Cerebrospinal fluid studies of bipolar disorder

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Gothenburg 2022

Cover illustration: Coronal section of brain by Anniella Isgren. Cerebrospinal fluid occupies the ventricles and surrounds the brain.

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ISBN 978-91-8009-747-5 (PRINT)
ISBN 978-91-8009-748-2 (PDF)
http://hdl.handle.net/2077/70532

Printed in Borås, Sweden 2022
Printed by Stema Specialtryck AB

Perplexity is the beginning of knowledge – Kahlil Gibran

Keywords: Biomarkers, bipolar disorder, cerebrospinal fluid, cytokines, etiology, inflammation, microglia, neuroimmune, pathophysiology, and proteomics.

ABSTRACT

The pathophysiology of bipolar disorder remains to be elucidated. One approach to studying brain processes is analysis of cerebrospinal fluid (CSF). This thesis comprises five studies based on the Sankt Göran Bipolar Project (SBP), a naturalistic and longitudinal study investigating patients from different demographic and social backgrounds, regarding associations between bipolar disorder and biological and clinical factors.

In the first two studies (*studies I* and *II*), we found increased CSF concentrations of the immune-related proteins interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), and chitinase-3-like protein 1 (CHI3L1/YKL-40) in patients in a bipolar disorder cohort compared with controls. In a later study (*study IV*), we could, however, not replicate these findings in an independent cohort. In *study III*, we longitudinally investigated associations between prospective clinical outcomes (from a 6–7-year follow-up) and the immune-related CSF proteins which we had found to differ between bipolar disorder patients and controls at baseline in *studies I* and *II*. We found a negative association between YKL-40 concentrations and manic/hypomanic episodes, but no other significant associations. In *studies IV* and *V*, we measured a large number of CSF proteins to explore case–control-associated proteins and their relation to clinical features in our two independent bipolar disorder cohorts. We found and replicated lower concentrations of growth hormone (GH) and testican-1, and higher concentrations of C-type lectin domain family 1 member B (CLEC1B) in patients compared with controls. Also, two other proteins were lower in patients with bipolar I disorder compared with controls: draxin and tumor necrosis factor receptor superfamily member 21 (TNFRSF21). All these proteins, except for CLEC1B, have known important functions in the brain.

Due to limited effect sizes, the identified proteins cannot on their own serve as biomarkers for diagnostics or prognostics. However, they give clues to the biological processes underlying bipolar disorder. In regard to immune aberrations in CSF from patients with bipolar disorder, our findings were contradictory.

This work brings to light challenges in CSF studies of bipolar disorder. The high intercorrelation between proteins, and the fact that many proteins are associated with demographic factors, emphasize the need for careful study designs and consideration of potential confounders.

SAMMANFATTNING PÅ SVENSKA

Bipolär sjukdom är en livslång psykiatrisk sjukdom som innebär perioder av förhöjt stämningsläge (manier och hypomanier) och perioder av sänkt stämningsläge (depressioner). Sjukdomen diagnostiseras genom samtal med patient och närstående och det finns i nuläget inga objektiva tester som stöd för diagnosen.

Det är fortfarande mycket som är oklart kring vad som orsakar bipolär sjukdom. Ett sätt att studera sjukdomsmekanismer i hjärnan är att undersöka likvor (cerebrospinalvätska), som är den vätska som omger hjärna och ryggmärg. I den här avhandlingen undersöktes likvor från patienter med bipolär sjukdom och från friska kontrollpersoner som samlades in i Sankt Göranprojektet – en longitudinell studie av personer med bipolär sjukdom. Målet med avhandlingen var att identifiera biomarkörer som är associerade med bipolär sjukdom för att bättre förstå sjukdomens patofysiologi, samt att hitta diagnostiska och prognostiska biomarkörer. Ett specifikt syfte var att undersöka om immunrelaterade proteiner i likvor är kopplade till bipolär sjukdom.

I *studie I* och *II* fann vi att koncentrationerna av tre immunrelaterade proteiner i likvor – interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) och chitinase-3-like protein 1 (YKL-40) – var högre hos patienter jämfört med kontroller. Dock replikerades inte dessa fynd i en fristående kohort (*studie IV*). I *studie III* undersöktes samband mellan sjukdomsutveckling över tid och proteinkoncentrationer i likvor för de proteiner vi identifierat med en koppling till bipolär sjukdom i de två första studierna. Vi fann ett negativt samband mellan koncentrationen av YKL-40 och maniska skov, men inga andra associationer mellan proteinnivåer och sjukdomsutveckling. I de sista delstudierna (*studie IV* och *V*) mätte vi ett stort antal proteiner i likvor i två fristående kohorter, med syftet att identifiera sjukdomsrelaterade proteiner. Vi fann att tillväxthormon (GH) och testican-1 var lägre i likvor hos patienter jämfört med kontroller i båda kohorterna, samt att C-type lectin domain family 1 member B (CLEC1B) var högre. När vi jämförde patienter med bipolär sjukdom typ 1 med kontroller fann vi ytterligare två proteiner som var lägre hos patienter jämfört med kontroller: draxin och tumor necrosis factor receptor superfamily member 21 (TNFRSF21). Samtliga proteiner, förutom CLEC1B, har kända funktioner i hjärnan, även om deras uppgifter inte är fullständigt kartlagda.

Sammanfattningsvis identifierade vi några likvorproteiner med koppling till bipolär sjukdom. Dessa proteiner kan inte enskilt användas i diagnostiken av bipolär sjukdom eftersom gruppskillnaderna var relativt små. Däremot ger fynden ledtrådar till patofysiologin vid bipolär sjukdom. Vad gäller immunologiska avvikelser i likvor vid bipolär sjukdom var våra fynd motstridiga.

List of Papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Isgren A**, Jakobsson J, Pålsson E, Ekman CJ, Johansson AG, Sellgren C, Blennow K, Zetterberg H, Landén M. *Increased cerebrospinal fluid interleukin-8 in bipolar disorder patients associated with lithium and antipsychotic treatment*. Brain, Behaviour, and Immunity 2015; 43: 198-204.

II. Jakobsson J, Bjerke M, Sahebi S, **Isgren A**, Ekman CJ, Sellgren C, Olsson B, Zetterberg H, Blennow K, Pålsson E, Landén M. *Monocyte and microglial activation in patients with mood-stabilized bipolar disorder*. Journal of Psychiatry and Neuroscience 2015; 40(4):250-258.

III. **Isgren A**, Sellgren C, Ekman CJ, Holmén-Larsson J, Blennow K, Zetterberg H, Jakobsson J, Landén M. *Markers of neuroinflammation and neuronal injury in bipolar disorder: Relation to prospective clinical outcomes*. Brain, Behaviour, and Immunity 2017; 65: 195-201.

IV. **Isgren A**, Göteson A, Holmén-Larsson J, Pelanis A, Sellgren C, Joas E, Sparding T, Zetterberg H, Smedler E, Jakobsson J, Landén M. *Cerebrospinal fluid proteomic study of two bipolar disorder cohorts* (submitted).

V. Göteson A, **Isgren A**, Jonsson L, Sparding T, Smedler E, Pelanis A, Zetterberg H, Jakobsson J, Pålsson E, Holmén-Larsson J, Landén M. *Cerebrospinal fluid proteomics targeted for central nervous system processes in bipolar disorder*. Molecular Psychiatry 2021; 26: 7446-7453.

List of Abbreviations

ADE	Affective Disorder Evaluation
ADHD	Attention deficit hyperactivity disorder
BMI	Body mass index
CGI	Clinical Global Impression
CHI3L1/YKL-40	Chitinase-3-like protein 1
CI	Confidence interval
CLEC1B	C-type lectin domain family 1 member B
CNS	Central nervous system
CSF	Cerebrospinal fluid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ELISA	Enzyme-linked immunosorbent assay
GAF	Global Assessment of Functioning
GH	Growth hormone
GWAS	Genome-wide association study
IFN	Interferon
IL	Interleukin
MADRS	Montgomery-Åsberg Depression Rating Scale
MCP-1	Monocyte chemoattractant protein 1
M.I.N.I.	Mini International Neuropsychiatric Interview
OR	Odds ratio
PCA	Principal component analysis
PEA	Proximity extension assay
SBP	Sankt Göran Bipolar Project
TNF	Tumor necrosis factor
TNFRSF21	Tumor necrosis factor receptor superfamily member 21
YMRS	Young Mania Rating Scale

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INTRODUCTION

Bipolar disorder is a lifelong psychiatric condition that affects more than 1% of the world's population (Merikangas et al., 2011). Since bipolar disorder is mainly diagnosed in young adulthood, and often entails functional impairment, the societal costs are high (Ekman, Granström, Omérov, Jacob, & Landén, 2013; Gardner et al., 2006). Indeed, the disorder is one of the leading causes of days out of role (Alonso et al., 2011), and it is often associated with a significant reduction in quality of life (IsHak et al., 2012). Although the knowledge of the etiology and pathophysiology of bipolar disorder has increased over the past decades, the disease mechanisms are to a large extent still unknown. This thesis is concerned with studies of cerebrospinal fluid (CSF) from patients with bipolar disorder and control participants, and aims to investigate pathophysiological mechanisms and search for biomarkers for diagnostics and prognostics.

BIPOLAR DISORDER

HISTORY OF BIPOLAR DISORDER

The characterization of bipolar disorder as a separate illness dates back to the French psychiatrist Jules Falret, who in 1854 described a condition called *folie circulaire* (circular madness) including alternating moods of depression and mania. In 1882, the German psychiatrist Karl Kahlbaum described depression and mania as stages of the same illness, which he called *cyclothymia*. A few years later, in 1899, the German psychiatrist Emil Kraepelin introduced the term *manic-depressive insanity*, a broad group including manic-depressive psychosis as well as recurrent depression. According to Kraepelin, manic-depressive psychosis differed from *dementia praecox* (later known as *schizophrenia*) by the absence of a dementing course (Angst & Sellaro, 2000; Sadock, Sadock, & Ruiz, 2015). The first versions of the psychiatric classification system The Diagnostic and Statistical Manual of Mental Disorders (DSM), DSM-I (1952) and DSM-II (1962), contained disorders that resemble bipolar disorder (*manic depressive reactions* and *manic-depressive illness*), but it was not until the launch of the DSM-III (1980) that the term *bipolar disorder* was

introduced (Mason, Brown, & Croarkin, 2016). The definition of *bipolar disorder* has since undergone minor changes in the DSM classification system. In the latest version, the DSM-5, a previous or current manic episode is required for a bipolar I disorder diagnosis, and a previous or current hypomanic episode in addition to a depressive episode is required for a bipolar II disorder diagnosis (American Psychiatric Association, 2013).

Clinical course

Fluctuations in mood, with periods of more elevated mood, alternating with periods of less energy and happiness, are common. However, severe mood swings that either cause an unequivocal change in a person's behavior, or a marked impairment in occupational or social functioning, could indicate an underlying bipolar disorder (American Psychiatric Association, 2013; Grande, Berk, Birmaher, & Vieta, 2016). The main characteristic differentiating bipolar disorder from other affective disorders is the periodic manic or hypomanic episodes, alternating with depressive episodes (Carvalho, Firth, & Vieta, 2020), as illustrated in Figure 1.

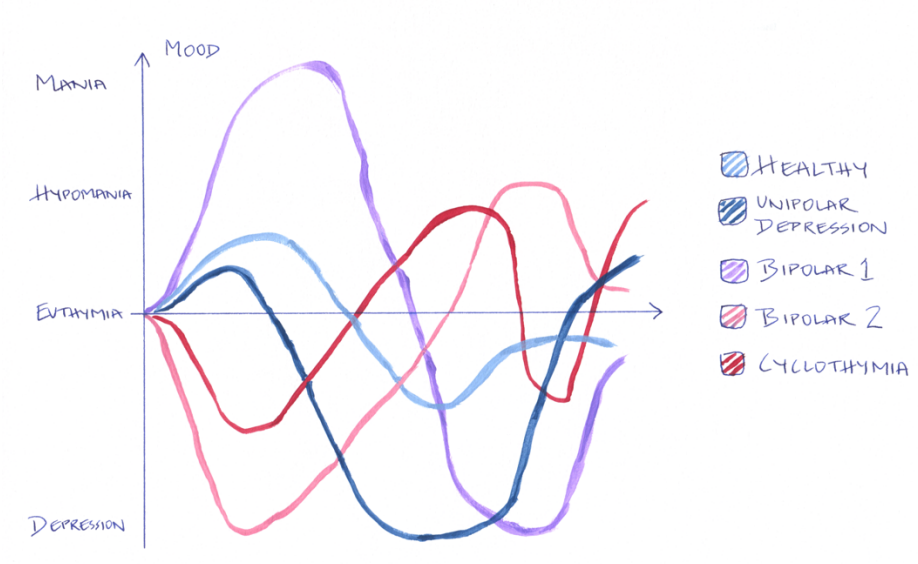

Figure 1. Illustration of mood fluctuations in bipolar spectrum disorders and unipolar depression.

A manic episode is a distinct period of abnormally and persistently elevated, expansive, or irritable mood coupled with abnormally and persistent goal-directed behavior or energy. The episode is accompanied by symptoms and signs such as inflated self-esteem, decreased need for sleep, increased speech flow, and distractibility. Other typical features are increased activity levels—for instance, engaging extensively in work assignments or showing markedly increased social interactions—and excessive involvement in activities that have a high potential for negative consequences, such as unrestrained shopping or sexual indiscretions (American Psychiatric Association, 2013). Psychotic symptoms, such as hallucinations or delusions, are present in up to 75% of manic episodes, and many episodes impair social functioning to the point that hospitalization is needed (Carvalho et al., 2020). A hypomanic episode is similar to a manic episode, but is not severe enough to cause marked impairment in social or occupational functioning or to necessitate hospitalization (American Psychiatric Association, 2013). In bipolar disorder, these manic or hypomanic episodes alternate with depressive episodes featuring depressed mood, loss of interest or pleasure in activities, sleep disturbances, fatigue, impaired ability to concentrate, a sense of worthlessness, and recurrent thoughts of death (American Psychiatric Association, 2013).

For a DSM-5 diagnosis of *bipolar I disorder*, a patient has experienced at least one manic episode; for a diagnosis of *bipolar II disorder*, a patient has experienced at least one hypomanic *and* one depressive episode (American Psychiatric Association, 2013). The bipolar spectrum disorders, according to DSM-5, are summarized in Table 1, where also the main differences between a manic and hypomanic episode are outlined.

Table 1. Differences between a manic and hypomanic episode and summary of the DSM-5 classification of bipolar and related disorders. Adapted from Diagnostic and statistical manual of mental disorders (American Psychiatric Association, 2013).

Episode type	**Mania**	**Hypomania**
Duration	≥ 7 days	≥ 4 days
Severity	Higher (functional impairment, need for hospitalization, or psychotic symptoms)	Lower (no functional impairment, need for hospitalization, or psychotic symptoms)
Bipolar disorder	Bipolar I disorder	Bipolar I and II disorders

Disorder	Episodes	Other criteria
Bipolar I disorder	≥ one manic episode (fulfilling criteria)	
Bipolar II disorder	≥ one hypomanic and ≥ one depressive episode (fulfilling criteria)	Symptoms of depression or the unpredictability caused by frequent alternation between depression and hypomania causes clinically significant distress or impairment in social, occupational, or other important areas of functioning
Cyclothymic disorder	Several episodes with symptoms of hypomania and of depression (but not fulfilling criteria for these episodes)	Episodes present for ≥ two years
Substance/ medication-induced bipolar and related disorder	≥ one episode with prominent and persistent disturbances in mood characterized by elevated, expansive, or irritable mood, with or without depressed mood	Evidence that the symptoms developed during or soon after substance intoxication or withdrawal, or after exposure to a medication
Bipolar and related disorder due to another medical condition	≥ one episode with persistent elevated, expansive, or irritable mood and high energy	Symptoms are a direct pathophysiological consequence of another medical condition
Other specified bipolar and related disorder	Symptoms do not meet the full criteria for any other bipolar disorder but cause significant distress or impaired functioning	Includes short-duration hypomanic episodes and major depressive episodes, hypomanic episodes with insufficient symptoms or without accompanying major depressive episodes, or short-duration cyclothymia
Unspecified bipolar and related disorder	Symptoms do not meet the full criteria for any other bipolar disorder but cause significant distress or impaired functioning	Is diagnosed when the clinician chooses not to specify why symptoms do not meet bipolar criteria

Affective disorders are sometimes classified along a spectrum defined by the type and severity of the mood shift—from unipolar depression to bipolar II to bipolar I disorder (Grande et al., 2016; Phillips & Kupfer, 2013). It should, however, be noted that several lines of research indicate that bipolar II disorder is not a milder form of bipolar I disorder but a distinct bipolar disorder category. One large study comparing patients with bipolar I and II disorder found that the bipolar II group had higher rates of depressive episodes and suicide attempts, and also higher prevalence of psychiatric comorbidity. On the other hand, they were more likely to have completed higher education and to be self-sufficient than the bipolar I group (Karanti et al., 2020). Also, the genetic architecture seems to differ between bipolar I and II disorders: A recent genome-wide association study (GWAS) of bipolar disorder found that although the two subtypes are highly genetically correlated, the correlation is not perfect and bipolar I disorder is more genetically correlated with schizophrenia, whereas bipolar II disorder is more correlated with major depressive disorder (Mullins et al., 2021).

The onset of bipolar disorder typically occurs around 15–25 years of age. The first episode is often depressive type, with the consequence that many patients are first diagnosed with a major depressive disorder (Carvalho et al., 2020). The natural course of bipolar disorder typically includes periods of remission, with a euthymic mood state, but frequent relapse into mood episodes is common, especially if adherence to treatment is low (Grande et al., 2016). Long-term follow-up studies of patients with bipolar I (Judd et al., 2002) and II (Judd et al., 2003) disorders have shown that patients are symptomatically ill approximately half of the time, with predominantly depressive symptoms. Apart from the burden caused by acute mood episodes, bipolar disorder is also associated with cognitive deficits across all mood states (Martínez-Arán et al., 2004; Sparding et al., 2015). Comorbid psychiatric disorders are common, with anxiety disorders being the most prevalent. Other frequent comorbidities are substance or alcohol use disorder, attention deficit hyperactivity disorder (ADHD), and personality disorders (McIntyre et al., 2020). Risk of death by suicide is significantly increased, being up to 20–30 times greater than in the general population. Further, bipolar disorder is associated with somatic comorbidities such as cardiovascular disorders, obesity, and diabetes (Grande et al., 2016; McIntyre et al., 2020).

DIAGNOSTICS

The diagnosis of bipolar disorder is based on interviews with the patient and, if possible, with relatives or other persons in the patient's social network. For differential diagnostic purposes, a somatic and neurological examination, including blood and toxicology tests, are included in the diagnostic work-up. Sometimes further medical investigations are indicated in the differential diagnostic procedure, such as magnetic resonance imaging (MRI) of the brain, or lumbar puncture. The diagnostic process and prediction of disease progression relies exclusively on clinical assessment of symptoms and signs, and there are no objective tests to aid the diagnosis. The mean delay between illness onset and diagnosis has been estimated to be 3–16 years for different age groups (Berk et al., 2007). Hence, there is a great need for biomarkers to aid the diagnostic process.

In *studies I*, *II*, *IV*, and *V*, we studied associations between CSF protein concentrations and the bipolar disorder diagnosis, and between CSF protein concentrations and measures of disease severity at baseline. In *study III*, we investigated associations between CSF protein concentrations at baseline and measures of disease severity during a follow-up period of 6–7 years.

TREATMENT

Treatment of bipolar disorder includes pharmacological treatments, psychological and lifestyle interventions, and, under certain conditions, electroconvulsive therapy (ECT). The therapeutic objectives are to prevent and treat acute mood episodes, prevent and treat interepisodic subsyndromal depressive symptoms, improve and preserve cognitive functioning, normalize circadian disturbances (e.g., sleep disturbances), prevent and treat psychiatric and somatic comorbidities, improve patient-reported outcomes (e.g., related to quality of life), and reduce suicidality (McIntyre et al., 2020). The drugs used to prevent mood episodes and treat acute mood episodes are lithium, some anticonvulsant drugs (e.g., valproate and lamotrigine), and some antipsychotics (e.g., olanzapine, quetiapine, and

aripiprazole). Along with the pharmacological treatment, psychotherapy—including cognitive behavioral, interpersonal, and social rhythm therapies—and psychoeducation are important treatment strategies (Beynon, Soares-Weiser, Woolacott, Duffy, & Geddes, 2008; Colom et al., 2009; Grande et al., 2016; Novick & Swartz, 2019). ECT is sometimes used for treatment-resistant acute mood episodes (Grande et al., 2016; Schoeyen et al., 2015).

ETIOLOGY AND PATHOPHYSIOLOGY

Knowledge of the etiology and pathophysiology of bipolar disorder has progressed rapidly over the past decades, but the specific disease mechanisms remain elusive. There is a large genetic component, and twin studies have estimated the heritability to be approximately 60–85% (Fabbri, 2021; Johansson, Kuja-Halkola, Cannon, Hultman, & Hedman, 2019). The disorder is highly polygenic, and a recent GWAS of over 40,000 individuals with bipolar disorder identified 64 genomic loci associated with bipolar disorder, each with a small effect size (Mullins et al., 2021). Interestingly, risk alleles for bipolar disorder were enriched in brain-expressed genes, particularly those with high expression in neurons of hippocampus and prefrontal cortex, and in genes in synaptic signaling pathways (Mullins et al., 2021).

Besides genetic influences, associations between environmental factors and the onset and clinical course of bipolar disorder have been shown. These include prenatal (e.g., maternal infections) and early-life (e.g., childhood trauma) factors, and factors across the lifespan related to life events and social support (Aldinger & Schulze, 2017). However, there are methodological challenges in the research field and causality between environmental factors and the onset of bipolar disorder is often unclear. One key issue is that environmental factors such as adverse life events are not independent of genetic vulnerability for mood disorders. Interestingly, a study assessing risks for a later bipolar disorder diagnosis, after exposure to negative life events in children with and without parental psychopathology (i.e., having a parent with any psychiatric diagnosis), found that the most important risk factor for bipolar disorder in the offspring was parental psychopathology (Bergink et al., 2016). The additional risk conferred by early-life events during childhood was only modest. The authors concluded that this reinforces the strong genetic component in bipolar disorder and challenges the notion of negative early-life events as key determinants, particularly

in individuals with a genetic susceptibility for bipolar disorder (Bergink et al., 2016).

Examples of biological mechanisms that have been suggested to be associated with bipolar disorder include mitochondrial dysfunction, aberrations in neural plasticity, epigenetic changes, oxidative stress, disturbances in the circadian rhythm, and immune aberrations (Grande et al., 2016; McIntyre et al., 2020). The latter mechanism is a particular focus of this thesis and is elaborated in a separate section below.

Finally, bipolar disorder is associated with morphological alterations of the brain. Findings include smaller subcortical gray matter brain volumes (e.g., of hippocampus, amygdala, and thalamus) (Hibar et al., 2016), larger lateral ventricles, lower cortical thickness (Hibar et al., 2018), and altered white matter integrity (Favre et al., 2019) in patients with bipolar disorder compared with controls. In a recent longitudinal study of structural brain changes in bipolar disorder, faster ventricle enlargement, but not accelerated cortical thinning, was shown in patients with bipolar disorder compared with controls. Frequent manic episodes were, however, associated with increased cortical thinning, primarily in the prefrontal cortex (Abé et al., 2021).

IMMUNOLOGY OF BIPOLAR DISORDER

Evidence for a contributing role of the immune system in the pathogenesis of bipolar disorder has expanded in recent years. The hypothesis that immune dysfunction may be involved in disease progression in bipolar disorder was first proposed in the 1980s by the researchers David F. Horrobin and Julian Lieb. They suggested that immune modulation may underlie lithium's mood-stabilizing properties, and that manic-depressive psychoses are associated with fluctuating levels of prostaglandin E1 in immune cells, and fluctuating T suppressor lymphocyte activity (Horrobin & Lieb, 1981; Rosenblat & McIntyre, 2017). Another early connection between mood disorders, including bipolar disorder, and the immune system relates to so-called "sickness behavior." This refers to the behavioral changes observed in inflammatory conditions such as infections and autoimmune disorders, including depressed mood, anxiety, and loss of energy. Sickness behavior is mediated by proinflammatory cytokines, including interleukin (IL)-1α/-1β, tumor necrosis factor-α (TNF-α), and IL-6 (Benedetti, Aggio, Pratesi, Greco, & Furlan, 2020; Dantzer, O'Connor, Freund, Johnson, &

Kelley, 2008). In support of this notion, several cytokine treatments may cause depressive symptoms, e.g., interferon-α (IFN- α) treatment of chronic hepatitis C (Bonaccorso et al., 2001), as well as IL-2 and IFN-α treatment of various cancer forms (Capuron et al., 2001). In line with this, mood disorders are more common in patients with conditions associated with chronic inflammation, such as rheumatoid arthritis, cardiovascular diseases, and type 2 diabetes, than in the general population (Dantzer et al., 2008). An increased risk for developing bipolar disorder has also been seen after receiving an autoimmune diagnosis (Eaton, Pedersen, Nielsen, & Mortensen, 2010). This connection seems to be bidirectional, and we have previously shown a higher prevalence of hyperthyroidism, hypothyroidism (not related to lithium), rheumatoid arthritis, and polymyalgia rheumatica in patients with bipolar disorder compared with controls (Cremaschi et al., 2017). Further, it has been shown that patients with mood disorders, but without symptoms of an autoimmune disorder, have higher prevalence of autoantibodies such as thyroperoxidase (TPO) antibod-ies (associated with thyroid diseases), hydrogen–potassium adenosine triphos-phatase (H^+/K^+ ATPase) antibodies (associated with chronic autoimmune atrophic gastritis), and glutamic acid decarboxylase-65 (GAD65A) antibodies (associated with type 1 diabetes) (Kupka et al., 2002; Padmos et al., 2004). Interestingly, positive TPO antibody titers have also been found more frequently in female bipolar offspring compared with controls (Hillegers et al., 2007). In conclusion, the high comorbidity between bipolar disorder and autoimmune or chronic inflammatory diseases suggests common underlying immune abnormalities (Bergink, Gibney, & Drexhage, 2014).

Another line of evidence for implications of the immune system in bipolar disorder is altered concentrations of peripheral immune-related proteins, such as *cytokines*, in patients with bipolar disorder. Cytokines are small proteins secreted by immune cells in response to different stimuli. They are important for cell-to-cell signaling, and are also involved in cell development, migration, differentiation, and death (Benedetti et al., 2020). In a meta-analysis of blood cytokine alterations in psychiatric patients, bipolar disorder patients displayed alterations in blood cytokine levels corresponding to inflammation and T cell activation (Goldsmith, Rapaport, & Miller, 2016). In manic patients, findings included increased concentrations of the two cytokines IL-6 and TNF-α, as well as soluble interleukin-2 receptor (sIL-2R) and interleukin-1 receptor antagonist (IL-1RA). In euthymic patients, increased levels of IL-6, IL-1β and sIL-2R were seen. There were remarkable similarities between bipolar disorder, major depressive disorder, and

schizophrenia, suggesting common mechanisms shared across these disorders. However, the effect sizes were small to moderate, most likely reflecting that immune system involvement applies only to a subgroup of patients with these disorders (Goldsmith et al., 2016).

Another inflammatory-related protein that has been studied in bipolar disorder is C-reactive protein (CRP), an acute-phase response protein of inflammatory activity. A meta-analysis concluded that peripheral CRP concentrations are higher in bipolar disorder in all mood states, and that manic patients have the highest concentrations (Fernandes et al., 2016).

Defective peripheral immunoregulation through a decrease in regulatory immune cells and/or through dysfunctional immune cells has been proposed to explain the chronic low-grade inflammation sometimes observed in mood disorders, including bipolar disorder. For T cells, findings include a reduced number of IL-10 expressing regulatory T cells (IL-10 Treg cells) (Barbosa et al., 2014) and reduced proportions of natural T regulatory cells in patients with bipolar disorder compared with controls (do Prado et al., 2013). Further, in a longitudinal study of offspring of patients with bipolar disorder—i.e., a population at high risk for developing bipolar disorder—and controls, there was a dynamic change in natural T regulatory cells in bipolar offspring, with reduced levels during adolescence, which normalized in adulthood. This was also accompanied by an inflammatory state during adolescence that subsided during young adulthood and nearly normalized at adulthood (Snijders et al., 2016). Finally, aberrations in monocyte gene expression have also been shown to be associated with bipolar disorder (Becking et al., 2015; Padmos et al., 2008).

The question is, how is the proposed impaired immunoregulation linked to the pathophysiological processes of bipolar disorder? There is no definitive answer to this question, but some suggested mechanisms are that the link is through: (1) failure to develop adaptive stress-related responses; (2) lack of trophic support and pro-cognitive functions in the brain; (3) dysbiosis; (4) microglia activation; and (5) dysregulation of the kynurenine pathway (Bauer & Teixeira, 2021; B. C. M. Haarman, Stam, Borkent, Ioannou, & Drexhage, 2021).

Evidence for the proposed mechanism related to an *aberrant stress response* includes a study reporting a reduced stress response—as shown by heart rate and salivary cortisol levels—in patients with bipolar disorder compared with healthy controls.

This was further associated with a decrease in the proportion of regulatory T cells and an increase in activated T cells compared with controls (Wieck et al., 2013).

The background for the second mechanism—*lack of trophic support and pro-cognitive functions in the brain*—is that cognitive deficits are part of the clinical picture of bipolar disorder (Martínez-Arán et al., 2004; Sparding et al., 2015) and that cognitive deficits are associated with aberrant immunoregulation (Bauer & Teixeira, 2021). Experimental work has shown that peripheral T cells play an important role in hippocampal neurogenesis and spatial learning abilities (Ziv et al., 2006). The crosstalk between peripheral T cells and the central nervous system (CNS) has further been shown to take place through activated T cells in meningeal spaces (Bauer & Teixeira, 2021; Derecki et al., 2010). After animal cognitive tasks, activated meningeal T cells have been observed to express IL-4. This leads to transformation of meningeal myeloid cells to a more anti-inflammatory state, and to upregulation of brain-derived neurotrophic factor (BDNF), an important molecule in learning and memory functions (Bauer & Teixeira, 2021; Derecki et al., 2010).

Dysbiosis—compositional and functional alterations of the gut microbiome—is a third suggested link between the immune system and bipolar disorder (Levy, Kolodziejczyk, Thaiss, & Elinav, 2017). Emerging evidence suggests that dysregulation of the so-called "gut–brain axis" is involved in the pathophysiology of psychiatric disorders, including bipolar disorder (Safadi, Quinton, Lennox, Burnet, & Minichino, 2021; Sublette et al., 2021). It is hence hypothesized that increased intestinal permeability, associated with gut microbiome disturbances, leads to translocation of food and bacterial antigens, further resulting in chronic low-grade inflammation in bipolar disorder (B. C. M. Haarman et al., 2021). Studies have demonstrated an important role for the gut microbiota in brain development and behavior, and the immune system has been shown to be an important regulator of these interactions. The gut microbiota is suggested to both modulate functions of CNS immune cells (e.g., microglia and astrocytes), and activate peripheral immune cells, which in turn affect neurogenesis and neuroinflammatory processes (Fung, Olson, & Hsiao, 2017). With respect to bipolar disorder, a recent review concluded that there is evidence for a role of the gut microbiome in bipolar disorder, with a low microbiome diversity and dysbiosis (abundance of the genera *Faecalibacterium* and *Bacteroides*) being linked to bipolar disorder (Sublette et al., 2021).

A fourth possible link between the immune system and the pathophysiology of bipolar disorder is through activated *microglia*. Microglia are cells of mesodermal/mesenchymal origin, which migrate into the CNS to become resident macrophages (Rubenstein & Rakic, 2013). Microglia were discovered as a cell type in 1919 by the Spanish neuroscientist Pío del Río Hortega, but the immune surveillance functions of microglia were not recognized until many decades later. Microglia are the tissue resident macrophages of the brain parenchyma, interacting with many other cell types including neurons, oligodendrocytes, and astrocytes (Prinz, Jung, & Priller, 2019). In the developing brain, microglia are involved in the establishment of neuronal architecture, and have been shown to participate in shaping neuronal circuits. Further, microglia have been reported to stimulate neurogenesis by affecting proliferation and maturation of neuronal progenitor cells and to interact with synapse formation, including removal of non-functional synapses. Microglia also have numerous functions in the adult brain, including interaction with neighboring cells, clearing dead and excessive cells, and continuously surveying the CNS and reacting to changes in the microenvironment (Kettenmann, Kirchhoff, & Verkhratsky, 2013; Nimmerjahn, Kirchhoff, & Helmchen, 2005; Prinz et al., 2019). A microglia micrograph is shown in Figure 2.

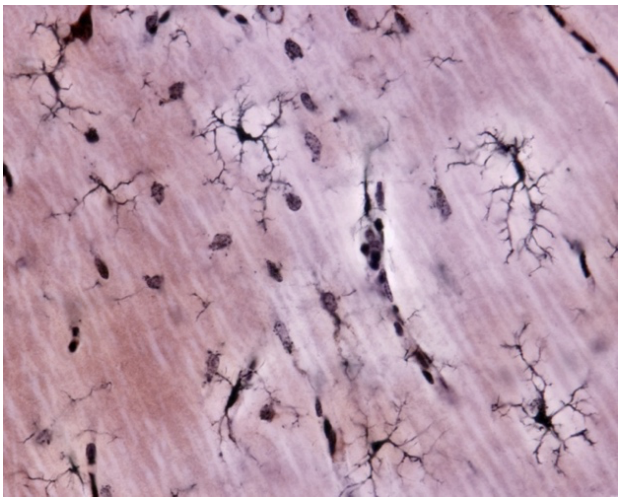

Figure 2. Microglia stained with silver carbonate. This type of microglia is called ramified or resting and appears in normal brain tissue. Courtesy of Jose Luis Calvo Martin and Jose Enrique Garcia-Maurino, iStockphoto.com.

Microglia are also known to be important responders to CNS pathology. After acute injury, microglia migrate towards the site of CNS damage, performing phagocytosis and mediating neuroinflammation by secreting important immune molecules such as the proinflammatory cytokines IL-1β, IL-6, IL-18, and TNF-α. Depending on the incoming signals, microglia can, however, be activated into a variety of phenotypes with different functions. Some phenotypes are characterized by upregulation of inflammatory mediators such as TNF-α and IL-1β, whereas others instead are characterized by secretion of anti-inflammatory molecules such as IL-10 and transforming growth factor beta (TGF-β) (Liu, Liu, Bao, Bai, & Wang, 2020; Orihuela, McPherson, & Harry, 2016). Microglia activation is proposed to be caused by several different stimuli such as stress, dysbiosis, glucocorticoids, bacterial endotoxin (lipopolysaccharide, LPS), and cytokines including TNF-α and IFN-γ (Lively & Schlichter, 2018; Zhang, Zhang, & You, 2018). Microglia have further been associated with several neurological and psychiatric disorders such as Parkinson's and Alzheimer's disease (Baecher-Allan, Kaskow, & Weiner, 2018; Bartels, De Schepper, & Hong, 2020). In bipolar disorder, evidence for a role of microglia is less compelling but there is some evidence for a pathophysiological role, including a study visualizing activated microglia in the right hippocampus of patients with bipolar disorder using positron emission tomography (PET) (B. C. Haarman et al., 2014).

Finally, the *kynurenine pathway* has been proposed to link the immune system to bipolar disorder. This pathway mediates breakdown of the amino acid tryptophan (also a precursor of the neurotransmitters serotonin and melatonin). In this pathway, tryptophan is metabolized into kynurenine by two enzymes called indoleamine 2, 3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). After this step, the kynurenine pathway divides into two pathways. One is considered to be mainly mediated by astrocytes and implicated in neuroprotection, resulting in production of kynurenic acid—an antagonist of the glycine site of the N-methyl-D-aspartate (NMDA) receptor and an α7-nicotinic acetylcholine receptor antagonist. The other is proposed to be mostly mediated by microglia in the brain and implicated in neurotoxicity, resulting in production of quinolinic acid—an NMDA receptor agonist and an oxidative stressor. Under physiological conditions, the neuroprotective branch is more active. Under inflammatory conditions, the metabolism is, instead, shifted towards the more neurotoxic branch through increased expression of the enzyme kynurenine-3-monooxygenase (KMO) in microglia and in peripheral immune cells such as monocytes and macrophages. An upregulation of the kynurenine pathway has

been hypothesized to be involved in bipolar disorder (B. C. M. Haarman et al., 2021). Elevated concentrations of kynurenic acid have been found in CSF of patients with bipolar disorder compared with controls (Olsson et al., 2010). An association with recent manic symptoms and a history of psychotic symptoms was also reported (Olsson, Sellgren, Engberg, Landén, & Erhardt, 2012). Table 2 presents examples of previous studies showing an association between bipolar disorder and the immune system.

Table 2. Examples of original studies linking immune aberrations to bipolar disorder.

Area	Findings
Cytokines	
BD and peripheral cytokines	Case–control study: IL-6 and TNF-α higher in BD compared with controls. IL-6 decreased after treatment (Kim, Jung, Myint, Kim, & Park, 2007).
BD and CRP/cytokines	Case–control study: CRP, TNF-α, IFN-γ, and IL-6 increased in BD during mania and decreased after treatment (Uyanik, Tuglu, Gorgulu, Kunduracilar, & Uyanik, 2015).
BD, cytokines, and cognition	Case–control study: Higher plasma levels of sTNFR1, TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, and IL-17 in BD compared with controls. IL-6 levels negatively correlated with global cognitive performance (Barbosa et al., 2018).
Autoimmune disorders	
BD and autoimmune disorders	Population-based study: A 70% increased risk of developing BD within four years after receiving an autoimmune disease diagnosis. A 20% increased risk in the time span from five years and onwards after the diagnosis compared with the background population (Eaton et al., 2010).
BD and autoimmune disorders	Population-based study: A prior hospital contact because of an autoimmune disease increased the risk of a subsequent BD diagnosis with 25% (Benros et al., 2013).
BD and autoimmune disorders	Case–control study: Hyperthyroidism, hypothyroidism regardless of lithium effects, rheumatoid arthritis, and polymyalgia rheumatica were more common in BD compared with controls (Cremaschi et al., 2017).

T lymphocytes	
BD and T cells	Case–control study: Reduced proportions of natural T regulatory cells and higher cytokine concentrations in BD patients compared with controls. T cells of BD patients had an increased p-ERK signaling, indicating lymphocyte activation (do Prado et al., 2013).
Monocytes	
BD and monocyte inflammatory gene expression	Case–control study: Monocytes from BD patients, and from offspring of BD patients, showed an inflammatory gene expression signature (Padmos et al., 2008).
BD and monocyte/lymphocyte activation	Case–control study: BD patients had changes in monocyte and lymphocyte subsets, indicating an immune imbalance (Barbosa et al., 2014).
BD and monocyte inflammatory gene expression	Case–control study: Patients in a mood episode had higher total gene expression score compared with both controls, euthymic patients, and their own scores when they were euthymic (Becking et al., 2015).
Microglia	
BD and microglia activation	Case–control study: Increased [(11)C]-(R)-PK11195 binding potential, indicative of neuroinflammation, was found in the right hippocampus of BD patients compared with controls using positron emission tomography (B. C. Haarman et al., 2014).

Abbreviations: BD, bipolar disorder. CRP, C-reactive protein. IFN, interferon. IL, interleukin. TNF, tumor necrosis factor.

A concluding remark on immunology and bipolar disorder is that the associations are very complex and have by no means been elucidated. It is as yet undecided to what degree neuroimmune mechanisms reflect pathogenic mechanisms or adaptive responses to pathological processes (Bhattacharya, Derecki, Lovenberg, & Drevets, 2016; Mesman et al., 2015). As discussed above, microglia can be activated into different states—some with a more proinflammatory phenotype and some with a more anti-inflammatory phenotype (Nakagawa & Chiba, 2015). Hence, it is possible that the immune system and microglia contribute *both* to pathological mechanisms of bipolar disorder *and* to maintaining homeostasis and antagonizing disease processes.

Finally, a comment on the terminology of CNS immune mechanisms. The term *neuroinflammation* is widely used, including in the first papers of this thesis. In initial studies using the term, neuroinflammation was often clearly defined as referring to processes corresponding to the features of peripheral inflammation, including macrophage (microglia) activation, recruitment of peripheral immune cells, increased cytokines, and local tissue damage. Examples of diseases including these processes are CNS infections and ischemic stroke. However, the term has broadened and now often includes *any* immune-related processes in the CNS, even in cases where only a single classic hallmark of inflammation is seen (Estes & McAllister, 2014; Woodburn, Bollinger, & Wohleb, 2021). Due to the various definitions of the term, some authors suggest using the term neuroinflammation only in contexts where multiple signs of inflammation are present. The purpose of this is to avoid incorrectly classifying processes as inflammatory when they might instead represent immune processes with adaptive roles, or even represent non-immune processes (Estes & McAllister, 2014). The term *immune signaling* has been proposed to be more appropriate to describe isolated release of immune-relevant molecules without concurrent expression of other signs of neuro-inflammation (Xanthos & Sandkuhler, 2014). In the later papers of this thesis, we have avoided the term neuroinflammation and instead used *immune-related processes*.

In *studies I*, *II* and *IV*, we investigated if CSF concentrations of immune-related proteins in patients with bipolar disorder differed from concentrations in controls. In *study III*, we investigated whether case–control associated immune-related proteins were associated with prospective clinical outcomes.

CEREBROSPINAL FLUID

PRODUCTION AND PHYSIOLOGY

Cerebrospinal fluid is a clear and colorless fluid occupying the spinal and cranial subarachnoid spaces between the arachnoid and pia mater, hence enclosing the brain and spinal cord (Tumani, Huss, & Bachhuber, 2017; Wright, Lai, & Sinclair, 2012). The knowledge of a fluid existing in the brain probably dates back to 3,000 BC, when the Egyptian physician Imhotep was the first to discover intracranial CSF. Later, the Greek physician Hippocrates (460-370 BC) described the existence of a liquid around the falx cerebri, and also described hydrocephalus as being caused by excess fluid, although he could not provide evidence for where inside the head this excess liquid was located. The localization of CSF is attributed to the Swedish scientist and philosopher Emmanuel Swedenborg in the 18th century, who referred to CSF as "spiritual lymph" and "highly gifted juice that is dispensed from the roof of the fourth ventricle to the medulla oblongata and the spinal cord" (Hajdu, 2003). It took until 1911, however, before the first complete description of the chemical composition of CSF was provided by the French physician William Mestrezat (Zambito Marsala, Gioulis, & Pistacchi, 2015).

An important function of CSF is to mechanically protect the brain in situations of fast head movements, preventing collision between the brain and skull. Cerebrospinal fluid is also important for maintaining metabolic homeostasis, and acid–base and electrolyte balances. It further supplies glial cells and neurons with nutrients, and removes degradation products of cellular metabolism. Finally, CSF enables transportation of neurotransmitters, hormones, and neuropeptides within the CNS (Brinker, Stopa, Morrison, & Klinge, 2014; Tumani et al., 2017).

Cerebrospinal fluid is mainly produced by the choroid plexus in the ventricles, but also in the meninges and brain parenchyma. The total volume of CSF in adults is between 125 mL and 150 mL, and the amount of CSF produced in 24 hours is approximately 500 mL. The CSF turnover rate therefore ranges between three and five times per day (Tumani et al., 2017; Wright et al., 2012). The secretion is a combination of a passive process (i.e., filtration of plasma from the fenestrated capillaries into the connective tissue of the choroid plexus) and an active process involving multiple ion channels (Wright et al., 2012). The path of circulation is from the choroid plexus through the ventricles, cisterns, and subarachnoid space.

The fluid is eventually absorbed into the venous circulation at the arachnoid villi (small protrusions of the arachnoid mater), and into the peripheral lymphatic vessels of the nasal mucosa and neck by transport along cranial nerves (Tumani et al., 2017). Cerebrospinal fluid flow in the subarachnoid space is shown in Figure 3.

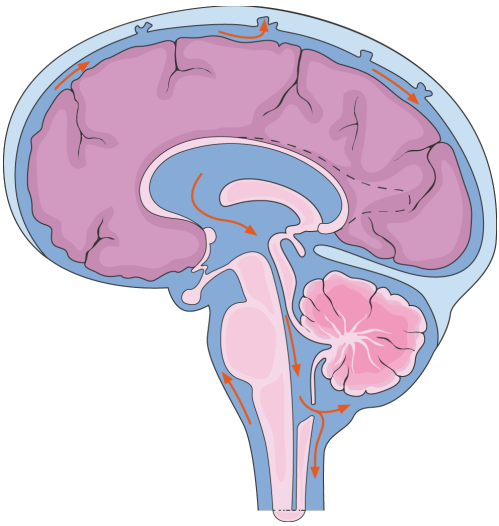

Figure 3. Illustration of cerebrospinal fluid flow in the subarachnoid space. Reprinted with permission from smart.servier.com.

BARRIERS OF THE CENTRAL NERVOUS SYSTEM COMPARTMENTS

The CNS and the circulatory system are separated by two barriers: the blood–brain barrier and the blood–CSF barrier. Both these barriers are important for maintaining the CNS environment and protecting the brain from the systemic circulation. The *blood–brain barrier* is an anatomical barrier, separating blood from the extracellular space of the brain. It consists of endothelial cells connected by tight junctions, basal membranes, pericytes, and astroglial endfeet and is located in CNS microvessels. The blood–brain barrier hinders transport of most molecules between blood and brain parenchyma, except for some small and lipophilic molecules, or if the transport is carried out through active transport mechanisms (Tumani et al., 2017).

The *blood–CSF barrier* separates the circulating blood from the CSF space. It consists of choroidal epithelial cells connected by gap junctions, basal membranes, and endothelium of the pia mater capillaries (Tumani et al., 2017). It is located at the choroid plexus—structures consisting of fenestrated capillaries, connective tissue, and epithelial cells—in the lateral, third, and fourth brain ventricles (Solar, Zamani, Kubickova, Dubovy, & Joukal, 2020). Transportation mechanisms across the blood–CSF barrier include passive transport of hydrophilic compounds, and active transport by carrier molecules and receptors. No barrier between the brain parenchyma and the CSF compartment has as yet been found. The interstitial fluid enclosing the cells in the brain parenchyma therefore closely resembles CSF (Nicholson & Hrabetova, 2017; Tumani et al., 2017).

Blood–brain barrier dysfunction has been of great interest in relation to CNS disorders, and has been reported in multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and Parkinson's and Alzheimer's disease (Sweeney, Sagare, & Zlokovic, 2018). The evidence is weaker for mental disorders, but a recent review and meta-analysis suggests that blood–brain barrier pathology is implicated also in psychiatric disorders such as schizophrenia and mood disorders. However, the review also reports that findings were divergent and important confounders were often not accounted for, hampering firm conclusions (Futtrup et al., 2020).

As there is no precise method for assessing blood–brain barrier and blood–CSF barrier function, the assessment is based on indirect measures. One common method is to determine the CSF/serum albumin ratio. Albumin is produced in the liver and not in the CNS (Brettschneider, Claus, Kassubek, & Tumani, 2005; Tibbling, Link, & Ohman, 1977; Tumani et al., 2017) and an increased ratio therefore indicates blood–brain barrier or blood–CSF barrier dysfunction allowing albumin to leak into the CSF. Other indirect measures are S100 calcium-binding protein B (S100B, a protein abundant in astrocytes, which under normal conditions is found at a low level in blood), radiolabeled markers, and radiological techniques (Futtrup et al., 2020; Kadry, Noorani, & Cucullo, 2020).

COMPOSITION OF CEREBROSPINAL FLUID

The total protein concentration of CSF is less than 0.5% of the protein concentration in blood (Rapoport, 1983) and CSF contains fewer than five cells per microliter under physiological conditions (Schilde et al., 2018). Since CSF is a filtrate of blood produced by the choroid plexus, approximately 80% of the proteins are blood-derived, and of these, albumin is the most abundant. The remainder are intrathecally produced or brain-derived proteins. Blood-derived proteins can penetrate the CSF compartment via passive diffusion across the blood–CSF barrier. This diffusion is related to the blood concentrations and molecular size. Different blood-derived CSF proteins have varying CSF/blood ratios, where larger proteins have higher concentration gradients between blood and CSF. With blood–CSF barrier dysfunction, as measured by an elevated CSF/serum albumin ratio, the concentration of blood-derived proteins in CSF increases (Reiber, 1994). By contrast, brain-derived proteins, such as those produced by glial cells or neurons, are not affected by blood concentration or blood–CSF barrier function (Reiber, 2001; Tumani et al., 2017).

So, what determines the concentration of proteins in CSF? The CSF total protein concentration, as well as concentrations of blood-derived proteins, is partly determined by the CSF flow/turnover rate in the subarachnoid space. Factors leading to a reduced CSF flow rate, such as a spinal block or a purulent meningitis, can lead to increased CSF concentrations of mainly blood-derived CSF proteins such as albumin (Brettschneider et al., 2005). For blood-derived proteins in CSF, concentrations are further affected by permeability of the blood–CSF barrier, as seen in meningitis (Brettschneider et al., 2005). Additionally, CSF protein concentrations can be influenced by circadian variations (with a higher CSF production rate during nighttime), site of sampling (lumbar vs. ventricular CSF), head movements (CSF flow rate and production are increased by head movements), and clearance via specific transporters (Nilsson et al., 1992; Tumani et al., 2017).

CEREBROSPINAL FLUID IN DISEASE AND DIAGNOSTICS

The CSF that occupies the lumbar cistern—from the termination of the spinal cord to the level of the first or second lumbar vertebrae—can be accessed by a procedure called *lumbar puncture*, in which a needle is inserted into the subarachnoid space to sample CSF. The first lumbar punctures were performed in 1891

by the German physician Heinrich Quincke and the British physician Walter Essex Wynter to remove excess fluid in patients with hydrocephalus and meningitis (Zambito Marsala et al., 2015). Figure 4 shows a lumbar puncture performed in the beginning of the 20th century.

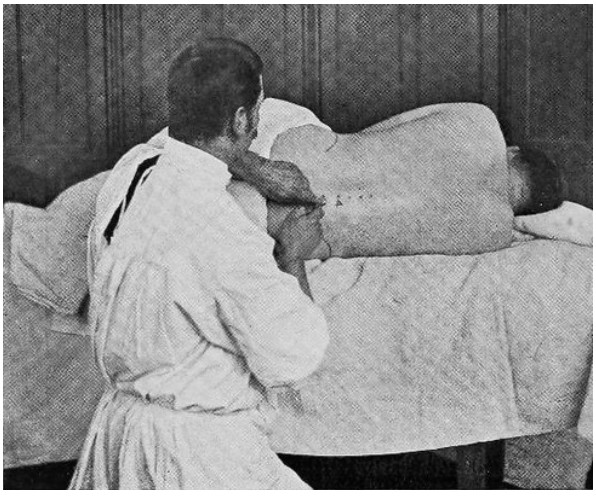

Figure 4. Image from Cerebro-spinal Fever (1916) by Medical Heritage Library, Inc., p 60. Copy of license: https://creativecommons.org/licenses/by-nc-sa/2.0/?ref=openverse&atype=rich.

Today, lumbar puncture is used in diagnostics and disease monitoring of a variety of CNS disorders such as infections, inflammatory diseases such as autoimmune encephalitis and Guillain-Barré syndrome, subarachnoid hemorrhage, lepto-meningeal metastases, Alzheimer's disease, and narcolepsy (Costerus, Brouwer, & van de Beek, 2018; Deisenhammer et al., 2009; Wright et al., 2012). Absolute and relative contraindications for the procedure are local infection at the lumbar puncture site, coagulation defects, and raised intracranial pressure (Wright et al., 2012). Complications include spinal hematoma, meningitis, persistent CSF leak, and headache following the procedure, i.e., post-dural puncture headache. All these complications are rare—for bleeding and infection, the risk is estimated to be less than 0.01%—except post-dural puncture headache, which occurs in approximately 9% of procedures with atraumatic needles (Wright et al., 2012). The headache is caused by a low-pressure condition, most commonly because the puncture site of the dura mater—the outermost of the three meninges—did not seal on withdrawal of the needle. The headache is characterized by worsening in

sitting or standing position, and improvement after lying down. In cases of persistent headache, caffein orally or, occasionally, intravenously can be used. If symptoms do not resolve, an epidural patch can be used, i.e., an injection of a small portion of the patient's own blood into the epidural space at the level of the lumbar puncture (Wright et al., 2012). Figure 5 shows the procedure of lumbar puncture.

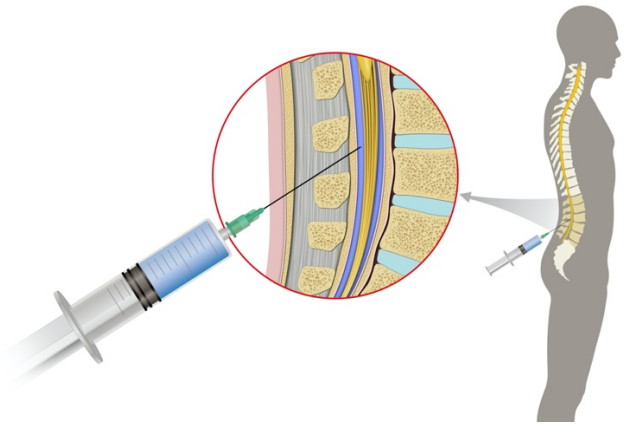

Figure 5. Illustration of lumbar puncture, a medical procedure in which a needle is inserted into the spinal canal to collect cerebrospinal fluid. Courtesy of Aldona, iStockphoto.com.

PREVIOUS CEREBROSPINAL FLUID STUDIES OF BIPOLAR DISORDER

Figure 6 shows the number of published studies accessible on PubMed with "bipolar disorder" and "CSF" in the title or abstract, yielding 358 results. However, not all of these studies include patients with bipolar disorder exclusively and some are meta-analyses and review articles. The very first study, published in 1960, investigated the protein-bound polysaccharide hexone in CSF in patients with epilepsy, manic-depressive disorder, and other psychotic disorders (Sakurada, Tanaka, & Takase, 1960). Between 1960 and 2008, between zero and ten studies per year were published on the topic, but in the past 15 years, the number of published papers in the field has accelerated, with a peak of 25 new studies in 2021. The studies have investigated a broad variety of proteins and other molecules, e.g., neuropeptides, neurotransmitters, hormones, viral antibodies, neurodegenerative markers, and immune markers.

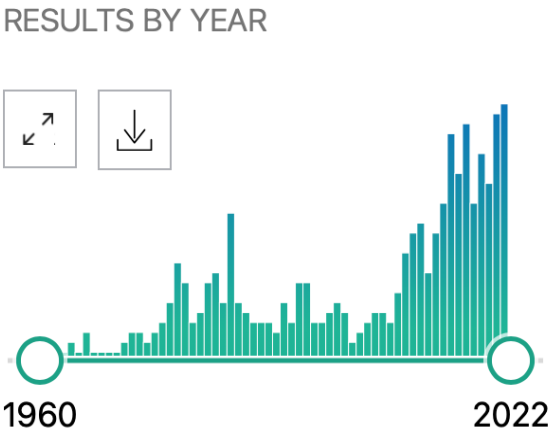

Figure 6. Bar chart of number of published studies in the field of cerebrospinal fluid (CSF) and bipolar disorder per year between 1960 and 2022. Results are from the search engine PubMed (https://pubmed.ncbi.nlm.nih.gov). The following search terms were used: ("bipolar disorder" OR "bipolar disorders" OR "manic-depressive" OR "manic depressive" OR "mania" OR "manic") AND ("CSF" OR "cerebrospinal fluid").

Earlier CSF studies were mainly hypothesis-driven, measuring a few analytes. The field has in recent years been complemented with semi-targeted studies that measure a number of prespecified analytes simultaneously. An example of such a publication is a study of patients with bipolar disorder and controls, measuring 22 CSF neuroplasticity-associated proteins using a multiplex immunoassay. The study found lower concentrations of neuronal cell adhesion molecule (NCAM)-1 and amyloid precursor protein (APP) in patients compared with controls (Hidese et al., 2020). So far, one study used a completely untargeted approach—mass spectrometry—to examine CSF from eleven patients with bipolar disorder. It identified a difference in CSF concentrations between patients and controls for 197 proteins; however, none of the results remained significant after correction for multiple comparisons (Al Shweiki et al., 2020).

AIMS

The aims of this doctoral study were to:

1. Identify CSF protein biomarkers[1] related to bipolar disorder to gain insights into pathophysiological processes (*studies I-II* and *IV-V*);
2. Identify diagnostic (*studies I-II* and *IV-V*) and prognostic (*study III*) CSF biomarkers for bipolar disorder;
3. Determine if concentrations of immune-related proteins are different in CSF from patients with bipolar disorder compared with controls (*studies I-II* and *IV*), and test if immune-related proteins are associated with prospective clinical outcomes (*study III*).

[1] Indicators of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention. Biomarkers may include molecular, histologic, radiographic, or physiological characteristics (Fda-Nih Biomarker Working Group, 2016).

PATIENTS AND METHODS

STUDY COHORTS

GENERAL DESCRIPTION

The studies in this thesis are based on a longitudinal Swedish study comprising two cohorts of patients with bipolar disorder and healthy controls—the Sankt Göran Bipolar Project Stockholm (SBP Stockholm) cohort and the Sankt Göran Bipolar Project Gothenburg (SBP Gothenburg) cohort. This is a naturalistic study, meticulously investigating patients at baseline and after 6–7 years, with respect to demographic, social, and clinical factors, as well as somatic and cognitive parameters. The study enrolls patients at the Northern Stockholm Psychiatric Clinic, Stockholm, and at the Bipolar Clinic, Sahlgrenska University Hospital, Gothenburg. Both catchment areas include neighborhoods with a range of socioeconomic strata. New patients under investigation for bipolar disorder, as well as existing patients with a diagnosis of bipolar disorder, were invited to participate.

The recruitment process and work-up procedures are summarized in Figure 7. The patients and controls participating in the studies in this thesis were enrolled between 2005 and 2015. The 6–7-year follow-up is ongoing and data from the follow-up is only used in one of the studies in this thesis (*study III*, in which we investigated clinical outcomes in the SBP Stockholm cohort).

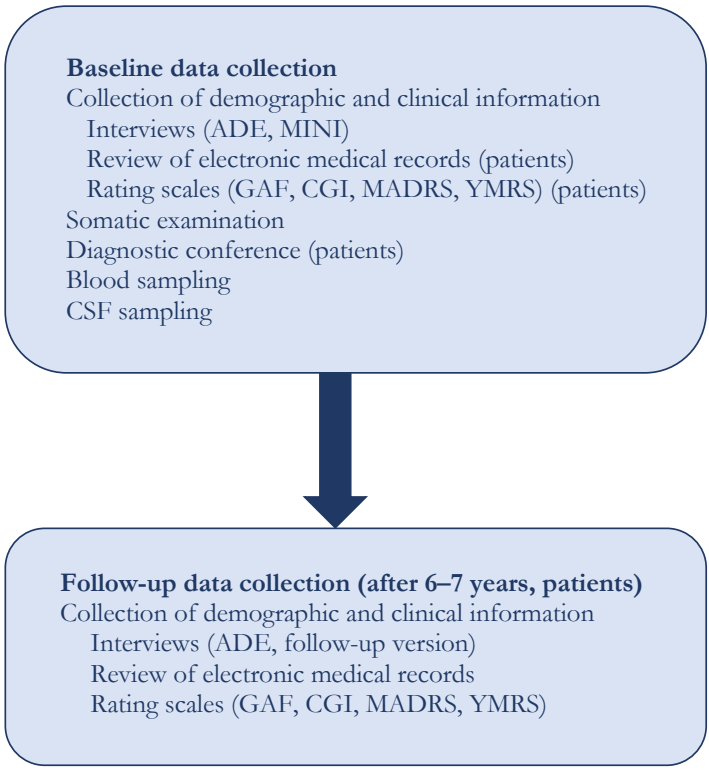

Figure 7. Summary of data collection. The Sankt Göran Bipolar Project also collects other types of information such as brain imaging data, but this figure shows the data collected and used for the studies in this thesis.
Abbreviations: ADE, Affective Disorder Evaluation. CGI, Clinical Global Impression. CSF, cerebrospinal fluid. GAF, Global Assessment of Functioning. MADRS, Montgomery-Åsberg Depression Rating Scale. MINI, Mini International Neuropsychiatric Interview. YMRS, Young Mania Rating Scale.

ETHICAL APPROVAL

All studies in this thesis were conducted in accordance with the Declaration of Helsinki and have been approved by the Regional Ethics Committee in Stockholm. All participants have given oral and written consent to participate in the study.

PATIENTS

General inclusion criteria for the study were age ≥18 years old and being under psychiatric evaluation for a suspected bipolar syndrome. At baseline, the patients were interviewed using a semi-structured interview called the Affective Disorder Evaluation (ADE). This instrument includes modified versions of the mood module of the Structured Clinical Interview for DSM-IV (SCID), and was developed for the Systematic Treatment Enhancement Program of Bipolar Disorder (STEP-BD) project (Sachs et al., 2003). The interview collects information about demographic and clinical factors, including measures of disease severity and medications, and aids the diagnostic process. To screen for comorbid psychiatric disorders, the Mini International Neuropsychiatric Interview (M.I.N.I) was used (Sheehan et al., 1998). The ADE and M.I.N.I. interviews were conducted by psychiatrists or residents in psychiatry in the SBP Stockholm and by psychiatrists, residents in psychiatry, a research nurse, and a psychologist in the SBP Gothenburg. A diagnostic decision was made by a consensus panel of board-certified psychiatrists specialized in bipolar disorder in Stockholm, and by at least one board-certified psychiatrist in Gothenburg. The decision was based on a best estimate procedure (Leckman, Sholomskas, Thompson, Belanger, & Weissman, 1982) using all available information from the ADE, the M.I.N.I., previous medical records, and interviews with next of kin where possible.

Several rating scales were used to assess symptom severity and level of functioning at baseline. Disease severity was assessed using the Clinical Global Impression (CGI) rating scale, and the Global Assessment of Functioning (GAF) scale, divided into a symptom severity (GAF-s) and a functional level (GAF-f) subscale. To assess manic and depressive symptoms at lumbar puncture, the Young Mania Rating Scale (YMRS) and the Montgomery–Åsberg Depression Rating Scale (MADRS) were used. Patients also underwent a somatic examination, blood tests, and a lumbar puncture. The study did not interfere with patients' treatment.

CONTROLS

Control participants were randomly selected by Statistics Sweden (SCB, www.scb.se) from the general population living in the same catchment area as enrolled patients. Controls were age- and sex-matched to the subset of patients who were enrolled when recruiting of controls started. Controls were interviewed by telephone by research nurses and eligible controls were scheduled for a personal examination. At the physical visit, a M.I.N.I. interview was conducted to screen for psychiatric conditions, and selected parts of the ADE were used to collect information about demographic factors, medications, and past psychiatric disorders. Exclusion criteria for controls were overconsumption of alcohol, substance abuse, untreated endocrinological disorders, pregnancy, dementia, neurological conditions other than mild migraines, coexisting psychiatric conditions other than past minor depressive episodes, isolated episodes of panic disorder, previous eating or obsessive–compulsive disorder that had remitted spontaneously or with brief psychotherapy counseling, and schizophrenia or bipolar disorder in first-degree relatives. Control participants underwent a somatic examination, blood tests, and lumbar puncture.

LUMBAR PUNCTURE

A lumbar puncture for patients and controls was performed at baseline between 9 and 10 a.m. after fasting overnight. Twelve mL of CSF was collected with a fine disposable spinal needle inserted into the L3/L4 or L4/L5 interspace. The tube was inverted to avoid concentration gradients, divided into 1.0–1.6 mL aliquots, and immediately stored at -80ºC until analyzed. All samples at the SBP Gothenburg were centrifuged prior to freezing. At the SBP Stockholm, samples from only six controls were centrifuged.

FOLLOW-UP

During the follow-up period, patients received usual psychiatric care at their bipolar outpatient unit, and were followed-up annually and when needed. After 6–7 years, patients were scheduled for a comprehensive follow-up. This included an interview with board-certified psychiatrists using a modified version of the ADE, to collect information about disease-related events and present status. Prior to the interview, patients' electronic medical records were reviewed.

In this thesis, only follow-up data from the SBP Stockholm is used—the follow-up for the SBP Gothenburg is currently ongoing.

CEREBROSPINAL FLUID ANALYSES

CYTOKINE MEASUREMENTS

In *study I*, CSF cytokine concentrations were measured using electrochemiluminescence enzyme-linked immunosorbent assays (ELISAs). Briefly, capture antibodies coat the surface of a plate. Samples are then incubated on the plate, allowing the analyte to bind to the capture antibody. Electrochemiluminescence tagged detection antibodies are then added, binding to the analyte and leading to a reaction in which an intermediate causes these molecules to enter an excited state. When re-entering a lower energy level, these molecules emit a photon of light. The fluorescent regions—in which specific interactions have occurred between the antibodies and the analyte—are quantified and correspond to the analyte concentration in the sample (Forster, Bertoncello, & Keyes, 2009; Keustermans, Hoeks, Meerding, Prakken, & de Jager, 2013).

For IL-6, a singleplex assay (Human IL-6 Ultra-sensitive kit; Meso Scale Discovery, Rockville, MD, USA) was used. For the other cytokines (IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, TNF-α, and IFN-γ), a multiplex assay was used: the Meso Scale Discovery (MSD) 96-well multi-array and multi-spot human cytokine assay (Human Cytokine Assay Ultra-Sensitive Kit, Meso Scale Discovery, Rockville, MD, USA).

ANALYSIS OF MARKERS OF MICROGLIAL AND ASTROCYTE ACTIVATION

The aim of *study II* was to assess markers of microglial and astrocyte activation in CSF in patients and controls. Monocyte chemoattractant protein 1 (MCP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) were determined by electrochemiluminescence ELISA (Human MCP-1 Ultra-Sensitive Kit and Human TIMP-1 kit; Meso Scale Discovery, Rockville, MD, USA). Chitinase-3-like protein 1 (YKL-40/CHI3L1), tissue inhibitor of metalloproteinases-2

(TIMP-2), and soluble cluster of differentiation 14 (sCD14) were analyzed by colorimetric ELISA (Human sCD14 quantikine ELISA kit, Human chitinase-3 quantikine ELISA kit, and Human TIMP-2 quantikine ELISA kit; R&D Systems, Inc., Minneapolis, MN, USA). This method is based on a color reaction instead of a light reaction. Briefly, tetramethylbenzidine (TMB) substrate solution is added after the initial steps of complex formation reaction between the capture antibody, analyte, and detection antibody. A blue color develops in proportion to the amount of analyte in the sample. Finally, the absorbance of the color is measured. The analyses were performed at the Clinical Neurochemistry Laboratory in Mölndal, Sweden.

EXPLORATORY CEREBROSPINAL FLUID ANALYSES

In *studies IV* and *V*, a large number of CSF proteins was analyzed simultaneously using multiplex immunoassays designed for protein biomarker discovery based on so-called "proximity extension assay (PEA)" technology (Olink® Proteomics, Uppsala, Sweden). Briefly, a pair of oligonucleotide-labeled antibodies (PEA probes) binds to the respective proteins in the sample. When two deoxyribonucleic acid (DNA) tags are brought into close proximity, they hybridize and addition of a DNA polymerase leads to formation of a polymerase chain reaction (PCR) template. This complex is amplified and quantified using microfluidic real-time PCR, enabling a multiplex setup with low cross-reactivity. The number of PCR cycles is related to the protein concentration in the sample. Due to the relative quantification method, protein concentrations are represented by normalized protein expression values on an arbitrary log2-scale (Assarsson et al., 2014).

In *study IV*, the Olink Proseek Multiplex Oncology I, Inflammation I, and Cardiovascular I panels (Olink® Proteomics, Uppsala, Sweden) were used. Together these three panels include 201 unique assays. Samples of CSF from both the SBP Stockholm and the SBP Gothenburg were analyzed. For the SBP Stockholm, analyses were performed at the Clinical Biomarker Facility at SciLifeLab Uppsala, Sweden. For the SBP Gothenburg, analyses were performed at Olink Bioscience Uppsala, Sweden. Samples from the SBP Stockholm were analyzed on three plates whereas samples from the SBP Gothenburg were analyzed on two plates.

In *study V*, CSF samples were analyzed using the Olink Proseek Neurology I panel (Olink® Proteomics, Uppsala, Sweden) containing 92 proteins related to neurobiological processes, immunology, metabolism, development, and cellular

regulation. Again, CSF from both the SBP Stockholm and the SBP Gothenburg was analyzed. The study subjects overlapped with study subjects in *study IV* and, for the SBP Stockholm cohort, the subjects also overlapped with individuals included in *studies I–III*.

Initial preprocessing and quality controls were performed by SciLifeLab, Uppsala (SBP Stockholm), and Olink Bioscience, Uppsala (SBP Gothenburg) in *studies IV-V*. No samples from either cohort failed the initial quality control. Further preprocessing steps and quality controls were performed by us. Proteins for which >25% of subjects had values below the limit of detection in both the patient and the control group were excluded from further analyses. For the remaining proteins, values below the detection limit were included. Associations between time of CSF sampling and protein concentrations were tested using Spearman correlation and no protein concentration was significantly correlated with time of CSF sampling in both cohorts. The staff performing the analyses were blinded to all phenotype information.

Analysis of albumin concentrations

Albumin concentrations were analyzed by immunonephelometry on a Beckman IMMAGE Immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA) at the Clinical Neurochemistry Laboratory in Mölndal, Sweden. To assess blood–brain and blood–CSF barrier function, we calculated the ratio between the albumin concentration in CSF and serum (Tibbling et al., 1977).

Statistical methods

Associations between protein concentrations and bipolar disorder

Group differences in CSF concentrations between patients with bipolar disorder and controls were established by analysis of covariance (ANCOVA) in *study I*. In *study II*, linear regression models were built, with group (patient/control) and selected covariates as independent variables and CSF protein concentration as the dependent variable. In *studies IV* and *V*, logistic regression models were used.

Protein concentrations in CSF and selected covariates were independent variables and group (patient/control) was the dependent variable.

Associations between protein concentrations and clinical features

To investigate associations between protein concentration and demographic and clinical features, Spearman correlation, and linear and logistic regression models were used (*studies I-V*).

Selection of covariates for analyses

Several demographic and clinical factors were found to be associated with protein concentrations and with bipolar disorder. Hence, it was important to carefully select the variables to be used as covariates in the analyses, in order to reduce confounding effects in the analyses assessing relations between CSF protein concentrations and bipolar disorder. Even though a true confounder is associated with both exposure and outcome (P. H. Lee, 2014), we chose to be somewhat overinclusive in our selection of covariates in some studies. The reason for this was that we estimated the risk for bias to be larger if we failed to include an important confounder in an analysis, than if we sometimes included a covariate that perhaps did not represent a confounder. In summary, the methods used to select confounding variables as covariates in the statistical analyses were: (1) including variables shown to be associated with CSF protein concentrations in linear regression models (*study I*); (2) including variables shown to be associated with CSF protein concentrations and/or clinical outcome (*study III*); (3) including variables associated with bipolar disorder (*studies IV* and *V*); and (4) including preselected variables based on reasoning (*study II*).

Other notes on statistics

To correct for multiple testing in *studies I–III*, statistical adjustment for multiple comparisons was performed using the Bonferroni method or false discovery rate (FDR) adjustments. By contrast, in *studies IV* and *V*, we did not perform statistical adjustment for multiple comparisons because we worked with two independent cohorts (the SBP Stockholm and the SBP Gothenburg). To decrease the risk for type I errors, we required statically significant findings in the same direction in both the SBP Stockholm and the SBP Gothenburg cohorts.

In *studies IV* and *V*, multivariate methods were used as a complement to univariate methods. We used principal component analyses (PCA) to summarize the variance in the included protein concentrations. In *studies I–III*, SPSS Statistics software (IBM Corp., Armonk, NY, USA) was used whereas R, version 3.6.3 (R Core, Brisbane, Australia), was used in *studies IV* and *V*.

GENETIC ANALYSES

In *study V*, we conducted genome-wide association analyses of proteins that were associated with case–control status. A subset of the study population had previously been genotyped at the Broad Institute of Harvard and Massachusetts Institute of Technology (MIT) using the Affymetrix 6.0 chip (Affymetrix, Santa Clara, CA, USA), the Illumina OmniExpress BeadChip (Illumina, San Diego, CA, USA), and the Infinium PsychArray-24 v1.2 BeadChip (Illumina, San Diego, CA, USA). The Rapid Imputation for COnsortias PipeLIne (RICOPILI) was used for quality control (Lam et al., 2020). Genotypes were imputed to the Haplotype Reference Consortium (HRC) 1.1 reference panel on the Sanger Imputation Server (SIS) (McCarthy et al., 2016). Genome-wide association analyses were performed using PLINK (Purcell & Chang). Additive regression models were built to test the association between genotype and CSF concentration of the selected proteins.

RESULTS AND DISCUSSION

DESCRIPTION OF THE STUDY COHORTS

Data and samples from the SBP Stockholm were included in *studies I-V* and from the SBP Gothenburg in *studies IV* and *V*. Table 1 in *study IV* presents demographics and clinical characteristics of the study cohorts, showing some important differences between the two cohorts. These included a larger proportion of patients with bipolar I disorder, fewer total lifetime episodes, less comorbid anxiety disorders, and a different pattern of prescribed psychiatric medications (more patients on lithium and fewer on anticonvulsants) in the SBP Stockholm compared with the SBP Gothenburg.

IMMUNE MARKERS IN CEREBROSPINAL FLUID

CYTOKINES AND MARKERS OF MICROGLIAL AND ASTROCYTE ACTIVATION (STUDIES I, II, AND IV)

In *study I*, we analyzed CSF cytokines from 121 SBP Stockholm patients with bipolar disorder and 71 controls. The aim was to test the hypothesis that bipolar disorder is associated with immune activation, as reflected by altered CSF cytokine concentrations. Using immunoassays, we analyzed eleven different cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, TNF-α, and IFN-γ). In a covariate-adjusted model, we found that CSF concentrations of IL-8 were higher in patients compared with controls. The values for all other cytokines were under or close to the detection limit in a large proportion of both patients and controls. We therefore limited further analyses to IL-8, a chemotactic cytokine that has several known functions in the peripheral immune system, and some known functions in the brain. Cerebrospinal fluid IL-8 concentrations were positively associated with age and CSF/serum albumin ratio. In the patient group, IL-8 concentrations were further positively associated with lithium and

antipsychotic treatment. The CSF concentrations of IL-8 in patients and controls are shown in Figure 1 in *study I* (Isgren et al., 2015).

Interleukin-8 has several functions in the peripheral immune system, including activation of and chemotactic effects on immune cells (Murphy et al., 2000; Stuart & Baune, 2014). Knowledge of the role of IL-8 in the CNS is scarce, but it is suggested to have immune functions and has been shown to be involved in synaptic plasticity, neuronal electric activity, and neurotransmitter release (Giovannelli et al., 1998; Y. B. Lee, Nagai, & Kim, 2002; Semple, Kossmann, & Morganti-Kossmann, 2010; Stuart & Baune, 2014). A few previous studies have also linked IL-8 to psychiatric disorders. These include a gene set analysis of post-mortem brain tissue, showing increased IL-8 expression in patients with major depression (Shelton et al., 2011).

We cannot disentangle the role of medication status in this study due to the naturalistic and cross-sectional design. Is a higher CSF concentration of IL-8 related to bipolar disorder or to the use of psychotropic drugs? The association with lithium and antipsychotic treatments, as well as our failure to find significant associations between CSF IL-8 concentrations and measures of disease severity, suggest that increased IL-8 concentrations and treatment with lithium and antipsychotics are directly associated. However, we cannot rule out "confounding by severity," where those patients with more severe forms of bipolar disorder are the ones receiving treatment with lithium and/or antipsychotic drugs. In summary, *study I* showed that patients with bipolar disorder had higher CSF concentrations of IL-8 compared with controls, with an association with lithium and antipsychotic treatment. We concluded that this might reflect an immune aberration in bipolar disorder or be secondary to pharmacological effects.

In *study II*, we analyzed five markers of monocyte, microglial, and astrocyte activation as well as associated tissue remodeling processes in CSF from 125 SBP Stockholm bipolar disorder patients and 87 controls: MCP-1, YKL-40, sCD14, TIMP-1, and TIMP-2. The aim was similar to the aim of *study I*, i.e., to test the hypothesis that bipolar disorder is associated with immune activation, as reflected by altered CSF concentrations of monocyte, microglial, and astrocyte markers. We found higher CSF concentrations of MCP-1 and YKL-40 in patients compared with controls after controlling for potential confounders. We also found some associations between psychotropic drugs and CSF protein concentrations: use of lithium, antipsychotics, and benzodiazepines was positively

associated with CSF MCP-1, whereas use of antidepressants was negatively associated with MCP-1 concentrations. These drug associations were, however, not significant when adjusting for multiple comparisons. The CSF concentrations of MCP-1 and YKL-40 in patients and controls are shown in Figure 1 in *study II* (Jakobsson et al., 2015).

Monocyte chemoattractant protein 1 is expressed in the brain by several cell types in response to proinflammatory cytokines including microglia, astrocytes, and neurons. This can lead to recruitment of microglia to sites of injury or inflammation (Hinojosa, Garcia-Bueno, Leza, & Madrigal, 2011). Moreover, MCP-1 also has neuromodulatory functions (Melik-Parsadaniantz & Rostene, 2008). On the other hand, YKL-40 is, in the CNS, primarily expressed by astrocytes, but also by microglia (Bhardwaj et al., 2015; Bonneh-Barkay et al., 2008), and has been associated with many peripheral chronic inflammatory conditions, and also with Alzheimer's disease (Lananna et al., 2020; Rathcke & Vestergaard, 2006). In a study of ours (not included in this thesis), we have shown a negative association between concentrations of CSF YKL-40 and executive cognitive performance in mood-stable bipolar disorder patients (Rolstad et al., 2015). The conclusion of *study II* was that neuroimmune processes may play a role in the pathophysiology of bipolar disorder, as reflected by higher CSF concentrations of MCP-1 and YKL-40 in bipolar disorder.

In *study IV*, where we analyzed a broad set of CSF proteins using multiplex immunoassays in the SBP in both Stockholm and Gothenburg, the markers IL-8, MCP-1, and YKL-40 were included. However, the findings of higher CSF IL-8, MCP-1, and YKL-40 in patients with bipolar disorder compared with controls were not replicated in the SBP Gothenburg cohort (see Table 3). Further, only YKL-40 differed between cases and controls in the SBP Stockholm cohort—for IL-8 and MCP-1, the difference between patients and controls was not quite statistically significant in this study.

There are several possible reasons for the discrepancies between the study cohorts. Firstly, the findings of higher CSF concentrations of IL-8, MCP-1, and YKL-40 in patients in the SBP Stockholm in *studies I* and *II* may have been false positive findings or may have been related to unmeasured confounding factors. Also, there are several clinical differences between the two cohorts, which may be associated with protein concentrations and lead to divergent results.

Table 3. Summary of results from our studies of CSF concentrations of IL-8, MCP-1, and YKL-40 and associations with bipolar disorder.

	SBP Stockholm		**SBP Gothenburg**
	ELISA	**PEA**	**PEA**
	(study I-II)	*(study IV)*	*(study IV)*
IL-8	**BD>CTRL**[1]	BD>CTRL[3]	BD<CTRL[3]
	(p=0.042)	(p=0.08)	(p=0.66)
MCP-1	**BD>CTRL**[2]	BD>CTRL[3]	BD>CTRL[3]
	(p=0.004)	(p=0.07)	(p=0.82)
YKL-40	**BD>CTRL**[2]	**BD>CTRL**[3]	BD<CTRL[3]
	(p=0.014)	**(p=0.03)**	(p=0.54)

[1]Analysis of covariance (ANCOVA) with age and CSF/serum albumin ratio as covariates.
[2] Linear regression with CSF protein concentration as dependent variable and age, sex, body mass index, nicotine use, CSF/serum albumin ratio, and C-reactive protein as covariates.
[3] Logistic regression with group (BD/CTRL) as dependent variable and age, sex, body mass index, nicotine use, and CSF/serum albumin ratio as covariates.
Results in bold are significant at a $p<0.05$ level.
Abbreviations: BD, bipolar disorder. CTRL, controls. ELISA, electrochemiluminescence enzyme-linked immunosorbent assays. IL-8, interleukin-8. MCP-1, monocyte chemoattractant protein 1. PEA, proximity extension assay. SBP, Sankt Göran Bipolar Project. YKL-40, chitinase 3-like protein 1.

IMMUNE MARKERS AND ASSOCIATIONS WITH PROSPECTIVE OUTCOMES (STUDY III)

Study III was a longitudinal study, investing associations between prospective clinical outcomes and the CSF proteins that we had found to differ between bipolar disorder patients and controls in *studies I* and *II*. The aim was to investigate whether higher concentrations of these proteins were associated with poorer outcomes, as it is still unclear whether immune and microglia activation reflect pathological mechanisms, adaptive responses to disease mechanisms, or vulnerability to mood disorders (Bhattacharya et al., 2016; Mesman et al., 2015).

A total of 77 SBP Stockholm patients who had their CSF analyzed at baseline were followed for 6–7 years. Associations between baseline concentrations of CSF proteins and clinical outcomes at follow-up (mood episodes, psychotic symptoms, suicide attempts, inpatient care, and GAF score change) were assessed. After correction for multiple testing, we found a negative association between YKL-40 concentrations and manic/hypomanic episodes. We found no other significant associations between baseline CSF protein concentrations and

prospective outcomes. The results from the analyses are shown in Table 2 in *study III* (Isgren et al., 2017). We concluded that high concentrations of these selected CSF markers of neuroimmune processes were not consistently associated with poor clinical outcomes, and that these proteins may be involved in adaptive immune processes or may be associated with bipolar disorder, without having predictive value for disease progression.

OTHER IMMUNE-RELATED MARKERS (STUDY IV)

In *study IV*, we measured 105 proteins in CSF from patients with bipolar disorder and controls in the SBP Stockholm and SBP Gothenburg cohorts. One-third of the proteins were from a panel developed for measuring proteins associated with inflammatory diseases and related biological processes. There were no differences in CSF concentrations between patients and controls for any of these immune-related proteins that replicated across cohorts.

EXPLORATORY CEREBROSPINAL FLUID STUDY (STUDY IV)

As previously described, *study IV* was an exploratory study of CSF concentrations in two independent bipolar disorder cohorts, the SBP in Stockholm and in Gothenburg. The aim was to investigate if the CSF concentration of any of the investigated proteins was associated with bipolar disorder. A total of 201 protein concentrations were measured using multiplex immunoassays. After quality control and removal of proteins with a low detection rate, 105 proteins were included in the analyses. We found that CSF concentrations of growth hormone (GH) were lower in patients with bipolar disorder compared with controls in both cohorts, using covariate-adjusted logistic regression models. For the remaining proteins, there were no statistically significant differences between patients and controls that were replicated between cohorts. In the multivariate analysis (PCA), there were no group differences between patients with bipolar disorder and controls in either cohort (see Supplementary figure 2 in *study IV*). In a secondary analysis of patients with bipolar I disorder and controls, GH was again the only protein for which a group difference was found across cohorts, with patients having lower CSF concentrations compared with controls. The effect sizes were larger than in the analysis that included all bipolar disorder spectrum patients. Cerebrospinal

fluid concentrations of GH in patients and controls are shown in Figure 1 in *study IV* and the association between GH and bipolar disorder is displayed in Table 2 in *study IV*.

We performed *post hoc* analyses of CSF GH to investigate relationships with demographic and disease-specific features. We found that GH was negatively associated with male sex, age, CSF/serum albumin ratio, and body mass index (BMI) in both cohorts. In the patient group, CSF GH was negatively associated with antipsychotic treatment in the SBP Gothenburg cohort, but not in the SBP Stockholm cohort. When excluding patients with antipsychotic treatment, there was still a significant difference in CSF GH concentrations between patients and controls in the SBP Stockholm, but the difference was not significant in the SBP Gothenburg after excluding these patients. A limited sample size contributes to this finding, and it is also possible that the negative association seen between GH and antipsychotic treatment in Gothenburg was driven by "confounding by indication" including "confounding by severity," i.e., confounding that is due to clinical factors affecting what type of mood-stabilizing drug the patient is prescribed. But even so, it cannot be ruled out that psychotropic drugs have an impact on CSF GH concentrations.

So, is GH of relevance for brain disorders? Indeed, it is known that GH has many effects in the CNS. The hormone has been shown to support glial differentiation, neuronal plasticity, and brain growth, and is also known to influence higher brain functions, such as memory, mood, and behavior (Hampl, Bicikova, & Sosvorova, 2015; Schneider, Pagotto, & Stalla, 2003). There are a few previous studies investigating associations between serum GH and bipolar disorder (da Silva et al., 2017; Duval et al., 2020). However, our study is the first to study CSF GH in relation to bipolar disorder.

In conclusion, in *study IV*, we found lower CSF concentrations of GH in bipolar disorder, but no other disease specific aberrations.

CENTRAL NERVOUS SYSTEM MARKERS IN CEREBROSPINAL FLUID (STUDY V)

In *study V*, we analyzed CSF from the SBP Stockholm and the SBP Gothenburg cohorts, using multiplex immunoassays designed for protein biomarker discovery in the CNS field. The aim was to identify CNS-related proteins associated with bipolar disorder. Ninety-two proteins were analyzed, but after initial pre-processing, 80 proteins remained in the dataset for further analyses. For two proteins, there was a significant association with bipolar disorder that replicated across cohorts: CSF concentrations of C-type lectin domain family 1 member B (CLEC1B) were higher in patients than in controls, whereas CSF concentrations of testican-1 were lower in patients than in controls. In a secondary analysis, patients with bipolar I disorder were compared with controls. Here, we identified two additional CSF proteins that were associated with bipolar disorder in both cohorts: tumor necrosis factor receptor superfamily member 21 (TNFRSF21) and draxin, both with lower CSF concentrations in patients than in controls. A forest plot showing effect sizes for case–control association tests is shown in Figure 1 in *study V* (Göteson et al., 2021).

We further tested if the case–control-associated proteins were associated with measures of disease severity, and found that none of them were associated with illness duration, total lifetime mood episodes, or lifetime CGI score. In relation to psychiatric drugs, testican-1 was negatively associated with antipsychotic treatment. However, the concentrations of testican-1 were significantly lower in patients with bipolar disorder also after excluding patients who used antipsychotic drugs, indicating that the case–control association with testican-1 cannot be explained by antipsychotic drug treatment.

This raises the question how these case–control-associated proteins identified in *study V* are relevant to psychiatry in the light of previously published data? Starting with testican-1, this is a CNS-enriched extracellular matrix protein that has been proposed to be involved in neuronal cell–cell and cell–matrix interactions even though the biological functions have not been extensively explored (Alliel, Perin, Jolles, & Bonnet, 1993; Y. Lee et al., 2018). Testican-1 has previously been implicated in neurodegenerative disorders such as multiple system atrophy (Jabbari et al., 2019) and Alzheimer's disease (Barrera-Ocampo et al., 2016). By contrast, CLEC1B is not known to be expressed in the CNS (Uhlen et al., 2015).

The two additional proteins that were significantly lower in bipolar I disorder patients compared with controls—draxin and TNFRSF21—do have important functions in the brain. Draxin is implicated in axon guidance during development of forebrain commissures (Islam et al., 2009; Shinmyo et al., 2015), whereas TNFRSF21 is important in formation of neuronal connections in the developing brain by regulating neuronal apoptosis and axonal pruning (Nikolaev, McLaughlin, O'Leary, & Tessier-Lavigne, 2009).

To identify regulatory generic variants for our case–control-associated proteins, we finally performed genome-wide association analyses of the four proteins that differed between cases and controls. We found no association between single nucleotide polymorphisms (SNPs) and protein concentrations at the genome-wide significance level ($p<5x10^{-8}$). In addition, we performed gene-based analysis, which is a test using the aggregated effects of multiple SNPs within a gene, to test associations between specific genes and protein concentrations. We found associations between the CSF concentrations of TNFRSF21 and the voltage-gated calcium channel subunit *CACNG4,* and between the CSF concentrations of CLEC1B and the lipid droplet-associated gene *PLIN5*.

To conclude, *study V* reports four novel CSF proteins associated with bipolar disorder, three of which have previously known, important CNS functions.

ALBUMIN RATIO IN BIPOLAR DISORDER

As outlined in the Introduction, blood–brain barrier pathology has been implicated but not yet proven in bipolar disorder (Futtrup et al., 2020). The CSF/serum albumin ratio distribution in patients and controls in our study cohorts is shown in Figure 8.

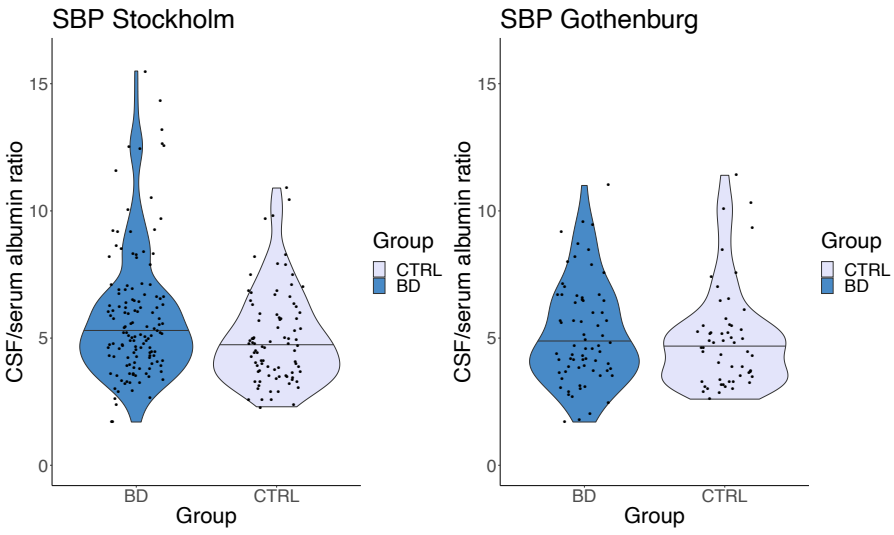

Figure 8. Cerebrospinal (CSF)/serum albumin ratio in patients and controls. Abbreviations: BD, bipolar disorder. CTRL, controls. SBP, Sankt Göran Bipolar Project.

We found that the CSF/serum albumin ratio was higher in patients with bipolar disorder than in controls in the SBP Stockholm, but not in the SBP Gothenburg. The covariate-adjusted linear regression analyses are shown in Table 4 below. The divergent findings may be partly due to clinical differences between the cohorts; at any rate, the findings speak against pronounced blood–brain barrier pathology in bipolar disorder.

Table 4. Linear regression models investigating associations between CSF/serum albumin ratio and bipolar disorder.

	SBP Stockholm			**SBP Gothenburg**		
	β	SE	p	β	SE	p
Patient/control	0.06	0.02	**0.005**	0.04	0.03	0.132
Age	0.003	0.001	**<0.001**	0.004	0.001	**<0.001**
Sex (male)	0.09	0.02	**<0.001**	0.11	0.027	**<0.001**

Log10 transformed CSF/serum albumin ratio are dependent variable.
Abbreviations: SBP, Sankt Göran Bipolar Project. SE, standard error.

CEREBROSPINAL FLUID PROTEINS AND RELATION TO DEMOGRAPHIC AND CLINICAL FACTORS

In both *studies IV* and *V*, we found that most CSF proteins were positively intercorrelated, with a median protein–protein correlation coefficient (Spearman's rho) of 0.5–0.6. The first principal component in a PCA explained 50–60% of the variation in the protein datasets. This is in line with the results of a study of CSF biomarkers of Alzheimer's disease using the same PEA technology, where a large proportion of CSF proteins were positively intercorrelated (Whelan et al., 2019). Further, the first principal component correlated with age, CSF/serum albumin ratio, and male sex in both *studies IV* and *V* (see Supplementary figure 1 in *study IV*). This suggests that these factors should be included as covariates in the analyses to minimize the risk for false positive findings when investigating associations between CSF protein concentrations and bipolar disorder.

CEREBROSPINAL FLUID PROTEINS AND RELATION TO PSYCHOTROPIC DRUGS

Several CSF proteins in this thesis were associated with psychotropic drugs. For example, IL-8 concentrations were positively associated with lithium and antipsychotic treatment in *study I*. In *study IV*, seven out of 105 proteins were associated with one of the psychotropic drug groups (lithium, antipsychotics, antiepileptics, antidepressants) in the SBP in both Stockholm and Gothenburg. These associations between CSF proteins and psychiatric drug groups are shown in Supplementary figure 4 in *study IV*. The findings highlight that these psychotropic drugs are associated with some CSF protein concentrations. However, it is difficult to disentangle the causal mechanisms between protein concentrations and psychotropic drugs due to the cross-sectional study design.

CEREBROSPINAL FLUID PROTEINS AND ASSOCIATIONS WITH SERUM PROTEIN CONCENTRATIONS

In a previously published study, we had measured serum protein concentrations in the SBP Stockholm and the SBP Gothenburg using the same Olink Proseek Multiplex panels (Oncology I, Inflammation I, and Cardiovascular I, Olink® Proteomics, Uppsala, Sweden) (Göteson et al., 2022) as we used to measure CSF protein concentrations in *study IV*. CSF and blood sampling were performed on the same day and we could therefore investigate between–fluid correlations for the 105 proteins that were measured both in CSF and serum.

We found that the CSF–serum correlations were generally low (median Spearman's rho 0.18 in the SBP Stockholm and 0.12 in the SBP Gothenburg). For GH, the between–fluid correlation coefficient was 0.59 in the SBP Stockholm and 0.55 in the SBP Gothenburg. Correlations between CSF and serum protein concentrations are shown in Figure 9. The results are similar to an Alzheimer's disease study, where 190 proteins were analyzed in CSF and serum. Also in that study, the strongest protein–protein correlations were seen within fluids, and the median correlation coefficient between CSF and serum for the analyzed proteins were 0.18 (Whelan et al., 2019).

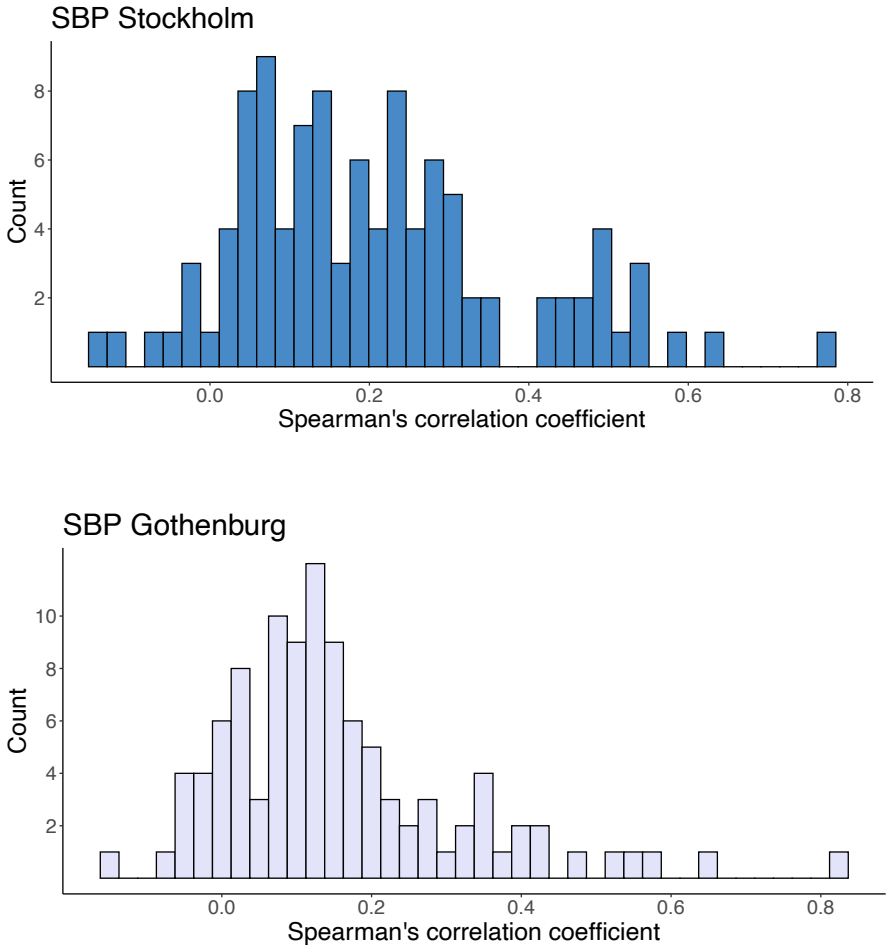

Figure 9. Cerebrospinal fluid (CSF)–serum correlations for the 105 proteins analyzed in CSF in *study IV* and in serum in a previous study (Göteson et al., 2022). Abbreviations: SBP, Sankt Göran Bipolar Project.

GENERAL DISCUSSION

SUMMARY OF CEREBROSPINAL FLUID STUDIES OF BIPOLAR DISORDER

OUR CEREBROSPINAL FLUID STUDIES OF BIPOLAR DISORDER

In *studies I* and *II*, we found increased CSF concentrations of some immune-related proteins (IL-8, MCP-1, and YKL-40) in patients compared with controls at the SBP in Stockholm. However, in *study IV*, we failed to replicate these findings at the SBP in Gothenburg.

Study III investigated associations between the case–control-associated proteins at baseline, and clinical outcomes at the 6–7-year follow-up at the SBP Stockholm. We found no significant associations between baseline CSF protein concentrations and prospective outcomes, except for a negative association between YKL-40 concentrations and manic/hypomanic episodes. We concluded that high concentrations of these CSF markers of neuroimmune processes are not consistently associated with poor clinical outcomes.

For *studies IV* and *V*, we used two independent cohorts of patients with bipolar disorder and explored a large number of CSF proteins to study case–control-associated proteins and their relation to disease-related parameters. Here, we found and replicated lower CSF concentrations of GH and testican-1—and higher concentrations of CLEC1B—in patients compared with controls. Additionally, two other proteins were lower in patients compared with controls when comparing bipolar I disorder patients with controls: draxin and TNFRSF21. All proteins, except for CLEC1B, have important functions in the brain. The studies are summarized in Table 5. It should, however, be stressed that, for all case–control-associated proteins, the effect sizes were fairly small—similar to the results of most other studies in this area of research—and there was therefore a large overlap between patients and controls.

Table 5. Summary of studies and findings.

Study	Cohorts	Main research question	Assay	Results
Study I	SBP-S	BD–CTRL differences?	Cytokines (ELISA)	↑ CSF IL-8 BD compared with controls. IL-8 ⇔(+) lithium, antipsychotics.
Study II	SBP-S	BD–CTRL differences?	Markers of microglia, astrocyte and monocyte activation (ELISA)	↑ CSF MCP-1 and YKL-40 BD compared with controls. MCP-1 ⇔(+) lithium, benzodiazepines, antipsychotics. MCP-1 ⇔(–) antidepressants.
Study III	SBP-S	Associations between proteins and clinical outcomes?	IL-8, MCP-1, YKL-40, NF-L	CSF YKL-40 ⇔(–) manic episodes. No other associations.
Study IV	SBP-S SBP-G	BD–CTRL differences?	201 (105 in data analyses) proteins (PEA)	↓ CSF GH BD compared with controls. GH ⇔(–) antipsychotics in SBP-G. Findings of *studies I-II* not replicated.
Study V	SBP-S SBP-G	BD-CTRL differences?	92 (80 in data analyses) proteins (PEA)	↓ CSF testican-1, draxin (BD I), TNFRSF21 (BD I) BD compared with controls. ↑ CSF CLEC1B BD compared with controls. testican-1 ⇔(–) antipsychotics.

Abbreviations: BD, bipolar disorder. CTRL, controls. CLEC1B, C-type lectin domain family 1 member B. CSF, cerebrospinal fluid. ELISA, electrochemi-luminescence enzyme-linked immunosorbent assays. IL-8, interleukin-8. MCP-1, monocyte chemoattractant protein 1. NF-L, neurofilament light chain. SBP-S, Sankt Göran Bipolar Project Stockholm. SBP-G, Sankt Göran Bipolar Project Gothenburg. PEA, proximity extension assay. TNFRSF21, tumor necrosis factor receptor superfamily member 21. YKL-40, chitinase 3-like protein 1.
⇔(+) indicates a positive association. ⇔(–) indicates a negative association.

The later studies (*studies IV* and *V*) in this thesis have thus highlighted the great advantage of using two independent cohorts in this field, where a low replication rate is an issue (Knorr et al., 2018). Indeed, in *study IV*, we showed significant differences in CSF protein concentrations between patients and controls for fourteen and nine proteins, respectively, in the two cohorts. Only one protein, GH, was replicated across cohorts.

CEREBROSPINAL FLUID STUDIES OF BIPOLAR DISORDER IN GENERAL

The first review to summarize all CSF biomarker studies in patients with bipolar disorder compared with healthy controls was published in 2018 (Knorr et al., 2018). It identified 410 studies, investigating a total of 117 biomarkers, and included *studies I* and *II* of this thesis. In these 410 studies, 40 different biomarkers had concentrations that were significantly different between patients with bipolar disorder and healthy controls in single studies. Only eleven biomarkers were measured in more than one study, and the only finding that was replicated across studies was elevated homovanillic acid (HVA) and 5-hydroxy-indoleacetic acid (5-HIAA) in patients compared to controls. The review points out that there was a risk of bias in many studies due to small sample sizes, lack of data on mood state, and not considering potential confounders such as age, sex, BMI, smoking, and psychotropic medications.

Divergent study results and poor replication rates are not a problem specific to studies of CSF in bipolar disorder—reproducibility is also lacking in proteomic CSF studies of other brain disorders such as Parkinson's disease (Halbgebauer, Ockl, Wirth, Steinacker, & Otto, 2016). This is not unexpected, since CNS disorders are very complex, and caused by a large number of genetic, epigenetic, and environmental factors. Still, bipolar disorder seems to differ from some neurodegenerative CNS disorders, where more disease-associated CSF biomarkers have been found in studies. For example, more case–control-associated CSF aberrations have been found, and replicated, in MS and Alzheimer's disease using the same PEA proteomic technology (Huang et al., 2020; Whelan et al., 2019). A contributing reason for this might be that the diagnostic processes of some of these disorders are aided by objective methods—such as brain imaging in MS and CSF biomarkers in Alzheimer's disease—making these patient cohorts more homogenous when it comes to underlying pathophysiological mechanisms, compared with bipolar disorder cohorts.

CEREBROSPINAL FLUID IMMUNE MARKERS IN BIPOLAR DISORDER

The previously mentioned review of CSF biomarkers in bipolar disorder included a total of eleven CSF inflammatory markers (Knorr et al., 2018). For five of these markers (three of which were from *studies I* and *II* of this thesis: IL-8, MCP-1, and YKL-40), differences in concentrations between patients and controls were detected, but none of these findings have been replicated so far.

Two meta-analyses have been performed on CSF markers of inflammation and infections, in bipolar and other psychiatric disorders. The first investigated CSF cytokine and tryptophan catabolite alterations in 28 studies, four of which included patients with bipolar disorder (one was *study I* of this thesis). Here, it was concluded that concentrations of IL-1β—but no other cytokine—were higher in patients with bipolar disorder compared with controls (Wang & Miller, 2018). The second meta-analysis included 32 studies and investigated markers of inflammation and infections in affective disorders and schizophrenia (Orlovska-Waast et al., 2019). Four studies of bipolar disorder patients were included (one of these was *study I* of this thesis). In affective disorders (mainly major depressive disorder but also bipolar disorder), the CSF/serum albumin ratio and total CSF protein were increased, but no other inflammatory biomarker was found to be associated with the disorders. It was concluded that affective disorders might implicate CSF abnormalities including signs of inflammation and blood–brain barrier dysfunction, but also, that no firm conclusions could be drawn since all studies were hampered by some degree of bias (Orlovska-Waast et al., 2019). It should also be stressed that, for the CSF biomarkers assessed in these meta-analyses of bipolar disorder patients, the number of studies for each biomarker was never more than two.

To summarize the studies of immune markers in this thesis, we have not presented convincing evidence for immune aberrations reflected in CSF in bipolar disorder: The aberrations presented in *studies I* and *II*, which were conducted at the SBP Stockholm, were not replicated in the SBP Gothenburg (*study IV*). Nor did we find any case–control differences in other immune-related proteins in *study IV*, where an immune-targeted immunoassay panel was used.

CHALLENGES IN CEREBROSPINAL FLUID STUDIES OF BIPOLAR DISORDER AND FUTURE PERSPECTIVES

Below follows a discussion on challenges in CSF studies of bipolar disorder in general, and some limitations—and strengths—in our studies specifically. Although some of these challenges are hard to overcome, they need to be considered when interpreting study results. Other issues can be handled in different ways and are discussed below as future perspectives.

ETHICAL CONSIDERATIONS

An ethical consideration that is important particularly in the psychiatric research field concerns the informed consent process. Decision-making ability can be affected by severe psychiatric symptoms and therefore it is even more crucial that the informed consent process in psychiatric research should receive special attention. The informed consent process of the SBP study included written and oral informed consent. Patients were in a euthymic state, as assessed by the treating physician, when the question about research participation was posed.

SELECTION BIAS AND GENERALIZABILITY

The two SBP cohorts were recruited in the two largest cities of Sweden. The catchment areas include all socioeconomic neighborhoods, which should make the sample reasonably representative of the Swedish population. Both patients under investigation for bipolar disorder, and existing patients at the clinics with a diagnosis of bipolar disorder were included, making the study sample representative of different disease stages. However, the invasive nature of lumbar puncture may have led to some selection bias.

There is probably a trade-off between generalizability and chances to find disease-related aberrations in CSF studies of bipolar disorder. Several studies performed by other researchers mix patients with bipolar disorder and major depressive disorder (Knorr et al., 2018), while we in our studies included only patients with bipolar spectrum diagnoses. Since bipolar disorder probably encompasses a pathophysiologically heterogeneous group of diseases that we currently diagnose as "bipolar disorder," we can assume that differences between patients and

controls would be easier to detect when further limiting the inclusion criteria, e.g., by including only patients with bipolar I disorder. This argument is strengthened by the fact that we identified larger effect sizes and a larger number of case–control-associated CSF proteins when restricting our analyses to patients with bipolar I disorder and controls in *studies IV* and *V*. On the other hand, such approaches lead to decreased generalizability and less power.

Another issue in our and other researchers' CSF studies of bipolar disorder is related to psychiatric and somatic comorbidities. In our studies, there were no patient exclusion criteria regarding other psychiatric diagnoses, and fairly high frequencies of psychiatric comorbidities such as anxiety disorders and alcohol abuse were seen in our patient cohorts. For the controls, on the other hand, exclusion criteria were stricter in this regard, and only controls with no or insignificant previous psychiatric conditions, such as past minor depressive episodes, were included. This may have induced bias, as detected differences between patients and controls may have been driven by psychiatric conditions other than bipolar disorder. On the other hand, using psychiatric comorbid diagnoses as exclusion criteria in the patient group would have decreased the generalizability due to the high prevalence of psychiatric comorbidities in bipolar disorder (McIntyre et al., 2020). A different approach would have been to relax exclusion criteria in controls with respect to current and previous psychiatric conditions. However, this may also have been problematic due to shared genetic and pathophysiological features of several psychiatric conditions. Also, bipolar disorder can present with other psychiatric symptoms such as anxiety and the mean delay between illness onset and diagnosis has been estimated to 3–16 years, as previously mentioned (Berk et al., 2007).

MOOD STATE AND CEREBROSPINAL FLUID STUDIES OF BIPOLAR DISORDER

Bipolar disorder is characterized by an episodic course, and many patients have few or no symptoms between mood episodes. The dynamic nature of bipolar disorder implies careful consideration of the study design. Biomarkers can be classified into "trait" and "state markers." *Trait markers* represent biological processes that play a causal role in the pathophysiology of a disorder, and reflect susceptibility to the disease. They can therefore be used to detect disorders early and to study disease mechanisms. On the other hand, *state markers* represent current clinical manifestations in patients and reflect dynamic changes in the disease course (Galbaud du Fort, Newman, & Bland, 1993). The CSF collection

in our studies was performed when patients were in a stable state, i.e., not suffering from an acute mood episode. This strategy could imply better chances to find trait-specific aberrations. However, it may be hypothesized that CSF aberrations are more prominent during mood episodes. Although studies in which CSF sampling is done during acute mood episodes may have more problems with confounders due to temporary changes in lifestyle—such as increased alcohol consumption or sleep deprivation—they are also of great value.

It should finally be mentioned that inclusion of patients across different mood states could contribute to the divergent results and low replication rate between studies in the field. Indeed, a meta-analysis of peripheral biomarkers, such as cytokines and oxidative stress mediators, in patients with bipolar disorder compared with controls, indicated specific mood-phase signatures (Rowland et al., 2018).

LIMITATIONS OF CEREBROSPINAL FLUID AS A SUBSTRATE FOR STUDIES OF CENTRAL NERVOUS SYSTEM DISORDERS

Not all CNS disorders are reflected by CSF changes, since not all brain areas contribute to the CSF composition. Examples of locations for CNS pathologies that are typically reflected in CSF are the meninges, temporobasal regions, periventricular areas, and spinal roots. By contrast, processes in cortical areas remote from the ventricles may not be reflected by changes in CSF composition (Tumani et al., 2017). Another difficulty in CSF studies is that the protein concentrations are low; the total protein concentration of CSF is less than 0.5% of the total blood protein concentration (Rapoport, 1983). This is illustrated by our studies, in which many proteins had values below the limit of detection for a large proportion of patients and controls.

Finally, measuring concentrations of an analyte in CSF, or in any biofluid, in an adult person with bipolar disorder is a crude method of studying pathophysiological mechanisms. If it were possible, it would be of interest to measure analyte concentrations in all parts of the brain and on many occasions throughout the fetal period, childhood, adolescence, and adulthood. However, since this is not yet possible, CSF is an important study substrate, at least to some extent reflecting brain biochemistry. Indeed, in some CNS disorders—such as Alzheimer's disease, MS, and narcolepsy—CSF studies have contributed greatly

to our pathophysiological understanding, and CSF analyses have even been implemented as diagnostic tools in some disorders (Deisenhammer et al., 2009).

CONFOUNDING ISSUES

A general limitation of CSF and other biofluid studies is that factors unrelated to—or unclearly related to—bipolar disorder may affect the CSF protein concentrations. Indeed, the previously discussed review on CSF studies of bipolar disorder highlights the risk of bias when not considering potential confounders including age, sex, comorbid diagnoses, BMI, smoking, and psychotropic medications (Knorr 2018).

In *studies IV* and *V*, we found that the vast majority of proteins were positively intercorrelated, with a median correlation coefficient of around 0.5–0.6, and that the first principal component in the PCA explained 50–60% of the variation in the protein datasets. Further, the first principal component correlated with age, CSF/serum albumin ratio, and sex in both these studies. This advocates the need to include these factors as covariates in the analyses to minimize the risk for false positive, but also false negative, findings. However, even when correcting for potential confounders, it may be hard to detect disease-specific aberrations with small or moderate effects when the proteins are associated with these general factors. It is also possible that unmeasured factors—such as lifestyle factors—are related to CSF protein concentrations and bipolar disorder and lead to residual confounding. In addition, it is possible that genetic components associated with bipolar disorder, and with CSF turnover, introduce bias in the studies.

One paper on CSF in neurological protein biomarker research discusses that one reason why only a small number of biomarkers have been validated in independent studies may be the high variability in the protein composition between—as well as within—individuals (Schilde et al., 2018). To investigate this possibility, they measured the inter- and intra-individual variability in the CSF proteome. They found an inter-individual coefficient of variation of up to 102% and an intra-individual coefficient of variation of up to 29% and concluded that natural variability impacts CSF protein biomarkers and can lead to unreliable results (Schilde et al., 2018). On the other hand, another study investigating intra-individual stability of CSF biomarkers in Alzheimer's disease found intra-individual coefficients of variation of around 8% and concluded that intra-individual biomarker levels are notably stable over two years (Zetterberg et al.,

2007). Nevertheless, in CSF biomarker research, inter- and intra-individual variability is a challenge of varying degrees, depending on the protein assessed.

A key issue in our and many other researchers' studies is the use of psychotropic drugs. This is a potential confounder that we cannot address by adding covariates to our analyses. Instead, we have performed subgroup analyses in controls and patients without a specific medication. However, few patients are completely drug-free and the sample sizes shrink in these subanalyses. Also, as discussed above, such subgroup analyses could introduce "confounding by severity," inducing new methodological problems when removing patients on psychiatric drugs from the analyses. Further, this strategy decreases the generalizability of results. It is difficult to study a large group of unmedicated patients with bipolar disorder due to the strong indication for mood-stabilizing treatments in bipolar disorder. However, one possibility is to study patients recently diagnosed with bipolar disorder, who are drug-naïve or at least not on current medication.

CROSS-SECTIONAL VS. LONGITUDINAL STUDIES

Cross-sectional case–control studies have the drawback that causality cannot be addressed. Is the presence of a CSF biomarker a cause of bipolar disorder—or is it caused by bipolar disorder? Or does the causality involve a combination of these two? Hypothetically, the biomarker could contribute to causing the disorder, or reflect a compensatory mechanism, but also be a consequence of the disease process. Longitudinal study designs can aid in disentangling causality, and can investigate temporal dynamics of disease-related processes. Longitudinal CSF studies of bipolar disorder are, however, scarce (Knorr et al., 2018). The SBP is a longitudinal project, but the studies in this thesis are mainly cross-sectional, since the follow-up has not yet been completed.

As well as giving clues to causal relationships, a longitudinal approach can minimize some of the confounding issues of cross-sectional study designs. In a cross-sectional design, individual factors potentially related to CSF protein concentrations, such as genetic or lifestyle factors, might confound patient–control comparisons. For CSF proteins with high inter-individual variability, problems with residual confounding should decrease when comparing *changes* in CSF protein concentrations over time between patients and controls, analogous with within-individual designs in epidemiological research. Apart from following patients longitudinally over several years, as in the SBP study, it would also be of

great interest to longitudinally investigate CSF from patients across different mood states.

ALTERNATIVE STUDY DESIGNS

Case–control studies of patients with a certain psychiatric diagnosis—or several different diagnoses—according to the DSM classification are common in psychiatric research. Indeed, the majority of CSF studies of bipolar disorder, including ours, compare patients with a DSM diagnosis of bipolar disorder to healthy controls, and several studies also pool patients with bipolar disorder and with other psychiatric diagnoses such as major depressive disorder. Based on the previous statement that bipolar disorder probably encompasses several different diseases, this approach may not be optimal for finding disease-associated aberrations. Some biomarkers might further be associated with symptoms and signs (e.g., auditory hallucinations or mental fatigue) that are shared between several psychiatric diagnoses. Hence, other strategies besides comparing patients with a DSM diagnosis of bipolar disorder with healthy controls are appealing. One approach would be to focus on specific signs or symptoms, such as presence of auditory hallucinations during manic episodes, or psychomotor retardation in depressive episodes. Such clinical features and their relation to CSF protein concentrations could be used to give clues to distinct pathophysiological entities within the bipolar spectrum. Further, they could be used for inclusion criteria to studies, alone or in combination with DSM diagnoses. To this end, constructs and subconstructs from the Research Domain Criteria (RDoC), e.g., "auditory perceptions" or "motor actions," could be used (Insel et al., 2010).

STATISTICAL CONSIDERATIONS

In the proteomic field in general, and in this thesis in particular, there are issues related to potential type I and type II errors. All studies in this thesis included several statistical tests. Especially in *studies IV* and *V*, where large numbers of proteins were analyzed simultaneously, with several *post hoc* tests performed, there were issues of multiple testing. In statistical hypothesis testing, there is always a trade-off between type I and type II errors. A *type I error* occurs when the null hypothesis of no difference between groups, or no association between variables, is rejected even though it is in fact true (a so-called "false positive result"). The likelihood of making a type I error is equivalent to the cutoff value for statistical significance (i.e., $p<0.05$ in this thesis), and the major issue that increases the

likelihood of a type I error is related to multiple testing, i.e., when researchers perform many statistical tests, with different endpoints, on the same data (Akobeng, 2016).

There are several ways to reduce the problem of *type I errors*. These include: (1) avoiding multiple testing; (2) abstaining from referring to results as "statistically significant" or "not statistically significant," and simply describing results in terms of estimates with confidence intervals; (3) adjusting for multiple testing by choosing a more stringent p-value as the cutoff level for statistical significance (e.g., Bonferroni or FDR adjustment) (Akobeng, 2016); and (4) using an independent validation cohort to replicate findings before publishing data. In this thesis, the third strategy was used for some analyses in *studies I-III*, and the fourth strategy was used in *studies IV* and *V*, where two independent cohorts were used. Indeed, in *study IV*, the concentrations of 14 proteins in the SBP Stockholm and nine proteins in the SBP Gothenburg differed significantly (p-value<0.05) between patients and controls. For eight of these proteins, there was a significant difference in one cohort, and an estimate in the same direction (however, not statistically significant) in the other cohort, indicating that this could be a "true" finding obscured by low statistical power. By contrast, for the 15 remaining proteins, the estimate was in one direction in one cohort and in the opposite directions in the other cohort, and the statistically significant difference between patients and controls in one cohort was probably due to factors not related to bipolar disorder.

Type II errors, by contrast, occur when a study has been unable to detect a true difference between groups or an association between variables because of the play of chance (so-called "false negative findings") (Akobeng, 2016). A type II error can be caused by poor statistical power (due to small sample size) or by too strict a method when correcting for multiple comparisons. In our studies, a problem was the small sample size in some analyses (e.g., for some clinical outcomes such as suicide attempts in *study III*). Also, for some analyses (*studies I-III*), Bonferroni or FDR corrections might have caused some type II errors.

BOTTOM-UP AND TOP-DOWN APPROACHES

Research methods can be divided into deductive or inductive, even though the approaches can be seen to exist on a spectrum (Varpio, Paradis, Uijtdehaage, & Young, 2020). *Deductive* research is a top-down approach, where hypotheses

arising from a previous theory are tested to either prove previous conceptualizations wrong or support them. *Inductive* research, on the other hand, is a bottom-up and data-driven approach, where the researcher starts from collected data and searches for patterns to generate an understanding of a phenomenon (Varpio et al., 2020).

There are challenges to both approaches. In the deductive strategy, there are generally fewer problems with false positive findings due to more reasoning when choosing what statistical analyses should be performed (e.g., testing associations between two proteins, as opposed to 2,000 proteins, and bipolar disorder). On the other hand, a drawback is that the hypothesis chosen to be tested (e.g., that there is an association between a specific protein and bipolar disorder, which has elsewhere been proposed to be of relevance) can be non-relevant, e.g., due to previous false positive findings. The inductive strategy, by contrast, is more flexible and open to unexpected findings, and, hence, suitable for research areas where not much is known. A drawback is, as previously discussed, that there is a risk of false positive findings when numerous statistical analyses are performed.

In CSF studies of bipolar disorder, there is a trend of going from smaller, hypothesis-driven studies investigating a few analytes, to larger and more explorative (i.e., inductive) studies. This tendency is partly driven by laboratory technological advances, and parallels can be drawn with the genetic field. Here, decades of candidate gene studies have gradually been almost abandoned in favor of hypothesis-free, genome-wide analyses of large samples, i.e., GWASs (Duncan, Ostacher, & Ballon, 2019). Even though there are many features differentiating genetics from proteomics, it will be interesting to see if recent advances in commercial multiplex assays and mass spectrometry technologies can advance the research field of CSF biomarkers for bipolar disorder, just as GWAS has revolutionized the field of genetics for psychiatric disorders (Tam et al., 2019).

SUMMARY OF FUTURE PERSPECTIVES

A summary of proposed future perspectives for CSF studies of bipolar disorder is provided in Figure 10 below.

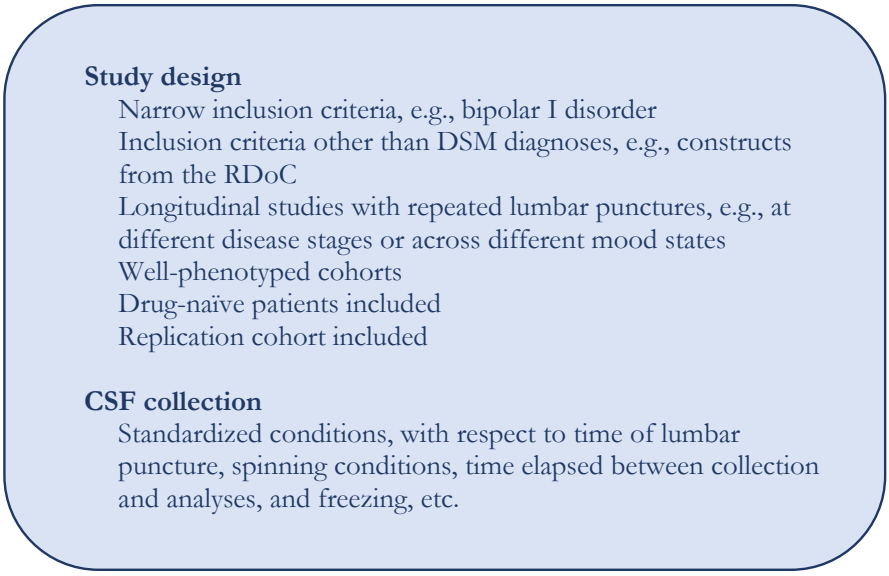

Figure 10. Summary of proposed future perspectives for cerebrospinal fluid studies of bipolar disorder.

SUMMARY AND CONCLUSIONS

In the first two studies of this thesis (*studies I* and *II*), we found increased CSF concentrations of a few immune-related proteins (IL-8, MCP-1, and YKL-40) in patients compared with controls in a bipolar disorder cohort. In a later study (*study IV*), we did not replicate these findings in an independent cohort. In *study III*, we longitudinally investigated associations between the immune-related CSF proteins from *studies I* and *II* and clinical outcomes at the 6–7-year follow-up. We found a negative association between YKL-40 concentrations and manic/hypomanic episodes, but no other significant associations, and concluded that high concentrations of these CSF markers of neuroimmune processes are not consistently associated with poor clinical outcomes.

In the final two studies of the thesis (*studies IV* and *V*), we used two independent cohorts of patients with bipolar disorder and measured a large number of CSF proteins to explore case–control-associated proteins and their relation to disease-related parameters. Here, we found, and replicated, lower concentrations of GH and testican-1 and higher concentrations of CLEC1B in patients compared with controls. Additionally, two proteins were lower in patients with bipolar I disorder than in controls: draxin and TNFRSF21. All these proteins, except CLEC1B, have important known functions in the brain. However, the findings need further replication, including assessments in longitudinal studies, to elucidate potential causal mechanisms. Due to the relatively small effect sizes, the identified proteins are not candidate biomarkers for diagnostics or prognostics, but they do have the potential to serve as groundwork for future pathophysiological research. In regard to immune aberrations in CSF from patients with bipolar disorder, our findings were contradictory and we did not find convincing evidence of immune aberrations in CSF from patients with bipolar disorder.

An important takeaway from the work of this thesis is that CSF studies of bipolar disorder are challenging. Some reasons behind this are the complex nature of psychiatric disorders, together with confounding factors that are associated with both the CSF proteome and bipolar disorder, but unrelated to disease mechanisms. The work of this thesis emphasizes the need for stringent approaches to designing studies, and cautious interpretation of results. Finally, it highlights the need for careful consideration of potential confounders.

The research methods we currently have for studying the pathophysiology and etiology of bipolar disorder are too crude to readily capture disease mechanisms of such a complex condition. An ideal, but currently unachievable, design of a proteomic study of bipolar disorder would be to assess the concentrations of all proteins in all brain areas across all life stages. Awaiting this unlikely-to-happen scenario, we meanwhile have to rely on the methods we have, and, where possible, evolve them. In spite of all the challenges discussed above, CSF studies of bipolar disorder are still relevant. Since CSF can reflect brain processes in living humans, and because proteins are more directly involved in pathophysiological processes than genes, CSF proteomic studies should continue to be an important ingredient in bipolar disorder research. Future CSF studies of bipolar disorder should preferably combine targeted methods, like measuring immune-related proteins, with more unbiased approaches, such as mass spectrometry-based proteomics and metabolomics.

ACKNOWLEDGEMENTS

Above all, I am very grateful to my supervisor **Mikael Landén**, who has started and managed the Sankt Göran Bipolar Project which made the studies in this thesis possible. Thank you for always having time for my never-ending questions, and for sharing your expertise with me. And also, for your optimistic mindset and sense of humor that can lighten up the dullest day (or the most pessimistic PhD student).

I am also very thankful to my co-supervisors **Joel Jakobsson** (for giving me a great introduction to research when I started in the group and knew nothing about research), **Jessica Holmén-Larsson** (for always being supportive and for helping me with laboratory questions), **Carl Sellgren** (for answering all my immune-related questions), and **Erik Smedler** (for helping me with my later studies and for being encouraging).

I want to thank my whole **research group Affective disorders** in Gothenburg and fellow group members in Stockholm for helping me during my PhD period in different ways, and for creating such an inspiring workplace. Specifically, I would like to thank: **Andreas Göteson** (for being an invaluable partner in crime in my struggles with study IV, and for helping me enter the mysterious world of R programming—I would not have survived this mentally without you…), **Timea Sparding** (for being a very important person to me during my PhD journey, and for being so fun to be with), **Anne Snellman** (for always being so supportive and for helping with all practical matters), **Elin Hörbeck** (for being a great companion in both research and clinical work), **Lina Jonsson** (for always being supportive and for explaining genetics to me), **Mathias Kardell** (for helping with data base and statistical questions and always being so kind), **Erik Joas** (for not being tired (?) of my never-ending statistical questions), **Erik Pålsson** (for introducing me to research and always being helpful), **Alina Karanti** (for being a source of inspiration, combining research with being a great psychiatrist), **Benita Gezelius** (for being both helpful and encouraging), and **Kristina Annerbrink** (for always being supportive and for having made it easy for me to combine research and clinical work).

I further want to acknowledge **all the patients and controls** that have participated in the Sankt Göran Bipolar Project and made this thesis, and numerous other studies, possible. I am also very grateful to **everybody working with the Sankt Göran Bipolar Project**, both in Gothenburg and Stockholm, and to my **co-authors** of the papers included in this thesis.

Financial support for my PhD study period was provided by the Swedish State Support for Clinical Research (Agreement for Medical Education and Research, ALF), which I am very grateful for.

To my beloved children **Ingrid** and **Mauritz**: Thank you for forcing me to work a little bit less than I wanted to, due to endless pandemic-related caring-for-sick-child days. Most of all, I owe everything to my husband **Joakim**. Thank you for at least pretending to enjoy being married to a MacBook-accessory during the past half-year. I am also incredibly grateful to my beloved parents **Mats** and **Karin** and my sister **Ellinor** who is always there for me. I also want to thank **Annika**, **Bertil**, **Svante** and **Lena** for always being supportive and helpful.

Finally, I wish to thank my wonderful friends who always encourage me. **Charlotte** (for having the talent to make every one-minute little thing on the phone evolve to a one-hour-conversation, but mostly for being an amazing friend and colleague). The rest of my treasured medical school gang: **Emma**, **Carin**, **Jaana**, **Malin**, and **Anna**. My dear childhood friends **Johanna**, **Karin**, and **Sofia**. My lovely colleague and friend **Maria S**. My precious friend **Styrsö-Maria**. My valuable colleague and friend **Lydia**. My close friend **Helena**. **Jenny Karlsson** for helping me edit illustrations. And finally, my **Asperö-friends** who help making my island-life lovely.

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