non-stimulated serum.
[b] Medication-free for 86,2% ($n=25$).
[c] Rapid cycling BD group.
[d] Levels detectable only in a minority of the study group (MF-BD $n=5$; HCs $n=8$), lower IL-2 levels in MF-BD compared to HCs not reaching significance.
[e] The authors report an increased trend not reaching significance.

Table 2
Methodological details on the included *ex vivo* studies.

References	Cell type	Stimulant	Duration of stimulation (h)	Culture medium	Assay	Cytokines measured
Kim et al., 2007	Whole blood	4 μg/ml PHA & 20 μg/ml LPS	48	RPMI	ELISA	IL-2, IL-4, IL-6, TNF-α, IFN-γ
Liu et al., 2004	Mononuclear blood cells	5 μg/ml/PHA	48	RPMI	ELISA	IL-2, IL-4, IL-10, IFN-γ
Rapaport, 1994	Lymphocytes	50 μg/ml PHA	48	NA	EIA	IL-2, sIL-2R
Knijff et al., 2007	Monocytes	1 μg/ml LPS	24	RPMI	ELISA	IL-1β, IL-6

PHA: phytohemagglutinin; LPS: lipopolysaccharide; RPMI: Roswell Park Memorial Institute; NA: not available; ELISA: enzyme-linked immunosorbent assay.
EIA: enzyme immunoassay; IFN: interferon; IL: interleukin; sIL-R: soluble interleukin receptor; TNF: tumor necrosis factor.

(≥5 years) lithium-treated patients and HCs have been reported in a retrospective study (Remlinger-Molenda et al., 2012). In the latter only 10 out of 45 patients were on lithium monotherapy, nevertheless, subgroup analysis showed no differences between lithium monotherapy and lithium combined with other mood-stabilizing drugs. No other significant differences were found between either euthymic patients with BD on lithium for ≥2months, medication-free euthymic patients or HCs concerning the IL-1β, IL-2, IL-5, IL-6, IL-10, IFN-γ, and sIL-2R levels (Guloksuz et al., 2010; Rapaport, 1994; Remlinger-Molenda et al., 2012). A longitudinal study in patients with bipolar depression with elevated levels of sTNFR1&2 at baseline, showed no changes in these cytokine receptor levels after a 6-week course of lithium, despite significant improvement of clinical symptoms (Teixeira et al., 2015).

Finally, 2 studies have been conducted in lithium-treated patients with BD in various mood states (Knijff et al., 2007; Rapaport et al., 1999). In a rapid cycling group, levels of sIL-2R and sIL-6R normalized (*i.e.* decreased) after 30 days of lithium treatment, whereas in HCs a slight, but non-significant increase was seen compared to baseline (Rapaport et al., 1999). Others compared the production of IL-1β and IL-6 by monocytes (both LPS-stimulated and non-stimulated) of patients with BD on lithium for ≥6 months to HCs (Knijff et al., 2007). These results do not show significant differences in IL-1β and IL-6 production between long-term lithium users and HCs.

3.3.2. Anti-epileptics

Two studies report on the effect of valproate on cytokine levels in patients with BD (Lee et al., 2014; Maes et al., 1995a). In 10 patients with mania, the effect of a 2-week trial with valproate on IL-6, sIL-2R and sIL-6R levels was evaluated (Maes et al., 1995a). A non-significant reduction in sIL-6R and sIL-2R was observed, but IL-6 levels were unaltered despite significant improvement of clinical symptoms. A study in patients with bipolar depression could not find an effect of a 12-week valproate trial on the levels of IL-1β, IL-6, IL-8 and TNF-α (Lee et al., 2014). We found no studies reporting on the effect of carbamazepine or lamotrigine on cytokine levels in BD.

3.3.3. Antipsychotics

We found no studies reporting on the effect of antipsychotic drugs on cytokine levels in BD.

In summary, clinical studies providing information on the effect of mood-stabilizing drugs on cytokine levels in BD mainly focus on the effect of lithium. Altogether, the results do not suggest strong effects of lithium use on cytokine levels in BD, but most of these studies are done in euthymic patients with ≥2 months lithium use. Likewise, the limited results on the effect of valproate do not indicate an effect on cytokine levels in BD.

4. Discussion

In this systematic review, we summarize the results of 17 original clinical research reports studying cytokine levels in medication-free patients with BD and the effect of mood-stabilizing drugs on these cytokine levels. Fourteen of these reports concern cytokine levels in medication-free patients, 8 concern the effect of a single mood-stabilizing drug on cytokine levels.

Characterizing the correlation between cytokine levels and mood state in medication-free patients with BD is an important base for studying how mood-stabilizing drugs affect cytokine levels. However, the clinical reality of BD often confronts us with a need for rapid pharmacological intervention and rarely allows medication-free patients to be followed longitudinally across different mood states. Several studies report on cytokine levels in medication-free patients with BD, mostly manic. Typical proinflammatory cytokines (TNF-α and IL-6) seem to be elevated in medication-free patients during a manic or depressive episode and to be similar to HCs during euthymia. These findings in medication-free patients suggest a state-related proinflammatory immune system activation. This is in line with previous findings demonstrating mood state-related cytokine level alterations (Fiedorowicz et al., 2015; Ortiz-Dominguez et al., 2007), mania-related increase in C-reactive protein (CRP) levels (Cunha et al., 2008; Dickerson et al., 2007) and a state-specific proinflammatory monocyte gene expression signature (Becking et al., 2015). The increase in proinflammatory cytokines is also observed in 2 meta-analyses in general (predominantly treated) BD populations (Modabbernia et al., 2013; Munkholm et al., 2013). Both meta-analyses report elevated TNF-α, sTNFR1, sIL-2R, sIL-6R and IL-4 levels; Modabbernia et al. (2013) additionally found elevated IL-10 levels. The latter provides evidence for a state-related increase in TNF-α, sTNFR1 and sIL-2R, which is in support of our current findings. However, the collated data in medication-free patients are too heterogeneous and insufficiently replicated to further compare between medication-free patients and the general population with BD.

Although the increase in proinflammatory cytokines seems a state-related phenomenon, we cannot conclude on the causality of this association. A bidirectional relationship, in which mood symptoms contribute to an increase in proinflammatory cytokines and *vice versa* is possible (Rosenblat et al., 2014).

Most data on how mood-stabilizing drugs affect cytokine levels in BD relate to lithium. Between lithium users, medication-free patients, and HCs no differences in cytokine levels have been found. Importantly most of these studies are done in euthymic patients with BD and ≥2 months lithium use. Since limited data is available on shorter-term lithium use within an acute mood episode, we cannot make conclusive statements regarding the effect of lithium use on cytokine levels in BD. Indeed, past research in several domains provides ample evidence for immune modulating effects of lithium. In the early 1980s, its immune enhancing effects in response to viral infections have been described extensively (Harvey et al., 2002; Lieb, 1981; Skinner et al., 1980) and its capacity to induce leukocytosis has put forward lithium in the treatment of neutropenia (Carmen et al., 1993; Petrini and Azzara, 2012). More recently, lithium use in refractory depressive and manic patients was found to attenuate the acute phase immune response (Maes et al., 1997; Sluzewska et al., 1997). Furthermore,

Table 3
Cytokine levels in Li-BD compared to MF-BD and HCs.

Mood state	Study design	MF-BD (*n*)	Li-BD (*n*)	HCs (*n*)	Mean age BD/HCs	Duration Li use (*mean*)	Li-BD vs.	Comedication	PHA/ LPS	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	sIL-2R	sIL-6R	sTNF-R1&2	TNF-α	References
Euthymia	CS	16	15	16	32/32	> 2 m (*36m*)	MF-BD & HCs	n	n	↔		↔	↑	↔		↔				↑	(Guloksuz et al., 2010)
	CS	12	14	34	41/39	> 2 m (*?*)	MF-BD & HCs	n	y/n[a]			↔					↔				(Rapaport, 1994)
	CS	/	45[b]	78	58/35	> 5y (*14y*)	HCs	n	n	↔	↔	↔			↔	↔				↔	(Remlinger-Molenda et al., 2012)
Depression	FU	29	29	27	28/28	1.5 m	MF-BD baseline	y	n										↔[c]		(Teixeira et al., 2015)
Various	CS	/	64	59	44/38	> 6 m (*74m*)	HCs	y	y/n[d]		↔				↔		↔[e]	↔[e]			(Knijff et al., 2007)
	FU	17	17	18	39/36	1 m	MF-BD baseline	?	n												(Rapaport et al., 1999)

CS: cross-sectional; FU: follow-up; MF: medication-free; BD: bipolar disorder; Li: lithium; HCs: healthy controls; PHA: phytohemagglutinin (mitogen-induced cytokine production); LPS: lipopolysaccharide (mitogen-induced cytokine production); IFN: interferon; IL: interleukin; sIL-R: soluble interleukin receptor; TNF: tumor necrosis factor.

[a] IL-2 levels produced by PHA-stimulated lymphocytes, sIL-2R determined in non-stimulated serum.

[b] Total Li-BD group ($n=45$) of which Li-monotherapy ($n=10$) and Li combined with other drugs ($n=35$). No significant difference in cytokine levels between HCs and total Li-BD group ($n=45$), neither between Li-monotherapy and Li combined with other drugs.

[c] Significant elevated levels at baseline (MF-BD) compared to HCs.

[d] Analysis of cytokines produced by both LPS-stimulated and non-stimulated monocytes, same results in comparison to HCs.

[e] Trend towards normalization reported.

in a retrospective analysis decreased CRP levels were observed in affective disorder patients on lithium monotherapy (Hornig et al., 1998). This decrease was more evident in lithium responders than in refractory affective disorder patients. Similarly, a retrospective study in patients with BD showed higher TNF-α levels in euthymic patients with a generally poor clinical response to lithium treatment compared to those with good response (Guloksuz et al., 2012). These findings could suggest a positive correlation between the clinical response to lithium and normalization of immune system activity. Indeed, some *in vitro* studies suggest a biphasic proinflammatory effect of lithium after short-time use (Kleinerman et al., 1989; Petersein et al., 2015) and an attenuating anti-inflammatory effect after longer-term use (Boufidou et al., 2004; Knijff et al., 2007; Maes et al., 1999; Rapaport and Manji, 2001). While some of these *in vitro* findings have been countered, the immunoregulatory properties of lithium are still mainly accepted (Agrawal et al., 2013; Himmerich et al., 2013, 2014).

Lithium has been proposed to exert its immunomodulatory role at the gene expression level. Several researchers have described downregulation of proinflammatory gene expression after treatment with lithium in both patients with BD (Haarman et al., 2014; Padmos et al., 2008) and HCs (Watanabe et al., 2014). Other possible mechanisms are the effects of lithium on different intracellular signaling pathways. Lithium modulates several second messenger systems that are involved in the regulation of cytokine production (Jope, 1999; Rapaport and Manji, 2001).

Findings from both clinical and preclinical studies thus support an immunomodulatory role of lithium. The collated evidence in this review suggests state-related cytokine alterations in medication-free patients as well as cytokine levels similar to HCs in medication-free euthymic patients and longer-term lithium-treated euthymic patients with BD. It remains to be determined whether these normalized cytokine levels can be attributed to the euthymic mood state or to immunomodulatory effects of longer-term lithium use. Alternatively, the normalized cytokine levels during lithium use could be explained as both an indirect consequence of mood symptom stabilization and a direct consequence of the immunomodulatory properties of lithium.

Insufficient studies are available to make conclusions on the effect of anti-epileptic drugs on cytokine levels in BD. The effect of both carbamazepine and valproate on cytokine levels has been investigated more extensively in epileptic patients and HCs, but results are conflicting (Mlodzikowska-Albrecht et al., 2007). Preclinical studies have repeatedly shown a predominantly, anti-inflammatory effect of valproate (Himmerich et al., 2014; Ichiyama et al., 2000; Suda et al., 2013).

We found no studies on the effect of antipsychotic drugs on cytokine concentration in patients with BD. The effect of antipsychotic drugs on cytokine levels in schizophrenia has been reviewed earlier (Drzyzga et al., 2006; Miller et al., 2011; Tourjman et al., 2013). In schizophrenia, antipsychotic drugs seem to have an anti-inflammatory effect positively correlated to the clinical antipsychotic response. These anti-inflammatory properties of both first and second generation antipsychotics are supported by findings from preclinical studies (Moots et al., 1999; Sugino et al., 2009; Yamamoto et al., 2016). Contrary, some studies report a proinflammatory effect related to the well-established metabolic side effects. The authors do emphasize limitations in our current understanding of the complex relationship between antipsychotic treatment and inflammation. (Drzyzga et al., 2006; Miller et al., 2011; Tourjman et al., 2013).

4.1. Limitations and future perspectives

This systematic review unveils a number of limitations in the current body of literature.

Table 4
Cytokine levels in VPA-BD compared to MF-BD.

Mood state	Study design	MF-BD (*n*)	VPA-BD (*n*)	Mean age	Duration VPA use (m)	VPA-BD vs.	Comedication	IL-1β	IL-6	IL-8	sIL-2R	sIL-6R	TNF-α	References
Depression	FU	117	76	30	3	MF-BD baseline	y	↔	↔	↔			↔	(Lee et al., 2014)
Mania	FU	10	10	40.5	0.5	MF-BD baseline	n		↔		↔	↔		(Maes et al., 1995a)

VPA: valproate; FU: follow-up; MF: medication-free; BD: bipolar disorder.

First, there is a lack of properly designed studies that specifically investigate the effect of single mood-stabilizing drugs on cytokine levels as primary endpoint. Included studies are characterized by a broad heterogeneity regarding several potentially confounding factors: demographic characteristics, mood state, symptom severity, duration of medication-free period or duration of mood-stabilizing drug use, concomitant drug use, illness duration and comorbidities among others. Many of these factors are known to influence cytokine levels (Haack et al., 1999; Haarman et al., 2014; O'Connor et al., 2009). Moreover, methodological differences such as sample type (plasma vs. serum) and cytokine assay type (enzyme-linked immunosorbent assay vs. enzyme immunoassay vs. flow cytometry) further increase heterogeneity (Leng et al., 2008; Zhou et al., 2010). The studies collated in this review are insufficient to make conclusive statements on possible confounding effects of methodological differences. To minimize the risk of bias due to this broad heterogeneity, we adopted a strict and thoughtful use of in- and exclusion criteria, performed detailed data acquisition and carefully interpreted the retrieved data. However, to be able to assess the interaction between the evolution of mood, immune system activity and mood-stabilizing drug use, longitudinal studies of cytokine levels in well characterized and more homogenous patient samples with randomized controlled treatment regimens are required. Recommendations on the use of in- and exclusion criteria have been proposed earlier (O'Connor et al., 2009). Although monotherapy for BD is rather uncommon in clinical practice, single mood-stabilizing drug studies are required for further clarification whether the mechanism of action of specific mood-stabilizing drugs influences immune activity.

Secondly, the data in medication-free populations retrieved by this systematic review cannot be extrapolated to the general BD population. It can be assumed that a medication-free population differs from a medicated population in characteristics such as mood symptom severity, duration of illness, number of mood episodes, general health status; all elements with a possible impact on the immune system activity. Furthermore, the duration of the medication-free period varies from 1 week to medication-naïve patients and medication history is very heterogeneous. The impact of wash-out effects and past mood-stabilizing drug use on immune activity is unknown but should be considered.

A third limitation is the wide variety in cytokines analyzed. The analysis of most cytokines is insufficiently replicated to draw strong conclusions; hence, replication studies are needed.

Fourthly, as we included 4 *ex vivo* studies on mitogen-stimulated blood cells (Kim et al., 2007; Knijff et al., 2007; Liu et al., 2004; Rapaport, 1994), the comprehensiveness of data was increased along with the methodological heterogeneity. In medication-free euthymic patients and lithium treated patients, the *ex vivo* results are in line with the *in vivo* data. During mania, results concerning TNF-α and IL-6 levels are again in line with the available *in vivo* data, but conflicting findings are seen regarding IFN-γ, IL-2 and IL-4. Although these results could suggest a divergent immune reaction between *in vivo* and *ex vivo* conditions, we want to emphasize the limited number of studies in every group.

Finally, the correlation and/or possible causality between cytokine levels, mood-stabilizing treatment and the severity of mood symptoms remain unclear. Clinical status is insufficiently documented in the current literature. Preliminary research suggests that immune system modulation is important for a beneficial clinical response in at least a subgroup of patients with BD (Guloksuz et al., 2012; Hornig et al., 1998). Identifying the clinically relevant immune system alterations would be a big step forward for personalized medicine and drug development in BD.

To conclude, this systematic review shows state-related cytokine level alterations in medication-free BD, with most evidence pointing to a proinflammatory cytokine response in mania. Euthymia and long-term lithium use are associated with cytokine levels similar to HCs. Included studies are characterized by methodological heterogeneity and replication is limited. Longitudinal studies with medication-free baseline measurements and randomized controlled treatment protocols with single drug use and close mood state monitoring are needed.

Author disclosure - contributors

SvdA, LvD, WS, GD and MM developed the review protocol. SvdA, LvD and WS evaluated the eligibility of articles and performed the data acquisition according to the protocol. SvdA drafted the paper. SvdA, LvD and WS contributed to the writing of the paper. VC and MM supervised the whole review process. All authors critically revised the manuscript and gave final approval for the submitted version of the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jad.2016.06.016.

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The Effect of Mood-Stabilizing Drugs on Cytokine Levels in Bipolar Disorder: A Systematic Review

S van den Ameele, L van Diermen, W Staels, V Coppens, G Dumont, B Sabbe, M Morrens

Supplementary Materials

Content

Review Protocol

Title

The effect of mood-stabilizing drugs on cytokine levels in bipolar disorder: a systematic review

Background

Cytokine level alterations suggest a role for the immune system in the pathophysiology of bipolar disorder (BD). Pharmacotherapy is an important confounding factor in clinical research on cytokine levels. This systematic review will focus on cytokine levels in medication-free BD patients and the influence of single mood-stabilizing drugs on cytokine levels in BD. We discuss limitations in the current body of literature and formulate suggestions for future research.

Review questions

Primary research questions

Q1: Is there a difference in cytokine levels between medication-free BD patients and HCs?

Q2: Do have specific mood-stabilizing drugs an effect on cytokine levels in BD patients?

Secondary research questions

Q3: Do have specific mood-stabilizing drugs a different effect on cytokine levels in BD patients according to the duration of treatment?

Q4: Do have specific mood-stabilizing drugs a different effect on cytokine levels in BD patients according to the mood state of patients?

Methods

This systematic review will be conducted and reported according to the PRISMA-P (preferred reporting items for systematic review and meta-analysis protocols) guideline (Moher et al., 2015).

Eligibility criteria

In order to obtain original studies on cytokine levels in medication-free BD patients as well as original studies reporting on the effect of single mood-stabilizing drugs on blood cytokine levels in BD, we apply the following eligibility criteria:

1) Patients with BD type I or II as confirmed by DSM-III-R, DSM-IV, DSM-IV-TR, DSM-5, ICD-9 or ICD-10 criteria.

2) Studies assessing levels of cytokines or soluble cytokine receptors in peripheral blood.

3) Studies evaluating cytokine levels in medication-free BD with a cross-sectional design comparing cytokine levels in BD patients to those in healthy controls (HCs), or;

4a) Studies evaluating the effect of mood-stabilizing drugs on cytokine levels with a longitudinal design reporting cytokine levels pre- and post-treatment; or with a cross-sectional design reporting cytokine levels in medication-free compared to medicated patients in similar mood state or to HCs; and,

4b) Results concerning the effect of a single mood-stabilizing drug on cytokine levels are provided or could be obtained by contacting the authors, and;

4c) The evaluated mood-stabilizing drug is recommended by the current international standards on the pharmacological treatment of BD (i.e. lithium, valproate, carbamazepine, lamotrigine or atypical antipsychotics) (Cipriani et al., 2011; Geddes and Miklowitz, 2013; Soares-Weiser et al., 2007; Yatham et al., 2013).

Information sources

A search was conducted in the Pubmed database (January 1946 – January 2016) and Embase database (1947 - January 2016) for English language articles using the following search terms: (bipolar OR mania OR manic) AND (cytokine OR interferon OR interleukin OR "tumor necrosis factor" OR TNF OR "transforming growth factor" OR TGF) AND (“medication-free” OR unmedicated OR lithium OR valproate OR carbamazepine OR lamotrigine OR antipsychotic). Specifying the product names of antipsychotic drugs in the search string did not change the number of hits. Reference lists of retrieved articles were searched by hand.

Study selection

The titles and abstracts were screened independently by SvdA and LvD to eliminate obvious irrelevant publications. Full-texts of the potential relevant articles were further screened for eligibility. Disagreements were resolved by discussion between SvdA and LvD.

Data extraction

We developed a data extraction sheet with following data: mood state, number of subjects, age, sex, study design, severity of mood symptoms, mood-stabilizing drug-use, duration of medication-free period, duration of treatment with mood-stabilizing drug, concomitant drug use, duration of illness, age of onset, body mass index (BMI), smoking status, sample type, type of cytokine assay, specific cytokines and cytokine receptors analyzed, correlations between cytokine levels and clinical characteristics. If cytokines were measured after stimulation of blood cells with mitogens, the

following data were additionally extracted: type and concentration of mitogen, duration of exposure to mitogen, cell type and culture medium.

SvdA initially extracted these predefined data, LvD checked the data extraction sheet. Disagreements were resolved by discussion between SvdA, LvD and a third independent researcher WS. If no agreement was obtained, we further discussed with two senior researchers, VC and MM. If needed, authors were contacted for further information or clarification.

We assessed risk of bias in individual studies by carefully reviewing study designs, methodology and interpretation of data. Heterogeneity between studies was evaluated in detail. Risk of bias and limitations were extensively discussed with all authors and are described in the discussion and limitations sections below.

Data extraction tables

Supplementary Table 1: Cytokine levels in MF-BD: characteristics of the included studies

Supplementary Table 2: Cytokine levels in Li-BD: characteristics of the included studies

Supplementary Table 3: Cytokine levels in VPA-BD: characteristics of the included studies

Supplementary table 1: Cytokine levels in MF-BD: characteristics of the included studies

Author, year	Subjects	N	BD type (n)	Age[a]	Male (%)	YMRS[a]	HDRS[a]	Medication naive/free (n)	MF period (range)	Duration of illness (y)[a]	Age of onset[a]	BMI[a]	Smokers (%)	Sample	Assay	PHA /LPS	Cytokines	Correlations with cytokine & cytokine receptor levels
Guloksuz et al., 2010	E	16	I/II: 12/4	32.3 (6.5)	75	<8	<8	0/16	>4w (4-44)	9.9 (5.3)	22.2 (5.9)	26.6 (5.5)	NA	Serum	CBA	n	IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α	NS: duration of illness, duration of euthymia, age
	C	16		31.8 (4.8)	75							23.8 (3.1)	NA					
Kim et al., 2002	M	25	I	28.6 (8.0)	48	32.6 (6.9)	NA	18/7	>4m (NA)	1.6 (3.3)	26.7 (9.1)	21.9 (2.7)	NA	Plasma	ELISA	n	IL-12	NS: BMI, smoking, clinical ratings, age of onset, number of admissions & episodes, duration of illness, age
	C	85		32.5 (9.6)	33							22.1 (2.6)	26					
Kim et al., 2004	M	70	I	32.9 (12.2)	39	30.8 (9.8)	NA	41/29	>4m (NA)	2.0 (5.2)	29.6 (12.0)	22.5 (3.1)	NA	Plasma	ELISA	n	IL-4, IFN-γ, TGF-β	NS: smoking, clinical ratings, age of onset, number of admissions & episodes, duration of illness, age, psychotic symptoms
	C	96		30.3 (10.1)	44							22.0 (2.8)	NA					
Kim et al. 2007	M	37	I	37.8 (12.0)	38	35.1 (9.5)	NA	20/17	>4m (NA)	7.3 (14.6)	31.6 (11.1)	22.0 (2.8)	NA	Plasma	ELISA	y	IL-2, IL-4, IL-6, TNF-α, IFN-γ	NS: smoking, clinical ratings, age of onset, number of admissions & episodes, duration of illness, age, psychotic symptoms
	C	74		37.5 (9.5)	38							22.2 (3.0)	NA					
Li et al., 2015	M	41	I	37.5 (13.6)	39	31.2 (5.0)	NA	28/13	>4w (NA)	NA	NA	NA	NA	Plasma	ELISA	n	IL-10, IL-17, IL-23, TGF-β1, TNF-α	NA
	C	36		36.9 (6.3)	39							NA	NA					
Liu et al., 2004	M	18-25	I	32.8 (8.9)[b]	NA	34.5 (5.9)[b]	NA	NA	>2w (NA)	7.3 (5.6)[b]	NA	NA	NA	Plasma	ELISA	y	IL-2, IL-4, IL-10, IFN-γ	NA
	C	29-45		26.8 (3.9)	NA							NA	NA					
Maes et al., 1995	M	10	I	40.5 (13.4)	30	31.8 (4.6)[c]	NA	NA	>7d (7-60)	NA	NA	NA	NA	Plasma	EIA	n	IL-6, sIL-2R, sIL-6R	NS: clinical ratings, sex, age, ethnicity, time of sampling
	C	21		33.0 (10.1)	38							NA	NA					
Ortiz-Dominguez, 2007	M	10	I	28.9 (8.5)	30	30.2 (5.9)	NA	7/3	>3w (NA)	NA	27.1 (9.5)	NA	NA	Serum	ELISA	n	IL-1β, IL-2, IL-4, IL-6[d], TNF-α	NA
	D	10	I	39.7 (11.4)	20	NA	23.2 (1.8)	2/8	>3w (NA)	NA	28.8 (12.4)	NA	NA					
	C	33		32.3 (10.8)	10							NA	NA					
Rapaport, 1994	E	12	I/II: 16/10[b]	Free: 42.7 (8.2) Naive: 41.1 (12.3)	35[b]	NA	NA	8/4	>60d (NA)	NA	NA	NA	NA	Serum	EIA	y/n[e]	IL-2, sIL-2R	NS: sex
	C	34		39.4 (12.1)	NA							NA	NA					
Rapaport et al., 1999	M-D-E	17	I/II: 3/14	38.8 (13.0)	47	2.4 (3.2)	8.6 (7.2)	NA	>2w (NA)	NA	NA	NA	NA	Serum	NA	n	IL-2, IL-4, IL-6, IL-10, IFN-γ, sIL-2R, sIL-6R[f]	NS: mood state, clinical ratings, sex
	C	18		36.3 (10.8)	56	0.3 (0.6)	0.2 (0.6)					NA	NA					
Teixeira et al., 2015	D	29	I/II: 11/18	28.4 (5.5)	28	NA	22.6 (3.6)	21/4 (4 not MF)	>6w (NA)	3.1 (1.5)	NA	NA	NA	Plasma	ELISA	n	sTNFR1, sTNFR2	NS: clinical ratings S: duration of illness (sTNFR1)
		27		28.0 (7.7)	30							NA	NA					
Tsai et al., 2003	M	7	I	33.6 (9.4)[b]	53[b]	32.8 (8.7)[b]	NA	NA	>2w (NA)	8.7 (6.7)[b]	23.2 (8.1)[b]	23.8 (4.5)[b]	47[b]	Plasma	ELISA	n	sIL-2R, sIL-6R	NA
	C	36		NA	NA							NA	NA					
Uyanik et al., 2015	M	30	I	33.4 (8.6)	53	28.7 (5.1)	NA	3/18 + 9 irregular users	>1m (NA)	NA	NA	NA	63	Plasma	ELISA	n	IL-4, IL-6, IL-10, IFN-γ, TNF-α	NS: clinical ratings
	C	28		33.1 (7.8)	60						24.5 (7.1)	NA	77					

MF: medication-free; BD: bipolar disorder; YMRS: Young Mania Rating Scale; HDRS: Hamilton Depression Rating Scale; BMI: body mass index; PHA: phytohemagglutinin; LPS: lipopolysaccharide; M: manic; D: depressed; E: euthymic; C: controls; NA: not available; NS: non-significant; S: significant; CBA: cytometric bead array; ELISA: enzyme-linked immunosorbent assay; EIA: enzyme immunoassay; y: yes; n: no; IL: interleukin; sIL-R: soluble interleukin receptor; IFN: interferon; TNF: tumor necrosis factor; TGF: transforming growth factor.

[a] Mean (SD)
[b] Data from total patient group are presented. No data available for medication-free subgroup only.
[c] Mania subscale of SDAS (Schedule for Affective Disorders and Schizophrenia)
[d] IL-6 not detectable in manic subgroup
[e] IL-2 levels produced by PHA-stimulated lymphocytes, sIL-2R determined in non-stimulated serum.
[f] IL-4, IL-6, IL-10 and IFN-γ not detectable. IL-2 detectable in a minority (5 patients & 5 controls), not allowing an interpretation of the significance of the findings.

Supplementary table 2: Cytokine levels in Li-BD: characteristics of the included studies

Author, year	Study design	Subjects	BD type (n)	N	Age[a]	Male (%)	YMRS/ HDRS baseline[a]	YMRS/ HDRS FU[a]	Duration MF or Li[b] (range)	Comedi-cation	Duration of illness (y)[a]	Age of onset[a]	BMI[a]	Smokers	Sample	Assay	PHA /LPS	Cytokines	Correlations with cytokine & cytokine receptor levels
Guloksuz et al., 2010	CS	Euthymic MF	I/II: 12/4	16	32.3 (6.5)	75	<8/<8		>4w (4-44)	n	9.9 (5.3)	22.2 (5.9)	26.6 (5.5)	NA	Serum	CBA	n	IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α	NS: duration of illness, duration of euthymia, age, serum Li, duration of Li-monotherapy
		Euthymic Li	I/II: 14/2	15	31.8 (7.1)	73	<8/<8		>8w (8-712)	n	11.5 (6.6)	20.3 (4.4)	23.6 (3.1)	NA					
		Controls		16	31.8 (4.8)	75							23.8 (3.1)	NA					
Knijff et al., 2007	CS	Various mood Li	I&II	64	44 (21-60)	50	NA		>6m (NA)	Various	19.4 (2-42)	NA	24.9 (3.3)	NA	Plasma	ELISA	y[c]	IL-1β, IL-6	NS: mood state, duration of illness, sex, age, duration of lithium, diagnosis, BMI
		Controls		59	38 (22-58)	44							NA	NA					
Rapaport, 1994	CS	Euthymic MF	I/II: 16/10[d]	12	Free: 42.7 (8.2) Naive: 41.1 (12.3)	35[d]	NA		>60d (NA)	NA	NA	NA	NA	NA	Serum	EIA	y/n[e]	IL-2, sIL-2R	NS: sex
		Euthymic Li		14	39.1 (12.4)		NA		>60d (NA)	NA	NA	NA	NA	NA					
		Controls		34	39.4 (12.1)	NA							NA	NA					
Rapaport et al., 1999	FU	Various mood MF → Li	I/II: 3/14[f]	17	38.8 (13.0)	47	2.4 (3.2)/ 8.6 (7.2)	1.8 (2.1)/ 8.2 (2.0)	>2w MF, 4w FU on Li	NA	NA	NA	NA	NA	Serum	NA	n	IL-2, IL-4, IL-6, IL-10, IFN-γ, sIL-2R, sIL-6R	NS: mood state, clinical ratings, sex
		Controls		18	36.3 (10.8)	56							NA	NA					
Remlinger-Molenda et al., 2012	CS	Euthymic Li	NA	45[g]	58 (26-81)	33	<9/<8		>5y (5-25)	n[g]	NA	NA	NA	NA	Serum	CBA	n	IL-1β, IL-2, IL-6, IL-10, IFN-γ, TNF-α	NA for long-term Li-group
		Controls		78	35 (21-63)	45							NA	NA					
Teixeira et al., 2015	FU	Depressive MF → Li	I&II	29	28.4 (5.5)	28	NA/ 22.6 (3.6)	NA/ 7.2 (6.0)	>6w MF, 6w FU on Li	BZD, zolpidem	3.1 (1.5)	NA	NA	NA	Plasma	ELISA	n	sTNFR1, sTNFR2	NS: baseline & change in clinical ratings, response to lithium, serum lithium, comedication S: duration of illness (sTNFR1)
		Controls		27	28.0 (7.7)	30							NA	NA					

Li: lithium; BD: bipolar disorder; YMRS: Young Mania Rating Scale; HDRS: Hamilton Depression Rating Scale; FU: Follow-up; MF: medication-free; BMI: body mass index; PHA: phytohemagglutinin; LPS: lipopolysaccharide; CS: cross-sectional study; NA: not available; BZD: benzodiazepine; CBA: cytometric bead array; ELISA: enzyme-linked immunosorbent assay; EIA: enzyme immunoassay; y: yes; n: no; IL: interleukin; sIL-R: soluble interleukin receptor; IFN: interferon; TNF: tumor necrosis factor; S: significant; NS: non-significant.

[a] Mean (SD) or Mean (range)
[b] Duration of MF period is indicated for MF subjects. Duration of Li-use is indicated for Li-users.
[c] Analysis of cytokines produced by both LPS-stimulated and non-stimulated monocytes, no significant differences between both conditions.
[d] Data of total patient group, no separate data on MF and Li patient group available.
[e] IL-2 levels produced by PHA-stimulated lymphocytes, sIL-2R determined in non-stimulated serum.
[f] Rapid cycling BD
[g] Total Li-BD group (*n*=45) of which Li-monotherapy (*n*=10) and Li combined with other drugs (*n*=35). No significant difference in cytokine levels between Li-monotherapy and Li combined with other drugs.

Supplementary table 3: Cytokine levels in VPA-BD: characteristics of the included studies

Author, year	Study design	Mood state	Subjects	N	Age[a]	Male (%)	BD type	Clinical ratings[a]	MF period (range)	Duration FU	Comedi-cation	Duration of illness	Age of onset	BMI	Smokers	Sample	Assay	PHA /LPS	Cytokines analyzed	Correlations with cytokine & cytokine receptor levels
Lee et al., 2014	FU	Depressed	Baseline MF	117	30.7 (11.1)	56	II	YMRS 9.5 (4.6) HDRS 19.2 (5.4)	Naive	12w	BZD/ AD	NA	NA	NA	NA	Plasma	Antibody pair-based assay	n	IL-1β, IL-6, IL-8, TNF-α	S: change in YMRS (TNF-α), change in HDRS (IL-1β & IL-6)
			FU VPA	76	29.8 (10.6)	62		YMRS 5.80 (3.9) HDRS 9.44 (6.5)					NA	NA						
Maes et al., 1995	FU	Manic	Baseline MF	10	40.5 (13.4)	30	I	Mania subscale SADS 31.8 (4.6)	>7d (7-60)	2w	n	NA	NA	NA	NA	Plasma	EIA	n	IL-6, sIL-2R, sIL-6R	NS: baseline & change in clinical ratings, sex, age, time of blood sampling, ethnicity
			FU VPA	10	40.5 (13.4)	30		Mania subscale SADS 19.1 (7.3)					NA	NA						

VPA: valproate; BD: bipolar disorder; MF: medication-free; FU: follow-up; BMI: body mass index; PHA: phytohemagglutinin; LPS: lipopolysaccharide; YMRS: Young Mania Rating Scale; HDRS: Hamilton Depression Rating Scale; SADS: Schedule for Affective Disorders and Schizophrenia; BZD: benzodiazepine; AD: antidepressant; NA: not available; EIA: enzyme immunoassay; y; yes; n: no; IL: interleukin; sIL-R: soluble interleukin receptor; TNF: tumor necrosis factor; S: significant; NS: non-significant.

[a] Mean (SD)

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	7
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	7
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	7
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	7
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	7

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	NA
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8 + Fig 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7 + Tables in text & Suppl
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Tables text & Suppl
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Tables text & Suppl
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	14-15
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	14-15
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	14-15
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Conflict of interest & acknowledgements

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

CHAPTER 4

Inflammation and kynurenine metabolism

van den Ameele S, van Nuijs ALN, Lai FY, Schuermans J, Verkerk R, van Diermen L, Coppens V, Fransen E, De Boer P, Timmers M, Sabbe B, Morrens M. A mood state specific interaction between kynurenine metabolism and inflammation is present in bipolar disorder. Under review.

A mood state-specific interaction between kynurenine metabolism and inflammation is present in bipolar disorder

Seline van den Ameele[1, 2, 3], Alexander LN van Nuijs[4], Foon Yin Lai[4], Jeroen Schuermans[1 ,2], Robert Verkerk[5], Linda van Diermen[1 ,2], Violette Coppens[1, 2], Erik Fransen[6], Peter de Boer[7], Maarten Timmers [7, 8], Bernard Sabbe[1, 2] and Manuel Morrens[1, 2]*

[1] Collaborative Antwerp Psychiatric Research Institute (CAPRI), Faculty of Medicine and Health Sciences, University of Antwerp, Universiteitsplein 1, Antwerp, Belgium

[2] SINAPS, University Psychiatric Hospital Duffel - VZW Emmaüs, Stationsstraat 22c, 2570 Duffel, Belgium

[3] Department of Psychiatry, CHU Brugmann, Van Gehuchtenplein 4, 1020 Brussels, Belgium

[4] Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, Antwerp, Belgium

[5] Laboratory of Medical Biochemistry, University of Antwerp, Universiteitsplein 1, Antwerp, Belgium

[6] StatUa Centre for Statistics, University of Antwerp, Universiteitsplein 1, Antwerp, Belgium

[7] Janssens Research and Development, a division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse, Belgium

[8] Reference Center for Biological Markers of Dementia (BIODEM), Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, Antwerp, Belgium

ABSTRACT

Objectives: Cytokines are thought to contribute to the pathogenesis of psychiatric symptoms by kynurenine pathway activation. Kynurenine metabolites affect neurotransmission and can cause neurotoxicity. We measured inflammatory markers in patients with BD and studied their relation to kynurenine metabolites and mood.

Methods: Patients with BD suffering from an acute mood episode were assigned to the depressive (n=35) or (hypo)manic (n=32) subgroup. Plasma levels of inflammatory markers [cytokines, C-reactive protein] and kynurenine metabolites [tryptophan (TRP), kynurenine (KYN), 3-hydroxykynurenine (3-HK), quinolinic acid (QA), kynurenic acid (KYNA)] were measured on 6 time points during 8 months follow-up. Biological marker levels in patients were compared to controls (n=35) and correlated to scores on mood scales. Spearman correlations and linear mixed models were used for statistical analysis.

Results: Twenty patients of the manic subgroup, 29 of the depressive subgroup, and 30 controls completed the study. The manic subgroup had a rapid remission of mood symptoms, but in the depressive subgroup subsyndromal symptoms persisted. No differences in inflammation were found between groups. A strong correlation between tumor necrosis factor-α and KYN, KYN/TRP, 3-HK and QA (*rho*>0.60) was specific for the manic group, but only at baseline (during mania). The depressive subgroup had a lower neuroprotective ratio (KYNA/3-HK, p=0.0004) and a strong association between interferon-γ and kynurenine pathway activation (p<0.0001). KYNA was low in both patient groups versus controls throughout the whole follow-up (p=0.0008).

Conclusions: Mania and chronic depressive symptoms in BD are accompanied by a strong interaction between inflammation and a potentially neurotoxic kynurenine metabolism.

KEYWORDS

Bipolar disorder, inflammation, kynurenine pathway, quinolinic acid, kynurenic acid, 3-hydroxykynurenine, neuroprogression

1 Introduction

Activation of the kynurenine pathway by cytokines is thought to contribute to the pathogenesis of psychiatric disorders (1, 2). The initial step in this pathway (Figure 1) is the conversion of tryptophan (TRP) into kynurenine (KYN) by indoleamine 2,3-deoxygenase (IDO-1). IDO-1 is specifically activated by cytokines as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). The KYN/TRP ratio is often used as a measure of IDO-1 activity (a higher ratio indicative for more IDO-1 activity). Several neuroactive metabolites are generated along 2 branches downstream in the pathway: a branch taking place mainly in microglia, and a branch preferentially taking place in astrocytes. 3-hydroxykynurenine (3-HK) and quinolinic acid (QA) are formed in microglia and are thought to have neurotoxic properties. Both metabolites increase oxidative stress by increasing the production of free radicals. QA also has excitotoxic effects through its N-methyl-D-aspartate receptor (NMDA-R) agonism. In astrocytes, KYN is metabolized into kynurenic acid (KYNA). KYNA is assumed to have a neuroprotective role via competitive NMDA-R antagonism and its anti-oxidant properties. However, KYNA is also thought to contribute to psychosis and cognitive deficits by antagonizing respectively the NMDA and the alpha 7 nicotinic (α7nACh)-receptor. (1, 2)

Elevated inflammatory markers have been found in different psychiatric disorders and these are thought to underlie an upregulation of IDO-1 which is often described in both schizophrenia and mood disorders (3, 4). However, multiple studies on postmortem brains, cerebrospinal fluid (CSF) and peripheral blood have shown differences in kynurenine metabolites (in particular those formed downstream of KYN) between specific psychiatric disorders (1).

In major depressive disorder (MDD) an upregulation of the microglial branch leading to increased 3-HK and QA levels along with decreased KYNA levels and decreased neuroprotective ratios (KYNA/3-HK, KYNA/QA) have been described (1, 3, 5). However, unchanged plasma levels (6) and even a downregulation of both branches in postmortem brain have been shown (7). In schizophrenia, studies in CSF and postmortem brain showed increased activation of astrocytes and elevated KYNA levels (8, 9). Studies on peripheral levels of kynurenine metabolites in schizophrenia have yielded inconsistent results (8, 10-12).

In bipolar disorder (BD), the kynurenine pathway has been less studied. As in schizophrenia, elevated KYNA levels have been found in CSF, postmortem brain and fibroblast cultures of

patients with BD, and are mainly associated with a history of mania or psychosis (8, 13-16). Studies in peripheral blood could not find differences in KYNA levels between patients with mania and healthy controls (17) or showed decreased KYNA levels in affective psychosis (18). Findings in bipolar depression are similar to those in MDD. Savitz (19) found a decreased neuroprotective ratio KYNA/QA in patients with bipolar depression, but no differences in absolute kynurenine metabolite levels. Decreased KYNA and increased neurotoxic ratios also have been reported in euthymic to mild depressive patients with BD (16, 20, 21). A recent meta-analysis on kynurenine metabolites in mood disorders found no significant differences between patients with BD and controls (22), but included a limited number of studies with high heterogeneity.

Pro-inflammatory cytokines are hypothesized to be the main trigger for kynurenine pathway activation leading to psychiatric symptoms (1, 3). A mood state-related immune system activation has indeed been described in BD (23, 24). In animal models, immune stimulation has been shown to induce depressive-like behavior that was mediated by increased IDO-1-activity (25, 26). In humans, several studies in patients undergoing cytokine therapy report the onset of depressive symptoms concurrent with changes in kynurenine metabolites (27, 28). However, in patient populations with psychiatric disorders, the interaction between inflammation, kynurenine metabolism and clinical symptoms is little investigated. In CSF of patients with schizophrenia, IL-6 levels were negatively correlated with the TRP/KYNA ratio (29), however a study with larger sample size could not confirm an effect of cytokines levels on kynurenine metabolism disturbances (12). In depressive populations such a link between inflammation, kynurenine metabolism and depressive symptoms has not yet been shown (6, 30).

The aim of this study was to further explore the relation between inflammation, kynurenine metabolism and mood symptoms in BD. We hypothesized to find elevated levels of pro-inflammatory cytokines in patients with BD compared to controls, and more so during mood episodes resulting in a mood-specific kynurenine metabolism.

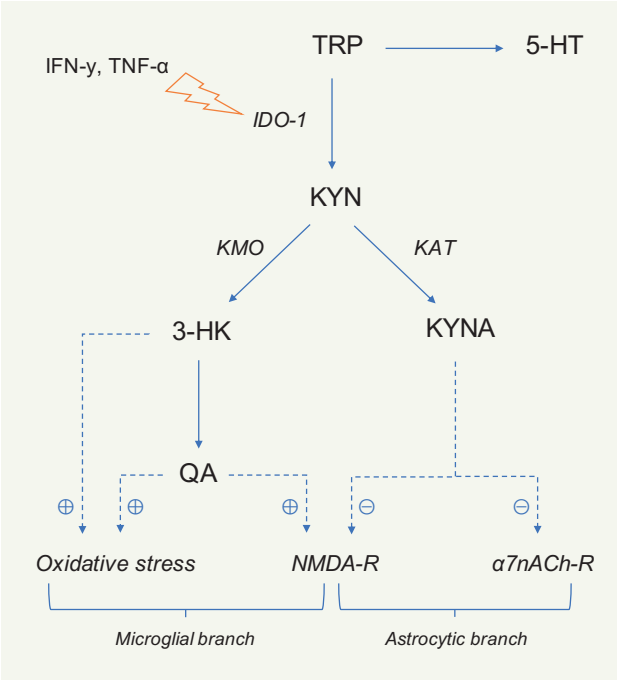

Figure 1 : The kynurenine pathway.
Cytokines as interferon-y (IFN-y) and tumor necrosis factor-alfa (TNF-α) activate indoleamine 2,3-deoxygenase (IDO-1) to metabolize tryptophan (TRP) into kynurenine (KYN) at the expense of serotonin (5-HT) formation. Further downstream the pathway, KYN is metabolized along 2 branches. 3-hydroxykynurenine (3-HK) and quinolinic acid (QA) are preferentially formed in microglia and have neurotoxic properties by increasing oxidative stress and the NMDA-R agonism by QA. Kynurenic acid (KYNA) is formed in astrocytes and thought to be neuroprotective by its NMDA-R antagonism. The pathway has been simplified for a better representation of the main metabolites.

2 Methods

2.1 Participants

Inpatients were recruited in three psychiatric centers in the region of Antwerp (Belgium). Outpatients were recruited via the Flemish patient association 'Ups&Downs'. The inclusion criteria were age 18-65 years, DSM-IV-TR diagnosis of BD type I, type II or schizoaffective disorder and suffering from a depressive or (hypo)manic episode at time of inclusion. Clinical assessments are described below (see 2.3 Clinical Assessments). Age and gender matched controls were recruited among staff members of the participating centers. We ensured an equal distribution of inclusions of patients and controls throughout the year to account for seasonality in immune system activity (31). Exclusion criteria for patient and control group were: substance abuse, use of anti-inflammatory drugs within 2 weeks preceding 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 complete blood count, electrolytes, 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-TR criteria and BD or psychotic syndrome in first-degree relatives and current use of psychopharmacological drugs. 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 and assigned to either the depressed (BD-D) or (hypo)manic (BD-M) subgroup. In both patients and controls, screening was followed by a first test day after 1-5 days. Subsequent test days were planned after respectively 1, 2, 4, 6 and 8 months, resulting in 6 test days per participant over a follow-up period of 8 months. Every test day included the same clinical and laboratory assessments described below. During the study period, patients received treatment as usual without therapeutic 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 controls because of its accurate structured DSM-IV diagnosis and its practical convenience (32). In patients, the severity of mood symptoms was assessed by the 17-item Hamilton Depression Rating Scaling (HDRS-17) (33) and the Young Mania Rating Scale (YMRS) (34) at screening and on all test days. At screening, threshold score for inclusion was set at ≥17 for the HDRS-17 or ≥13 for the YMRS, corresponding to moderate depression or hypomania respectively (35, 36). Psychotic symptoms were evaluated on test days using the positive subscale of the Positive and Negative Syndrome Scale (PANSS) (37). In the control group, the occurrence of mood episodes during follow-up was evaluated on all test days based on a short screening questionnaire. For all participants, we assessed medication use and the occurrence of any of the exclusion criteria on every test day. All clinical assessments were done by a psychiatrist in training (SvdA) and supervised by a psychiatrist (MM).

2.4 Bioanalytical measurements

Blood was drawn by venipuncture between 08.00 A.M. and 10.30 A.M. Plasma tubes were immediately stored at 4°C, centrifuged at 2000g and 4°C for 10 minutes within 2h after blood draw, and plasma was aliquoted and stored at -70°C until assayed. EDTA plasma was used for analysis of inflammatory markers, citrate plasma for kynurenine metabolites.

TNF-α, IFN-γ, IL-1b, IL-4, IL-6 and CRP were measured in duplicate by an electrochemiluminescence immunoassay technique developed by Mesoscale Discovery

(Rockville, USA) according to the manufacturer's instructions. Kits used for detection were V-plex Pro-inflammatory Panel I for TNF-α, IFN-γ, IL-1b, IL-4 and IL-6 and Human Vascular Injury Panel for CRP. The lower limits of detection (LLOD) were respectively 0.04, 0.2, 0.04, 0.02, 0.06 and 1.3 pg/mL. Sample signals were fitted on a 4-parametric logistic calibration curve.

Patient and control samples were analyzed in randomized sequence with both samples of a single subject on the same plate and an equal distribution of patients and controls per plate. Samples with a coefficient of variation (CV) >20% were excluded from statistical analyses. Because >80% of the samples were below the LLOD for IL-1b and IL-4, these cytokines were not included in statistical analyses. Excluded samples were equally distributed among patients and controls. For TNF-α, IFN-γ, IL-6 and CRP, >80% of the samples were included for analysis. The mean CV (%) and standard deviation were 6.3 (4.7), 8.4 (5.5), 7.6 (5.5), 5.4 (4.5) respectively.

Collected plasma samples were extracted and analyzed for the five target kynurenine metabolites (KYNA, TRP, KYN, 3-HK and QA). Prior to solid-phase extraction (SPE), 250µL plasma was combined with 1650µL ultrapure water, 25µL formic acid and 50µL of a mixture containing mass-labelled internal standards (concentrations between 0.5-20ng/µL). The mixture was then vortexed for 3 minutes for homogenization. SPE cartridges (Oasis® HLB, 60 mg, 3cc, Waters®) were pre-conditioned with methanol (2mL) and ultrapure water containing 1% formic acid (2.5mL). The sample mixture was then loaded onto the cartridges and extracted under gravity. The cartridges were dried under vacuum for 40min. Target compounds were finally eluted with methanol (3mL) and methanol containing 5% ammonium hydroxide (3mL). The eluent was evaporated under a high purity nitrogen stream to almost dryness. The final extract was obtained through reconstitution with 100µL of a 5% acetonitrile/95% ultrapure water mixture.

Calibration curves (KYNA, 2-1000ng/mL; TRP, 200-40000ng/mL; KYN, 100-10000ng/mL; 3-HK, 2-1000ng/mL; QA: 2-1000ng/mL) and sample extracts were analyzed using ultra high pressure liquid-chromatography coupled to tandem mass spectrometry (Agilent 1290 Infinity LC system coupled to an Agilent 6460 Triple Quadrupole MS system). Chromatographic separation of the target analytes was achieved on a Waters® ACQUITY HSS T3 analytical column (100mm x 2.1mm, 1.8µm) with a mobile phase composed of (A) 0.1% formic acid in water and (B) acetonitrile, in gradient. Injection volume was 2µL. Ionisation was performed with electrospray ionization in positive mode. For each analyte, three multiple reaction monitoring

(MRM) transitions in the MS were included. The isotope dilution approach was used to quantify concentrations of the target compounds, in which potential compound loss during SPE and matrix effects during instrumental analysis were compensated with the corresponding mass-labelled compounds.

In every batch of sample extraction, quality control and assurance samples were added, including ultrapure water blanks, plasma samples (horse plasma) and spiked samples with native target compounds in ultrapure water and the plasma samples. No contamination was observed throughout the procedure and over batches. The average day-to-day accuracy of the procedure was 98-106% (KYNA, 99-112%; TRP, 98-118%; KYN, 92-119%; 3-HK, 91-121%; QA: 89-109%) with the spiked ultrapure water samples and was 92-101% (KYNA, 91-113%; TRP, 83-101%; KYN, 84-110%; 3-HK, 85-108%; QA: 87-106%) with the spiked plasma samples. The day-to-day precision (CV%) of the procedure for measuring the target compounds in plasma matrix was 5.0-8.0%.

2.5 Statistical analysis

Normality of outcome variables and homoscedasticity of residuals were evaluated by visual inspection. For the regression modelling, IFN-γ, IL-6, TNF-α, CRP, 3-HK, QA and KYNA concentrations were log-transformed to obtain a normal distribution. Homogeneity of variances was assessed by Levene's test and further analyses were adapted accordingly.

Baseline differences in biological, clinical and demographic parameters between the patient and control group were examined by two-tailed independent t-tests for continuous variables and Pearson chi-square test for categorical variables. Baseline differences between controls, the depressive subgroup and the manic subgroup were examined by one-way Anova, followed by a posthoc analysis with Tukey HSD correction for multiple comparison.

Longitudinal data were examined using linear mixed model analysis with the kynurenine metabolites and ratio's as outcome variables (TRP, KYN, 3-HK, QA, KYNA, KYN/TRP and KYNA/3-HK). Subject ID was added as random intercept in all mixed models to account for the non-independence between observations from the same individual. To compare the change in outcome over time between the groups, we included following factors as fixed effects: group (BD-D *vs*. BD-M *vs*. HC or BD-total *vs*. HC), moment (subsequent test days), and the interaction group*moment. To compare the relation between the outcome variables and inflammatory markers between groups, following fixed effects were entered in the model:

inflammatory markers, group status and the interaction inflammatory marker*group. Adjusted models were fitted with sex, age, smoking status, albumin levels and BMI added as covariates. For post hoc assessments on same outcome variables, several clinical characteristics were separately entered as fixed effects in the mixed model. The output from the mixed model analysis, is reported as "F-ratio (DF); *p*-value; *b*".

The relation among biological parameters and the relation between symptom severity scores and biological parameter levels were studied by pairwise correlations. Correlations are reported by the Spearman's rho for non-parametric distributions.

Since we have 7 main outcome variables, we applied a Bonferroni correction for multiple testing. *P*-values below 0.00714 were considered as statistically significant. All statistical analyses were performed in JMP Pro 14 (JMP, Marlow, UK).

3 Results

3.1 Participants

Sixty-seven patients with BD or schizoaffective disorder and 35 controls were included. At screening, 35 patients were assigned to the BD-D group, 32 patients to the BD-M group. Demographic and metabolic characteristics are shown in Table 1. Patients and controls were matched by sex and age. The percentage of smokers was higher in BD-D and BD-M compared to controls. The BD-M group had a higher BMI and lower albumin concentration. No other significant differences in demographic or metabolic parameters were found between patients and controls. Clinical characteristics of patients are shown in Table 2. The BD-D group had a higher proportion of BD type II, a younger age of onset with longer duration of illness and less psychotic features as compared to the BD-M group.

Forty-nine patients and 30 controls completed the 8-month follow-up. Drop-out in the patient group was due to: chronic use of low-dose acetylsalicylic acid (n=5), substance abuse (n=3), ECT (n=2) and loss of contact or lack of motivation for further participation (n=8). Drop-out in the control group was due to difficult blood draws (n=1) and repeated orthopedic surgery (n=1). Three controls were only included for baseline testing. The mean number of test days

per participant was 5.0 in patients and 5.4 in controls. Over 8 months, the total number of blood samples included for analyses was 336 in patients and 188 in controls.

Table 1: Demographic and metabolic characteristics of participants

	HC	BD-D	BD-M	*p*-value[†]
N	35	35	32	
Gender, female	19 (54.3)	24 (68.6)	15 (46.9)	n.s.
Age, years	42.7 ± 11.6	43.7 ± 9.7	42.4 ± 12.7	n.s.
Caucasian	34 (97.1)	33 (94.3)	30 (93.8)	n.s.
Smokers	6 (17.1)	18 (51.4)	14 (43.8)	*0.006*
BMI, kg/cm^2	23.7 ± 2.6	24.3 ± 3.5	26.3 ± 4.7	*0.013*[‡]
Waist, cm	84.4 ± 9.6	87.1 ± 11.7	91.2 ± 12.4	n.s.
Fasting glucose, mg/dl	87.5 ± 6.8	88.9 ± 8.8	91.1 ± 9.8	n.s.
Cholesterol, mg/dl				
Total	190.9 ± 42.4	189.1 ± 42.7	183.7 ± 47.3	n.s.
HDL	62.0 ± 18.4	61 ± 15.6	56.0 ± 19.9	n.s.
LDL	109.0 ± 33.4	108.4 ± 41.5	102.4 ± 40.6	n.s.
Albumin, g/L	46.6 ± 2.6	45.4 ± 3.0	44.9 ± 2.3	*0.033*[‡]

Data presented as mean ± SD (range) or n (%).
HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein.

[†] P-values of one-way anova for continuous data and chi-square for categorical. P-values < .05 are italicized; n.s. = not significant (p > .05).

[‡] Tukey HSD: BMI: BD-M>HC; Albumin: BD-M<HC

Table 2: Clinical characteristics and baseline data of patients		
	BD-D	**BD-M**
N	35	32
Diagnosis		
BD type I	16 (45.7)	26 (81.3)
BD type II	19 (54.3)	4 (12.5)
Schizoaffective disorder	0	2 (6.3)
Age of onset, years	22.5 (10.9)	27.5 (11.8)
Duration of illness, years	20.6 (10.2)	14.3 (11.8)
First episode: depression	23 (67.7)	17 (53.1)
Lifetime psychotic features	13 (37.1)	24 (75.0)
Total number of hospitalizations		
0	6 (17.1)	3 (9.4)
1-5	23 (65.7)	22 (68.8)
6-10	6 (17.1)	7 (21.9)
Lifetime substance abuse		
Alcohol	11 (31.4)	7 (21.8)
THC	6 (17.1)	7 (21.8)
Hard drugs	4 (11.4)	1 (3.1)
Baseline medication use		
Medication-free	3 (8.6)	3 (9.4)
Lithium	13 (37.1)	11 (34.4)
Valproate	6 (17.1)	3 (9.4)
Carbamazepine	1 (2.9)	2 (6.3)
Lamotrigine	7 (20.0)	1 (3.1)
Antipsychotic	19 (54.3)	23 (71.9)
Antidepressant	25 (71.4)	6 (18.8)
Benzodiazepine	8 (22.9)	16 (50.0)
Baseline mood scales		
HDRS-17	21.2 (4.0)	8.1 (5.7)
YMRS	5.5 (4.8)	20.9 (5.1)
PANSS positive subscale	9.8 (3.4)	15.0 (4.7)
Psychotic features present	5 (14.3)	12 (37.5)

Data presented as mean ± SD (range) or n (%).
BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline BD: bipolar disorder; THC: tetrahydrocannabinol; HDRS-17: 17 item Hamilton depression rating scale; YMRS: Young mania rating scale; PANSS: Positive and negative syndrome scale.

3.2 Course of biological markers in patients versus controls

In line with our previous report (38), no significant differences in inflammatory markers (CRP, IFN-γ, IL-6 and TNF-α) were found between patients and controls at baseline or during follow-

up, see Table S1-S3. Kynurenine metabolites did differ between patients and controls. At baseline, KYNA levels were significantly lower in BD-D and BD-M compared to controls. This difference remained significant after adjustment for sex, age, BMI and smoking status, while the *p*-value increased to 0.03 after adding the factor albumin concentration. Baseline levels of 3-HK in both patient groups and QA in the BD-D group were also lower compared to controls, but not significantly after Bonferroni correction (Table 3). Follow-up data are shown in Figure 2 and Table S4. Similar to baseline, the overall follow-up data showed lower KYNA levels in both patient groups compared to controls ($F(96.4)=7.7$; $p=0.0008$). The interaction group*moment for KYNA was not significant, indicating stable low levels in patients compared to controls throughout all test moments. In contrast to the baseline data, the neuroprotective ratio KYNA/3-HK was significantly decreased in the BD-D group compared to both the controls and the BD-M group ($F(98.5)=8.5$; $p=0.0004$). We saw a significant group*moment interaction for both 3-HK ($F(367.8)=2.7$; $p=0.0031$) and KYNA/3-HK ($F(368.5)=2.6$; $p=0.0041$). These interaction effects were driven by an increase in 3-HK at 2 months in the BD-D group approaching the levels in the control group. The statistical models on follow-up data remained significant after adjustment for sex, age, BMI, smoking status and albumin concentration ($p<0.00714$).

No other significant differences in kynurenine metabolites were found between patients and controls at baseline or during follow-up.

Table 3: Baseline concentration of kynurenine metabolites

	HC (n=35)	**BD-D** (n=35)	**BD-M** (n=32)	Statistics[‡]
TRP	10901.19 (408.46)	9931.16 (402.58)	10775.65 (434.83)	n.s.
KYN	350.58 (16.02)	301.02 (15.79)	311.24 (17.06)	n.s.
3-HK[†]	9.30 (0.08)	7.12 (0.08)	6.89 (0.08)	F(2)=4.2; *p=0.0180*[§]
QA[†]	4.88 (0.05)	4.03 (0.05)	4.25 (0.06)	F(2)=3.7; *p=0.0297*[§]
KYNA[†]	7.15 (0.07)	5.18 (0.07)	5.47 (0.08)	F(2)=5.5; ***p*=0.0056**[§]
KYN/TRP[†]	0.03 (0.04)	0.03 (0.04)	0.03 (0.04)	n.s.
KYNA/3-HK[†]	0.76 (0.08)	0.71 (0.08)	0.80 (0.09)	n.s.

Data presented as mean ± standard error and concentrations are given in ng/mL.
HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; TRP: tryptophan; KYN: kynurenine; 3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid.
Significant *p*-values passing Bonferroni correction are in bold; p-values < .05 are italicized; n.s. = not significant (p > .05)

[†] Statistics on log-transformed data

[‡] One-way anova

[§] Tukey HSD: 3-HK and KYNA: BD-D&BD-M<HC ; QA: BD-D<HC

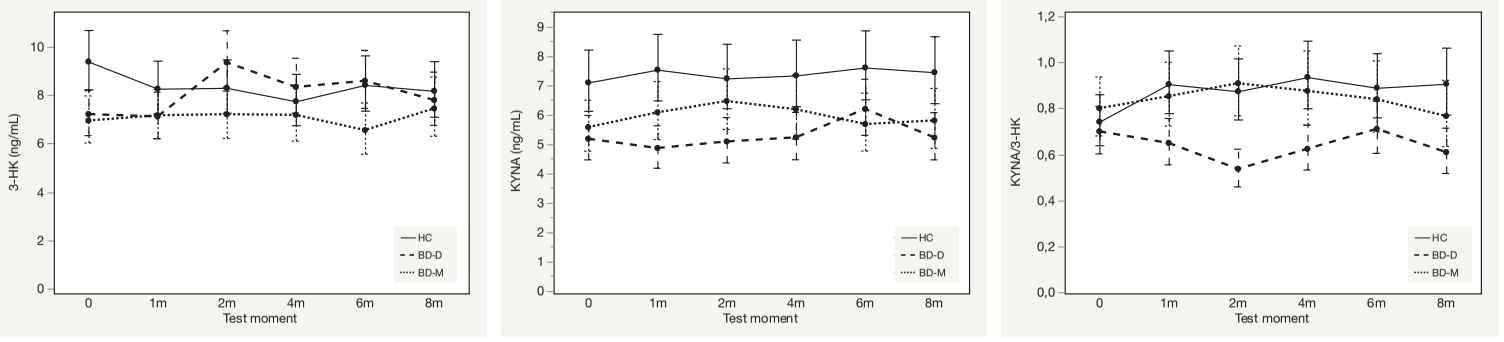

Figure 2: Least square means interaction plots of 3-HK, KYNA and KYNA/3-HK.

Main lines indicate the progression of the mean concentrations and error bars represent 95% confidence interval.

HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; 3-HK: 3-hydroxykynurenine; KYNA: kynurenic acid.

3.3 Mood, inflammation and kynurenine metabolites

The evolution of mood scale scores is shown in Figure 3. Manic symptoms rapidly disappeared in the BD-M group with a YMRS at 2 months of 3.9 (SD 7.7). Subsyndromal depressive symptoms persisted in the BD-D group with a HDRS score at 8 months of 12.4 (SD 7.4). As reported earlier (38) and shown in Table S5, correlations between clinical scores (HDRS, YMRS and PANSS positive subscale) and inflammatory markers were very weak. Apart from a weak negative correlation between KYNA and HDRS (-0.21), no relevant correlations were found between clinical scores and kynurenine metabolites (Table S5). We see slightly higher KYNA levels in euthymic patients (HDRS&YMRS<8, 64 euthymic test moments in 30 patients) compared with patients with mood symptoms, however this finding is not significant after Bonferroni correction ($F(294.3)=5.4$; $p=0.0211$; $b=-0.12$). No other significant differences in levels of biological markers were found between euthymic patients and patients with mood symptoms.

Correlations between baseline inflammatory markers and kynurenine metabolites are shown in Table 4. In the BD-M group, strong correlations were found between TNF-α and kynurenine metabolites. Correlations of TNF-α with KYN, KYN/TRP and the neurotoxic metabolites 3-HK and QA showed spearman's $\rho \geqslant 0.60$. These correlations decrease during follow-up, i.e. along with remission of manic symptoms (see Table S6). In contrast to controls, inflammatory markers during acute mood episodes (both depression and (hypo)mania) were negatively correlated with the neuroprotective ratio KYNA/3-HK. Correlations between inflammatory markers and kynurenine metabolites in patients and controls of overall follow-up data are presented in Table S7. Higher correlation coefficients of TNF-α with neurotoxic ratios became less pronounced ($\rho<0.40$) than in the acute manic group at inclusion. Negative correlations between inflammatory markers and neuroprotective ratios were comparable to those during acute mood episodes.

Finally, we compared the relation between kynurenine metabolites and inflammatory markers between groups and found some significant interaction effects (see Figure 4). In contrast to the evolution in controls and the BD-M group, increasing concentrations of IFN-y were strongly related to increases in KYN ($F(330.3)=8.6$; $p=0.0002$), QA ($F(328.6)=9.8$; $p<.0001$) and KYN/TRP ($F(331.4)=24.8$; $p<.0001$) in the BD-D group. IL-6 also appeared to have specific effects in the BD-D group as it correlated with decreased TRP ($F(402.6)=5.6$; $p=0.0040$),

increased KYN/TRP (F(386.9)=5.4; p=0.0050) and decreased KYNA/3-HK (F(358.7)=7.1; p=0.0009). All interaction effects remained significant when accounting for sex, age, BMI and albumin. However, accounting for smoking status resulted in non-significant associations, which is, at least partially, due to the confounding between smoking status and group. As shown in table 1, the patient group includes significantly more smokers that the control group.

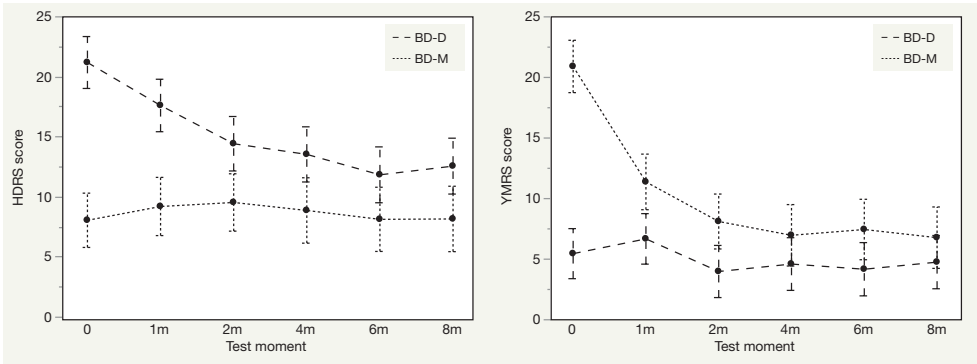

Figure 3: Course of mood symptoms by test moment in BD-D and BD-M group
Horizontal lines indicate the progression of the mean mood scale scores and standard deviations.
BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; HDRS: Hamilton Depression Rating Scale; YMRS: Young Mania Rating Scale

Table 4: Baseline correlations between inflammatory markers and kynurenine metabolites in HC, BD-D and BD-M (Spearman's ρ)

	HC				BD-D				BD-M			
	IFN-γ	IL-6	TNF-α	CRP	IFN-γ	IL-6	TNF-α	CRP	IFN-γ	IL-6	TNF-α	CRP
TRP	0.16	0.00	0.25	-0.16	-0.07	-0.26	-0.01	0.06	0.07	0.06	0.23	-0.18
KYN	0.11	-0.09	0.52	-0.26	-0.08	-0.20	0.21	-0.05	0.04	0.14	0.61	-0.16
3-HK	0.09	-0.01	0.23	-0.25	0.33	0.09	0.36	0.07	-0.07	0.15	0.70	-0.17
QA	-0.03	-0.14	0.29	-0.06	-0.05	-0.13	0.19	0.14	-0.22	0.29	0.60	0.20
KYNA	0.07	-0.28	0.10	-0.42	-0.12	-0.11	0.14	-0.31	-0.21	-0.10	0.32	-0.20
KYN/TRP	-0.01	-0.14	0.40	-0.18	-0.09	0.07	0.29	-0.11	0.16	0.22	0.62	0.02
KYNA/3-HK	-0.05	-0.22	-0.01	-0.12	-0.27	-0.32	-0.13	-0.27	-0.42	-0.33	-0.24	-0.19

Correlation coefficients <-0.20 or > 0.20 are shaded in grey.
HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline;
IFN-γ: interferon-gamma; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; TRP: tryptophan; KYN: kynurenine;
3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid

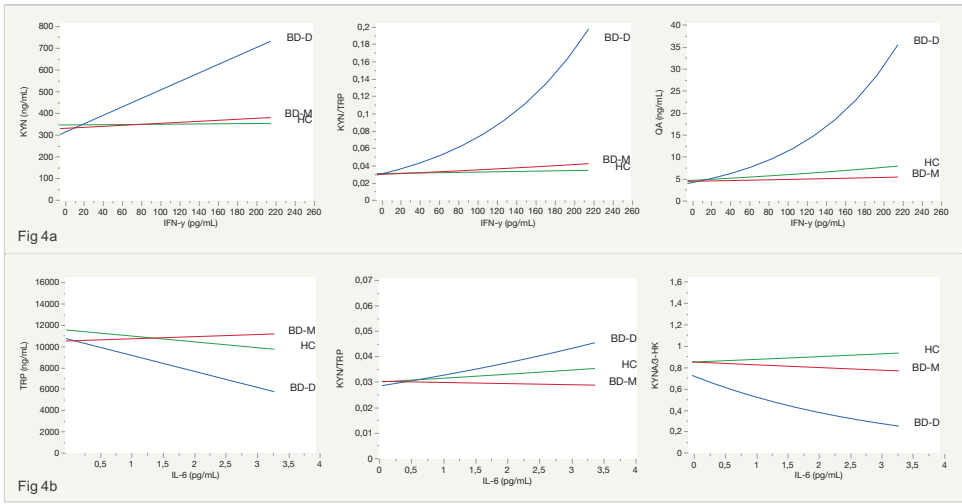

Figure 4: Interaction plots of IFN-γ and IL-6 on kynurenine metabolites by group
(a) Increasing IFN-γ associated with an increase in KYN, KYN/TRP and QA in the BD-D group.
(b) Increasing IL-6 associated with decreasing TRP, increasing KYN/TRP and decreasing KYNA/3-HK in the BD-D group.
HC: healthy controls (green), BD-D: patients with bipolar disorder and depression at baseline (blue); BD-M: patients with bipolar disorder and (hypo)mania at baseline (red). TRP: tryptophan; KYN: kynurenine; 3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid.

Post hoc assessments were done to evaluate relations between kynurenine metabolites and several clinical characteristics: current and lifetime psychotic features, BD type I or II, number of depressive or manic episodes, number of hospitalizations, duration of illness, age of onset, experiencing euthymia during study period and medication use. We found increased KYN/TRP with a longer duration of illness (p=0.015), and decreased QA with lifetime psychotic features (p=0.036) and a higher hospitalization rate (p=0.009). None of these findings however remain significant after Bonferroni correction. We observed no effect of psychopharmacological drugs in general, neither of lithium, antipsychotics or antidepressants. Though based on small subgroups (N=6 in BD-D, N=3 in BD-M), valproate was associated with a decrease of almost all kynurenine metabolites: TRP ($F(216.1)=59.8$; $p<.0001$), KYN ($F(277.7)=33.1$; $p<.0001$), QA ($F(218.3)=13.8$); $p=0.0003$), KYNA ($F(9.7)=271.2$; $p=0.0020$) and KYNA/3-HK ($F(254.7)=12.6$; $p=0.0005$).

4 Discussion

In this study, we investigated inflammation in patients with BD and its relation to kynurenine metabolism and mood symptoms. Although we found no difference in inflammatory markers between patients and controls, our data do suggest a shift of kynurenine metabolism in patients towards the microglial branch, especially in response to inflammation and in the presence of mood symptoms.

BD-D patients suffered from moderate to severe depression at inclusion and, in contrast to a rapid remission in the BD-M group, subsyndromal depressive symptoms persisted during follow-up.

Specifically in the BD-D group, a decreased neuroprotective ratio KYNA/3-HK is seen during follow-up. Also, a strong interaction between IFN-y and KYN, KYN/TRP and QA is exclusive to the BD-D group. A disbalance in kynurenine metabolism in favor of the microglial branch has been reported in both BD and MDD (5, 10, 19, 39). We are the first however to document a strong relation between inflammatory cytokines (mainly IFN-y), IDO-1 activation and neurotoxic kynurenine metabolites in a depressive patient population (6, 30).

Specifically in the BD-M group, at baseline we see strong correlations between TNF-α and both neurotoxic kynurenine metabolites (3-HK, QA) and IDO-1 activation (KYN, KYN/TRP). Similar to findings in the BD-D group, inflammation during acute mania seems to be accompanied by a an increased microglial kynurenine metabolism. While in the BD-D group (with chronic depressive symptoms) a major role is attributed to IFN-y, our data indicate that during mania TNF-α drives this neurotoxic shift. TNF-α is produced by macrophages and microglia and is involved in the rapid, innate immune response. TNF-α is known to upregulate the IFN-y-stimulated activation of IDO-1 by increasing IDO-1 mRNA transcription (40). However, TNF-α alone has also been shown to have stimulatory effects on the kynurenine pathway (10, 40). At 2 months of follow-up most manic symptoms are gone and, in contrast to our findings at baseline, we found no differences between the BD-M group and controls during follow-up.

The course of mood symptoms in our study population is typical for patients with BD. Depressive episodes can be severe with often long-term subsyndromal residual symptoms. Manic episodes are acutely very intense, but full remission is reached much sooner. (41) Our results indicate that in both situations of altered mood, i.e. chronic subsyndromal depressive

symptoms and acute mania, the kynurenine pathway is susceptible to inflammation and may produce a neurotoxic metabolic response.

Besides these mood state-specific findings, some differences between controls and patients seem to persist across mood states. First, KYNA levels were decreased in both the BD-D and BD-M group compared to controls and this on all test moments. Although we observe a weak inverse correlation between KYNA levels and HDRS scores and a trend to further decreased KYNA levels in patients showing mood symptoms versus euthymic patients, our data strongly suggest a decreased kynurenine metabolism downstream the astrocytic branch in both euthymic and non-euthymic patients. This decrease in KYNA contrasts with previous reports in CSF and postmortem brain tissue where increased KYNA levels in patients with BD were related to manic episodes and psychotic symptoms (8, 13, 15, 16). In line with our findings, studies using peripheral blood samples showed unchanged or decreased KYNA levels in patients with a history of mania or psychosis. (8, 12, 16) Decreased peripheral KYNA levels have also been shown in euthymic and depressive patients with BD (16, 20, 21). As further discussed below, this discrepancy between central and peripheral results may reflect the difficult passage of KYNA across the blood-brain barrier (BBB). Secondly, a negative correlation between inflammatory markers and the neuroprotective ratios are found in patients only and appear across all mood states. This is in line with findings in MDD were a decreased neuroprotective ratio (KYNA/QA) has been described in both remitted and non-remitted depressive patients (39). Both findings, i.e. the decreased KYNA levels and the negative correlation between inflammatory markers and the neuroprotective ratio in patients, indicate a hypo-activated astrocytic branch of the kynurenine pathway in patients and this across all mood states.

BD is considered to be a neuroprogressive disorder in which recurrent severe mood episodes and chronic subsyndromal symptoms contribute to a pathological rewiring of the brain leading to increased risk for relapse, chronicity, cognitive and functional impairment (42). A pro-inflammatory status and neurotoxic kynurenine metabolites would, among others, underlie this pathological process (19, 21, 42, 43). No differences in inflammatory markers has been shown in our study sample at baseline or during follow-up, which may be due to insufficient power to demonstrate subtle inflammatory differences. However, the different interactions that we found between inflammation and kynurenine metabolism in patients vs. controls may represent a loss in resilience of patients to recover from the neurotoxic effects of

inflammation. A chronic mild pro-inflammatory state could result in an increased baseline microglial activity and the preferential breakdown of kynurenine into the microglial branch at the expense of KYNA formation (1, 44).

Our study has multiple strengths: (1) we included patients in manic and depressive episodes and completed 6 test moments during a follow-up of 8 months, (2) the longitudinal design enables a within-person assessment of diverse mood states and (3) the high number of assessments increases the power of the study. (4) The impact of methodological bias was minimized by standardized blood sampling and uniform, meticulous laboratory procedures. (5) All clinical assessments were done by the same clinician-researcher, excluding interrater bias. (6) Data on illness course and medication use were collected carefully. (7) We used robust, transparent statistical methods. Mixed model analysis enables correction for missed moments, drop-out and a random variation in time and subject. The statistical models were adjusted for influences of sex, age, BMI, smoking status and albumin levels.

Some limitations should be mentioned nevertheless. First, biological markers are measured in peripheral plasma. Cytokines communicate across the BBB by several routes (45), the influence of peripheral kynurenine metabolites on the brain is less investigated. TRP, KYN and 3-HK easily cross the BBB by active transport, but passage of QA and KYNA is more difficult (46). A recent study by Sellgren et al. (16) in a large sample of patients with BD and healthy controls, could not show a relevant correlation between CSF and plasma KYNA levels. As shown earlier by the same group, history of psychosis was related to a significant increase in central KYNA levels, which was not reflected in plasma levels. Similar to our results, depressive symptoms were associated with decreased KYNA levels in both plasma and CSF (the levels were however not correlated) (16). The correlation of central and peripheral QA levels has not yet been studied. We want to emphasize that findings in peripheral samples cannot give a direct or quantitative representation of central processes, but we believe they contain important information on the systemic pathophysiology of psychiatric disorders. Although peripheral immune alterations led to the hypothesis of BD as a multi-system inflammatory disorder (47), the systemic impact of alterations in kynurenine metabolism is largely unknown and needs further exploration. Also in view of monitoring disease activity and staging, peripheral measurements may be of great value, but further research is needed. Meanwhile, in order to better understand the relation between central and peripheral markers, further

study on the specific central and peripheral dynamics of kynurenine metabolites and their ways to communicate across the BBB throughout the different stages of illness remain essential. Secondly, the ratio KYN/TRP was used as measure for IDO-1 activity. This ratio should be interpreted with caution, since both TRP and KYN can be metabolized in several other pathways and their bioavailability is influenced by albumin binding. In our sample, correction with albumin did not impact on results of KYN/TRP.

Questions on causality cannot be answered using our study design. We did not intend to make conclusions on the causality of changes in kynurenine metabolism found in our study. A kynurenine pathway upregulation in response to inflammatory stimuli has been shown in both animal studies and humans, so causality can be defended (48). Also, mood symptoms have been shown to appear after immune stimulation in both animal and clinical studies (49). We however believe the changes found in our sample should be integrated in a bigger picture of the pathophysiology of BD with multiple interacting processes (50).

Finally, 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. The BD-D group was characterized by a higher rate of BD type II and an earlier age of onset, possibly influencing the results. Post-hoc, we assessed the impact of these factors on biological markers and could not find a significant effect. Apart from the use of anti-inflammatory medication and ECT, there were no treatment restrictions during follow-up. Since patients were included during an acute mood episode, nearly all had changes in psychopharmacological treatment. The effect of psychopharmacological treatment on inflammatory markers and kynurenine metabolites has scarcely been studied (24). Although only prescribed in a small subgroup, valproate was associated with low levels of almost all kynurenine metabolites. A negative correlation between valproate and peripheral KYNA has been shown before, although again in a small subgroup of patients (16). This repeated observation underlines the need for further investigation on the effect of psychopharmacological treatment on biological markers.

5 Conclusion

Our study reveals that inflammation in patients with BD is associated with a shift of kynurenine metabolism towards the microglial branch and this especially in presence of mania and chronic depressive symptoms. Treatment strategies should be identified in order to counterbalance the strong relation between mood symptoms, low-grade inflammation and the possible neurotoxic impact of kynurenine metabolites.

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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.

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This study was supported by a grant from Janssen Research and Development, a division of Janssen Pharmaceutica N.V. The funding source has no role in the design and the conduct of the study.

Authors' contributions

MM, VC and SvdA developed the study protocol. SvdA did the patient recruitment, screening and clinical assessments and first drafted the manuscript. JS and SvdA did the laboratory analyses for cytokines, AvN and FYL for kynurenine metabolites. Statistical analyses were done by SvdA supervised by EF. RV assisted in the interpretation of data. All authors contributed to the development of the manuscript and have approved the final version of the manuscript.

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Supplementary tables S1-S7

Table S1: Baseline levels of inflammatory markers in HC, BD-D and BD-M

	HC	BD-D	BD-M	*Statistics (linear mixed model)*
IFN-γ (pg/ml)	5.18 (0.15)	4.04 (0.14)	6.44 (0.17)	F(75)=2.4; p=0.101
IL-6 (pg/ml)	0.43 (0.11)	0.42 (0.11)	0.53 (0.11)	F(86)=1.4; p=0.247
TNF-α (pg/ml)	1.80 (0.06)	1.93 (0.06)	1.91 (0.06)	F(95)=0.4; p=0.656
CRP (mg/L)	1.24 (0.21)	1.37 (0.20)	2.11 (0.20)	F(93)=1.9; p=0.156

Data presented as mean (standard error). Standard error on log-transformed data.
HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; IFN-γ: interferon gamma; IL: interleukin; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein

Table S2: Differences in levels of inflammatory markers between subgroups, all test moments

	HC	BD-D	BD-M	*Statistics (linear mixed model)*
IFN-γ (pg/ml)	4.87 (0.10)	4.24 (0.10)	4.73 (0.11)	F(92.0)=0.5; p=0.582
IL-6 (pg/ml)	0.41 (0.09)	0.46 (0.09)	0.53 (0.09)	F(96.1)=1.9; p=0.154
TNF-α (pg/ml)	1.77 (0.05)	1.92 (0.05)	1.90 (0.06)	F(98.4)=0.7; p=0.493
CRP (mg/L)	1.27 (0.17)	1.90 (0.17)	1.95 (0.18)	F(99.9)=1.9; p=0.162

Data presented as mean (standard error). Standard error on log-transformed data.
HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; IFN-γ: interferon gamma; IL: interleukin; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein

Table S3: Differences in levels of inflammatory markers between total patient group & controls, all test moments

	HC	BD-total	*Statistics (linear mixed model)*
IFN-γ (pg/ml)	4.87 (0.10)	4.46 (0.07)	F(92.7)=0.5; p=0.465; b=0.09
IL-6 (pg/ml)	0.41 (0.09)	0.50 (0.06)	F(96.2)=2.6; p=0.111; b=0.18
TNF-α (pg/ml)	1.77 (0.05)	1.91 (0.04)	F(99.3)=1.4; p=0.237; b=0.08
CRP (mg/L)	1.27 (0.17)	1.92 (0.13)	F(100.9)=3.7; p=0.056; b=0.41

Data presented as mean (standard error). Standard error on log-transformed data.
HC: healthy controls; BD-total: total patient group; IFN-γ: interferon gamma; IL: interleukin; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein

Table S4: Kynurenine metabolites during follow-up: effect of group (HC, BD-D, BD-M), moment and interaction			
	Group	Moment	Group*moment
TRP	F(95.2)=4.1; *p=0.0193*[b]	n.s.	n.s.
KYN	n.s.	n.s.	n.s.
3-HK[a]	n.s.	n.s.	F(367.8)=2.7; ***p*=0.0031**
QA[a]	n.s.	n.s.	n.s.
KYNA[a]	F(96.4)=7.7; ***p*=0.0008**[b]	n.s.	n.s.
KYN/TRP[a]	n.s.	n.s.	n.s.
KYNA/3-HK[a]	F(98.5)=8.5; ***p*=0.0004**[b]	n.s.	F(368.5)=2.6; ***p*=0.0041**

HC: healthy controls, BD-D: patients with bipolar disorder and depression at baseline; BD-M: patients with bipolar disorder and (hypo)mania at baseline; TRP: tryptophan; KYN: kynurenine; 3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid.
Significant *p*-values passing Bonferroni correction are in bold ; *p*-values < .05 are italicized ; n.s. = not significant (*p*>.05)
[a] Statistics on log-transformed data
[b] Tukey HSD: TRP: BD-D < HC; KYNA: BD-M&BD-D < HC; KYNA/3-HK: BD-D < BD-M&HC

Table S5: Correlations between symptom scales and biological markers (Spearman's ρ)				
		HDRS	YMRS	PANSS-P
Inflammation	IFN-γ	-0.11	0.02	-0.00
	IL-6	-0.08	0.04	0.00
	TNF-α	-0.02	0.08	0.08
	CRP	0.02	0.05	0.03
Kynurenine metabolism	TRP	-0.13	0.13	0.07
	KYN	-0.13	0.13	0.06
	3-HK	-0.08	0.07	-0.02
	QA	-0.16	0.08	0.02
	KYNA	-0.21	0.09	0.05
	KYN/TRP	-0.04	0.03	-0.01
	KYNA/3-HK	-0.17	0.07	0.06

Correlation coefficients <-0.20 or > 0.20 are shaded in grey.
HDRS: Hamilton depression rating scale; YMRS: Young mania rating scale; PANSS-P: Positive and negative syndrome scale – positive subscale; TRP: tryptophan; KYN: kynurenine; 3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid.

Table S6: Correlations between inflammatory markers and kynurenine metabolites in HC all moments, BD-M baseline and BD-M follow-up (Spearman's ρ)

	HC (all moments)				BD-M baseline				BD-M follow-up (1m-8m)			
	IFN-γ	IL-6	TNF-α	CRP	IFN-γ	IL-6	TNF-α	CRP	IFN-γ	IL-6	TNF-α	CRP
TRP	-0.03	-0.04	-0.03	-0.27	0.07	0.06	0.23	-0.18	-0.02	-0.1	0.04	-0.14
KYN	0.22	0.05	0.25	-0.14	0.04	0.14	0.61	-0.16	0.22	0.12	0.43	-0.15
3-HK	0.06	-0.03	0.09	-0.11	-0.07	0.15	0.70	-0.17	0.15	-0.05	0.20	-0.05
QA	0.21	0.04	0.23	0.25	-0.22	0.29	0.60	0.20	0.30	0.21	0.48	0.07
KYNA	0.07	-0.02	0.07	-0.30	-0.21	-0.10	0.32	-0.20	-0.09	-0.20	0.12	-0.30
KYN/TRP	0.25	0.09	0.25	0.05	0.16	0.22	0.62	0.02	0.28	0.16	0.47	-0.17
KYNA/3-HK	-0.07	0.02	0.04	-0.13	-0.42	-0.33	-0.24	-0.19	-0.17	-0.27	-0.03	-0.30

Correlation coefficients <-0.20 or > 0.20 are shaded in grey.
HC: healthy controls, BD-M: patients with bipolar disorder and (hypo)mania at baseline; IFN-γ: interferon-gamma; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; TRP: tryptophan; KYN: kynurenine; 3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid.

Table S7: Correlations between inflammatory markers and kynurenine metabolites in controls and patients overall (Spearman's ρ)

	HC (all moments)				Patients (all moments)			
	IFN-γ	IL-6	TNF-α	CRP	IFN-γ	IL-6	TNF-α	CRP
TRP	-0.03	-0.04	-0.03	-0.27	-0.02	-0.16	0.04	-0.07
KYN	0.22	0.05	0.25	-0.14	0.16	0.03	0.40	-0.09
3-HK	0.06	-0.03	0.09	-0.11	0.14	0.07	0.29	0.07
QA	0.21	0.04	0.23	0.25	0.21	0.15	0.33	0.16
KYNA	0.07	-0.02	0.07	-0.30	-0.15	-0.19	0.08	-0.24
KYN/TRP	0.25	0.09	0.25	0.05	0.20	0.18	0.40	-0.06
KYNA/3-HK	-0.07	0.02	0.04	-0.13	-0.24	-0.27	-0.16	-0.31

Correlation coefficients <-0.20 or > 0.20 are shaded in grey.
HC: healthy controls, IFN-γ: interferon-gamma; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; TRP: tryptophan; KYN: kynurenine; 3-HK: 3-hydroxykynurenine; QA: quinolinic acid; KYNA: kynurenic acid.

CHAPTER 5

Inflammation and BH_4-dependent monoamine metabolism

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Markers of Inflammation and Monoamine Metabolism Indicate Accelerated Aging in Bipolar Disorder

Seline van den Ameele[1,2], Dietmar Fuchs[3], Violette Coppens[1,2], Peter de Boer[4], Maarten Timmers[4,5], Bernard Sabbe[1,2] and Manuel Morrens[1,2]*

[1] Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium, [2] University Psychiatric Hospital Duffel - VZW Emmaüs, Duffel, Belgium, [3] Division of Biological Chemistry, Biocenter, Medical University of Innsbruck, Innsbruck, Austria, [4] Janssen Research and Development, A Division of Janssen Pharmaceutica N.V., Beerse, Belgium, [5] Reference Center for Biological Markers of Dementia, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium

Background: A mild pro-inflammatory status accompanies bipolar disorder (BD). Inflammation can cause a shift in monoamine metabolism, thereby activating more cytotoxic pathways. The extent to which low-grade inflammation in BD interacts with monoamine metabolism and how this accords to aging and clinical course is unknown.

Objectives: We evaluated the presence of alterations in inflammation and monoamine metabolism in BD throughout different mood states and the role of aging therein.

Methods: Sixty-seven patients with BD were included during an acute mood episode, either depressive (n = 29), (hypo)manic (n = 29), or mixed (n = 9). Plasma levels of inflammatory markers [tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin-6 (IL-6), and C-reactive protein (CRP)] and markers of monoamine metabolism (neopterin, tryptophan, kynurenine, phenylalanine, and tyrosine) were measured repeatedly during a follow-up of 8 months. Levels in patients were compared to controls (n = 35) and correlated to HDRS-17 and YMRS scores. Spearman correlations and linear mixed model analysis were used for statistical analysis.

Results: Forty-nine patients and 30 controls (age range: 22–62 years) completed the study. No significant differences in inflammatory markers were found between patients and controls overall. Tryptophan, tyrosine, and phenylalanine levels were lower in patients. In both patients and controls, markers of inflammation correlated only weakly with markers of monoamine metabolism, but correlations representative for activity of cytotoxic pathways in monoamine metabolism were more pronounced in patients. In patients, but not in controls, older age was associated with increases in inflammatory markers (IL-6, CRP, neopterin) and the kynurenine/tryptophan ratio. None of the biological markers correlated significantly with mood symptom severity.

Conclusion: Our data suggest an increased susceptibility of patients with BD to develop a pro-inflammatory state and to shift monoamine metabolism toward more cytotoxic pathways. These findings are in support of the theory of neuroprogression and accelerated aging in BD. Since associations between biological markers and clinical characteristics are limited, it remains to be determined if alterations in biological markers are due to a disease effect or rather are a consequence of confounding factors.

Keywords: bipolar disorder, inflammation, monoamines, neopterin, neuroprogression, accelerated aging

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Błażej Misiak,
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Erasmus University Rotterdam, Netherlands
Janusz K. Rybakowski,
Poznan University of Medical Sciences, Poland

****Correspondence:***
Seline van den Ameele
seline.vandenameele@gmail.com

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INTRODUCTION

Low-grade inflammation has been documented extensively in bipolar disorder (BD). Increased levels of pro-inflammatory cytokines and acute phase proteins have been shown during acute mood episodes (1–5) and in later stages of the disease (4, 6). Even during euthymia, isolated monocytes were found to express more pro-inflammatory genes, and the activity of hippocampal microglia was increased (7–9). According to the theory of accelerated aging, the early onset of a chronic low-grade inflammation underlies neuroprogression in BD by affecting monoamine synthesis and increasing the production of cytotoxic metabolites (10, 11).

Rising evidence suggests that immune system pathways act on monoamine biosynthesis (See **Figure 1**) (12–14). In chronic inflammation, activation of guanosine triphosphate cyclohydroxylase 1 (GTP-CH1) by the pro-inflammatory cytokines interferon gamma (IFN-y) and tumor necrosis factor-alfa (TNF-α) results in increased neopterin production at the expense of tetrahydrobiopterin (BH_4). Neopterin is a marker of activated cell-mediated immunity and increased oxidative stress (15, 16), while BH_4 is an essential cofactor in the synthesis of dopamine, noradrenaline, adrenaline and serotonin (17–19). IFN-y and TNF-α also stimulate indoleamine 2,3 dioxygenase 1 (IDO-1) activity. Upon immune activation, IDO-1 converts tryptophan to kynurenine and thus depletes tryptophan for serotonin synthesis. Kynurenine metabolites have several downstream cytotoxic or neuroactive effects (14, 20).

In viral infections, cancer and autoimmune diseases, inflammatory markers have already been correlated with monoamine synthesis and neopterin production (16). As seen in healthy aging, low-grade inflammation correlates to increased neopterin levels and IDO-1-mediated induction of tryptophan metabolism. In otherwise healthy elderly IDO-1 and GTP-CH1 activity have been associated with depressive symptomatology (17). Decreased levels of BH_4 have been found in patients with major depression and schizophrenia (21, 22). Although changes in neopterin levels and IDO-1 activity were found in patients with BD (23, 24), it remains unclear whether these changes in monoamine synthesis correlate with inflammatory alterations.

The aim of this study was to evaluate a possible association between inflammation and monoamine metabolism in bipolar disorder and its relation to aging and clinical course. We hypothesized pro-inflammatory cytokines to be increased in patients with BD compared to healthy controls, and more so during mood episodes and in older patients with a longer duration of illness, resulting in an activation of GTP-CH1 and IDO-1.

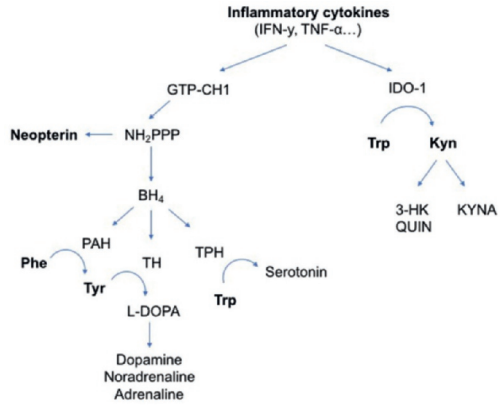

FIGURE 1 | Influence of inflammatory cytokines on neurotransmitter metabolism. The pro-inflammatory cytokines IFN-y and TNF-α can activate enzymatic pathways that change neurotransmitter metabolism. IFN-y stimulates GTP-CH1, resulting in the synthesis of BH_4 and neopterin. TNF-α further enhances IFN-y-stimulated GTP-CH1 activity. Neopterin is almost exclusively synthesized in and released by activated cells of the monocyte/macrophage system and used as a marker for activated cell-mediated immunity. Increased neopterin levels also indicate increased oxidative stress induced by immune-mediated processes. In chronic inflammation, mainly neopterin is released at the expense of BH_4 production. BH_4 is an essential cofactor in the synthesis of dopamine, noradrenaline, adrenaline and serotonin. IFN-y and TNF-α also stimulate IDO-1 activity. Upon immune activation, IDO-1 converts Trp to Kyn and thus depletes Trp for serotonin synthesis. Downstream kynurenine metabolites have several cytotoxic or neuroactive effects. Arg, L-Arginine; BH_4, tetrahydrobiopterin; GTP-CH1, guanosine triphosphate cyclohydroxylase 1; IDO-1, indoleamine 2,3 dioxygenase 1; IFN-y, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; Kyn, kynurenine; KYNA, kynurenic acid; L-DOPA, L-3,4-dihydroxyphenylalanine; NH_2PPP, dihydroneopterin triphosphate; NO, nitric oxide; PAH, phenylalanine hydroxylase; Phe, phenylalanine; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor alpha; TPH, tryptophan hydroxylase; Trp, tryptophan; Tyr, tyrosine; QUIN, quinolinic acid; 3-HK, 3-hydroxykynurenine.

METHODS

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 at time of inclusion. Clinical assessments are described below (see section Clinical Assessments). Age and gender matched controls were recruited mainly among staff members of the participating centers. We ensured an equal distribution of inclusions of patients and controls throughout the year to account for seasonality in immune system activity (25). Exclusion criteria for both patient and control group were: substance abuse, use of anti-inflammatory drugs within 2 weeks preceding 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 complete blood count, electrolytes, 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 major depressive disorder, BD or psychotic syndrome as defined by DSM-IV criteria and BD or psychotic syndrome in a first-degree family member and current use of psychopharmacological drugs. 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.

Study Design

Patients were recruited during an acute mood episode, either depressed, (hypo)manic or mixed. In both patients and controls, screening was followed by a first test day after 1–5 days. Subsequent test days were planned after, respectively 1, 2, 4, 6, and 8 months of follow-up, resulting in 6 test days per participant over the course of 8 months. Every test day included the same clinical and laboratory assessments as described below. During the study period, patients received treatment as usual without intervention of the investigators.

Clinical Assessments

The M.I.N.I.-plus, International Neuropsychiatric Interview, version 5.0.0 was chosen as diagnostic instrument in patients and controls because of its accurate structured DSM-IV diagnosis and convenience to administer (26). In patients, the severity of mood symptoms was assessed by the 17-item Hamilton Depression Rating Scaling (HDRS-17) (27) and the Young Mania Rating Scale (YMRS) (28) at screening and on all test days. At screening, threshold score for inclusion was set at $\geq$17 for the HDRS-17 or $\geq$13 for the YMRS, corresponding to moderate depression or hypomania, respectively (29, 30). On all subsequent test days, the mood state of patients was classified as "depressive," "(hypo)manic," "mixed," or "euthymic" according to the HDRS and YMRS scores. Psychotic symptoms were evaluated on test days using the positive subscale of the Positive and Negative Syndrome Scale (PANSS) (31). In the control group, the occurrence of mood episodes during follow-up was evaluated on all test days based on a short screening questionnaire. For all participants, we assessed medication use and the occurrence of any of the exclusion criteria on every test day. All clinical assessments were done by a psychiatrist in training (SvdA) and supervised by a psychiatrist (MM).

Laboratory Assessments

Blood was drawn by venipuncture between 08.00 and 10.30 a.m. into a citrate vacuum tube (2.7 ml). Tubes were immediately stored at 4°C, centrifuged at 2 g and 4°C for 10 min within 2 h after blood draw, and plasma was aliquoted and stored at −70°C until assayed.

TNF-α, IFN-y, IL-1β, IL-4, IL-6, and CRP were measured in duplicate by an electrochemiluminescence immunoassay technique developed by Mesoscale Discovery (Rockville, USA) according to the manufacturer's instructions. Kits used for detection were V-plex Pro-inflammatory Panel I for TNF-α, IFN-y, IL-1β, IL-4, and IL-6 and Human Vascular Injury Panel for CRP. The lower limits of detection (LLOD) were, respectively 0.04, 0.2, 0.04, 0.02, 0.06, and 1.3 pg/ml. Sample signals were fitted on a 4-parametric logistic calibration curve to calculate concentrations.

Patient and control samples were analyzed in randomized sequence with both samples of a single subject on the same plate and an equal distribution of patients and controls per plate. Samples with a coefficient of variation (CV) >20% were excluded from statistical analyses. Because >80% of the samples were below the LLOD for IL-1β and IL-4, these cytokines were not included in statistical analyses. Excluded samples were equally distributed among patients and controls. For TNF-α, IFN-y, IL-6, and CRP, >80% of the samples were included for analysis. The mean CV and standard deviation were 6.3 (4.7), 8.4 (5.5), 7.6 (5.5), 5.4 (4.5), respectively.

Neopterin concentrations were determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (BRAHMS Diagnostics, Hennigsdorf, Germany). Tryptophan, kynurenine, phenylalanine, and tyrosine were determined by high-performance liquid chromatography, as described previously (32, 33). The ratios of Kyn/Trp and Phe/Tyr were calculated as indexes of IDO-1 and PHA activity, respectively. The Phe/Tyr ratio is also used as a reliable measure of BH_4 availability (34).

Statistical Analysis

Normality of outcome variables and homoscedasticity of residuals were evaluated by visual inspection. For the regression modeling, IFN-y, IL-6, TNF-α, and CRP concentrations were log-transformed to obtain a normal distribution. Homogeneity of variances was assessed by Levene's test and further analyses were adapted accordingly.

Baseline differences in clinical and demographic parameters between the patient and control group were examined by two-tailed independent *t*-tests for continuous variables and Pearson chi-square test for categorical variables.

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 subject ID as random intercept. We included subsequently group (patient vs. HC) and mood state [depression vs. (hypo)mania vs. mixed episode vs. euthymia vs. controls] as fixed effects. Smoking status and BMI were added as covariates to the adjusted models. As nutritional status affects amino acid levels, albumin concentrations were also added as covariates in the adjusted model of amino acid level prediction. Impact of age was assessed by adding age and the interaction between age and group as covariates in the linear mixed model. The output

from the mixed model analysis, is reported as "F-ratio (DF); *p*-value; *b*."

The relation among biological parameters and the relation between symptom severity scores and biological parameter levels were studied by pairwise correlations. Correlations are reported by the Spearman's rho for non-parametric distributions. *P*-values below 0.05 were considered statistically significant. All statistical analyses were performed in JMP Pro 12 (JMP, Marlow, UK).

RESULTS

Participants

Sixty-seven patients with BD and 35 controls were included. At screening, 29 patients had a depressive episode, 29 patients a hypomanic or manic episode, and 9 patients a mixed episode. Demographic and metabolic characteristics are shown in **Table 1**. Patients and controls were matched by sex and age. Body mass index (BMI) and the percentage of smokers were higher in patients compared to controls. No other significant differences in demographic or metabolic parameters were found between patients and controls. Clinical characteristics of patients are shown in **Table 2**.

Forty-nine patients and 30 controls completed the 8-month's study design. Drop-out in the patient group was due to: chronic use of low-dose acetylsalicylic acid ($n = 5$), substance abuse ($n = 3$), ECT ($n = 2$), and loss of contact or lack of motivation for further participation ($n = 8$). Drop-out in the control group was due to difficult blood draws ($n = 1$) and repeated orthopedic surgery ($n = 1$). Three controls were only included for baseline testing. The mean number of test days per participant was 5.0 in patients and 5.4 in controls. Over 8 months, the total number of blood samples included for analyses was 336 in patients and 188 in controls. In total, 178 test moments were during a depressive episode, 71 during a (hypo)manic episode, 23 during a mixed episode, and 64 test moments were during a euthymic episode. Symptom severity scores by mood state are shown in **Table 3** and Table S1.

TABLE 1 | Baseline demographic and metabolic characteristics.

	Patients	Controls	*p*-value[a]
N	67	35	
Gender, female	39 (58.2)	19 (54.3)	0.704
Age, years	43.3 ± 11.1 (23–62)	42.7 ± 11.6 (23–62)	0.883
Caucasian	63 (94.0)	34 (97.1)	0.489
Smokers	32 (47.8)	6 (17.1)	**0.002**
BMI, kg/cm^2	25.3 ± 4.2 (18–39)	23.7 ± 2.6 (20–29)	**0.025**
Waist, cm	89.1 ± 12.1 (66–122)	84.4 ± 9.6 (67–104)	0.059
Fasting glucose, mg/dl	90.0 ± 9.3 (69–116)	87.5 ± 6.8 (73–102)	0.176
Cholesterol, mg/dl			
Total	186.5 ± 44.7 (101–341)	190.9 ± 42.4 (132–283)	0.636
HDL	58.6 ± 17.8 (24–102)	62.0 ± 18.4 (28–118)	0.372
LDL	105.6 ± 40.9 (44–264)	109.0 ± 33.4 (57–184)	0.674

Data presented as mean ± SD (range) or n (%). BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein. [a]p-values of t-test or Chi-squared test. Bold values: significant p-values (p < 0.05).

Biological Markers in Patients and Healthy Controls

Biological Markers in Patients vs. Controls

The overall levels of inflammatory markers and amino acids in patients and controls are shown in Table S2. No significant differences in levels of inflammatory markers (IFN-y, IL-6, TNF-α, and CRP) were found between patients and controls. Patients had significantly lower levels of tryptophan [$F_{(92.4)} = 5.2$; $p = 0.026$; $b = 3.37$], tyrosine [$F_{(98.2)} = 7.8$; $p = 0.006$; $b = 9.56$], and phenylalanine [$F_{(95.0)} = 9.6$; $p = 0.003$; $b = 5.14$] as compared to controls. After adjustment for BMI and albumin levels, also kynurenine was found to be lower in patients

TABLE 2 | Clinical characteristics and baseline data of patients.

N	67
Diagnosis	
BD type I	42 (62.7)
BD type II	23 (34.3)
Schizoaffective disorder	2 (3)
Age of onset, years	24.9 ± 11.5 (8-55)
Duration of illness, years	17.6 ± 11.3 (0-49)
First episode: depression	40 (60.6)
Age first depression, years	25.9 ± 12.1 (8-55)
Age first mania/hypomania, years	28.9 ± 11.9 (8-59)
Lifetime psychotic features	37 (55.2)
Total number of hospitalizations	
0	9 (13.4)
1-5	45 (67.2)
6-10	13 (19.4)
Lifetime substance abuse	30 (44.8)
Alcohol	18 (26.9)
THC	13 (19.4)
Hard drugs	5 (7.5)
Baseline medication use	
Medication-free	6 (9.0)
Lithium	24 (35.8)
Valproate	9 (13.4)
Carbamazepine	3 (4.5)
Lamotrigine	8 (11.9)
Antipsychotic	42 (62.7)
Antidepressant	31 (46.3)
Benzodiazepine	24 (35.8)
Baseline mood episode	
Depression	29 (43.3)
(Hypo)mania	29 (43.3)
Mixed	9 (13.4)

Data presented as mean ± SD (range) or n (%). BD, bipolar disorder; THC, tetrahydrocannabinol.

[$F_{(95.6)} = 3.9$; $p = 0.0499$; $b = 0.13$]. After adjustment for smoking status, kynurenine remained significantly lower in patients [$F_{(99)} = 4.0$; $p = 0.048$; $b = 0.16$]. Neopterin and the Phe/Tyr and Kyn/Trp ratios did not differ significantly between patients and controls.

Differences between controls and different mood states in patients [i.e., depression vs. (hypo)mania vs. mixed episode vs. euthymia vs. controls] are shown in **Table 3**. No differences in levels of inflammatory markers were found. The decrease in tryptophan, tyrosine, and phenylalanine found in the total patient group is significantly more pronounced in depressive patients. These findings did not retain statistical significance after adjustment for smoking status.

Correlation Between Markers of Inflammation and Amino Acids in Patients vs. Controls

In both patients and controls, inflammatory parameters correlated positively to neopterin levels. IFN-y and TNF-α positively correlated to Kyn/Trp in both groups. In patients, but not in controls, IL-6 correlated positively to the Kyn/Trp ratio and negatively to tryptophan. Neopterin correlated positively to kynurenine and the Kyn/Trp and Phe/Tyr ratios, with a stronger correlation in patients. Further details on correlations are shown in **Table 4** and Table S3.

Impact of Age and Course of Illness on Biological Markers

Correlations between inflammatory markers and symptom severity scores were weak and not significant (see Table S4). Small inverse correlations were found between HDRS scores and tryptophan ($\rho = -0.13$), kynurenine ($\rho = -0.14$), and tyrosine ($\rho = -0.11$) levels, while positive correlations (ρ between 0.15 and 0.22) were found between YMRS and PANSS positive subscale scores and tryptophan, kynurenine, tyrosine, and phenylalanine levels. Neopterin levels and the Phe/Tyr and Kyn/Trp ratios were not significantly correlated to symptom severity scores, except for a weak positive correlation between Phe/Tyr and HDRS scores ($\rho = 0.13$). See Table S4 for further details. In line with the correlations, the presence of psychotic features was associated with an increase in phenylalanine levels [$F_{(318.4)} = 9.1$; $p = 0.003$; $b = 4.99$].

Longer duration of illness was associated with higher TNF-α [$F_{(65.2)} = 5.3$; $p = 0.024$; $b = 0.01$], kynurenine [$F_{(64.7)} = 4.3$; $p = 0.042$; $b = 0.01$], Kyn/Trp [$F_{(66.4)} = 8.0$; $p = 0.006$; $b = 0.17$], and neopterin [$F_{(70.2)} = 6.2$; $p = 0.015$; $b = 0.03$], effect sizes are rather small. Comparing the impact of aging on biological parameters between patients and controls, we observed a significant interaction effect between age and patient/control status for IL-6, CRP, neopterin, and the Kyn/Trp ratio. Patients have rising IL-6, CRP, neopterin, and Kyn/Trp ratio with older age, while these parameters are stable in controls (see **Figure 2**). A similar age effect is found when comparing biomarker levels in participants below and above 45 years of age (see **Table 5**). Only in the patient group we observed a significant difference between both age groups: the older patient group had higher levels of Kyn/Trp, neopterin, IL-6, and TNF-α and lower tryptophan levels. Comparing patients and controls, the <45 years group had lower levels of kynurenine, Kyn/Trp, and tyrosine in patients ($p = 0.012$, 0.018, and 0.040, respectively), while inflammatory markers were not different between patients and controls. Patients in the >45 years group had lower levels of tryptophan and phenylalanine (p-value of 0.009 and 0.006, respectively) compared to controls and higher IL-6, TNF-α, and CRP levels (p-values of 0.003, 0.047, and 0.007).

Other illness characteristics such as duration of current mood state, lifetime psychotic features, BD type I or II, number of mood episodes and number of hospitalizations revealed no significant relations with any of the biological markers ($p > 0.05$).

TABLE 3 | Mood symptom severity and differences in biological markers between mood states and controls.

	Depression	(Hypo)mania	Mixed	Euthymia	Controls	Effect	Tukey HSD
# Test moments (N)	178	71	23	64	188		
HDRS	16.6 (5.8)	6.6 (4.1)	20.5 (4.4)	3.9 (2.2)			
YMRS	3.5 (2.7)	18.3 (7.0)	15.2 (4.4)	2.7 (2.4)			
IFN-y (pg/ml)*	4.24 (0.08)	4.67 (0.10)	4.71 (0.15)	4.78 (0.10)	4.87 (0.10)	$F_{(403.4)} = 0.6$; $p = 0.655$	
IL-6 (pg/ml)*	0.49 (0.07)	0.52 (0.08)	0.42 (0.12)	0.52 (0.08)	0.41 (0.09)	$F_{(434)} = 1.6$; $p = 0.168$	
TNF-α (pg/ml)*	1.95 (0.04)	1.89 (0.04)	1.83 (0.06)	1.87 (0.04)	1.77 (0.05)	$F_{(498.4)} = 1.5$; $p = 0.198$	
CRP (mg/L)*	1.81 (0.15)	2.08 (0.15)	2.16 (0.21)	1.97 (0.15)	1.27 (0.17)	$F_{(487.7)} = 1.5$; $p = 0.205$	
Trp (μmol/l)	50.04 (0.99)	51.67 (1.24)	54.61 (2.02)	51.29 (1.28)	54.37 (1.18)	$F_{(333.2)} = 2.8$; **$p = 0.025$**	D < C
Kyn (μmol/l)	1.43 (0.04)	1.46 (0.05)	1.53 (0.08)	1.51 (0.05)	1.58 (0.05)	$F_{(350.7)} = 1.9$; $p = 0.116$	
Kyn/Trp (μmol/mmol)	28.89 (0.75)	28.89 (0.91)	28.54 (1.42)	29.98 (0.93)	29.41 (0.92)	$F_{(351.5)} = 0.5$; $p = 0.702$	
Neo (nmol/l)	4.96 (0.17)	5.00 (0.23)	5.12 (0.40)	5.44 (0.25)	5.04 (0.18)	$F_{(306.1)} = 0.8$; $p = 0.499$	
Tyr (μmol/l)	62.64 (2.25)	70.62 (2.78)	71.67 (4.45)	64.10 (2.87)	75.06 (2.71)	$F_{(345.3)} = 4.9$; **$p < 0.001$**	D < C & M; E < C
Phe (μmol/l)	49.47 (1.12)	52.08 (1.43)	56.13 (2.35)	50.54 (1.48)	55.93 (1.31)	$F_{(334.7)} = 4.9$; **$p < 0.001$**	D < C & Mx
Phe/Tyr	0.83 (0.02)	0.77 (0.22)	0.81 (0.03)	0.82 (0.02)	0.78 (0.02)	$F_{(346.3)} = 2.4$; $p = 0.053$	

*Data presented as mean (SE). SE, standard error; HDRS, Hamilton depression rating scale; YMRS, Young mania rating scale; IFN-y, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha; CRP, C-reactive protein; Trp, tryptophan; Kyn, kynurenine; Neo, neopterin; Tyr, tyrosine; Phe, phenylalanine; D, depression; C, controls; E, euthymia; M, (hypo)mania; Mx, mixed. *SE on log-transformed data. Bold values: significant p-values ($p < 0.05$).*

DISCUSSION

In this study we measured markers of monoamine synthesis and immune activity in patients with BD and controls. We found decreased levels of tryptophan, phenylalanine and tyrosine in patients, which were more pronounced during depressive episodes. We found no differences in inflammatory markers between the overall groups of patients vs. controls. Nonetheless, our results suggest a proneness of patients with BD for an increased pro-inflammatory state and its related cytotoxic effects as (i) correlations between inflammatory markers and monoamine metabolites diverge distinctly between patients and controls and (ii) a premature pro-inflammatory status arises in middle-aged patients and increases over the course of illness.

The positive correlation between Phe/Tyr and neopterin levels found in patients with BD is similar to the changes seen during chronic inflammation in cancer, HIV, and autoimmune diseases which are related to activation of the GTP-CH1 enzyme (18). Activation of GTP-CH1 results in increased neopterin synthesis in macrophages at the expense of BH_4, an essential cofactor for monoamine synthesis (17). We found that IL-6 levels correlated positively to Kyn/Trp and negatively to tryptophan only in patients, which suggests activation of the IDO-1 enzyme. Increased IDO-1 activity results in higher tryptophan breakdown in the kynurenine pathway. The consequence is a lower tryptophan availability for serotonin synthesis and increased levels of kynurenine metabolites that have multiple cytotoxic and neuroactive effects. These different interactions between markers of inflammation and monoamine metabolism in patients vs. controls are suggestive for a stronger interaction between inflammation, activation of IDO-1 and GTP-CH1, impaired monoamine synthesis and increased production of cytotoxic metabolites in patients.

Interestingly, exclusively in the patient group, aging and increased duration of illness were associated with a rise in levels of pro-inflammatory markers, neopterin and the Kyn/Trp ratio. A pro-inflammatory status accompanies normal aging and is thought to underlie the increased frailty and vulnerability for psychiatric disorders in elderly (35–37). Similar to the correlations found in our patient group, chronic low-grade inflammation in healthy elderly was related to increased Phe/Tyr, and to decreased tryptophan levels (17). As in our patient group, older age was also related to increasing IL-6, neopterin, and IDO-1 activation. The mean age of 79.9 years in the above study of Capuron et al. (17) contrasts with the mean age of 43 years in our study population. Previous research shows that in healthy subjects over 60 years of age the effects of aging on inflammation and IDO-1 activation become more apparent

TABLE 4 | Spearman correlations between markers of inflammation and monoamine metabolism in patients vs. controls.

	Neopterin		Kyn/Trp		Phe/Tyr	
	Patients	**Controls**	**Patients**	**Controls**	**Patients**	**Controls**
IFN-y	0.31***	0.35***	0.17**	0.34***	0.05	−0.11
IL-6	0.20**	0.28**	0.19**	0.15	−0.14*	−0.06
TNF-α	0.33***	0.26***	0.32***	0.27*	0.04	−0.04
CRP	0.23***	0.25***	−0.01	0.1	−0.04	−0.05
Neopterin	1	1	0.43***	0.27***	0.17**	−0.07

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Trp, tryptophan; Kyn, kynurenine; Tyr, tyrosine; Phe, phenylalanine; IFN-y, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha; CRP, C-reactive protein.

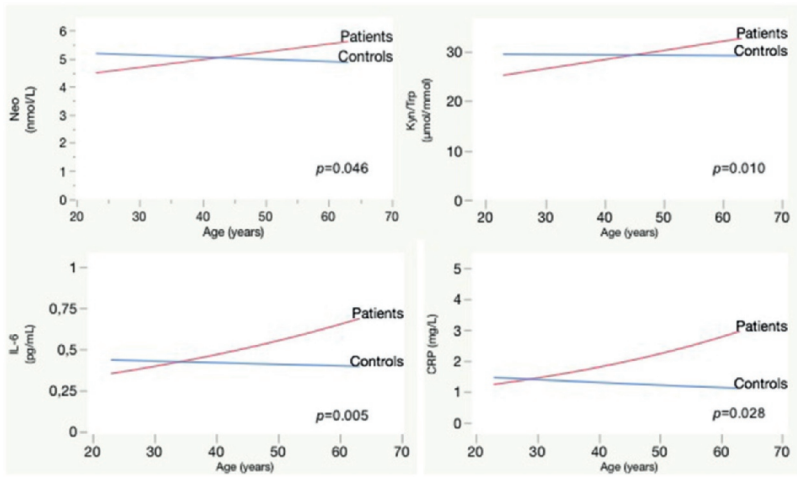

FIGURE 2 | Effect of aging on Neopterin, Kyn/Trp, IL-6, and CRP in patients vs. controls: interaction plots. CRP, C-reactive protein; IL, interleukin; Kyn, kynurenine; Neo, neopterin; Trp, tryptophan.

TABLE 5 | Differences in biological markers in patients and controls below and above 45 years of age.

	Patients			Controls		
	<45 y ($n = 36$)	≥45 y ($n = 31$)	Effect	<45 y ($n = 19$)	≥45 y ($n = 16$)	Effect
Trp (μmol/l)	52.91 (1.35)	48.70 (1.8)	$F_{(61.6)} = 5.3$; $\mathbf{p = 0.025}$; $b = 4.21$	53.81 (1.30)	55.15 (1.34)	$F_{(150)} = 0.8$; $p = 0.369$; $b = -1.34$
Kyn (μmol/l)	1.43 (0.05)	1.51 (0.06)	$F_{(63.5)} = 1.0$; $p = 0.311$; $b = -0.08$	1.57 (0.05)	1.59 (0.05)	$F_{(181)} = 0.1$; $p = 0.700$; $b = -0.02$
Kyn/Trp (μmol/mmol)	27.20 (0.91)	31.30 (0.99)	$F_{(65.0)} = 9.4$; $\mathbf{p = 0.003}$; $b = -4.09$	29.71 (0.87)	29.08 (0.90)	$F_{(180.5)} = 0.5$; $p = 0.468$; $b = 0.64$
Neo (nmol/l)	4.78 (0.18)	5.41 (0.20)	$F_{(67.5)} = 5.5$; $\mathbf{p = 0.022}$; $b = -0.63$	5.15 (0.22)	4.94 (0.23)	$F_{(88.8)} = 0.6$; $p = 0.457$; $b = 0.22$
Tyr (μmol/l)	64.05 (2.50)	66.34 (2.69)	$F_{(60.6)} = 0.5$; $p = 0.498$; $b = -2.28$	76.70 (4.00)	73.12 (4.14)	$F_{(179.7)} = 0.8$; $p = 0.378$; $b = 3.52$
Phe (μmol/l)	50.97 (1.26)	50.27 (1.37)	$F_{(58.2)} = 0.2$; $p = 0.695$; $b = 0.69$	55.89 (1.85)	55.88 (1.92)	$F_{(169.7)} = 0.0$; $p = 0.998$; $b = 0.01$
Phe/Tyr	0.83 (0.02)	0.79 (0.02)	$F_{(64.8)} = 1.3$; $p = 0.250$; $b = 0.04$	0.77 (0.02)	0.78 (0.03)	$F_{(160.7)} = 0.1$; $p = 0.757$; $b = -0.01$
IFN-γ (pg/ml)*	4.66 (0.10)	4.94 (0.09)	$F_{(56.7)} = 0.2$; $p = 0.683$; $b = -0.06$	5.55 (0.13)	4.29 (0.14)	$F_{(95.5)} = 2.0$; $p = 0.161$; $b = 0.26$
IL-6 (pg/ml)*	0.41 (0.08)	0.61 (0.08)	$F_{(62.8)} = 12.4$; $\mathbf{p < 0.001}$; $b = -0.41$	0.42 (0.12)	0.41 (0.12)	$F_{(136.3)} = 0.1$; $p = 0.774$; $b = 0.04$
TNF-α (pg/ml)*	1.77 (0.05)	2.08 (0.06)	$F_{(65.2)} = 4.4$; $\mathbf{p = 0.039}$; $b = -0.16$	1.74 (0.06)	1.81 (0.06)	$F_{(174.7)} = 0.8$; $p = 0.378$; $b = -0.04$
CRP (mg/L)*	1.65 (0.19)	2.29 (0.20)	$F_{(67.7)} = 1.4$; $p = 0.236$; $b = -0.33$	1.41 (0.16)	1.14 (0.16)	$F_{(154.2)} = 1.3$; $p = 0.260$; $b = 0.21$

Data presented as mean (SE).

*SE, standard error; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha; CRP, C-reactive protein; Trp, tryptophan; Kyn, kynurenine; Neo, neopterin; Tyr, tyrosine; Phe, phenylalanine. *SE on log-transformed d. Bold values: significant p-values ($p < 0.05$).*

(38, 39). Our study revealed increased pro-inflammatory markers in patients with BD above 45 years of age. Similarly, Drexhage et al. (40) demonstrated a higher proportion of regulatory T-cells in patients below 40 years, compared to controls. Regulatory T-cells temper the inflammatory response and maintain immune homeostasis and tolerance.

Both the stronger correlation between inflammation and GTP-CH1 and IDO-1 activation and the premature shift toward a pro-inflammatory status in our patient group strengthens the hypothesis of BD as a disease of accelerated aging (11). Due to both acute and chronic stress throughout the course of illness, the compensatory mechanisms in patients show a decreasing capacity to restore homeostasis, resulting in impaired resilience and neuroprogression (10, 11, 41).

However, the associations between biological parameters and characteristics of clinical course are rather small. It remains to be determined whether the differences in biological markers between patients and controls are inherent characteristics of the disease pathophysiology or rather a consequence of confounding factors such as psychopharmacological treatment, smoking status, or other lifestyle factors. Nearly all patients received psychopharmacological treatment that evidently affects monoamine metabolism. Differences in amino acid levels did not remain significant after adjustment for smoking status. Conflicting data on the effect of smoking on monoamine metabolism (42–44) and the high proportion of smokers in our patient group vs. the low proportion in controls make the interpretation of these results difficult.

STRENGTHS AND LIMITATIONS

We included patients in manic, depressive, and mixed episodes and completed approximately 6 test moments during a follow-up of 8 months. The longitudinal design enables a within-person assessment of diverse mood states and the high number of assessments by mood state increases the power of the study. The impact of methodological bias was minimized by standardized blood sampling and uniform, meticulous laboratory procedures. All clinical assessments were done by the same clinician-researcher, excluding interrater bias. Data on illness course and medication use were collected carefully. We used robust, transparent statistical methods. Mixed model analysis enables correction for missed moments, drop-out and a random variation in time and subject. The statistical models were adjusted for possible influences of BMI, smoking status, age, and albumin levels. 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. Apart from the use of anti-inflammatory medication and ECT, there were no treatment restrictions during follow-up. Since patients were included during an acute mood episode, nearly all had changes in psychopharmacological treatment.

CONCLUSION

We found stronger correlations between pro-inflammatory markers and cytotoxic pathways of monoamine metabolism in patients vs. controls. Middle-aged patients and patients with longer duration of illness had increased inflammatory and cytotoxic markers compared to young patients and controls. A pro-inflammatory proneness of patients and a subsequent shift of monoamine metabolism toward more cytotoxic pathways could underlie neuroprogression in BD. Since only few associations are found between biological markers and characteristics of clinical course, it remains to

be determined if alterations in biological markers are due to a disease effect or rather a consequence of confounding factors.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2018.00250/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material: Table S1-S4

Table S1: Symptom severity by mood state in patients (baseline and all test moments combined)				
	Euthymia	**Depression**	**(Hypo)mania**	**Mixed episode**
Baseline				
N		29	29	9
HDRS, mean (SD)		21.2 (3.8)	6.8 (4.2)	21.0 (4.6)
YMRS, mean (SD)		3.8 (3.3)	20.9 (5.3)	15.8 (4.7)
PANSS pos, mean (SD)		8.9 (2.8)	14.7 (4.6)	15.4 (4.3)
PANSS neg, mean (SD)		11.2 (4.9)	8.3 (3.1)	8.9 (2.4)
Psychotic features, N (%)		3 (17.7)	10 (58.8)	4 (23.5)
All test moments (baseline + follow-up)				
N	64	178	71	23
HDRS, mean (SE)	3.9 (2.2)	16.6 (5.8)	6.6 (4.1)	20.5 (4.4)
YMRS, mean (SE)	2.7 (2.4)	3.5 (2.7)	18.3 (7.0)	15.2 (4.4)
PANSS pos, mean (SE)	7.4 (0.8)	8.6 (2.3)	13.5 (4.7)	14.6 (4.2)
PANSS neg, mean (SE)	7.6 (1.3)	10.4 (4.0)	8.3 (2.4)	9.2 (2.4)
Psychotic features, N (%)	0	16 (9.0)	21 (29.6)	7 (30.4)

HDRS: Hamilton depression rating scale; YMRS: Young mania rating scale; PANSS: positive and negative symptom scale

Table S2: Levels of markers of inflammation and monoamine metabolism in patients and controls			
	Patients	Controls	
IFN-γ (pg/ml)*	4.46 (0.07)	4.87 (0.10)	F(92.7)=0.5; p=0.465; b=0.09
IL-6 (pg/ml)*	0.50 (0.06)	0.41 (0.09)	F(96.2)=2.6; p=0.111; b=0.18
TNF-α (pg/ml)*	1.91 (0.04)	1.77 (0.05)	F(99.3)=1.4; p=0.237; b=0.08
CRP (mg/L)*	1.92 (0.13)	1.27 (0.17)	F(100.9)=3.7; p=0.056; b=0.41
Trp (μmol/l)	50.99 (0.87)	54.36 (1.20)	F(92.4)=5.2; p=**0.026**; b=3.37
Kyn (μmol/l)	1.46 (0.04)	1.58 (0.05)	F(97.6)=3.6; p=0.061; b=0.12
Kyn/Trp (μmol/mmol)	29.06 (0.66)	29.41 (0.91)	F(99.3)=0.1; p=0.756; b=0.35
Neo (nmol/l)	5.07 (0.13)	5.04 (0.18)	F(99.6)=0.0; p=0.910; b=0.02
Tyr (μmol/l)	65.48 (2.01)	75.05 (2.77)	F(98.2)=7.8; p=**0.006**; b=9.56
Phe (μmol/l)	50.78 (0.98)	55.93 (1.34)	F(95.0)=9.6; p=**0.003**; b=5.14
Phe/Tyr	0.81 (0.02)	0.78 (0.02)	F(99.4)=1.3; p=0.259; b=0.03

Data presented as mean (SE).
IFN-γ: interferon gamma; IL: interleukin; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; Trp: tryptophan; Kyn; kynurenine; Neo: neopterin; Tyr: tyrosine; Phe: phenylalanine.
*SE on log-transformed data

Table S3: Correlations among biological markers in patients (lower left) and *controls (upper right*), spearman's ρ + significance levels

	Trp	Kyn	Kyn/Trp	Neo	Tyr	Phe	Phe/Tyr	IFN-γ	IL-6	TNF-α	CRP
Trp	1	*0,39***	*-0,29***	*-0,08*	*0,45***	*0,48***	*-0,23***	*-0,04*	*-0,09*	*-0,00*	*-0,20***
Kyn	0,43***	1	*0,72***	*0,19***	*0,27***	*0,26***	*-0,20***	*0,31***	*0,08*	*0,23***	*-0,01*
Kyn/Trp	-0,30***	0,68***	1	*0,27***	*0,00*	*-0,05*	*-0,07*	*0,34***	*0,15*	*0,27**	*0,1*
Neo	-0,07	0,37***	0,43***	1	*-0,02*	*-0,10*	*-0,07*	*0,35***	*0,28***	*0,26***	*0,25***
Tyr	0,38***	0,22***	-0,04	-0,15**	1	*0,72***	*-0,79***	*0,06*	*-0,07*	*-0,00*	*-0,07*
Phe	0,40***	0,19***	-0,10	-0,07	0,69***	1	*-0,21***	*-0,02*	*-0,16**	*-0,06*	*-0,14*
Phe/Tyr	-0,17**	-0,13*	-0,02	0,17**	-0,73***	-0,06	1	*-0,11*	*-0,06*	*-0,04*	*-0,05*
IFN-γ	-0,07	0,12	0,17**	0,31***	-0,09	-0,06	0,05	1	*0,39****	*0,51****	*0,22***
IL-6	-0,18**	0,04	0,19**	0,20**	0,06	-0,05	-0,14*	0,33***	1	*0,50****	*0,48****
TNF-α	0,04	0,33***	0,32***	0,33***	-0,06	-0,06	0,04	0,43***	0,47***	1	*0,17**
CRP	-0,08	-0,03	-0,01	0,23***	0,05	0,02	-0,04	0,17**	0,48***	0,03	1

*p<0.05; **p<0.01; ***p<0.001
Trp: tryptophan; Kyn; kynurenine; Neo: neopterin; Tyr: tyrosine; Phe: phenylalanine; IFN-γ: interferon gamma; IL: interleukin; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein.

Table S4: Correlations of biological markers to symptom severity, spearman's ρ + significance levels			
	HDRS	YMRS	PANSS pos
Trp	-0.13*	0.17**	0.15**
Kyn	-0.14*	0.17**	0.11*
Kyn/Trp	-0.05	0.03	-0.01
Neo	0.02	-0.01	-0.01
Tyr	-0.11*	0.21***	0.21***
Phe	-0.03	0.18**	0.22***
Phe/Tyr	0.13*	-0.10	-0.07
IFN-γ	-0.11	0.02	-0.00
IL-6	-0.08	0.04	0.00
TNF-α	-0.02	0.08	0.08
CRP	0.02	0.05	0.03

*p<0.05; **p<0.01; ***p<0.001

CHAPTER 6

Neurotrophic factors and mood state

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Neurotrophic and inflammatory markers in bipolar disorder: A prospective study

Seline van den Ameele[a,b,*], Violette Coppens[a,b], Jeroen Schuermans[a,b], Peter De Boer[c], Maarten Timmers[d,e], Erik Fransen[f], Bernard Sabbe[a,b], Manuel Morrens[a,b]

[a] Collaborative Antwerp Psychiatric Research Institute (CAPRI), Department of Biomedical Sciences, University of Antwerp, Belgium
[b] University Psychiatric Hospital Duffel, VZW Emmaüs, Duffel, Belgium
[c] Experimental Medicine Neuroscience, Janssen Pharmaceutica N.V., Beerse, Belgium
[d] Janssen Research and Development, a division of Janssen Pharmaceutica N.V., Beerse, Belgium
[e] Reference Center for Biological Markers of Dementia (BIODEM), Institute Born-Bunge, University of Antwerp, Belgium
[f] StatUa – Center for Statistics, University of Antwerp, Belgium

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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

* Corresponding author at: PZ Duffel – Wetenschappelijk Onderzoek, Stationsstraat 22c, 2570 Duffel, Belgium.
E-mail address: seline.vandenameele@gmail.com (S. van den Ameele).

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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.

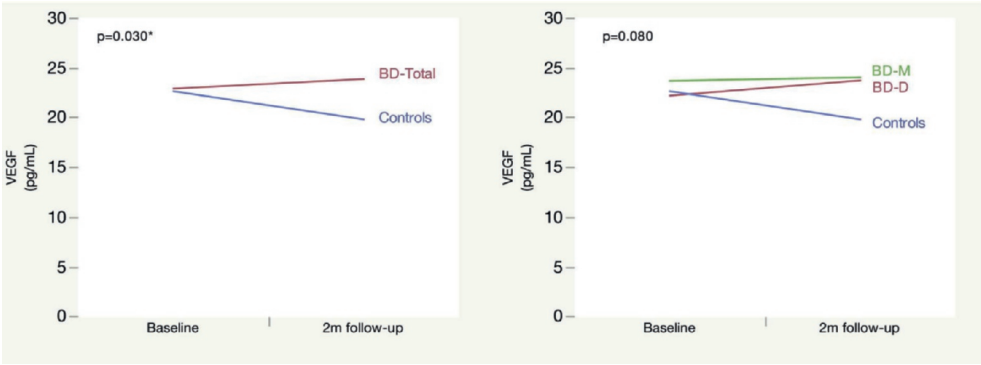

Fig. 1. Group by moment interaction VEGF.
Legend: BD-total: total patient group; BD-D: patient group with depressive episode at baseline; BD-M: patient group with (hypo)manic episode at baseline; VEGF: vascular endothelial growth factor.

$p = 0.030$) 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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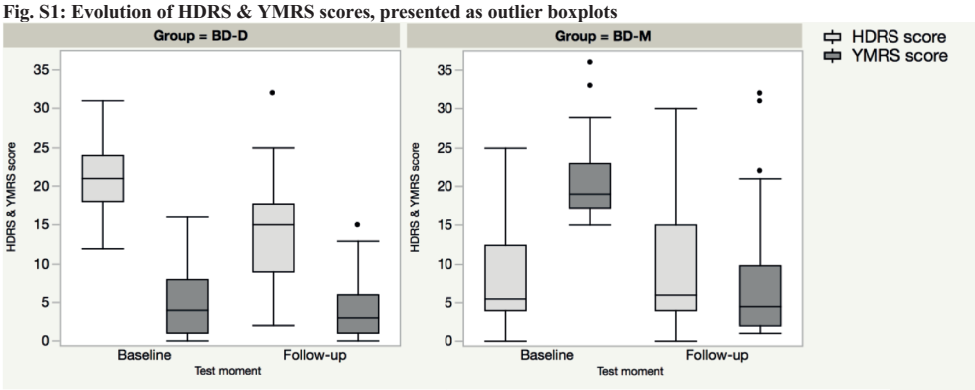
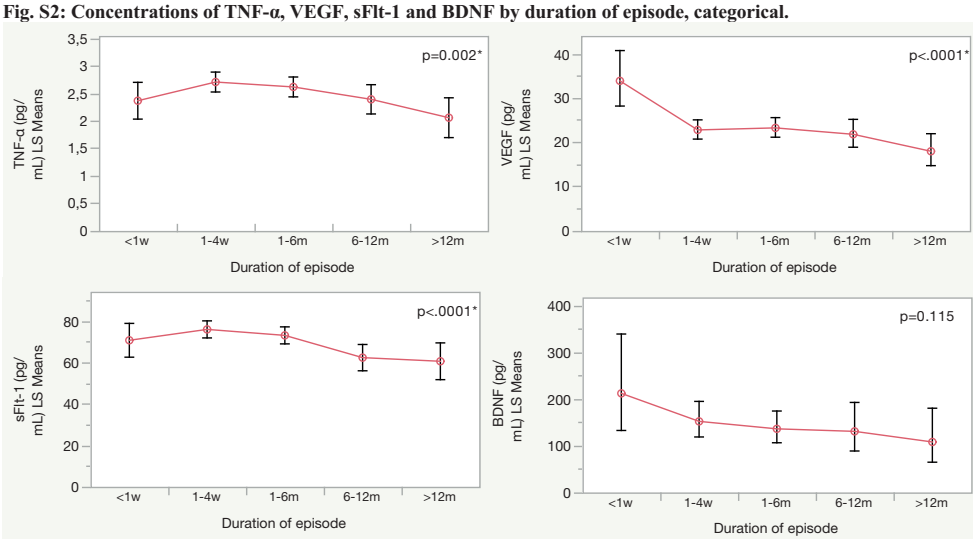
Supplementary material: Figure S1-S2 & Table S1-S5

Fig. S1: Evolution of HDRS & YMRS scores, presented as outlier boxplots



BD-D: patient group with depressive episode at baseline; BD-M: patient group with (hypo)manic episode at baseline; HDRS: Hamilton depression rating scale; YMRS: Young mania rating scale

Fig. S2: Concentrations of TNF-α, VEGF, sFlt-1 and BDNF by duration of episode, categorical.



LS means: least square means; TNF-α: tumor necrosis factor-alfa; VEGF: vascular endothelial growth factor; sFlt-1: soluble fms-like tyrosine kinase-1; BDNF: brain derived neurotrophic factor.

Table S1: Results of fixed effect tests assessing the impact of group (BD-D, BD-M, HC), moment (baseline, follow-up) and group*moment interaction on biological parameters.

	Group	Moment	Group*Moment
TNF-α	F(2, 95.2)=2.96; *p*=0.056	F(86.5)=0.08; *p*=0.777	F(2, 86.5)=0.12; *p*=0.121
BDNF[a]	F(2, 88.9)=0.41; *p*=0.664	F(78.1)=0.06; *p*=0.800	F(2, 78.1)=0.21; *p*=0.815
VEGF[a]	F(2, 92.7)=0.91; *p*=0.407	F(85.2)=0.22; *p*=0.641	F(2, 85.2)=2.60; *p*=0.080
sFlt-1	F(2, 94.8)=0.28; *p*=0.753	F(85.7)=0.46; *p*=0.501	F(2, 85.7)=2.22; *p*=0.115

TNF-α: tumor necrosis factor-alfa; BDNF: brain derived neurotrophic factor; VEGF: vascular endothelial growth factor; sFlt-1: soluble fms-like tyrosine kinase-1.
[a]Outcome variable was log-transformed.

Table S2: Results of fixed effect tests assessing the impact of group (BD-total, HC), moment (baseline, follow-up) and group*moment interaction on biological parameters.

	Group	Moment	Group*Moment
TNF-α	F(96.1)=4.75; *p*=**0.032**	F(87.5)=0.03; *p*=0.864	F(87.5)=0.00; *p*=0.984
BDNF[a]	F(89.3)=0.53; *p*=0.469	F(78.5)=0.02; *p*=0.885	F(78.5)=0.07; *p*=0.789
VEGF[a]	F(92.8)=1.65; *p*=0.203	F(85.3)=1.36; *p*=0.247	F(85.3)=4.89; *p*=**0.030**
sFlt-1	F(95.2)=0.01; *p*=0.912	F(86.4)=0.88; *p*=0.351	F(86.4)=1.44; *p*=0.234

TNF-α: tumor necrosis factor-alfa; BDNF: brain derived neurotrophic factor; VEGF: vascular endothelial growth factor; sFlt-1: soluble fms-like tyrosine kinase-1.
[a]Outcome variable was log-transformed

Table S3: Pairwise correlations: clinical scores vs. biological parameters in pg/ml, presented as Pearson r.

	HDRS	YMRS	PANSS pos. subscale
TNF-α	0.08	0.02	0.07
sFlt-1	0.04	0.02	0.18
BDNF*	-0.07	-0.10	0.04
VEGF*	-0.10	0.06	0.13

HDRS: Hamilton depression rating scale; YMRS: Young mania rating scale; PANSS: positive and negative symptom scale; TNF-α: tumor necrosis factor-alfa; sFlt-1: soluble fms-like tyrosine kinase-1; BDNF: brain derived neurotrophic factor; VEGF: vascular endothelial growth factor.
*Outcome variable was log-transformed.

Table S4: Pairwise correlations: changes in clinical scores vs. baseline biological parameters (pg/ml), presented as Pearson r.

	HDRS-change		YMRS-change		PANSS pos. subscale-change	
	BD-D	BD-M	BD-D	BD-M	BD-D	BD-M
TNF-α	-0.01	-0.11	-0.05	0.03	0.01	-0.07
sFlt-1	-0.001	-0.14	-0.05	0.06	0.18	-0.14
BDNF*	-0.22	-0.05	0.20	-0.30	-0.25	-0.44
VEGF*	-0.23	-0.21	0.10	0.04	-0.22	-0.28

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; sFlt-1: soluble fms-like tyrosine kinase-1; BDNF: brain derived neurotrophic factor; VEGF: vascular endothelial growth factor.
*Outcome variable was log-transformed.

Table S5: Pairwise correlations: changes in clinical scores vs. changes in concentration of biological parameters between baseline and follow-up, presented as Pearson r.

	HDRS-change		YMRS-change		PANSS pos. subscale-change	
	BD-D	BD-M	BD-D	BD-M	BD-D	BD-M
TNF-α change	-0.12	-0.25	-0.22	-0.34	-0.22	0.20
sFlt-1 change	-0.27	0.16	0.03	0.20	-0.12	0.11
BDNF* change	0.07	0.41	-0.25	0.11	0.21	0.55
VEGF* change	0.38	0.35	0.09	0.23	0.25	0.30

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; sFlt-1: soluble fms-like tyrosine kinase-1; BDNF: brain derived neurotrophic factor; VEGF: vascular endothelial growth factor.
*Outcome variable was log-transformed.

CHAPTER 7

Discussion

Discussion

With this project, we aimed to improve our understanding of the pathophysiological mechanisms underlying neuroprogression in BD. We focused on inflammation and its interaction with other pathological processes that are assumed to underlie neuroprogression: BH_4-dependent monoamine metabolism, kynurenine metabolism, and neuroplasticity. First, we will discuss our findings on these three individual pathological processes in relation to inflammation. Next, we will frame the whole of our findings within the theoretical concept of neuroprogression.

Low-grade inflammation in bipolar disorder: present or not?

Current evidence shows a pro-inflammatory state in at least a subset of patients with BD (Rosenblat and McIntyre, 2017). Some studies suggest that, on top of this pro-inflammatory state, there is a further increase in the levels of pro-inflammatory cytokines and C-reactive protein (CRP) during mood episodes. Acute mania has been most robustly linked to inflammation (Fernandes et al., 2016; Munkholm et al., 2013). The evidence for such increases in pro-inflammatory markers however is based on studies with quite a few limitations. There is limited standardization between studies regarding blood sampling conditions, resulting in high heterogeneity regarding the timing of blood sampling and fasting conditions. Confounding factors that influence inflammatory markers are only sporadically controlled for. Age, obesity, smoking habits, physical activity, poor diet, substance abuse, and sleeping problems possibly influence on cytokine levels, but these factors are often not or insufficiently documented (Fernandes et al., 2016; O'Connor, M.F. et al., 2009). While findings on TNF-α and CRP levels have been replicated, findings on other cytokines are often based on single or few studies (Modabbernia et al., 2013; Munkholm et al., 2013). Medication use is a common and practically inevitable confounder in clinical studies on BD. Our systemic literature review (van den Ameele et al., 2016) (Chapter 3) showed that TNF-α and IL-6 levels are consistently increased in medication-free patients during mania and depression. These elevated levels confirm that the increased inflammation during mood episodes is not purely the consequence

of medication use. Medication-free euthymic patients showed no significant differences in cytokine levels compared to controls. The absence of such inflammatory changes during euthymia support a mood state-specific increase in pro-inflammatory cytokines. Mood-stabilizing medication, such as lithium, is thought to have immunoregulatory properties (Nassar and Azab, 2014; Raghavendra et al., 2014). However, we found results on cytokine levels only in medicated patients that were both euthymic and under long-term lithium treatment. Therefore, it remains unclear whether the normalized cytokine levels in euthymic lithium users can be attributed to the euthymic mood state or to direct immunomodulatory effects of longer-term lithium use. To further unravel the complex interaction between mood symptoms, cytokine levels and mood-stabilizing medication, we need longitudinal studies with medication-free baseline measurements, randomized controlled treatment protocols using single drugs, and close monitoring of mood states.

In contrast to our findings in the systematic literature review (Chapter 3), in our study sample (Chapter 4, 5 and 6), we found no obvious differences in inflammatory markers between patients and controls. Both at baseline during an acute mood episode and during follow-up, levels of inflammatory markers remained comparable between patients and controls. While this is in contrast with our primary hypothesis, some findings regarding the inflammatory markers are worth highlighting. First, in our report on neurotrophic markers and TNF-α (Chapter 6), we performed analyses on a subset of samples at baseline and 2 months follow-up. In this subset, TNF-α levels were higher in patients than in controls, however after adjustment for smoking status this difference was no longer statistically significant. In those patients reaching euthymia at 2 months follow-up, TNF-α levels had significantly decreased compared to baseline. While this decrease suggests a mood state-related increase in TNF-α levels, we could not replicate these changes in the entire longitudinal dataset (Chapter 4 and 5), and therefore we cannot draw solid conclusions.

Second, in the entire longitudinal dataset, we found that in patients and not in controls pro-inflammatory markers were increased with older age (Chapter 5). A chronic subclinical inflammation and immune system dysfunctions are known phenomena accompanying healthy aging, termed immunosenescence (Franceschi et al., 2000). In our study sample, we found evidence for a premature onset of this pro-inflammatory shift in patients with BD.

In sum, in contrast to most current evidence, including the results of our systematic literature review (Chapter 3), we found no direct evidence for either the mild pro-inflammatory state or mood state-related changes in cytokine levels in patients with BD in our study sample. There are some possible explanations for these discrepancies.

First, it is good to point out that our findings are in line with quite a few studies on inflammation in BD. These studies also could not find differences between patients with BD and controls and also revealed that cytokine profiles are highly heterogeneous (Fiedorowicz et al., 2015; Jacoby et al., 2016; Rosenblat and McIntyre, 2017). Both publication and citation bias may thus overestimate the contribution of inflammation to the pathogenesis of BD (Cristea and Naudet, 2018). A more refined view would state that inflammation is present in at least a subset of patients with BD (Rosenblat and McIntyre, 2017). This 'subset' of patients presenting with low-grade inflammation remains to be characterized clinically (Rosenblat and McIntyre, 2017). We could not identify such a subset of patients with low-grade inflammation.

Second, since the pro-inflammatory state in patients with BD is assumed to be mild, we are prone to make type II errors due to insufficient power. Especially during euthymia, we expect to see only small differences (small effect size) between patients and controls and therefore, to avoid false negative results, we would need large sample sizes. In addition, our patient sample was characterized by large heterogeneity regarding several clinical aspects (illness duration, type of bipolarity, severity of mood symptoms, medication use, history of substance abuse, etc.) which makes it even more difficult to discern differences between patient and control groups. A larger or more homogeneous sample might have discerned different results.

The complex interaction between inflammation, kynurenine metabolism, BH_4 dependent monoamine metabolism and mood

By stimulation of the GTP-CH1 and IDO-1 enzymes, cytokines are able to respectively alter BH_4 dependent monoamine and kynurenine metabolism. Both pathways are known to impact neurotransmitter systems essential for mood regulation (Myint and Kim, 2014; Vancassel et al., 2018). In our research project, we aimed to better understand the complex interaction between inflammatory changes, changes in kynurenine and monoamine metabolism, and mood symptoms. We hypothesized to find mood state-related dynamics in levels of inflammatory markers, resulting in mood state-related dynamics in kynurenine metabolites

and monoamines. The lack of obvious differences in inflammatory markers between patients and controls hampers our ability to draw conclusions on downstream changes in kynurenine metabolites and monoamines.

Nevertheless, we did find stronger associations between pro-inflammatory markers and the cytotoxic pathways of both kynurenine and BH_4-dependent monoamine metabolism in patients compared to controls.

First, in patients we saw a higher IDO-1 activation with aging (Chapter 5) and a shift in kynurenine metabolism towards the neurotoxic branch when inflammatory markers were elevated (Chapter 4). This 'neurotoxic' interaction between inflammatory markers and kynurenine metabolites was even stronger in the presence of mood symptoms. While during chronic depressive symptoms a major role is attributed to IFN-γ, in the acute context of mania, TNF-α seems to drive this neurotoxic shift.

Regarding BH_4 dependent monoamine metabolism, we found a stronger correlation between PHE/TYR and neopterin in patients with BD. This pattern of monoamines is typically found during chronic inflammation and suggests increased GTP-CH1 activity in turn leading to increased neopterin synthesis in macrophages at the expense of BH_4 formation. The latter is an essential cofactor for monoamine synthesis (Neurauter et al., 2008) (Chapter 5). We indeed saw low TRP, TYR and PHE especially during depression, but our data do not allow us to conclude that these low levels are the consequence of decreased BH_4 availability. Our study did not show a clear relation between mood, inflammation and BH_4 dependent monoamine metabolism. Recent studies describe a link between kynurenine metabolism and BH_4 dependent monoamine metabolism resulting in a synergistic compromising effect on monoamine synthesis. Xanthurenic acid, a metabolite of 3-HK, directly inhibits BH_4 biosynthesis (Haruki et al., 2016) and due to the formation of free radicals by 3-HK and QA, BH_4 availability further decreases through an oxidative loss (Oxenkrug, 2011). BH_4 availability could thus be affected by inflammation both as a result of preferential neopterin synthesis and as a result of the adverse effects of several kynurenine metabolites. Indeed, BH_4 measurements would complement our findings as it would allow us to identify the underlying cause of the low levels of monoamine metabolites and the possible role of inflammation.

Neurotrophic markers: can methods be improved?

In Chapter 6, we investigated mood-specific changes in neurotrophic markers in patients with BD. In contrast to many studies reporting low BDNF levels in patients with BD (Fernandes et al., 2015; Munkholm et al., 2016), BDNF levels did not differ between patients and controls in our sample. Vascular endothelial growth factor (VEGF) and one of its soluble receptors (sFlt-1) were included as a screening for other neurotrophic factors, but no differences between patients and controls were found. Correlations between symptom severity scales and neurotrophic factors levels were weak, but we did find a significant opposed evolution in VEGF levels between patients and controls. VEGF in patients decreased from baseline till 2 months while in controls the trend was an increase. These unexpected findings (i.e. no difference in BDNF levels between patients and controls and the increasing VEGF trend in controls) have prompted us to devote special attention to possible methodological issues in this Chapter. Nevertheless, we did not find methodological problems leading to biased results. While several reports claim that neurotrophic markers improve our understanding of disease onset and activity, treatment response and illness progression (Frey et al., 2013), many of these studies have methodological problems. Along with other groups (Munkholm et al., 2016; O'Connor, M.F. et al., 2009; Zhou et al., 2010), we have highlighted the problem of weak standardization of clinical and laboratory procedures among these studies and stressed a need for methodological guidelines (van den Ameele et al., 2017).

In a systematic approach to future research on peripheral biomarkers in psychiatric disorders, we strongly advocate for methodological studies on biomarker stability. A strict assessment of blood sampling procedures, storage conditions, blood fraction, type of assay and data analysis is necessary for qualitative recommendations. Consensus on clinical and laboratory practices will increase the chance to find clinically relevant biomarkers.

What did we learn about neuroprogression in bipolar disorder?

Most of our results are in line with the concept of neuroprogression and allow us to unravel some dynamics of the underlying pathophysiology. In patients with BD we see an increase in pro-inflammatory markers with longer duration of illness and this mild pro-inflammatory state presents at an earlier age than expected in healthy aging controls (Chapter 5). Neopterin, as marker of inflammation, is associated with decreased BH_4 availability in patients, possibly

compromising the synthesis of monoamine neurotransmitters (Chapter 5). Furthermore, pro-inflammatory cytokines are associated with increased levels of neurotoxic kynurenine metabolites further exacerbated in the presence of mood symptoms (Chapter 4). Meanwhile, a decreased activity of the neuroprotective branch of kynurenine metabolism is seen in patients independent of mood state or inflammation (Chapter 4). Our systematic literature review indeed concludes that a mood state-related increase in pro-inflammatory markers is present in medication-free patients (Chapter 3). However, such inflammatory changes were not seen in our study sample. Overall, our results show that both the acute and chronic stress throughout the course of illness are accompanied by an increase in inflammatory and neurotoxic substances that possibly impact neurotransmission through effects on BH_4 dependent monoamine and kynurenine metabolism. Such processes would, according to the allostatic load theory, exert pressure on the compensatory mechanisms of the body, which results in neuroprogression (Berk et al., 2011; Kapczinski et al., 2008b; Rizzo et al., 2014).

Neuroprogression in BD: many questions remain

Our conclusions are mainly based on differences in associations seen between different (patho)physiological processes in the patient group as compared to the control group. Despite a proper power analysis and sample size determination, few quantitative differences in levels of biological markers have been identified. Our study on neurotrophic markers in particular could not confirm the contribution of such factors to neuroprogression. These negative data raised critical questions on the current evidence on peripheral biomarkers and the biological validity of the concept of neuroprogression. Clinical and methodological heterogeneity within and in-between existing studies indeed requires cautious interpretation of data, and our findings should be interpreted with similar precautions. This hesitating attitude gives rise to several questions, as discussed below.

1. *What can these peripheral findings tell us about central processes?*

Although neuroprogression has systemic implications, mainly central processes are assumed to contribute to the development of psychiatric symptoms, cognitive impairment, treatment resistance and chronicity. The extent to which peripheral biomarkers mirror central processes is largely unknown and studies to date have found conflicting results. Communication of

immune processes across the blood-brain barrier (BBB) is often studied and involves several pathways (Dantzer et al., 2008). First, there is a neural route whereby peripheral inflammation is communicated towards the brain through the activation of afferent (vagal) nerves. Second, there are humoral routes including both active transport of cytokines across the BBB and passive transport across leaky regions, e.g. the circumventricular organs. Third, a cellular route due to infiltration in the brain by activated peripheral monocytes and macrophages and the secretion of inflammatory products by cells that form the BBB (Dantzer et al., 2008; Miller et al., 2013; Quan and Banks, 2007). However, the specific effects of cytokines that pass across the BBB on brain and immune activity, especially in the context of stress or disease, are unknown and may be different for different cytokines (Munkholm et al., 2013; Quan and Banks, 2007). An important consideration in this context is that peripheral inflammation contributes to the pathophysiology of several somatic disorders with high prevalence in BD (i.e. cardiovascular disease, obesity, diabetes mellitus, and autoimmune disorders). Findings on peripheral cytokines may therefore also reveal relevant information on the systemic complications or associations of BD and contribute to the hypothesized view of BD as a multi-system inflammatory disorder (Leboyer et al., 2012; Munkholm et al., 2013).

While amino acids such as TYR, TRP and PHE readily cross the BBB by the large neutral amino acid transporter (Hawkins et al., 2006), BBB-crossing of neopterin is limited (Eisenhut, 2013). Regarding BDNF and kynurenine metabolites, studies on communication across the BBB are limited. BDNF has been demonstrated to cross the BBB, but correlations between its peripheral and central levels are conflicting (Klein et al., 2011; Lanz et al., 2012; Pan and Kastin, 1999; Sartorius et al., 2009). The kynurenine metabolites TRP, KYN and 3-HK readily cross the blood-brain barrier by active transport, but passage of QA and KYNA is again limited (Fukui et al., 1991; Schwarcz and Stone, 2017). A recent study by Sellgren et al. (2019) showed the absence of a relevant correlation between CSF and plasma KYNA levels.

Many questions regarding the analysis of peripheral biomarkers and their implication in central processes remain open. Analyzing central markers on the other hand is complicated in clinical populations. Peripheral markers offer several advantages: easy sample collection, lower cost, applicable at large scale. While peripheral markers may fail to quantitively represent central effects, a better understanding of their peripheral dynamics and their relation to illness characteristics could nevertheless contribute to the identification of relevant biomarkers for disease activity and progression.

1. *Can our findings be translated into a concrete pathophysiological process?*

As discussed above, meta-analytical findings of increased levels of TNF-α and CRP in patients with BD are rather robust and show increased inflammation in at least a subset of patients with BD (Modabbernia et al., 2013; Munkholm et al., 2013; Rosenblat and McIntyre, 2017). Increased levels of pro-inflammatory markers in mood disorders and schizophrenia are often linked with an abnormally activated mononuclear phagocyte system (Beumer et al., 2012).

In normal situations, microglial activation upon acute stress or brain trauma results in a balanced release of both pro-inflammatory cytokines and neurotrophic factors that will dampen tissue damage and stimulate repair. Because of a repeated microglial activation during several stressful mood episodes, the downregulatory capacities of the system get exhausted which results in a constant microglial activity with preferential production of pro-inflammatory cytokines at the expense of neurotrophic factors (Stertz et al., 2013). This response has been described as the polarization of activated microglia towards a more 'toxic' profile (M1-type), which contrasts with the resting-state microglia that bear a 'repair' profile (M2-type) (Ascoli et al., 2016). Microglial polarization towards the M1-type in situations of chronic or recurrent stress lead to a chronic elevation in pro-inflammatory cytokine levels which in turn inhibit neurogenesis and synaptic pruning, induce further microglial activation and eventually could lead to BBB-disruption and systemic toxicity (Ascoli et al., 2016; Stertz et al., 2013).

Increased activity of macrophages and microglia has indeed been described in schizophrenia and BD (Ascoli et al., 2016; Haarman et al., 2014; Marques et al., 2018; Monji et al., 2009; van Berckel et al., 2008), however in BD, data are very limited and poorly replicated. Haarman et al. (2014) showed by PET imaging an increased microglial activity in the right hippocampus. Increased microglial activity has also been demonstrated in patients with BD compared to healthy controls based on changes in biomarker levels in the CSF (Jakobsson et al., 2015; Rolstad et al., 2015). In peripheral blood monocytes a mood state-related pro-inflammatory gene expression has been shown in patients with BD (Becking et al., 2015). Results of post-mortem research are inconclusive. Increased microglial activity in the frontal cortex of patients with BD versus controls has been found (Rao et al., 2010), while in the dorsolateral prefrontal cortex, decreased glial density and glial hypertrophy have been described (Rajkowska et al., 2001). However, Steiner et al. (2011) could not confirm increased microglial activity in the anterior cingulate cortex of postmortem brains of patients with BD.

Although our study does not directly address markers that assess the activity of macrophages or microglia, our findings do support an aberrant activation of the mononuclear phagocyte system. In healthy aging, a pro-inflammatory state appears and is accompanied by dysfunctional microglia and macrophages (Franceschi et al., 2000; Rawji et al., 2016). Aging microglia are characterized by increased production of pro-inflammatory cytokines and decreased reparative activity (Rawji et al., 2016). The premature onset of such a pro-inflammatory state in patients with BD recruited in our study suggests that this chronic microglial activation with a rather 'toxic' profile, appears at an earlier age (Chapter 5). Also, the finding of a preferential activation of the neurotoxic, microglial, branch of kynurenine metabolism in response to inflammation, which is most pronounced during acute mood episodes, is in line with this theory and illustrates the imbalanced microglial activity towards a more toxic profile (Chapter 4). The stronger relation between increased neopterin levels and reduced BH_4 formation similarly suggests an increased activation of macrophages in patients compared to controls (Chapter 5).

Although solid data are absent, this increased activity of the mononuclear phagocyte system with polarization towards a more 'toxic' profile throughout the illness course and more pronounced during acute mood episodes, could increase allostatic load, induce a allostatic overload, eventually leading to reduced resilience and neuroprogression.

2. Can our findings be translated into clinical characteristics of neuroprogression?

Our study was designed to assess the impact of acute mood episodes on pathological processes that might contribute to neuroprogression. Acute mania and chronic subsyndromal depressive symptoms were associated with a neurotoxic shift in kynurenine metabolism in response to inflammation (Chapter 4). In addition, we found an increase in pro-inflammatory markers and IDO-1 activation at an early age when compared to healthy controls and even more so with longer duration of illness (Chapter 4). These findings suggest that the occurrence of mood symptoms, both acute severe symptoms and chronic mild symptoms, are associated with increased neurotoxicity and inflammation. These processes might contribute to increasing allostatic load and neuroprogression.

We found no other association between biological markers and characteristics of illness severity or progression as number of mood episodes and hospitalizations, BD type I or II, history of psychosis etc. Since investigating the latter associations was not a primary study

aim, it was only assessed by post hoc analyses. The complex interaction between multiple biological processes on one side and the high clinical heterogeneity on the other side hampers the identification of clinically relevant biomarkers. The translation of biological findings in clinical characteristics will require larger and more homogeneous study populations.

3. What about causality?

Increased inflammation and neurotoxicity could be the consequence of comorbidities of psychiatric disorders such as obesity, poor diet, smoking habits and sleeping problems. Ample evidence however indicates that inflammation may, at least in some cases, directly cause depression. Numerous pre-clinical studies have shown that inducing a pro-inflammatory state in animals results in 'sickness behavior' with depressive-like symptoms as lethargy, anhedonia, and anorexia (Moreau et al., 2008; Rosenblat et al., 2014). In humans, depressive symptoms are also seen after immune stimulation through vaccination or IFN therapy (Niemegeers et al., 2016; Raison et al., 2005; Udina et al., 2012). Animal studies also showed that IDO-activation in the brain is required to develop depressive-like symptoms upon immune stimulation (O'Connor, J.C. et al., 2009; Savitz, 2017), suggesting that IDO-activation is an essential intermediate between inflammation and mood symptoms. Since psychological stress also increases peripheral levels of inflammatory cytokines (Steptoe et al., 2007), a bidirectional link between mood and inflammation seems most likely (Rosenblat et al., 2014).

However, in this thesis, we did not intend to make statements on causality. We described a pathological process occurring along the course of illness which possibly contributes to increasing allostatic load, decreased resilience and illness progression.

4. How can our findings contribute to better prognosis?

The view of BD as a neuroprogressive disorder points out the need for early intervention and prevention of increased allostatic load and illness progression. Based on the results of our research, we see following therapeutic targets: the premature pro-inflammatory shift, a disbalanced kynurenine metabolism towards the neurotoxic branch, and a decreased availability of BH_4.

Several therapeutic interventions targeting these disease characteristics have been proposed in scientific literature.

Anti-inflammatory agents have been tested in clinical trials during different mood states, usually as adjunctive therapy to standard mood-stabilizing treatment. Effect sizes are moderate, but clinical applicability remains limited due to small study samples and little replication of the findings. Proposed anti-inflammatory agents are, among others, N-acetyl-cysteine, non-steroidal anti-inflammatory drugs, omega-3 polyunsaturated fatty acids, minocycline, and TNF-α inhibitors (Ayorech et al., 2014; Rosenblat et al., 2016). As suggested in our systematic literature review, conventional mood-stabilizing drugs as lithium and valproate are also thought to modulate the immune system, indeed possibly contributing to their mood-stabilizing effects (Rosenblat and McIntyre, 2017; Vancassel et al., 2018).

Restoring the imbalanced kynurenine metabolism is possible by several ways, mostly investigated in preclinical studies. Possible interventions are enzyme inhibitors (IDO and KMO inhibition), reducing kynurenine transport towards the brain, increasing KYNA availability or augmenting its neuroprotective effect by NMDA receptor antagonism (Vancassel et al., 2018). BH_4 supplementation has been poorly studied with inconsistent results and unknown effect on neurotransmitter synthesis (Vancassel et al., 2018). Some non-pharmacological interventions could also influence these pathways. Obesity for example is accompanied by chronic low-grade inflammation (Capuron et al., 2017; Hotamisligil, 2006) and weight loss has been shown to reduce inflammation and kynurenine pathway activation (Aleman et al., 2017) together with depressive symptom improvement (Capuron et al., 2011a). Since the high somatic comorbidity of cardio-vascular disease and insulin resistance, a strict screening for metabolic risk factors and the application of an earlier setpoint for intervention is likely needed (Kilbourne et al., 2004; Leboyer et al., 2012).

Finally, since the neurotoxic shift in kynurenine metabolism in response to inflammation is accompanying both acute severe and chronic subsyndromal mood symptoms, complete remission should be the first aim in the treatment of mood episodes.

CHAPTER 8

General conclusion and perspectives

General conclusion and perspectives

The concept of neuroprogression aims to explain the decreasing resilience often found in patients with BD that starts from the premorbid stage with normal functioning and progresses in the late-stage illness with chronic mood symptoms, somatic comorbidity, cognitive and functional impairment. Despite these clinical observations, the pathophysiological mechanisms underlying neuroprogression are still largely unknown. The proposed biological mechanisms involve several interacting systems both central and peripheral. In our research project we studied patients with BD during a short period of the diseases' course, i.e. an acute mood episode and the subsequent months. During this follow-up period, we assessed several biological markers to try to elucidate some of the underlying pathological processes that contribute to neuroprogression. We found some evidence for an increase in pro-inflammatory markers and neurotoxic substances along the chronic illness trajectory and during acute mood episodes.

Our findings are in line with the hypothetical framework in which an abnormally activated mononuclear phagocyte system leads to increased systemic and central toxicity with decreased neurogenesis and repair. Importantly, our findings raise several questions on methodological aspects of studies in this domain and their potential for translation into clinical tools. We therefore would like to propose some perspectives and ideas for future research:

Methodology

Biomarker studies in psychiatric disorders are becoming more popular in view of the potential of biomarkers in risk assessment, early detection and monitoring of illness activity and progression (Pfaffenseller et al., 2013). Awareness of confounding demographic and clinical factors is increasing. This awareness will remain essential for future studies (O'Connor, M.F. et al., 2009). However, most urgently needed is a consensus on several aspects of biomarkers: the biodynamics of individual markers, the correlation between central and peripheral levels, the use of ratios between markers to represent an enzyme activity, the appropriate laboratory methods regarding way of sampling, stability of markers, specificity and sensitivity of analyses,

and the interpretation of results. Intensive collaboration between psychiatric and biomedical researchers will be needed to improve current methods.

The risk of citation bias was mentioned above as it is particularly relevant to our research field with so many conflicting results and unanswered questions. We were also confronted with this problem as we interpreted our study findings in a context which can easily be biased to fit the results. We tried to present a balanced overview of the current evidence and our own findings. Critical review of original research papers, replication studies, and transparent meta-analyses and systematic literature reviews are needed to deal with citation bias.

Clinical translation

Since the associations between biological markers and clinical characteristics of illness progression are still limited, neuroprogression remains a rather theoretical concept. Most studies investigated BDNF as marker of illness progression and suggested an inverse correlation between BDNF and mood symptom severity (Cunha et al., 2006; Fernandes et al., 2015; Machado-Vieira et al., 2007), an increase in BDNF in treatment responders (de Sousa et al., 2011; Tramontina et al., 2009) and a negative correlation with duration of illness (Kauer-Sant'Anna et al., 2009). Meta-analyses however do not confirm unanimously the mood state-related BDNF changes (Fernandes et al., 2015; Munkholm et al., 2016). Inflammatory markers have been linked to acute mood states, but findings on their association with illness progression are limited (Fernandes et al., 2016; Grande et al., 2014; Kauer-Sant'Anna et al., 2009). Also regarding BH_4 dependent monoamine metabolism and kynurenine metabolism, the link between alterations in these pathways and illness progression in BD has been understudied. In MDD, Savitz (Savitz et al., 2015b) found negative correlations between a neuroprotective ratio in kynurenine metabolites (KYNA/QA) and illness severity, but these correlations were not replicated in BD (Savitz et al., 2015a). Our research shows an increased correlation between inflammation and neurotoxic kynurenine metabolism related to illness activity and an increase in pro-inflammatory markers at early age and with longer duration of illness. We could not find other associations between biological markers and illness progression. Longitudinal studies with detailed and comprehensive monitoring of both clinical characteristics and biological markers in patients with BD could further clarify this association and direct future research questions.

An increased pro-inflammatory state may be present in only a subset of patients with BD, as could be the case for neuroprogression which is present in most, but not all, patients with BD (Passos et al., 2016). Why some patients suffer from a severe progressive course while others can attain full remission is unknown. Identification of those subgroups of patients vulnerable for a progressive illness course could target early interventions. Again, careful longitudinal observation of both clinical and biological markers in patients with BD is needed.

Illness trajectories in BD are highly variable and no single model will fully explain the complex underlying pathophysiology. Hopefully, in future, a multifactorial model might have a role in disease monitoring and prediction of outcome. With our research project we aimed to integrate a few pathways that possibly contribute to neuroprogression. We hope future studies will replicate and validate our findings and that new pathways will be identified and incorporated in the neuroprogression model. Only through an improved understanding of the different pathways underlying neuroprogression and their role in the illness course of the individual patient, will we be able to develop novel therapies to halt the progressive illness course and improve the quality of life of patients with BD.

Summary

Summary

Bipolar disorder is a recurrent chronic disorder characterized by fluctuations in mood and energy. The course of illness is highly variable among individuals, but many patients experience a progressive course with shortening symptom-free intervals, increasing treatment resistance, psychiatric and physical comorbidities and cognitive deficits leading to functional impairment and reduced quality of life. Pathophysiological mechanisms underlying the disease and its progressive course are still largely unknown, which impedes the development of strategies for early intervention and treatment. Mechanisms as immune system dysregulation, disturbances in neurotransmitter synthesis, increased oxidative stress, and impaired neuroplasticity are proposed to contribute to the progressive course, but are still not fully understood. An abnormally activated mononuclear phagocyte system in mood disorders would lead to a chronic low-grade inflammatory state with increased levels of pro-inflammatory cytokines. Increased levels of pro-inflammatory cytokines have indeed been demonstrated in at least a subset of patients with bipolar disorder compared to controls.

Although the finding of an increased pro-inflammatory state in patients with bipolar disorder is rather robust according to results of meta-analyses, cautious interpretation of results remains warranted because of many confounding factors present in clinical research populations. For example, in bipolar disorder, often complex mood-stabilizing drug schemes are prescribed with unknown effect on cytokine levels. In our systematic literature review (Chapter 3), we assessed cytokine levels in medication-free patients with bipolar disorder and the effect of single mood-stabilizing drugs on these levels. Our results showed evidence for a mood state-related increase in pro-inflammatory cytokines (TNF-α and IL-6) with normalization during euthymia in medication-free patients. Euthymic lithium users had cytokine levels similar to healthy controls. Immunomodulatory properties are attributed to lithium in the literature. However, based on the results of our systematic literature review, it is uncertain whether the normalized cytokine levels in euthymic lithium users can be attributed to the euthymic mood state or to immunomodulatory effects of longer-term lithium use. A combined effect seems most plausible.

Cytokines are able to contribute to the development of mood and psychotic symptoms by stimulating key enzymes in both kynurenine metabolism and BH_4 dependent monoamine synthesis. Kynurenine metabolism gives rise to several neuroactive metabolites, some with neurotoxic and others with rather neuroprotective properties. Disbalances in these kynurenine metabolites are described in psychiatric disorders as schizophrenia, major depressive disorder, and to a lesser extent in bipolar disorder. By their impact on neurotransmission and oxidative stress, disbalances between neurotoxic and neuroprotective kynurenine metabolites could contribute to the development of psychiatric symptoms and increased neurotoxicity. In situations of chronic inflammation, neopterin is synthesized at the expense of BH_4, an essential cofactor in the synthesis of neurotransmitter monoamines. Neopterin is produced in activated macrophages and used as a marker of macrophage activity and oxidative stress. Decreased BH_4 availability results in impaired monoamine synthesis, possibly leading to disturbances in neurotransmitter synthesis.

The interaction between the immune system and these two pathways has frequently been described in reviews and investigated in pre-clinical research. Objectivation of this interaction in clinical populations with psychiatric disorders is however very limited and this in particular for bipolar disorder. Also, the association of these pathological processes to mood fluctuations and illness progression has scarcely been studied.

In our study, we aimed for an integrated and longitudinal evaluation of mood symptoms as well as inflammation, kynurenine metabolism and BH_4 dependent monoamine synthesis in patients with bipolar disorder versus healthy controls. We included patient with bipolar disorder during an acute mood episode (depression or (hypo)mania) and had 6 test moments over the course of 8 months follow-up.

No differences in inflammatory markers (CRP, IFN-γ, TNF-α and IL-6) were found between patients in different mood states and controls (Chapter 4 and 5). Similar to a pro-inflammatory status accompanying healthy aging, we saw increasing pro-inflammatory markers with age, but this with a premature onset in patients. This increase in pro-inflammatory markers was also apparent in patients with longer duration of illness (Chapter 5).

Independent of mood state, patients had low KYNA levels, a putative neuroprotective kynurenine metabolite. In patients with chronic subsyndromal depressive symptoms, we found a decreased neuroprotective ratio KYNA/3-HK during the whole follow-up period. In

presence of mood symptoms, i.e. acute manic as well as chronic subsyndromal depressive symptoms, we saw a strong association between inflammation and a neurotoxic shift in kynurenine metabolism. During mania, TNF-α was strongly correlated to neurotoxic kynurenine metabolites (3-HK and QA), while during chronic depressive symptoms, IFN-γ was related to increased neurotoxic QA and activation of IDO-1, the key enzyme in activation of kynurenine metabolism in response to pro-inflammatory cytokines. (Chapter 4)
Regarding BH_4 dependent monoamine synthesis (Chapter 5), monoamines levels (TYR, TRP and PHE), were lower in patients and this most pronounced during depression. Furthermore, we saw an increased correlation between neopterin and PHE/TYR in patients overall, typical for situations of chronic inflammation and suggesting increased neopterin formation at the expense of BH_4 synthesis and compromised monoamine synthesis. A clear link between mood state, inflammation and monoamine synthesis could however not been demonstrated.

In normal situations, pro-inflammatory and neurotoxic processes balance with neurotrophic processes, hence preserving homeostasis. In bipolar disorder however, the important neurotrophic factor BDNF is decreased. Since BDNF is shown to further decrease during acute mood episodes and with longer duration of illness, this trophic factor has also been put forward as a marker for illness activity and progression. However, as this finding is poorly replicated, more research is needed to understand the role of BDNF in illness progression and to identify other adequate neurotrophic markers.
We assessed BDNF, VEGF and sFlt (soluble VEGF receptor) in patients with bipolar disorder suffering from mania or depression at baseline and at 2 months follow-up (Chapter 6). Despite adequate sample size and rigorous methodology, we could not identify differences in levels of these trophic markers between patients and controls, neither identify mood state-related alterations. Better understanding and improvement of methodological issues as sampling methods, stability of markers and interpretation of data are needed to guide for future research on neurotrophic markers.

To conclude, our research shows evidence for increased neurotoxicity along the illness trajectory of bipolar disorder with more pronounced toxicity when mood symptoms were present. This increased neurotoxicity in patients was inferred from a premature pro-inflammatory state, a stronger association between neurotoxic kynurenine metabolites and inflammation, and a compromised monoamine synthesis. Our data should be interpreted with caution and will need replication. Finally, we formulate methodological suggestions for future research on biomarkers in bipolar disorder (Chapter 8).

Samenvatting

Samenvatting

Bipolaire stoornis is een chronische ziekte gekenmerkt door wisselingen in stemming en energieniveau. De ziekte kent een heterogeen verloop, maar vaak zien we een progressief beeld met steeds kortere symptoomvrije intervallen, toenemende behandelresistentie, het optreden van psychiatrische en somatische comorbiditeit, en het ontwikkelen van cognitieve functiestoornissen die een belangrijke impact hebben op het dagelijks functioneren en de levenskwaliteit. Omdat de onderliggende pathofysiologie van de ziekte en zijn progressief verloop nog grotendeels ongekend is, blijven de mogelijkheden op vlak van vroegtijdige interventie en behandeling beperkt. Een ontregeld immuunsysteem, verstoringen in neurotransmissie, toegenomen oxidatieve stress en verminderde neuroplasticiteit zouden volgens de huidige wetenschappelijke literatuur een rol spelen. Op vlak van het immuunsysteem wordt een abnormale activiteit van het mononucleair–fagocytair systeem beschreven, wat zou leiden tot een chronische mild proinflammatoire status. Gestegen concentraties van proinflammatoire cytokines werden inderdaad aangetoond bij personen met bipolaire stoornis ten opzichte van gezonde controlepersonen. Hoewel deze bevindingen bevestigd worden door de resultaten van verschillende meta-analyses, blijft een voorzichtige interpretatie aangewezen door talrijke mogelijks verstorende factoren die typisch aanwezig zijn bij klinisch naturalistisch onderzoek. Bij personen met bipolaire stoornis worden bijvoorbeeld vaak complexe medicatieschema's voorgeschreven. Een effect van medicatie op cytokineconcentraties wordt beschreven in de literatuur, maar is onvoldoende gespecifieerd. In een systematisch literatuuroverzicht (Hoofdstuk 3) evalueerden we cytokineconcentraties bij medicatievrije patiënten met een bipolaire stoornis en het effect van stemmingsstabiliserende geneesmiddelen op deze concentraties. De resultaten bij medicatievrije patiënten tonen een proinflammatoire immuunrespons tijdens manie. Euthymie en langdurig lithiumgebruik zijn geassocieerd met normale cytokineconcentraties. Op basis van dit literatuuronderzoek kan niet besloten worden of de normale cytokineconcentraties bij euthyme lithiumgebruikers te wijten zijn aan de euthyme stemmingstoestand of aan de mogelijks immunomodulerende effecten van lithium. Een gecombineerd effect lijkt het meest waarschijnlijk.

Cytokines kunnen bijdragen tot het ontwikkelen van stemmingssymptomen en psychose door het stimuleren van enzymen met een sleutelrol in twee biologische pathways: (1) de afbraak van kynurenine en (2) de BH_4-afhankelijke vorming van monoamines. (1) Afbraak van kynurenine leidt tot verschillende neuro-actieve producten. Sommige hebben neurotoxische eigenschappen, andere zijn eerder neuroprotectief. Een onevenwicht tussen deze afbraakproducten van kynurenine wordt beschreven bij psychiatrische ziektes als schizofrenie, depressie en in mindere mate ook bij bipolaire stoornis. Door hun impact op neurotransmissie en oxidatieve stress, kan een onevenwicht tussen neurotoxische en neuroprotectieve producten bijdragen tot het ontwikkelen van psychiatrische symptomen en neurotoxiciteit. (2) BH_4 is een essentiële cofactor voor de vorming van monoamine neurotransmitters. Bij chronische inflammatie, wordt preferentieel neopterin gevormd ten koste van BH_4. Neopterin wordt gevormd in geactiveerde macrofagen en is een merker van macrofaag-activiteit en oxidatieve stress. Verminderde aanwezigheid van BH_4 leidt tot verminderde aanmaak van monoamine neurotransmitters.

Een interactie tussen het immuunsysteem en deze twee pathways wordt frequent beschreven in reviews en werd onderzocht in preklinisch onderzoek. Bij personen met psychiatrische ziekten en zeker in het geval van bipolaire stoornis, zijn deze interacties zelden onderzocht. Ook het verband tussen deze biologische processen, stemmingswisselingen en het progressief ziekteverloop werd zelden onderzocht.

Met ons onderzoek beoogden we een geïntegreerde en longitudinale evaluatie van zowel stemmingssymptomen als merkers van inflammatie, kynurenine afbraak en BH_4-afhankelijke monoaminevorming bij personen met bipolaire stoornis en gezonde controlepersonen. We includeerden personen met bipolaire stoornis tijdens een acute stemmingsepisode (depressie of (hypo)manie). De deelnemers doorliepen 6 onderzoeksmomenten over het verloop van 8 maanden opvolging. We vonden geen verschillen in merkers van inflammatie (CRP, IFN-γ, TNF-α en IL-6) tussen patiënten in verschillende stemmingsepisodes en controlepersonen (hoofdstuk 4&5). Gelijkaardig aan de proinflammatoire status die optreedt bij normale veroudering, ontwikkelden patiënten in onze onderzoekspopulatie een proinflammatoire status met toenemende leeftijd. We zien deze proinflammatoire status in de patiëntengroep echter ontstaan op vroegtijdige leeftijd. De toename van proinflammatoire merkers was ook zichtbaar bij patiënten met langere ziekteduur (Hoofdstuk 5). Onafhankelijk van de stemmingsepisode, zagen we bij patiënten een lagere KYNA concentratie, een afbraakproduct

van kynurenine met vermoedelijk neuroprotectieve eigenschappen. In patiënten met chronische subsyndromale depressieve symptomen, vonden we een verminderde neuroprotectieve ratio KYNA/3-HK tijdens de hele follow-up periode. In de aanwezigheid van stemmingssymptomen, i.e. acute manie en chronisch subsyndromale depressieve symptomen, vonden we een sterk verband tussen inflammatie en een afbraak van kynurenine naar de meer neurotoxische afbraakproducten. Tijdens manie was TNF-α sterk gecorreleerd aan de neurotoxische kynurenine afbraakproducten (3-HK en QA), terwijl bij chronisch depressieve symptomen, eerder IFN-γ gerelateerd was aan een gestegen neurotoxisch QA en activatie van IDO-1. IDO-1 is het sleutelenzym in de afbraak van kynurenine en wordt geactiveerd door proinflammatoire cytokines. (Hoofdstuk 4)

In hoofdstuk 5 onderzochten we de BH_4-afhankelijke monoaminevorming. De concentratie van monoamines (TYR, TRP en PHE) was lager bij patiënten ten opzichte van controlepersonen en dit voornamelijk tijdens depressie. Daarnaast vonden we een sterkere correlatie tussen neopterine en PHE/TYR in de gehele patiëntengroep. Dit is een typische bevinding in situaties van chronische inflammatie en suggereert een verhoogde vorming van neopterine ten koste van BH_4 en bijgevolg een verminderde monoaminevorming. Een duidelijke link tussen stemmingssymptomen, inflammatie en monoaminevorming konden we echter niet aantonen.

In normale situaties wordt homeostase bewaakt door een evenwicht tussen enerzijds proinflammatoire en neurotoxische processen en anderzijds neurotrofe processen. Bij bipolaire stoornis is de concentratie van de neurotrofe factor BDNF gedaald. Aangezien in onderzoek werd aangetoond dat BDNF verder afneemt tijdens acute stemmingsepisodes en bij een langere ziekteduur, werd deze neurotrofe factor voorgedragen als een merker voor ziekteactiviteit en progressie. Echter, deze bevinding kon onvoldoende gerepliceerd worden, waardoor meer onderzoek nodig is om de rol van BDNF in ziekteprogressie beter te begrijpen en zo mogelijk andere neurotrofe merkers te identificeren.

We onderzochten BDNF, VEGF en sFlt (oplosbare VEGF receptor) bij personen met bipolaire stoornis tijdens een manische en depressieve episode en na 2 maanden opvolging (Hoofdstuk 6). Ondanks een adequate grootte van de onderzoekspopulatie en een rigoureuze methodologie, konden we geen stemmingsgerelateerde veranderingen in neurotrofe stoffen identificeren. Een beter begrip en optimalisering van methodologische aspecten zoals de procedure van bloedafnames, de biologische stabiliteit van de merkers, en de interpretatie

van de resultaten zijn nodig om toekomstig onderzoek aangaande neurotrofe merkers te sturen.

Ter conclusie, onze resultaten tonen een verhoogde neurotoxiciteit doorheen het ziekteverloop bij personen met bipolaire stoornis en dit meer uitgesproken in de aanwezigheid van stemmingssymptomen. Deze verhoogde neurotoxiciteit bij patiënten leiden we af uit een vroegtijdige proinflammatoire status, een sterker verband tussen inflammatie en neurotoxische kynurenine afbraakproducten, en een verminderde aanwezigheid van monoamine neurotransmitters. De resultaten moeten kritisch geïnterpreteerd worden en replicatie is aangewezen. Tot slot formuleren we methodologische suggesties voor toekomstig onderzoek naar biomerkers in bipolaire stoornis (Hoofdstuk 8).

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Abbreviations

3-HK	3-hydroxy-kynurenine
5-HT	serotonin
α7nACh-R	alpha 7 nicotinic receptor
BBB	blood-brain barrier
BD	bipolar disorder
BDNF	brain-derived neurotrophic factor
BH_4	tetrahydrobiopterin
CRP	C-reactive protein
CSF	cerebrospinal fluid
ECT	electric convulsive therapy
GTP-CH1	guanosine triphosphate cyclohydroxylase 1
HDRS	Hamilton depression rating scale
HPA	hypothalamo-pituitary-adrenal
IDO-1	indoleamine 2,3 deoxygenase
IFN-γ	Interferon gamma
KYN	kynurenine
KYNA	kynurenic acid
MDD	major depressive disorder
NMDA-R	N-methyl-D-aspartate receptor
PANSS	positive and negative syndrome scale
PET	positron emission tomography
PHE	phenylalanine
QA	quinolinic acid
sFlt	soluble fms-like tyrosine kinase-1 / soluble VEGF-receptor
TNF-α	tumor necrosis factor alfa
TRP	tryptophan
TYR	tyrosine
VEGF	vascular endothelial growth factor
YMRS	Young mania rating scale

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Curriculum vitae

Personal details

Name: Seline van den Ameele

Address: Louis Mettewielaan 9/35, 1080 Sint-Jans-Molenbeek, Belgium

E-mail: seline.vandenameele@gmail.com / seline.vandenameele@chu-brugmann.be

Phone: +32 478 28 77 86

Date of birth: August 2nd, 1986

Nationality: Belgian

Education

- 1998-2004: Secondary school - Sint-Lodewijkscollege, Bruges, Belgium.
- 2004-2011: Master in Medicine. Ghent University, Ghent, Belgium.
- Dissertation: "Role and position of the healthcare-sector in the prevention of sexual violence towards Sub-Saharan Transmigrants in Morocco: knowledge, attitudes and practices of healthcare workers". Promotor: Prof. Dr. M. Temmerman; Co-promotor: Dr. I. Keygnaert.
- 2012-2016: Postgraduate Integrative Psychotherapy. Antwerp University, Belgium.
- 2011-2018: Master of Specialist Medicine - Adult psychiatry. Antwerp University, Belgium.

Working experience

- 11/2018 - present: Resident in Psychiatry. Department of Psychiatry, Brugmann University Hospital, Brussels, Belgium.
- Training in psychiatry:

  08/2011-07/2012: Equator Foundation, psychiatric treatment program for traumatized refugees and victims of human traffic. Amsterdam, The Netherlands.

  08/2012-02/2014: University Psychiatric Hospital, Duffel, Belgium. Unit of observation & diagnosis - Unit for psychotic and bipolar disorders - Crisis intervention unit.

  02/2015-02/2017: Centre of Mental Health Brussels (CGG West), Sint-Jans-Molenbeek, Belgium. Psychotherapeutic and psychiatric consultations.

  10/2017-02/2018: Psychiatric Hospital Multiversum Boechout, Belgium. Addiction psychiatry.

  02/2018-08/2018: PAAZ St-Maarten, Mechelen, Belgium. General psychiatry.

International internships

- 9/2008-7/2009: Erasmus exchange at the University Victor Segalen, Bordeaux, France.
- 12/2010-03/2011: Internships in pediatrics and internal medicine in Mombasa and Kilifi, Kenya.

Research experience

- 2007-2009: Participation in the research project 'La Violence Sexuelle contre et parmi les trans-migrants Subsahariens au Maroc. Un partenariat participatif pour la prévention'.
  Supervision: Prof. dr. M. Temmerman, Dr. I. Keygnaert. International Centre of Reproductive Health (ICRH), Ghent University, Belgium.
- 2015-2016: Sub-Investigator on recruitment site PZ Duffel for a Johnson&Johnson initiated RCT: An Exploratory Multicenter, Double-Blind, Diphenhydramine- and Placebo-Controlled Safety, Efficacy and Biomarker Study with JNJ-42847922 in Subjects with Major Depressive Disorder.
- 2014-2017: PhD fellow CAPRI – Antwerp University: Research on Inflammation in Bipolar Disorder. Promotors: Prof. Dr. B. Sabbe and Prof. Dr. M. Morrens.

Publications

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Teaching

- 2013-2017: Communication skills with psychiatric patients. Master of medicine, Antwerp University, Belgium.
- 2015-2017: Psychiatric pathology: Bachelor Psychiatric Nursing. Thomas More College, Mechelen, Belgium.
- 2015-present: Co-promotor master theses: 'Cognition & Inflammation' and 'Cognitive Insight of Patients with Bipolar Disorder'. Master students in Medicine, University of Antwerp, Belgium.

Presentations

- Sap K, van den Ameele S. Need a break? The unit health & society in the first year of medical training. Keynote lecture & poster presentation. Annual conference of The Network: Towards Unity for Health. November 2005, Ho Chi Minh City, Vietnam.
- Seminary on 'Violence Sexuelle et Trans-Migrants Subsahariens au Maroc' in Faculté des Sciences de l'Education, University Mohammed V, May 2009, Rabat, Morocco.
  - Presentation: "Méthodologie et résultats de l'étude auprès des professionnels de santé".
  - Animator of the workshop "Le rôle du secteur de la santé dans la prévention de la violence sexuelle".
- Local presentations for colleagues:
  - Metabolic syndrome: 29/5/2013, Fase 2, PZ Duffel, Belgium.
  - Catatonia. 10/12/2013, Opname 1, PZ Duffel, Belgium.
  - Inflammation in Mood Disorders, 18/05/2018, PAAZ Mechelen, Belgium.
- Research Club & Postgraduate CAPRI, University of Antwerp, Belgium:
  - Inflammation in Bipolar Disorder, 9/9/2014.
  - Inflammation and Cognition in Bipolar Disorder: preliminary results, 20/12/2016.
  - Neuroprogression in Bipolar Disorder, 08/05/2018.
- RAP-evening (Reflection Evening Psychiatry) for medical students, Trauma in refugees and asylum seekers, 11/02/2015, University of Antwerp, Belgium.
- Ups&Downs monthly meeting Antwerp: 'The role of inflammation in mood disorders', 16/09/2015, Antwerp, Belgium.

- Poster presentation 'The effect of mood stabilizing drugs on cytokine levels in bipolar disorder' on the 'Research in Psychiatry Day', BCNBP, 02/10/2015, UZ Gasthuisberg, Leuven, Belgium and on the 18th Annual conference of the International Society of Bipolar Disorder, 13-16/07/2016, Amsterdam, The Netherlands.
- Oral presentation 'The clinical impact of immune system alterations in bipolar disorder'. Flemish Conference of Mental Health (GGZ congress), 20/09/2016, University of Antwerp, Belgium.

Extra courses

- Narrative Exposure Therapy Course, Centrum 45. 4/2012, Diemen, The Netherlands.
- European Psychiatry Association (EPA) Academia Summer School: Comorbidity between mental and physical disorders. 7-10/6/2013, Strasbourg, France.
- ECNP Workshop on Clinical Research Methods, 2-4/11/2016, Barcelona, Spain.
- Winter School on Statistics with JMP: Data visualization and exploration & Statistical inference: confidence intervals, tests and regression, 23-25/01/2017, Faculty of Bio-engineering, KUL, Leuven, Belgium
- 2014-2016: Antwerp Doctoral School, Antwerp University: Several language and statistical courses.

Voluntary work

- July- August 2007: Teaching on family planning and reproductive health for midwifes and high school students. Region around Quetzaltenango, Guatemala.
- 2009-2011: Mentor of a refugee-medical student at the Ghent University.