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CEREBROSPINAL FLUID BIOMARKERS IN TWINS WITH SCHIZOPHRENIA AND BIPOLAR DISORDER

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CEREBROSPINAL FLUID BIOMARKERS IN TWINS WITH SCHIZOPHRENIA AND BIPOLAR DISORDER

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ABSTRACT

Previously identified cerebrospinal fluid (CSF) biomarkers were studied in 37 twins with schizophrenia or bipolar disorder to determine their genetic and environmental properties. Further, comorbidities and sibling risks were investigated in patients with schizophrenia (n=22,781), bipolar disorder (n=30,761) and depression (n=172,479) in relation to multiple sclerosis (MS) (n=16,467) in a nationwide cohort

In S*tudy I*, CSF was analyzed using scanning electron microscopy in twins with schizophrenia and bipolar disorder to identify microscopic structures that had previously been established in patients with schizophrenia and bipolar disorder. Microscopic structures were found not only in the patients but also in the non-affected twin siblings to a higher degree than in healthy controls. The results indicate that genetic and shared environmental mechanisms might be involved in the development of CSF structures.

Study II was a case series in which microparticles were studied in the CSF of healthy controls and in patients with schizophrenia. Microparticles may be indicative of stress-induced cell activation. In the patients with schizophrenia microparticles that originated from leukocyte and endothelial cells were accumulated in higher levels as compared with the healthy controls.

In S*tudy III*, immune and amyloid biomarkers previously associated with psychotic disorders were analyzed in the CSF of twins. Soluble cluster of differentiation 14 (sCD14), a protein expressed by microglia in the central nervous system, was found to highly correlate within monozygotic twins. In the co-twin control analysis higher levels were observed in patients with schizophrenia and bipolar disorder compared with the non-affected co-twins. sCD14 was also associated with negative psychotic symptoms and schizotypal and paranoid personality traits. The results strengthen previous findings of microglia activation in psychotic disorders.

In S*tudy IV*, tryptophan metabolites and cytokines were analyzed in twins with schizophrenia and bipolar disorder. None of the substances correlated within the monozygotic twin pairs, which indicates the influence of environmental factors. Kynurenic and quinolinic acid were shown to be associated with schizotypal personality traits, strengthening previous results of an association between the tryptophan metabolites and psychosis.

In S*tudy V*, comorbidity between the neuroinflammatory disorder MS and schizophrenia, bipolar disorder and depression was analyzed in a nationwide cohort. Consistent with previous studies an increased MS risk was noted in patients with bipolar disorder and depression. An increased MS risk was also found in males with a diagnosis of bipolar disorder and previous manic episodes. Decreased MS risk was seen in patients with schizophrenia. No change in MS risk was detected in the siblings to patients with schizophrenia, bipolar disorder and depression. Possible inflammatory mechanisms may account for the comorbidity between affective disorders and MS, whereas the protective effect of schizophrenia on MS risk remains to be further investigated.

Biomarker analysis in CSF using twin methodology revealed some promising findings for future biomarker studies. However, larger twin cohorts are desirable for conclusive confirmation. The potential mechanisms underlying the associations between MS and psychiatric disorders require further study.

SAMMANFATTNING

Schizofreni och bipolär sjukdom är psykiska sjukdomar som antas drabba hjärnan. De bakomliggande orsakerna är till stor del okända. Ärftliga faktorer är av betydelse, men det saknas kunskap om vad som mer specifikt händer i hjärnan när sjukdomarna uppstår. Schizofreni och bipolär sjukdom tycks delvis ha en gemensam ärftlig grund och vissa symtom överlappar. Det finns inga objektiva sätt att diagnostisera psykiska sjukdomar idag, vilket kan göra att diagnosen inte ställs i tid och att behandlingen fördröjs. Ett sätt att förbättra diagnostiken är att identifiera biomarkörer. Exempel på biomarkörer kan vara resultatet från ett blodprov, en röntgenundersökning eller en psykologisk testning.

Syftet med denna avhandling har varit att undersöka potentiella biomarkörer för schizofreni och bipolär sjukdom genom att analysera ryggmärgsvätska som omger hjärnan och ryggmärgen. Vätskan fungerar bland annat som en stötdämpare för hjärnan och som transportör av vissa substanser och kan utvinnas genom att ett ryggvätskeprov tas med hjälp av en nål.

I forskningen om biomarkörer brukar man först jämföra nivåerna av en specifik markör mellan friska och sjuka individer. Om markören skiljer sig kan man gå vidare för att ta reda på om skillnaden beror på ärftliga eller miljöfaktorer (delade eller unika) genom att analysera tvillingpar. I avhandlingens första studier (I-IV) analyserades biomarkörer i ryggmärgsvätska från tvillingar med schizofreni eller bipolär sjukdom (totalt 37 individer från 18 kompletta tvillingpar). I den sista studien (V) analyserades samsjukligheten mellan psykisk sjukdom och den neurologiska sjukdomen multipel skleros. Tidigare studier har nämligen visat på en ökad risk för bipolär sjukdom, depression och psykos vid multipel skleros, vilket skulle kunna bero på nedärvda faktorer.

I *Studie I* analyserades ryggmärgsvätska med svepelektronmikroskop för att identifiera mikrometer-stora strukturer i ryggmärgsvätska hos tvillingar med schizofreni eller bipolär sjukdom. Den mikroskopiska undersökningen visade att tvillingarna med schizofreni eller bipolär sjukdom hade cirkulära strukturer i storleken 0,5-1 mikrometer i ryggmärgsvätskan i betydligt större utsträckning än när de jämfördes med friska kontrollindivider. Även de friska tvillingarna med ett sjukt syskon, som således hade en ökad ärftlighet för sjukdomen, uppvisade en ökad mängd strukturer. Resultaten kan tolkas som att strukturerna orsakas av ärftliga faktorer, men också av miljöfaktorer som delas inom ett tvillingpar.

Studie II är en fallrapport där mikropartiklar analyserades i ryggmärgsvätskan hos ett tvillingpar med schizofreni och jämfördes med friska kontrollpersoner. Mikropartiklar är små fragment av celler som frigörs från modercellen med syftet att sända ut signaler till sin omgivning och en hypotes är att mikropartiklarna utsöndras vid stress. Fallstudien visade att mängden mikropartiklar som utsöndrades från tvillingarna med schizofreni var betydligt högre jämfört med kontroller. Mikropartiklarna som med hjälp av antikroppar kunde spåras till vita blodkroppar och celler från kärlväggar var klart förhöjda, medan mängden mikropartiklar från blodplättar inte skilde sig från kontrollerna. Det var första gången en ökad mängd mikropartiklar kunde påvisas hos patienter med schizofreni.

I *Studie III* analyserades biomarkörer för immunologisk aktivitet i ryggmärgsvätskan hos tvillingarna. Flera av biomarkörerna hade tidigare visat sig vara förändrade vid schizofreni och bipolär sjukdom. Undersökningen visade intressanta resultat för lösligt CD14 som i nervsystemet tillverkas av celltypen mikroglia som i sin tur är en viktig del av immunförsvaret i hjärnan. Man fann att nivåerna av lösligt CD14 överensstämde ovanligt mycket mellan enäggstvillingar (som är 100% genetiskt lika), vilket tyder på hög ärftlig styrning av dess produktion. Likväl visade analysen mellan tvillingparen att sjukdomsspecifika faktorer i kombination med ärftliga faktorer (orsakade av schizofreni eller

bipolär sjukdom) gav ökade nivåer av lösligt CD14. Resultaten kan tolkas som att mikroglia-cellerna är aktiverade vid schizofreni och bipolär sjukdom.

I *Studie IV* undersöktes tvillingarnas ryggmärgsvätskenivåer av tryptofan och dess nedbrytningsprodukter samt cytokiner. Tryptofan är en aminosyra som bland annat omvandlas till serotonin som är vital för vårt välbefinnande. Tryptofan bryts också ner via kynurensyra-systemet som bland annat påverkar glutamat- och dopamin-nivåerna. Glutamat och dopamin har sannolikt en stor av betydelse för uppkomsten och utvecklingen av psykotiska symtom. Studien visade att tryptofan-metaboliterna kynurensyra och quinolinsyra var associerade till ökade psykossymtom hos patienterna.

Studie V är en registerstudie med hela Sveriges befolkning som underlag där sambandet mellan schizofreni, bipolär sjukdom, depression och den neurologiska sjukdomen multipel skleros undersöktes. I studien fann man som förväntat att risken för multipel skleros var förhöjd vid bipolär sjukdom och depression. Något oväntat så var risken för multipel skleros minskad hos patienterna med schizofreni. Syskon till patienter med bipolär sjukdom, depression eller schizofreni undersöktes också, men de visade inte någon ökad eller minskad risk för multipel skleros jämfört med kontrollpersoner utan ärftlighet för psykisk sjukdom.

Resultaten från denna avhandling visar att analyser av biomarkörer i ryggmärgsvätska hos tvillingar är av stort värde. Trots det mindre antalet tvillingar som har analyserats så är antalet insamlade prover av ryggmärgsvätska bland det största någonsin hos tvillingar med allvarlig psykisk sjukdom. Det är också av stort intresse att ta reda på orsaken till varför patienterna med schizofreni i viss utsträckning verkar vara skyddade mot multipel skleros.

LIST OF SCIENTIFIC PAPERS

I. VIKTORIA JOHANSSON, Rolf Nybom, Lennart Wetterberg, Christina M Hultman, Tyrone D Cannon, Anette G Johansson, Carl-Johan Ekman, Mikael Landén. Microscopic particles in two fractions of fresh cerebrospinal fluid in twins with schizophrenia or bipolar disorder and in healthy controls. PLoS One, 2012. 7(9): p. e45994.

II. Fariborz Mobarrez, Rolf Nybom, VIKTORIA JOHANSSON, Christina M Hultman, Håkan Wallén, Mikael Landén, Lennart Wetterberg. Microparticles and microscopic structures in three fractions of fresh cerebrospinal fluid in schizophrenia: Case report of twins. Schizophr Res, 2013. 143(1): p. 192-7.

III. VIKTORIA JOHANSSON, Joel Jakobsson, Becky Fortgang, Tyrone D Cannon, Lennart Wetterberg, Christina Hultman, Mikael Landén. Cerebrospinal fluid microglia and amyloid biomarkers in twins concordant or discordant for psychotic disorders. (Manuscript)

IV. Magdalena Kegel, VIKTORIA JOHANSSON, Lennart Wetterberg, Maria Bhat, Lilly Schwieler, Tyrone D Cannon, Ina Schuppe-Koistinen, Göran Engberg, Mikael Landén, Christina M. Hultman, Sophie Erhardt. Association between kynurenic acid and schizotypal personality traits in twin pairs with psychiatric morbidity. (Manuscript)

V. VIKTORIA JOHANSSON, Cecilia Lundholm, Jan Hillert, Tomas Masterman, Paul Lichtenstein, Mikael Landén, Christina M Hultman. Multiple sclerosis and psychiatric disorders: comorbidity and sibling risk in a nationwide Swedish cohort. Mult Scler, 2014. 20(14): p. 1881-91.

CONTENTS

LIST OF ABBREVIATIONS

AUDIT	Alcohol Use Disorders Identification Test
Aβ	Amyloid beta
BMI	Body mass index
CD144	Cluster of differentiation 144
CD42a	Cluster of differentiation 42a
CD45	Cluster of differentiation 45
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
DUDIT	Drug Use Disorders Identification Test
DZ	Dizygotic
fMRI	Functional magnetic resonance imaging
GAF	Global Assessment of Functioning
GWAS	Genome-wide associations study
HR	Hazard ratio
ICD	International Classification of Diseases
IFN	Interferon
Il	Interleukin
LPS	Lipopolysaccharide
MCP-1/ CCL2	Monocyte chemotactic protein 1/ Chemokine ligand 2
MINI	Mini International Neuropsychiatry Interview
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MZ	Monozygotic
NMDA	N-metyl-D-aspartate
OR	Odds ratio
P-tau	Phosphorylated tau
RNA	Ribonucleic acid
SANS	Scale for Assessment of Negative Symptoms
sAPP	Soluble amyloid precursor protein
SAPS	Scale for Assessment of Positive Symptoms
SBP	St. Göran Bipolar Project
sCD14	Soluble cluster of differentiation 14
SCID I	Structured Clinical Interview for DSM-IV Axis I Disorders
SCID II	Structured Clinical Interview for DSM-IV Axis II Disorders
SPQ-B	Schizotypal Personality Questionnaire-Brief

STAR	Schizophrenia Twins and Relatives
TNF	Tumor necrosis factor
T-tau	Total tau
YKL-40/ CHI3L1	Chitinase 3 like protein 1

1 INTRODUCTION

1.1 PSYCHIATRIC RESEARCH – POSSIBILITIES AND CHALLENGES

Psychiatry is a medical discipline treating patients with psychiatric disorders characterized by a suggested dysfunction of the central nervous system (CNS). However, there is a critical difference between psychiatry and most other medical disciplines. In psychiatry the diagnosis is based on observed behavior and the patient's own description of the symptoms sometimes combined with results from assessment scales and neuropsychological function tests. In most other medical disciplines the clinicians have access to various laboratory tests and radiological techniques, to establish the correct diagnosis, in addition to the physical symptoms that the patient presents.

In medicine objective biological measures (such as results from laboratory tests and radiological examinations) are called biological markers or biomarkers. Psychiatry has lagged behind other areas of medicine in the identification of biomarkers for clinical diagnosis and treatment. Instead, the foundation of the diagnostic nomenclature is the symptom criteria presented in the Diagnostic and Statistical Manual of Mental Disorders, (DSM) [1] and the International Classification of Diseases (ICD) by the World Health Organization [2]. The categorical classification system may be problematic because the psychiatric syndromes often display symptoms within a spectrum. Also, there are problems with validity and inter-rater reliability of today's nomenclature which is not fully satisfactory for the clinician [3].

The difficulty in measuring the biological processes of psychiatric disorders in the brain of living humans makes the psychiatric research more complicated compared with other areas of clinical medical research. It is more difficult to understand underlying pathophysiological processes and the weaknesses in the psychiatric diagnostic system make it harder to divide the patients into relevant groups for analysis. Even large-scale genetic studies, including thousands of study participants, that have scanned the whole genome for potential genetic biomarkers have generated less results than expected; one explanation may be the inherent weaknesses of today's diagnostic classification system.

The research field of biomarkers is intense and biomarkers may be useful for different purposes (e.g., risk prediction, treatment response and as a diagnostic aid) [4]. To develop biomarkers for psychiatric disorders examples of methods used are brain imaging techniques and genetic or biochemical analysis of various substrates such as blood, saliva and skin samples. In this context the cerebrospinal fluid (CSF) that is encircling the brain and the spinal cord is a useful substrate not only as a potential source of biomarkers but also to study the pathophysiology of the brain [5].

Nevertheless, it is a time-consuming procedure to collect samples from individual patients and in certain situations it may not even be ethical. In epidemiology research it is possible to avoid interference with patients by taking advantage of already existing data from population registers. Disease patterns of various disorders in large populations may be analyzed and associations found may not only generate new hypotheses, but will also answer important questions about causation.

This thesis will focus on biomarkers and the underlying pathophysiology of schizophrenia and bipolar disorder by analyzing CSF collected from a cohort of twins. By using twin pairs, it is possible to analyze the influence of hereditary factors as well as the effect of the shared and unique environment. Finally, in a nation-wide epidemiological study comorbidities and familiar influences between multiple sclerosis (MS) and schizophrenia, bipolar disorder and depression are analyzed using Swedish population registers.

1.2 DISORDERS OF THE CENTRAL NERVOUS SYSTEM

1.2.1 Schizophrenia and bipolar disorder

Schizophrenia and bipolar disorders are both severe mental disorders with a prevalence of approximately 0.4% and 2.4%, respectively [6, 7]. Schizophrenia is characterized by psychotic symptoms such as hallucinations, delusions and abnormal thought processes as well as social withdrawal and cognitive impairment [1]. Typically, its onset occurs in early adult life but the impairment of cognitive skills seems to develop already at a premorbid level [8]. Bipolar disorder is an affective disorder presenting symptoms such as episodic mood swings from mania to depression and psychotic symptoms that often appear during severe periods of mania and depression [1].

Similar to schizophrenia, bipolar disorder has its onset in early adulthood. Bipolar disorder is separated into bipolar disorder type I, characterized by manic and depressive episodes, and bipolar disorder type II, defined by less pronounced episodes of elevated mood called hypomania and depressive episodes. Cognitive deficits are also present in bipolar disorder [9-11], although milder compared with schizophrenia [12]. There are likely genetic differences in the cognitive deficits of schizophrenia and bipolar disorder: not-affected co-twins of schizophrenia patients performed worse in cognitive testing compared with not-affected bipolar co-twins whose results did not deviate from the control twins [13, 14]. Schizoaffective disorder is classified as a separate disorder characterized by psychotic symptoms (e.g., hallucinations and delusions) comparable to schizophrenia and mood instability as in bipolar disorder [1]. Therefore, schizoaffective-disorder has been suggested to be a phenotype in the spectrum between schizophrenia and bipolar disorder [15].

The etiology of schizophrenia and bipolar disorder is not fully understood but genes seem to play an important role and heritability has been estimated to approximately 60 % [16]. The risk for schizophrenia and bipolar disorder increases with greater kinship, being highest in identical twins. Further there is an increased overlapping risk as shown in the figure (Figure 1). Genetic linkage studies have given fewer results than expected over the years, indicating that schizophrenia and bipolar disorder are not caused by genes with large effect sizes. The recent large scale genome-wide association studies (GWAS) have been more successful and several loci associated with the respective disorder have been identified. The effect sizes have been small, indicating that many genes in combination may cause the disorders [17]. The overlapping genetic risks of schizophrenia and bipolar disorder have been explored with GWAS in combined samples of schizophrenia and bipolar patients and common risk loci have been identified (e.g., *CACNA1C* coding for proteins involved in voltage gated calcium channel activity and *ANK3* coding for ankyrin 3 with the function to connect membrane proteins to the spectrin-actin cytoskeleton) [18, 19]. The *CACNA1C* and *ANK3* risk genes have also been linked to aberrant results on functional magnetic resonance imaging (fMRI) scans in patients with bipolar disorder and their relatives [20, 21].

Finally, without forgetting to mention the environmental risk factors with substantial influence on the risk for schizophrenia and bipolar disorder. Environmental risk factors for schizophrenia include obstetric risk factors such as low birth weight and hypoxia at birth, early life viral infections and winter birth [22]. Similar risk factors seem to affect bipolar disorder (e.g., winter birth and prematurity increase the risk) [23].

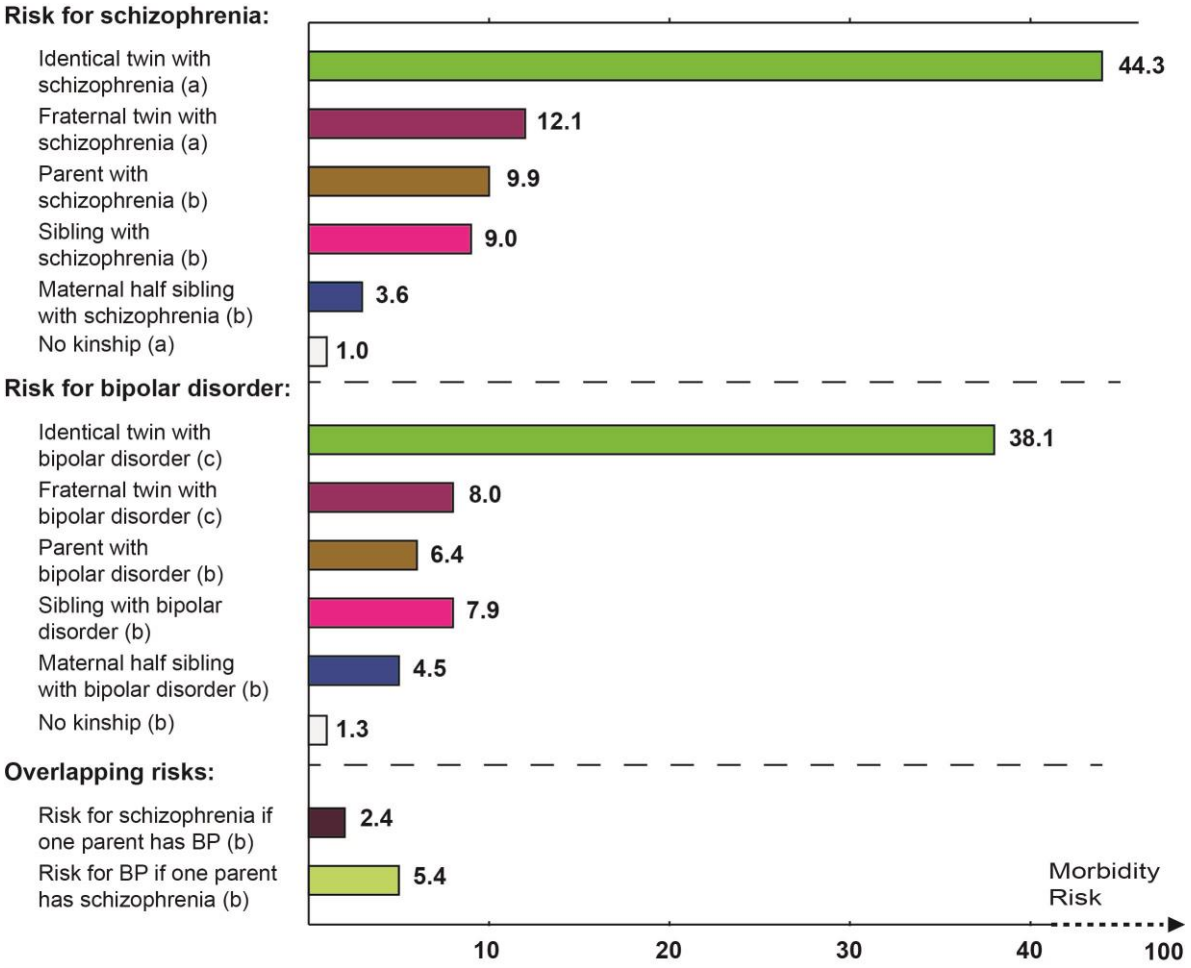

Figure 1. The morbidity risk of schizophrenia and bipolar disorder (BP) and overlapping risks. *Identical twins share 100% of their genes; fraternal twins, parent-child and siblings, on average, 50% of their genes; and half sibling 2%. a. Concordance rates in twins from McGue, 1991 [24]. b. Relative risks from Lichtenstein, 2009 [16]. c. Concordance rates from Edvardsen, 2008 [25].*

1.2.2 Depression

Major depressive disorder is characterized by low mood and loss of interest that may persist from weeks up to several months and even years. The disorder has an episodic course. Other symptoms include low self-esteem, feelings of guilt, low energy, worsened appetite and suicidal ideation. Severe forms may be accompanied by psychotic symptoms. [1, 3]. Depression may affect individuals once or several times during their lifetime and usually arises after puberty. Risk factors include heredity, negative life events, medical conditions, other psychiatric diagnosis and alcohol or substance abuse. The lifetime prevalence may vary from 8%-12% in most countries of the world and the heritability has been estimated to 37 % [26]. As with bipolar disorder and schizophrenia, it is likely that many genes with smaller effect sizes affect the depression risk and many GWAS studies have failed to detect any common risk genes [26, 27]. Only recently two risk loci on chromosome 10 were found in Chinese women with severe depression, indicating that today's classification of depression may have contributed to the failure of detecting risk genes previously [28].

1.2.3 Multiple sclerosis and psychiatric comorbidity

MS has a lifetime prevalence of 0.2% [29]. It is a disease affecting the brain and the spinal cord that may lead to disability with detrimental effects on quality of life. The disease is characterized by neuroinflammation that is mainly driven by lymphatic cells leading to demyelination and axonal loss. It has a relapse and remitting course but over time it often turns into a chronic disease. Environmental risk factors include infections (for instance, Epstein-Barr virus) smoking and reduced exposure to ultra-violet light. The incidence increases with distance from the equator (northward as well as southward) [30]. MS is more common in females than in males and the heritability has been estimated to 0.64 [31, 32]. The diagnosis is based on the physical symptoms that the patient presents in combination with CSF- and radiological examination [30].

Charcot, a neurologist, first described pathological abnormalities in patients with MS in 1868. Some years later he went on to describe psychiatric symptoms in MS patients [33] and since then several studies have demonstrated increased rates of depression and bipolar disorder in patients with MS [34-45].Associations have also been found between MS, schizophrenia and non-affective psychosis. [42, 46-48] The comorbidity of MS on the one hand and the major psychiatric disorders schizophrenia, bipolar disorder and depression, on the other, may indicate common underlying mechanisms. For example, shared pathogenic processes and environmental or familial risk factors may explain the simultaneous presence of MS and psychiatric illness. Previous studies have shown an increased risk for schizophrenia and depression in first-degree relatives to MS patients compared with controls. [38, 41, 48]. But, some studies have limited statistical power because of small sample sizes and some results have been contradictory and difficult to interpret.

1.3 NEUROINFLAMMATION, TRYPTOPHAN METABOLISM AND PSYCHIATRIC DISORDERS

1.3.1 Is inflammation linked to psychopathology?

The immune system is our primary defense against infections and is also activated in response to body injuries and ischemia to initiate reparation procedures. Thus, it may seem surprising that dysregulation of the immune system plays an important role in the etiology of schizophrenia and bipolar disorder. The immune system, however, is not only activated in response to foreign invaders and trauma, but is also activated in many somatic disorders (e.g., cardiovascular disease, diabetes type 2 and autoimmune disorders) [49]. Epidemiological data show that many somatic disorders mediated by inflammation come into clusters with psychiatric disorders. Register studies, for instance, have shown that patients with autoimmune disorders (e.g., MS, autoimmune thyrotoxicosis, systemic lupus erythematous, Guillain-Barré syndrome, and autoimmune hepatitis) are at increased risk of schizophrenia or bipolar disorder [50, 51]. Patients with bipolar disorder and schizophrenia also have an elevated risk to develop cardiovascular disease and diabetes type 2 [52]. Similarly, genetic studies provide evidence for involvement of the immune system, especially in schizophrenia in which many shared susceptibility genes have been found in the immune-related human leukocyte antigen region of the human genome [53, 54]. The C-reactive protein (CRP) is an inflammatory marker commonly used in medical practice and recent meta-analysis has shown that CRP is increased in schizophrenia as well as in bipolar disorder [55, 56].

1.3.2 The immune system

The peripheral immune system is divided into two parts: the innate and the acquired immune system. The innate (non-specific) immune system is mediated by dendritic cells and macrophages of monocyte origin that identify pathogens through the toll-like receptors with the capability to recognize patterns of foreign elements. When activated, phagocytosis of pathogens is induced and a cascade is initiated leading to a release of cytokines to recruit inflammatory cells to the site of action. The acquired (adaptive) immune system consists of T and B lymphocytes with the ability to remember previous infections through antigens that allows a more effective response if the body is invaded by the same intruder a second time [57].

The immune system of the CNS differs from the peripheral system. Because of the vital function of the CNS, it is well protected and the blood-brain-barrier regulates the transport into the CNS. Microglia, a specialized subtype of cells of myeloid origin within the CNS, is involved in many of the central immune functions, related to both the innate and acquired immune system, including phagocytosis, antigen presentation to activate T-cells and production of inflammatory compounds such as cytokines [58].

1.3.3 The tryptophan metabolism – essential for psychiatric well-being

Tryptophan is an essential amino acid in the diet and is probably best known to form serotonin. In the brain serotonin has important functions (such as regulation of mood and appetite) and serotonin may be converted into the hormone melatonin, a regulator of the circadian rhythm. Nevertheless, most of the tryptophan is degraded through the kynurenine pathway, where tryptophan is converted into kynurenine and transported from the periphery into the CNS. Kynurenine is then degraded into kynurenic acid or quinolinic acid both of which pass through the blood-brain barrier poorly [59]. Quinolinic acid is in turn degraded into the end product nicotinamide adenine dinucleotide with an essential role in the metabolism of the body [60]. Enzymes of importance for the kynurenine pathway of the tryptophan metabolism are indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase, and indoleamine 2,3-dioxygenase is known to be induced by cytokines (e.g., interferons) [61-63].

Kynurenic acid is a potent antagonist of the N-metyl-D-aspartate (NMDA) and α-7-acetylcholin receptors [60]. In recent years special interest has been directed towards the metabolite because it is the only endogenous antagonist of the NMDA receptor. The NMDA receptor, stimulated by glutamate and glycine, is of particular interest for psychosis because a blockade of the receptor by the exogenous NMDA antagonist phencyclidine has been shown to affect the extracellular dopamine levels in the brain and induce psychotic symptoms [64, 65].

1.3.4 Amyloids and neurocognitive dysfunction

Cognitive impairment is a major symptom in both schizophrenia and bipolar disorder [8-10] but the mechanisms underlying the cognitive decline are unknown. In a disorder such as Alzheimer's disease with severe progressive cognitive decline the main pathological mechanism is the accumulation of amyloid-β-containing (Aβ) plaques and intracellular neurofibrillary tangles consisting of tau proteins [66, 67]. The normal physiological function of tau proteins is to stabilize the neuronal microtubules while the function of Aβ is not exactly known. In Alzheimer's disease accumulation of Aβ is thought to induce innate

immune responses through activation of microglia, which may contribute to the progressive neurodegeneration [68].

Considering the cognitive dysfunction in psychosis, disturbances of the amyloid turnover may also be of interest to investigate in psychotic disorders. A few studies have shown deviations in the CSF levels of Aβ-related proteins in schizophrenia [69] and bipolar disorder [70]. In a study on elderly schizophrenia patients Aβ-42 was found to be lower compared with controls but higher compared with Alzheimer patients, whereas tau CSF levels were normal [69]. Aβ is generated through cleavage of the amyloid precursor protein. In a study on bipolar patients the soluble form of amyloid precursor protein α and β were found to be lower. No differences were seen in Aβ in bipolar patients compared to controls, but higher ratios of Aβ-42/Aβ-38 and Aβ-42/Aβ-40 were found in the bipolar patients [70]. Thus, there seem to be abnormalities in Aβ metabolism also in psychiatric disorders, although the pattern seems to differ from Alzheimer's disease.

1.4 CEREBROSPINAL FLUID BIOMARKERS IN SCHIZOPHRENIA AND BIPOLAR DISORDER

1.4.1 Cerebrospinal fluid

A useful substrate for biomarker discovery is CSF because of its proximity to the brain [5]. CSF is a colorless fluid, surrounding the neurons and glia of the CNS and is secreted predominantly by the choroid plexus in the lateral, third and fourth ventricles. The total volume remains constant at 100–150 ml and is located in the ventricular system, the subarachnoid space, brain parenchyma and the spinal canal. The fluid is turned over four times a day, as 500 ml is produced daily; the fluid is drained via arachnoid granulations into the bloodstream [71]. CSF may be obtained by lumbar puncture using a needle inserted in the vertebral interspace at the L3-L4 or L4-L5 level.

The blood-CSF barrier limits the passage of molecules into the CSF from the blood to maintain an optimal environment for the neurons of the CNS. Albumin exclusively originates from the blood [72]. Thus, increased leakage of albumin into the CSF may be used as a measure of a worsened function of the blood-CSF-barrier by calculating the CSF/serum quotient, an. albumin ratio [72, 73]. An increased albumin ratio is found in CNS infections, Guillan Barré syndrome, brain tumors and cerebrovascular disorders [74]. An increased albumin ratio is also present in bipolar disorder and chronic as well as anti-psychotic naïve schizophrenia patients [75, 76]. The evidence achieved so far indicates that there is a disturbed blood-CSF barrier function in schizophrenia and bipolar disorder that is not related to antipsychotic medication, although the study on bipolar patients could not exclude such an effect [75].

1.4.2 Neuroinflammatory biomarkers

Macrophages (in periphery) and microglia (in CNS) express the protein CD14, which is a co-receptor. They are accompanied by the toll-like receptors, with the ability to detect bacterial lipopolysaccharide. CD14 is attached to the membrane as mCD14 and after activation of the immune system it is shed from the membrane into the circulation in a soluble form, i.e. as sCD14 [77-80]. It has been shown to be increased in chronic inflammatory disorders (e.g., cardiovascular disease [81] and rheumatoid arthritis [82]). Thus, sCD14 may be used as a marker of inflammation; higher levels of sCD14 indicate inflammation. Another

inflammation marker is “Chitinase 3 like protein 1” (YKL-40 or CHI3L1), which is a protein secreted by macrophages and microglia, among others, and has a role in inflammation and tissue remodeling [83]. Increased serum levels of sCD14 are associated with schizophrenia and bipolar disorder [84] and elevated CSF-levels of sCD14 and YKL-40/CHI3L1 were previously found in patients with bipolar disorder [85].

Cytokines are small proteins with functions in cell signaling processes that include chemokines, interferons (IFN), interleukins (Il), lymphokines and the tumor necrosis factor (TNF) proteins. Many studies have focused on measuring various kinds of cytokines in the blood and CSF of schizophrenia and bipolar patients to learn more about potential immune function abnormalities in psychiatric disorders.

Studies on serum have shown that the cytokines Il-1β, Il-6 and TNF-α have a significant influence on psychiatric disorders and psychosis in particular. For instance, a meta-analysis of studies on drug-naïve first episode psychotic patients reported associations between increased serum levels of the cytokines Il-1β, Il-6 and TNF-α and psychosis [86]. A meta-analysis on bipolar disorder found significant associations with increased serum levels of TNF-α only; and instead another interleukin, Il-4, was significantly increased [87]. Studies on CSF are fewer than those on serum. A CSF study on Il-1β showed higher levels in first-episode schizophrenia patients compared with healthy individuals [88] and chronic schizophrenia patients displayed elevated CSF levels of Il-6 compared with controls [89, 90]. But, a number of studies did not show any group differences [91]. Higher levels of Il-1β and lower levels of Il-6 were also found in euthymic bipolar patients [88], whereas screening for eleven cytokines in 121 patients with bipolar disorder only showed increased levels of Il-8 with no difference found for Il-1β, Il-6, and TNF-α [92].

Chemokines also seem to be of interest for psychiatric disorders and the chemokine “monocyte chemotactic protein 1”, also called “chemokine ligand 2” (MCP-1 or CCL2) activates macrophages, T-lymphocytes and dendritic cells to the site of inflammation. It has been shown to be elevated in serum in bipolar disorder as well as in schizophrenia, although the study samples have been limited [93]. In bipolar patients and their offspring the expression of MCP-1 mRNA in monocytes was also upregulated [94].

Alterations in the tryptophan metabolism and more specifically in the kynurenic pathway have been found in numerous CNS disorders such as bacterial meningitis [95], Alzheimer’s disease [96] and MS [97] as well as in traumatic brain injury [98]. Elevated CSF levels of kynurenine and kynurenic acid have been found in patients with schizophrenia [99, 100] and elevated CSF levels of kynurenic acid have been found also in bipolar disorder [101]. A recent study suggests that an increased production of kynurenic acid in relation to quinolinic acid might be of significance for schizophrenia [62]. The tryptophan metabolism appears to be interconnected with the immune system for instance through the enzyme indoleamine 2,3-dioxygenase that is activated by the cytokines INF-γ and TNF-α [63].

1.4.3 Microscopic structures

Electron microscopy techniques to examine CSF have been successfully performed to identify infectious germs such as the human immunodeficiency virus [102]. In 2002, micrometer-sized structures were identified in fresh CSF from schizophrenia patients using the scanning electron microscopy technique [103] and some years later structures were also identified in patients with bipolar disorder [104]. However, in a separate investigation , in Finland using frozen CSF samples from schizophrenia patients and different methodologies no association was observed between structures in CSF and schizophrenia [105].

The identified structures have typically spherical shapes and the sizes average from 0.5-1 µm, (Figure 2). The structures are smaller than cells but larger than protein molecules and the shape may indicate high lipid contents. Yet, it has been difficult to learn more about the biochemical properties of the particles and staining with antibodies did not give any conclusive results (unpublished data). Possible hypotheses of the origin of the structures are that they are waste products (e.g., from inflammatory processes or degeneration of brain tissue) or that they result from a reduced cleaning function of the CSF.

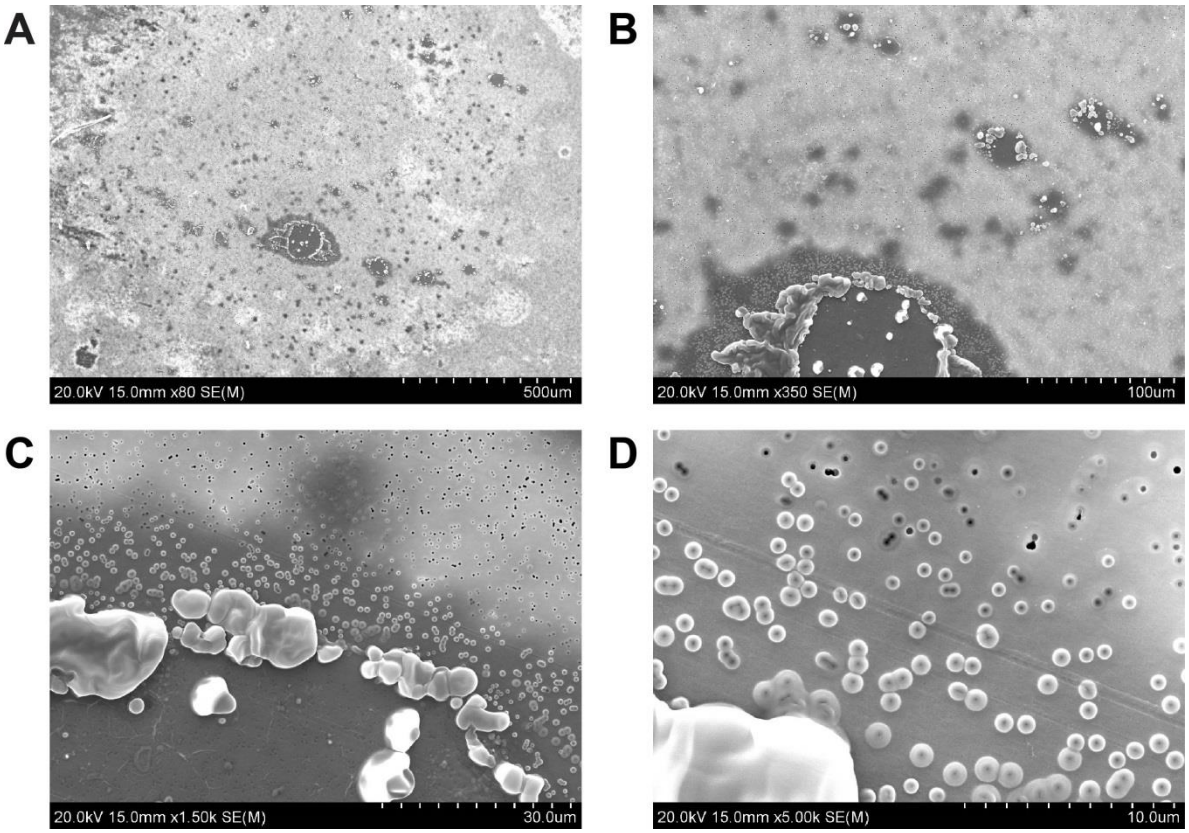

Figure 2 Example of structures in the CSF of a patient with schizophrenia. The same area on the filter is displayed in four magnifications*: In picture* ***A****. 80 times,* ***B****. 350 times,* ***C****. 1500 times and* ***D.*** *5000 times magnification. Structures with a donut shape and a diameter of 0.5-1 µm that seem to originate from the larger complex (in the lower left-hand corner) can be viewed in this picture. The dark circles in the upper part of the picture represent the pores in the filter with a size of about 0.5 µm in diameter. (Photos: Sten Johansson and Viktoria Johansson, unpublished data).*

1.4.4 Microparticles (microvesicles)

The phenomena of microparticles, also designated as microvesicles, were first described by Wolf in 1967 [106]. Microparticles are formed when cells, induced by activation, stress or apoptosis, release a part of the membrane forming small rounded particles. The small particles are thought to be involved in signaling processes and transportation procedures between cells. The size may range from 0.3-1 µm and the particles are composed by proteins, lipids and genetic material from its parent cell. It is thereby possible to analyze its original cell-type. [107]. The possibility to analyze its origin make microparticles suitable as a

biomarker for cell activation, but further research is needed to explore its potential role in psychiatric disorders.

1.5 GENETICALLY SENSITIVE STUDY DESIGNS

Epidemiology is a science in which health and disease conditions are studied in populations to learn about causes, risk factors and health outcomes. A study design is chosen to be able to answer a specific research question in the best way. Examples of study designs often used are cohort studies, case-control studies and case series [108]. Epidemiological studies are usually designed to measure the relationship between an exposure and an outcome. Another type of study design is the genetically sensitive designs used in twin and family studies that measure the environmental and genetic influence on the phenotype.

1.5.1 Twin studies

Of all births, 1-2 % are twin births and about 30% of the twin pairs born are monozygotic (MZ), which means that they have developed from the same ovum [109]. MZ twins, also known as identical twins, share 100% of their genes. Dizygotic twins (DZ), also called fraternal twins, share on average 50% of their genes and are not more alike than siblings [110]. In the DZ twin pairs about half have the same sex. For research purposes, it is most common to use same sexed twins.

Identical twins are assumed to share the genetic setup by 100% and fraternal twins by 50%. Both MZ and DZ twins share the intrauterine environment, and later on (if reared together) they share to a higher degree upbringing, childhood experiences and other environmental factors such as exposures to early infections. Therefore, MZ and DZ twins are assumed to completely share early environmental factors

In the classical twin model the incidence of a disorder in both twins of a pair (the concordance rate) is compared between MZ and DZ twins. Such a comparison makes it possible to estimate the heritability of the disorder of interest. Nowadays heritability estimation of a certain disorder or trait is performed by using structural equation modelling in the "ACE-model" where three components are estimated: additive genetics "A", shared early environmental factors "C", and unique environmental factors "E".

Co-twin control analysis is performed on twin pairs that are discordant to a disease or exposure. It means that one twin in a pair has a disease, whereas the other does not. By comparing the twins within a pair it is possible to investigate a risk factor while controlling for genetic and shared environmental confounding within the twin pair. The diseased twin in the pair is referred to as the "proband" and the healthy twin as the "co-twin". First, the probands may be compared with external control twins to determine whether there is an association between exposure and outcome. Second, a co-twin control analysis is performed in which the probands and co-twins are compared within the MZ and DZ pairs, which controls for confounding from shared environmental factors. In a third step the proband and co-twins are compared within the MZ pairs to control for genetic confounding [110, 111].

1.5.2 Sibling designs

Similar principles may be used in sibling design methods. Sibling methods are ideal to study unusual exposures and outcomes because, in comparison with twin studies, it is possible to investigate larger population samples. Siblings share on average 50% of their genes, but do not share early environmental factors as twins do because they were not born or reared at the same time. Suppose that there is an association between disease A and B and you want to ascertain whether the association is affected by genetic factors. Siblings to individuals with disease A may be considered as exposed to a genetic liability for disease A, whereas siblings to non-affected individuals are unexposed. Then the risk for disease B is compared in siblings exposed to A and the siblings that are not exposed. If there were differences in risks between the exposed and non-exposed siblings, genetic factors are of importance for the relationship between disease A and B.

2 AIMS

The overall aim of this thesis was to study the hereditary and environmental properties of potential biomarkers for schizophrenia and bipolar disorder in CSF by using twin pairs. In addition, the intention was to explore the comorbidity between schizophrenia, bipolar disorder, depression and MS. The aim of each individual study is as follows:

Study I: To study the hereditary and environmental properties of previously identified microscopic structures in the CSF of twins with schizophrenia and bipolar disorder.

Study II: To study the presence of microparticles/microvesicles in CSF from a twin pair with schizophrenia and healthy controls and to study over time the stability of the microscopic CSF structures from Study I.

Study III: To study the hereditary and environmental properties of microglia- and amyloid biomarkers in the CSF of twins with schizophrenia and bipolar disorder and their relation to previously identified microscopic structures.

Study IV: To study the hereditary and environmental properties of tryptophan metabolites and cytokines in twins with schizophrenia and bipolar disorder and their relation to psychometric scales.

Study V: To study comorbidity and sibling risk in schizophrenia, bipolar disorder and depression in relation to MS.

3 MATERIALS AND METHODS

3.1 SUMMARY OF STUDIES

Table 1. This thesis is based on the following studies. Note that the study samples are partly overlapping in Study I-IV. STAR: The Schizophrenia Twins and Relatives. CSF: Cerebrospinal fluid. SBP: St. Göran Bipolar Project. MS: Multiple sclerosis

	Type of study	Population	Materials & Methods	Statistical analysis
I	Twin study	37 twins from the STAR study 65 singletons from SBP	Structures in CSF were identified with scanning electron microscopy. *Exposure* : Microscopic structures *Outcome*: Presence of bipolar disorder /schizophrenia or liability to psychiatric disorder.	The association between a psychiatric disorder/ liability and CSF-structures were computed using conditional logistic regression.
II	Case series	One twin pair from the STAR study 4 + 10 singletons from SBP	Microparticles/ microvesicles were analyzed in CSF	Descriptive data of the findings in twins with schizophrenia and healthy controls.
III	Twin study	35 twins from the STAR study (17 complete pairs)	Immune/amyloid biomarkers were analyzed in CSF. *Exposure*: Bipolar disorder/schizophrenia *Outcome*: CSF-biomarker deviations	Paired t-test, Wilcoxon signed rank test and linear mixed models were used for group differences. Spearman's/ Pearson's coefficients for correlations.
IV	Twin study	23 twins from the STAR study (10 complete pairs)	Tryptophan metabolites and cytokines were analyzed in CSF. *Exposure*: Bipolar disorder/schizophrenia *Outcome*: CSF-biomarker deviations	Wilcoxon signed rank, Chi-square or Fisher exact tests, as well as linear mixed models were used for group differences. Spearman's coefficients for correlations.
V	Matched cohort study	71 134 schizophrenia/ bipolar disorder patients* 172 479 depression patients* 118 374 siblings to schizophrenia/ bipolar disorder patients* 280 577 sibling to depression patients* 16 608 MS-patients* 26 506 sibling to MS-patients*	*Exposure*: Psychiatric disorder/MS or Sibling with psychiatric disorder/ Sibling with MS *Outcome*: MS or Psychiatric disorder	The risk of MS/psychiatric disorder was estimated using Cox proportional hazards with robust sandwich estimates.

*10 controls were randomly selected per patient/sibling pair matched by birth year and sex

3.2 STAR – A SWEDISH TWIN COHORT

The Schizophrenia Twins and Relatives (STAR) study is a Swedish twin-cohort consisting of 383 same-sex twins (159 complete twin pairs) born between 1942 and 1986 with twin pairs concordant or discordant for schizophrenia, schizoaffective disorder or bipolar disorder and non-affected control pairs. All twins have undergone careful psychiatric assessment, neuropsychological testing, genotyping, magnetic resonance imaging (MRI) and fMRI scanning, all described in detail below. The cohort is one the largest of its kind with the intention to learn about the genetic and environmental mechanisms underlying schizophrenia and bipolar disorder.

3.2.1 Selection of twins

In total, 1213 twins, including 602 complete twin pairs, where both were alive, were identified from the Swedish Twin Register and the National Patient Register. If one member in a pair had a treatment episode of schizophrenia, schizoaffective disorder or bipolar disorder the pair was invited to participate. In addition, non-affected control twin-pairs (five pairs for each affected pair) were randomly selected and matched by birth year and sex (Figure 3).

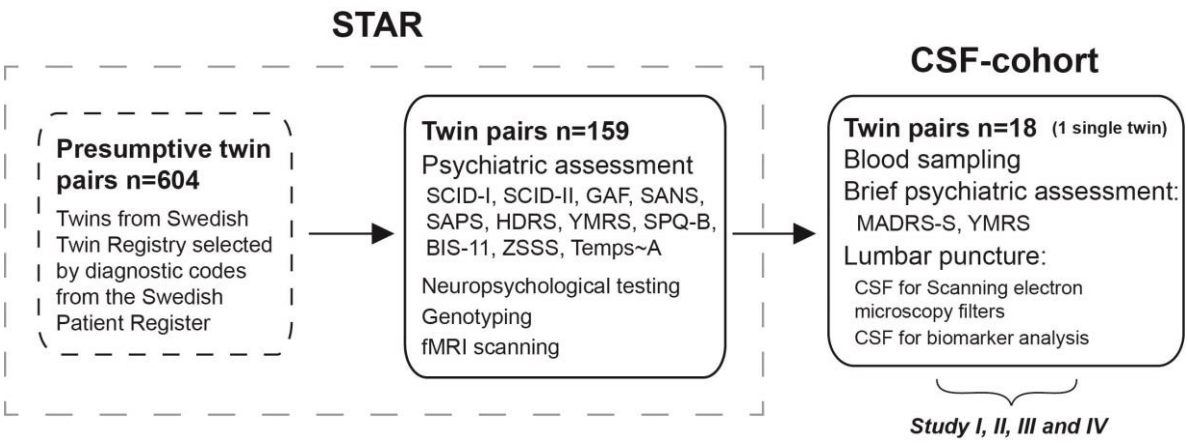

Figure 3. Flowchart of the recruitment of twins in STAR and the pairs recruited for the CSF-study. *SCID-I: Structured Clinical Interview for DSM-IV Axis I Disorders, SCID-II: Axis II Disorders, GAF: Global Assessment Function scale, SANS: Scale for Assessment of Negative Symptoms, SAPS: Scale for Assessment of Positive Symptoms, HDRS: Hamilton Depression Rating Scale, YMRS: Young Mania Rating Scale, SPQ-B: Schizotypal Personality Questionnaire-Brief, BIS-11: Barratt Impulsiveness scale, ZSSS: Zuckerman sensation seeking scale, Temps-A: Temperament scale, fMRI: Functional Magnetic Resonance Imaging, MADRS-S: Montgomery Åsberg Depression Rating Scale-Self assessment, CSF: Cerebrospinal fluid.*

3.2.2 Assessment of twins

In total, 159 complete twin pairs and 37 singe twins (where only one in the pair agreed to participate) took part in the psychiatric evaluation. Before the clinical assessment the study participants completed screening forms of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) and Axis II Disorders (SCID-II) and the following questionnaires: the Schizotypal Personality Questionnaire-Brief (SPQ-B) [112], the Barratt Impulsiveness scale [113], the Zuckerman sensation seeking scale [114] and the Temperament scale [115]. A psychiatrist or a doctor under psychiatric training administered the interviews of SCID-I [116], SCDI-II [117] and rated the participants with the Scale for Assessment of Negative

Symptoms (SANS) [118], the Scale for Assessment of Positive Symptoms (SAPS) [119], the Hamilton Depression Rating Scale [120], the Young Mania Rating Scale (YMRS)[121] and the Global Assessment Function scale (GAF) [122]. Information on socioeconomic status, smoking habits, and somatic status was collected. The twins were also tested with a neuropsychological battery and were scanned with MRI as well as fMRI. A blood sample was taken for zygosity determination and DNA and RNA analysis. Exclusion criteria included history of head injury with loss of consciousness, mental retardation, and substance use disorder within the past six months, inability to read or comprehend Swedish and any condition that precluded MRI scan of the brain (e.g., metal implants, shrapnel and certain heart operations). In addition control twins were excluded if they had a personal or family history of schizophrenia, schizoaffective disorder, or bipolar disorder.

3.2.3 Zygosity testing

In the Swedish twin register, zygosity (i.e. if a twin pair was MZ or DZ) is partly based on the following two survey questions:

1) *"During childhood, were you and your twin partner as alike as 'two peas in a pod' or not more alike than siblings in general?"*
2) "*How often did strangers have difficulty in distinguishing between you and your twin partner when you were children?"*

If both twins in a pair responded *"like two peas in a pod"* to the first question or *"always or almost always"* to the second question, the twins were determined as MZ. A validation study on DNA proved a correct result in 99% of the cases [110].

The most reliable method to determine zygosity is to compare genetic variation in the genome by DNA testing. A set of genetic markers is preferably chosen to show a large variation within the population and a low correlation between each other; the markers should not be associated with certain diseases [123]. In this thesis, based on a subset of STAR cases, twin zygosities are based on a method developed and validated by Hannelius and co-workers. The method consists of a highly multiplexed panel of 47 single nucleotide polymorphisms including one sex-specific marker [124]. The validation of the method showed that the frequency of false positives was < 0.01 when a genotype error of 1% was assumed. The analysis showed that 21 twins were MZ and 16 twins were DZ. Of the MZ twins one pair did not fulfill the criteria of being MZ according to the survey questions. All 16 twins determined as DZ in the DNA analysis fulfilled the DZ criteria according to the survey.

3.3 CEREBROSPINAL FLUID IN TWINS

3.3.1 Selection of twins for lumbar puncture

After participation in the STAR-study MZ and DZ pairs that were discordant for schizophrenia, schizoaffective disorder or bipolar disorder according to the SCID-I-interview were asked if they wanted to participate in the CSF-study. Some pairs were also recruited as non-affected control pairs and concordant pairs. Totally, 37 twins in 18 complete pairs agreed to participate in this substudy (Figure 4). One of the twins in a pair did not agree to participate in the lumbar puncture and was therefore not included in the study. For a complete listing of the twins, see table in Appendix (Appendix Table).

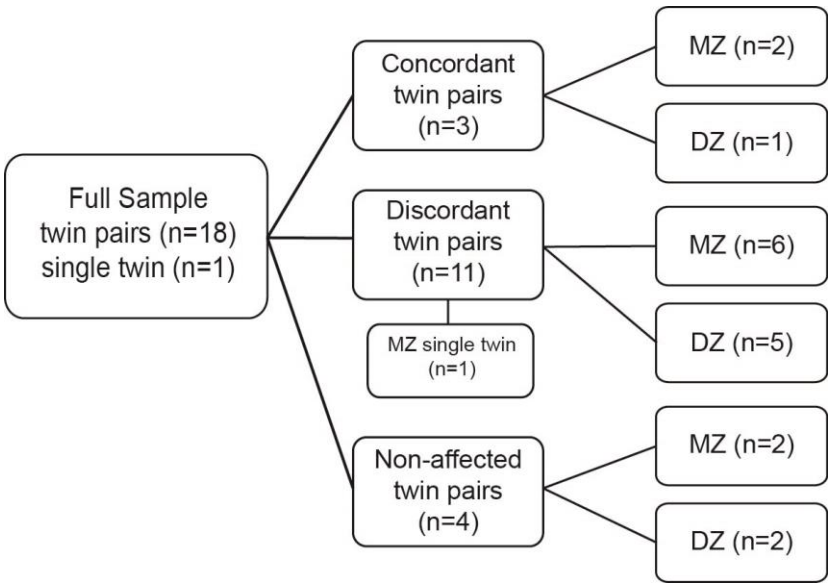

Figure 4 Overview of the twins that participated in the CSF study and number of concordant, discordant and non-affected twin pairs. *MZ: Monozygotic, DZ: Dizygotic*

3.3.2 Sampling of blood and cerebrospinal fluid

In Study I-IV paired samples of blood and CSF were collected between 8 and 11 am, in a fasting state. The samples were collected from March 2008 until September 2011 and were performed on the same day except for one pair who was examined within 43 days. Lumbar puncture was performed by an experienced neurologist with the patient in a sitting position. One of two types of fine disposable needle was used: Becton Dickinson 22 GA 3.00 IN, 0.70675 mm ("Quincke needle" used in 26 twins) or Whitaker Needle 25 GA 3.50 IN, 0.50690 mm ("Sprotte needle" used in 12 twins). The skin in the lumbar region was washed with sterile cotton swabs and chlorhexidine 5 mg/mL (Fresenius Kabi) before puncture. The needles were inserted in the vertebral interspace L3/L4, or L4/L5. The first drops of CSF, approximately 0.6 mL, were collected to be used for microscopic examination and (designated fraction I). The following 12 mL of CSF were then collected, gently inverted to avoid gradient effects. Another 0.6 ml were collected for microscopic examination (designated fraction II) and the remaining CSF was divided into 1.0–1.6 ml aliquots that were stored at 80 degrees Celsius awaiting analysis.

In Study II paired blood and CSF samples were collected a second time (October 2011) from one MZ twin pair that had agreed to participate in the study three years after the first visit. Lumbar punctures were performed as described above using a "Sprotte needle". The first two drops (100 µL) was collected for microscopic examination and the following 50 µL (designated fraction I) were collected in a sterile test tube for immediate flow cytometry analysis of microparticles described under 3.3.4. Two additional samples were collected for flow cytometry analysis the second one after approximately 7 ml of CSF had been drained (designated fraction II) and the third sample after approximately 14 ml of CSF (designated fraction III).

3.3.3 Preparation of cerebrospinal fluid and gold coating

To prepare for investigation with microscopy (Study I-III) the two separate CSF fractions (200 µL) were pipetted and dripped onto the surface of a polycarbonate filter (Nucleopore, Inc., Pleasanton, CA, USA) with 0.6 µm pores. The filters were specially prepared by GP Plastic AB (Gislaved, Sweden) and supplied by Sempore AB (Stockholm, Sweden). The filter was then fitted to an airtight device designed with flow channels that allow CSF to stream in the direction of the center of the filter when vacuum suction is applied from below. The design does not allow particles with sizes larger than 0.6 µm to pass through and the remaining structures in the CSF are allowed to concentrate in the center of the filter. After dehydration the filter was gold coated in a JEOL JFC-1200 Fine Coater (JEOL Tokyo, Japan) during two min with ionized gold to a thickness of 40 Å.

3.3.4 Scanning electron microscopy

The filters (Study I-III) were analyzed in a scanning electron microscope (Philips High Resolution SEM 515, Philips Electronic Instruments, Eindhoven, The Netherlands). The total area of each filter with a diameter of one cm was examined in the microscope and rated by an experienced researcher. The peripheral area outside the center was mostly free of structures. To standardize the procedure, microscopic images of the central areas of the filters were enlarged 50, 500 and 2000 times and saved for further reference.

A second researcher rated independently the same images with similar results. [6]. The microscopic quantity of the morphological structures on each filter was rated in the following four categories: 0= none, 1 =few, 2= several and 3= many structures as described previously [104]. Particles larger than 0.6 mm and some with smaller diameters (down to 0.1 mm) were revealed.

3.3.5 Flow cytometric analyses of microparticles in blood and cerebrospinal fluid

All blood and CSF samples in the analysis of microparticles were analyzed freshly within one hour after the collection procedure (Study II). Blood samples were centrifuged at 2000 xg for 20 min at room temperature to obtain platelet poor plasma. Before flow cytometric analysis samples were again centrifuged at 2000 xg for 20 min and the supernatant was then centrifuged again at 13,000 xg for 2 min. Twenty microliters of the sample were then incubated with the following antibodies: phalloidin-Alexa-660 (Invitrogen, Paisley, UK), lactadherin-FITC (Haematologic Technologies, VT, USA), CD42a-PE (BD,NJ, USA), CD144-APC (AH diagnostics, Stockholm, SWE) and CD45-PC7 (Beckman Coulter, CA, USA). The results in plasma are reported as absolute numbers of microparticles (106 MPs/L) (microparticles counted×standard beads added/L)/standard beads counted (FlowCount, Beckman Coulter, CA, USA).

Twenty microliters of the CSF samples were incubated for 20 min in dark with the same antibodies as described above. The microparticle-gate was determined using Megamix beads (BioCytex, Marseille, France), which is a mix of three beads with diameters of 0.5 µm, 0.9 µm and 3 µm. Microparticles were defined as particles less than 1.0 µm in size, negative to phalloidin (in order to exclude cell membrane fragments) and positive to lactadherin (which binds to phosphatidylserine). Later the microparticles were sorted into particles positive for CD42a (platelet origin; GPIX), CD144 (endothelial origin, VE-cadherin) or CD45 (leukocyte origin; pan-leukocyte antigen). Conjugate isotype-matched

immunoglobulin (IgG1-FITC, IgG1-PE, IgG1-APC and IgG1-PC7) with no reactivity against human antigens was used as a negative control to define background noise in the cytometry analysis. The results are presented as number of microparticle events in the microparticle-gate during 45 seconds of measurement.

3.3.6 Analysis of amyloid- and microglia related biomarkers

In Study III, amyloid and microglia markers were performed. CSF levels of Aβ38, Aβ40, Aβ42, sAPP-α and sAPP-β and MCP-1 were measured using the MSD ® Human/Rodent (4G8) Abeta-Triplex Assay, MSD® sAPP-α/sAPP-β Multiplex Assay, and MSD® Human MCP-1 Ultra-Sensitive Kit respectively, as described by the manufacturer (Meso Scale Discovery, Gaithersburg, MD, USA). CSF levels of total-tau (T-tau), hyperphosphorylated-Tau (P-tau), and Aβ1-42 were measured simultaneously by Luminex xMAP technology using the Inno-Bia AlzBio3 kit (Innogenetics, Zwijndrecht, Belgium). sCD14 and YKL-40 were measured by Human sCD14 quantikine ELISA kit and Human chitinase-3 quantikine ELISA kit, respectively (R&D systems, Inc, Minneapolis, MN, USA).

3.3.7 Analysis of tryptophan metabolites

In Study IV, to quantify the CSF concentrations of tryptophan, kynurenic acid and quinolinic acid, high-performance liquid chromatography and gas chromatography-mass spectrometry methods were used. The methods have been previously described in detail by Linderholm et.al. (2012) and in the Thesis of Magdalena Kegel (Dysregulation of the kynurenine pathway in psychotic disorders: immunological aspects Chapter 3.4, page 47-51) [100, 125].

3.3.8 Analysis of cytokines

Cytokines were analyzed in Study IV. The CSF samples had been thawed once before this analysis was carried out. IL-1β, IL-6, IL-8 and TNF-αwere quantified in CSF using a customized Human Ultra-Sensitive 4-Plex Kit (MesoScale Discovery®, Gaithersburg, MD, USA) in 2012. The assays were analyzed as per the manufacturers protocol (http://www.mesoscale.com), with modification of a longer primary incubation time (overnight at 4°C) and a sample volume of 50 µl. Intra-assay coefficient of variation was below 20% for all samples presented. The limit of detection in our analysis was: IL-1β (0.19 pg/ml), IL-6 (0.05 pg/ml), IL-8 (0.04 pg/ml) and TNF-α (0.08 pg/ml). The methods have previously been described in detail [125].

3.4 SINGLETON CONTROL SUBJECTS

In Study I, individuals serving as controls (n=65) were included from the St. Göran Bipolar Project (SBP), a prospective study on patients with bipolar disorder. In Study II, controls were included from the SBP (n=10).

3.4.1 Selection of singleton controls

Healthy volunteers residing in the Stockholm area were randomly selected for the study from the Total Population Register by Statistics Sweden. The participants were matched on sex and age with bipolar patients previously enrolled in the SBP. Exclusion criteria were any ongoing psychiatric disorder, current treatment with any psychotropic drugs, having a first-degree relative with schizophrenia, schizoaffective disorder or bipolar disorder and any condition that precluded MRI scan of the brain.

3.4.2 Assessment of controls

As first step research nurses with psychiatric training telephone interviewed eligible participants. The participants who fulfilled the screening criteria were scheduled for participation in the study. In a second step the participants were interviewed by a psychiatrist using a Swedish modified version of the Affective Disorder Evaluation assessment tool [126]. This interview includes, except for the screening for bipolar illness, questions about socioeconomic status, use of alcohol and psychoactive substances, family history of psychiatric disorders in first- and second-degree relatives, treatment history and somatic illnesses. To screen for psychiatric disorders other than bipolar illness The Mini International Neuropsychiatry interview (MINI) was used [127]. Further the controls were rated using the GAF scale [122]. Finally, the participants completed self-report questionnaires: the YMRS [121], MADRS [128], the Alcohol Use Disorders Identification Test (AUDIT) [129] and the Drug Use Disorders Identification Test (DUDIT) [130] (Figure 5).

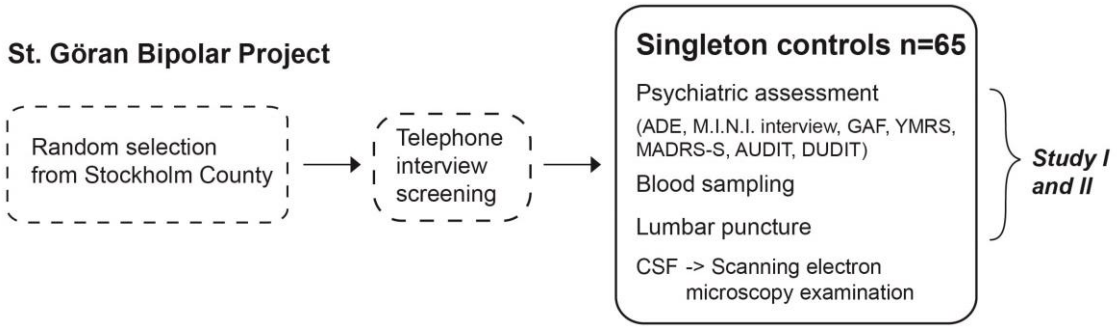

Figure 5. Flowchart of the recruitment of singleton controls in the St. Göran Bipolar Project. *ADE: Affective Disorder Evaluation, MINI: The Mini International Neuropsychiatry interview, GAF: Global Assessment Function scale, YMRS: Young Mania Rating Scale, MADRS-S: Montgomery Åsberg Depression Rating Scale-Self assessment, AUDIT: Alcohol Use Disorders Identification Test, DUDIT: Drug Use Disorders Identification Test.*

3.4.3 Sampling of cerebrospinal fluid and blood in controls

Sampling of CSF and blood in the controls was performed from November 2009 to December 2010; the sampling procedure followed the same scheme as described under 3.3.1. In Study II, in four of the controls the sampling of blood and CSF took place in October 2011 in order to analyze the blood and CSF with flow cytometry within an hour after sampling.

3.5 REGISTER DATA

Data from the national registers of Sweden have been used to identify participants in all studies of the thesis. The participants in the studies were identified from the personal identification number which is a ten digit code assigned to each Swedish resident.

3.5.1 The Swedish Twin Register

The Swedish Twin Register was established in the 1950s and is one of the world's largest twin databases, including more than 194,000 twins born from 1886 and onwards [131]. Among other information, the register includes information about twin status and sex.

3.5.2 The Swedish National Patient Register

The Swedish National Patient Register (National Board of Health and Welfare, http://www.socialstyrelsen.se) is a nationwide register that includes information on psychiatric inpatient care from 1973 and specialist care from 2001. Information on somatic diagnoses (e.g. MS) is covered from 1987. The diagnoses are recorded according to the ICD 8, 9 and 10 [2, 132, 133] and the register has a high level of agreement with medical and psychiatric diagnoses according to a validation study [134].

3.5.3 The Swedish MS Register

The Swedish MS Register [135] was started in 1996 and contained information on 13,558 unique individuals (up to January 1, 2010). All MS diagnoses are decided by consultant neurologists [136].

3.5.4 The Multi-Generation Register

The Multi-Generation Register (Statistics Sweden, http://www.scb.se) is a national database with information of the biological parents in individuals born in Sweden since 1932 and living in Sweden after 1960, as well as of immigrants who became Swedish citizens before the age of 18 [137].

3.5.5 The Cause of Death Register

The Cause of Death Register provides data on all deaths among Swedish citizens including death dates [138].

3.5.6 The Total Population Register

The Total Population Register contains information on place of birth, citizenship status, place of residence and immigration and emigration from the country. The register is administered by the Swedish Tax Agency [139].

3.6 STATISTICAL SOFTWARE

Statistical Analysis Software (SAS 9.3 or 9.4) or Statistical Package for the Social Sciences (IBM SPSS Statistics 21.0) were used for the analyses.

4 STUDY DESIGN AND STATISTICAL METHODS

4.1 STUDY I

4.1.1 Study design

CSF from 37 twins and 65 non-affected singleton controls was collected and examined with scanning electron microscopy. To be able to analyze the genetic and environmental properties of the microscopic structures in the CSF we constructed three groups: probands (twins with bipolar disorder, schizophrenia or schizoaffective disorder), co-twins (non-affected twins with liability of bipolar disorder, schizophrenia or schizoaffective disorder) and non-affected controls. We then predicted the risk of being a proband or a co-twin compared to the control group (reference) if the microscopic finding was positive.

We analyzed the influence of potential confounders such as albumin ratio, body mass index (BMI), lifetime alcohol abuse or dependence, lifetime anxiety syndrome, any medical disorder, smoking status and inflammatory markers in serum (high sensitivity CRP and white blood cell count). The overall effect of psychotropic medication and the different types (neuroleptics, lithium, anticonvulsants and antidepressants) were also analyzed. To determine the genetic and environmental properties of the CSF-structures differences between MZ and DZ twins were tested in the co-twin group.

4.1.2 Statistical method

An exact conditional logistic regression model was applied to analyze the presence of a microscopic finding as the independent (exposure) variable and the risk of being a proband or a co-twin compared with the control group as the dependent (outcome) variable. The results were expressed as odds ratios (OR) with Wald's confidence intervals (CI). A two-sided p-value of 0.05 was considered statistically significant. All analyses were adjusted for age and sex and non-independency of the twin pairs was controlled by removing either twin 1 or twin 2 in the analysis from the concordant pairs. Potential confounders were tested by adding them in sequence to the model as covariates. The effect of psychotropic medication was tested using logistic regression models with medication (along with age and sex) as the independent variable and the microscopic finding as the dependent variable. Fisher's exact test was applied to examine any association between a positive CSF finding and zygosity in the co-twins (n=12).

4.2 STUDY II

4.2.1 Study design

In this case series blood and CSF from one MZ twin pair concordant for schizophrenia and four non-affected controls were analyzed for microparticles, of leukocyte, endothelial and platelet origin, using flow cytometric analysis. The samples were analyzed within one hour after lumber puncture. CSF was analyzed with scanning electron microscopy already three years earlier in the twin pair and thus a second microscopic examination could be performed to investigate the stability of the structures over time. The scanning electron microscopy results were compared with the results from ten singleton controls previously included in Study I. The twins were in their 50s at the time of sampling and therefore controls were chosen to be as close in age as possible with age ranging from 46 to 64 years.

4.2.2 Statistical method

Only descriptive data are presented.

4.3 STUDY III

4.3.1 Study design

Biomarker concentrations of immune related markers (sCD14, YKL-40 and MCP-1) and amyloid markers (Aβ-38, Aβ-40, Aβ-42, Aβ-1-42, sAPP-α, sAPP-β and T-tau and P-tau) were measured in the CSF of 35 twins (17 complete pairs). The sample included 15 probands with either schizophrenia, schizoaffective disorder, bipolar I disorder or bipolar II disorder; 12 co-twins (of which 11 belonged to complete pairs) and 8 controls (from 4 complete non-affected pairs). In the main analysis the probands were collapsed into one group of psychotic disorders.

Correlations were computed in all complete twin pairs regardless of diagnostic status (n=17). Then a co-twin control analysis was performed in the disease discordant pairs (n=11) and in the MZ and DZ pairs separately. Associations between the biomarkers and potential confounders were analyzed: albumin ratio, medication (antipsychotics, lithium, antidepressants, and anticonvulsants), smoking, BMI and measures of peripheral inflammation (high sensitivity-CRP and white blood cell count). Associations between biomarkers and psychiatric assessment scales for psychosis was analyzed (SANS, SAPS, SPQ-B and the screening version of SCID-II – Cluster A part).

A positive association has previously been described between sCD14 and albumin ratio [85]. To test whether this association was causal we used a MZ twin intra-pair differences model, as described elsewhere [140]. The correlation of the intra-pair differences between sCD14 and albumin ratio was calculated in the MZ and DZ pairs regardless of diagnostic status. In addition to the classical co-twin control approach we performed an analysis comparing the biomarker concentrations between the probands, co-twins, and controls. However, the model was hazardous because of a limited number of controls (n=8). Finally, we also tested for associations between microscopic structures and microglia and amyloid biomarkers.

4.3.2 Statistical method

Correlations were computed using Pearson's coefficient if data were normally distributed (Spearman's correlation coefficient was applied for non-normal distributions) and Fishers Z-transformation was used to compute CIs. In the co-twin control analysis of the discordant pairs the paired t-test and Wilcoxon signed rank test were used. In the proband-, co-twin-, control approach, a linear mixed model with random intercepts shared within each twin pair was applied to compensate for the clustered nature of the twin data. The same model was applied to analyze the effect of potential confounders, psychiatric assessment scales and scanning electron microscopy results on the biomarker levels. In all linear mixed model analyses the biomarkers always entered the model as the dependent (outcome) variable. Age and sex were added as covariates. Log transformations were applied when the data were not normally distributed. Bonferroni correction was used to adjust for multiple testing.

4.4 STUDY IV

4.4.1 Study design

Kynurenic acid, quinolinic acid, tryptophan, and cytokines (IL-1β, IL-6, IL-8 and TNF-α) were analyzed in CSF from 23 twins (ten complete twin pairs). Within twin pairs, differences of the MZ and DZ pairs were calculated and potential differences between the MZ and DZ twins were computed by using the Wilcoxon signed-ranks test (Mann-Whitney U-tests). To obtain a normal distribution of the data all markers, except for IL-1β, were log transformed. Associations between the biomarkers and the results from psychiatric assessment scales were measured by using a linear mixed model analysis with random intercepts to account for the clustered nature of the data. The following assessment scales were studied: SPQ-B, the screening questions of SCID-II (cluster A part including questions for paranoid, schizoid and schizotypal personality traits), SANS and SAPS.

4.4.2 Statistical methods

Characteristics between proband and co-twins, and between DZ and MZ twins were compared using Wilcoxon signed-ranks test, Chi-square tests or Fisher exact tests. Log transformations were applied on all biomarkers, except for IL-1-α, to obtain a normal distribution of the data. Correlations were calculated using Spearman's correlation coefficient. To account for the clustered nature of the data, we fitted a linear mixed model with random intercepts shared between twins in a pair. Potential confounders, which were identified in the initial analyses (with a p-value of <0.1), were included as co-variates in the main model. Co-factors were subsequently removed based on significance of co-factors and Akaike's Information Criterion aiming for a final bestfitted model with fewer parameters. Age and sex were included as co-variates in all analyses and two-tailed probability values are reported.

4.5 STUDY V

4.5.1 Study design

Patients with schizophrenia, schizoaffective disorder, bipolar disorder, depression and MS were identified from the National Patient Register. To secure diagnostic specificity we required two or more diagnoses from the register. No diagnostic overlap was allowed between bipolar disorder, schizophrenia and schizoaffective disorder and patients with diagnostic overlap were classified in a separate mixed category designated "Mixed bipolar/psychosis". Subgroups were constructed for bipolar patients who had experienced one or more episodes of mania, designated "Bipolar disorder type I", and patients with depression who had experienced at least one episode of severe depression, designated "Severe depression". The Swedish MS Register was used to verify the quality of the MS diagnoses from the National Patient Register and 96% of the patients with two diagnoses of MS in the National Patient Register were registered as having MS in the Swedish MS register.

We used a matched cohort design based on individuals and pairs of siblings living in Sweden, identified from register linkages of the Multi-Generation Register, the Cause of Death Register and the Total Population Register. Follow-up started in 1987 (or age 18) when MS was the event and in 1973 (or age 18) when a psychiatric disorder was the event.

Participants were followed until an event occurred or until the participant was censored because of death, emigration or end of follow-up, on December 31, 2009 (Figure 6).

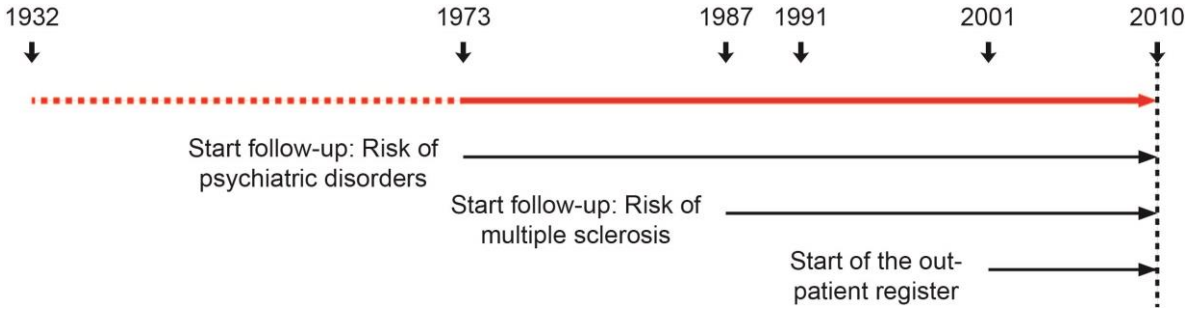

Figure 6. Timeline of Study V. *The patients were followed from 1987 when multiple sclerosis was the outcome, and from 1973 when any psychiatric disorder was the outcome. Sensitivity analysis was performed using data from the out-patient register starting in 2001. The patients were followed until the end of 2009.*

First associations between a psychiatric disorder and the risk of MS were studied in a "comorbidity cohort". Ten unexposed individuals were randomly selected per patient, matched by birth year and sex. Second associations between having a sibling with a psychiatric disorder and the risk of MS were studied in a "sibling cohort". All pairwise combinations of sibling pairs were considered; for each exposed pair ten unexposed sibling pairs were randomly selected, matched for birth year and sex for both individuals in the pair. The analysis of the comorbidity cohort and the sibling cohort was also performed in reverse order in order to assess the association between MS or having a sibling with MS (exposure) and the risk of a psychiatric disorder (outcome).

To address the influence of ascertainment bias in the comorbidity analysis we performed two analyses by time period. In the first analysis we explored the risk of MS in three time windows. MS risk was analyzed (1) from entry date until two years before date of onset of the psychiatric disorder, (2) from two years before until two years after date of onset of the psychiatric disorder and (3) from two years after date of onset of the psychiatric disorder until end of follow-up. In the second analysis MS risk was measured after date of onset of the psychiatric disorder until an event occurred or the participant was censored. Patients with an MS event before entry date were censored. All of the above analyses were performed in a reverse manner, whereby the risk of a psychiatric disorder was analyzed with MS as exposure.

4.5.2 Statistical methods

The risk of being diagnosed with an event among exposed individuals was obtained by calculating hazard ratios (HR) with 95% CI in a Cox regression model. The clustering effect in the sibling analysis was compensated by using robust sandwich estimates. Two-tailed probability values were calculated, with values less than 0.05 regarded as significant. Both in the comorbidity and the sibling analyses, we adjusted for immigration status (born in or born outside of Sweden); Effect modification was tested by including interaction terms between sex and exposure.

5 RESULTS AND COMMENTS

5.1 STUDY I: MICROSCOPIC PARTICLES IN TWO FRACTIONS OF FRESH CEREBROSPINAL FLUID IN TWINS WITH SCHIZOPHRENIA OR BIPOLAR DISORDER AND IN HEALTHY CONTROLS

5.1.1 Results

Spherical particles averaging from 0.1-8.0 µm were identified in CSF with scanning electron microscopy, for example see figure below (Figure 7). Probands, i.e. patients with schizophrenia, schizoaffective or bipolar disorder were more likely than healthy controls to have structures in the CSF, but also the co-twins were more likely to have CSF-structures (Table 2). Psychotropic medication, albumin ratio, BMI, lifetime anxiety syndrome, any medical disorder, smoking status and inflammatory markers in serum did not affect the results. Lifetime alcohol abuse or dependence was strongly associated with proband status, but when we removed the probands with previous or ongoing alcohol misuse (n=7) the association between the probands and CSF-structures remained (OR: 131, CI 9.0-999, p<0.0001). In the co-twins we found a greater proportion of positive CSF findings in the MZ co-twins (57%) than in the DZ co-twins (20%) though the difference was not found to be significant (Fisher's Exact Test: F=3, p=0.22).

Group status (n)	Positive CSF findings (%)	Odds ratio*	95% CI	p-value
Probands (n=17)	12 (70.6)	48.5	8.2-550.8	< 0.0001
Co-twins (n=12)	5 (41.7)	16.2	2.0-217.8	0.006
Healthy controls (n=65) and unaffected twins (n=8)	3 (4.1)	Reference	-	-
Total (n=102)	20 (19.6)			

Table 2. Distribution of scanning electron microscopic findings in cerebrospinal fluid (CSF) *The proband group includes patients with schizophrenia (n=7), schizoaffective (n=2) and bipolar disorder (n=8). CI: Confidence interval.* * Conditional logistic regression analysis with age and sex as covariates

5.1.2 Comments

Microscopic structures in the CSF were strongly associated with schizophrenia, schizoaffective disorder and bipolar disorder in the twin sample which confirms previous reports [103, 104]. The one study performed in Finland in 2003 that did not find any association between microscopic structures and schizophrenia analyzed frozen CSF using other methodological differences, which may explain the negative findings [105]. Moreover structures were found to be more common in the not affected co-twins with a liability for schizophrenia, schizoaffective disorder, or bipolar disorder compared with healthy controls. This result suggests a genetic influence on the microscopic structures but also an influence from common environmental factors. In the MZ co-twins CSF structures were more frequent

than in the DZ co-twins, and although not significant, one may speculate that the presence of CSF structures is genetically driven.

The structures were rare in the non-affected controls but a smaller proportion of the controls presented positive CSF-findings. For instance, CSF structures were found in patients with atrial fibrillation and Parkinson's disease. Moreover, in a previous case-report CSF-structures were found in a patient with amyotrophic lateral sclerosis [141], indicating that other medical conditions may affect the presence of structures in the CSF.

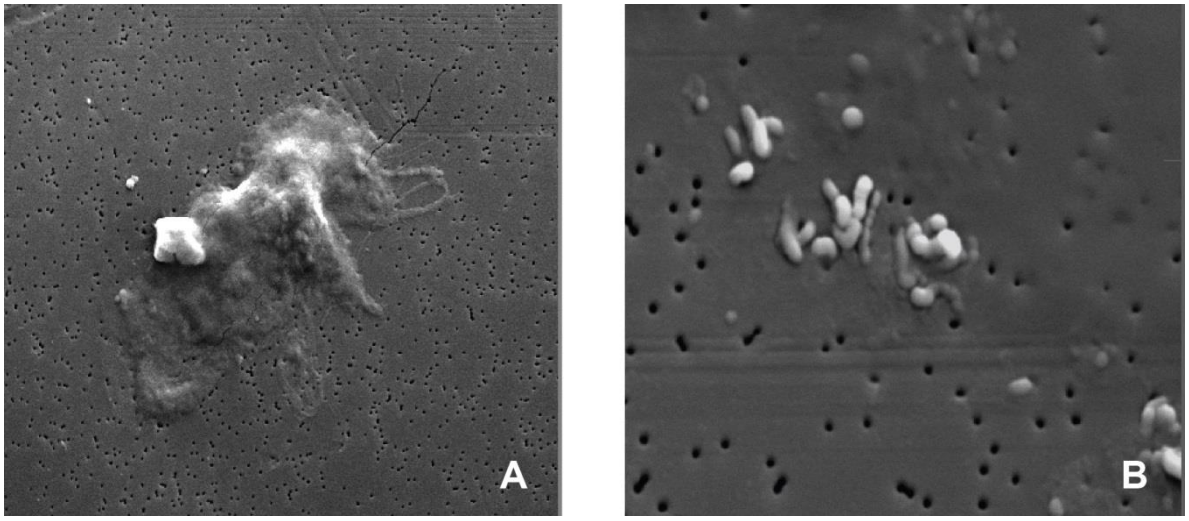

Figure 7. Scanning electron microscopy pictures of a filter showing the contents of cerebrospinal fluid from a twin with schizophrenia. *In picture* ***A:*** *Macrophage-like structure (magnification x1700) and* ***B:*** *Structures averaging between 1 to 4 µm in size (magnification x7000). The non-affected co-twin did not exhibit any similar structures in the cerebrospinal fluid. (Photos: Rolf Nybom, unpublished data)*

5.2 STUDY II: MICROPARTICLES AND MICROSCOPIC STRUCTURES IN THREE FRACTIONS OF FRESH CEREBROSPINAL FLUID IN SCHIZOPHRENIA: CASE REPORT OF TWINS.

5.2.1 Results

In the CSF of the twin pair with schizophrenia the overall rate of microparticles in CSF was higher compared with the controls. Microparticle levels of leukocyte (CD45) and endothelial origin (CD144) were observed to be higher than in the controls, whereas the levels of platelet origin (CD42a) were lower. In plasma the level of microparticles did not differ between the groups. The CSF blood ratio of platelet-derived microparticles was about 1:1000 indicating that the blood CSF barrier is dense not allowing an influx of microparticles from blood to the CSF.

CSF fraction I (representing the first 50 µl of CSF) may reflect the composition of the lumbar dural sac; CSF fraction II (representing the next 7 ml of CSF) may reflect the more rostral spinal fluid, and CSF fraction III (representing the next 14 ml of CSF) may possibly reflect the contents of ventricular CSF [142]. A possible gradient was seen between CSF fraction I and II in both twins while the microparticle levels in fraction III were clearly elevated in one of the twins but not in the other (Table 3). Three fractions of CSF were investigated with the

scanning electron microscopy technique in the twins with schizophrenia. The result from this examination was compared with a similar examination carried out three years earlier: a similar microscopic pattern was seen between the two examinations (Table 3).

	CSF or plasma fraction [a]	PS-MP	CD42a	CD144	CD45	SEM structures [b]
Twin 1	**I**	94	4	17	25	Many
	II	71	0	19	17	Many
	III	79	1	19	24	Many
	Plasma	11699	8388	806	2013	-
Twin 2	**I**	116	3	27	32	Many
	II	83	2	24	19	Many
	III	334	15	77	137	Many
	Plasma	19492	14356	864	2920	-
Controls n=4 [c]	**I**	32	9	2	6	None
	II	31	8	1	7	None
	Plasma	13298	11402	601	2044	-

Table 3 Microparticles in three fractions of cerebrospinal fluid (CSF), plasma and results from the scanning electron microscopic examination (SEM structures) in two fractions of CSF. *The results in CSF fraction I, II and III are presented as number of MP events in the microparticle gate during 45 seconds of measurement and in plasma as absolute numbers of microparticles (10^6/l)*

PS-MP: Phosphatidylserine activated MPs, CD42a: Platelet derived MPs, CD45: Leukocyte derived MPs, CD144-CSF: Endothelial derived MPs. a) CSF I: first CSF-drops (approximately 50 µl) sampled at lumbar puncture, CSF II after sampling of >7 mL and CSF III after sampling of >14 mL. b) Twin 1 and Twin 2 both displayed "Many" structures in CSF fraction I and II in the CSF-examination three years earlier. c) Presented as mean values of the results from the four controls

5.2.2 Comments

This is a case-report with interesting but possible spurious findings. Increased CSF levels of microparticles of leucocyte and endothelial, but not of platelet origin were found in patients with schizophrenia. In CSF a higher frequency of microparticles of leucocyte and endothelial origin may be an indicator of activation of those particular cell types (e.g., because of an ongoing inflammation, increased stress or apoptosis). Another possibility may be a worsened clearance process of the CSF. Although, disturbances of the CSF clearance processes as the only explanation would be less likely because increased levels of microparticles of platelet origin would then be expected as well. Thus, cell activation or an increase of the cell-decay would be more probable explanations that may reflect an increased stress exposure at the cell level. If it depends on schizophrenia or other factors such as antipsychotic medication remains to be determined. A disturbance of the endothelial and leucocyte function may be a target for future schizophrenia research.

5.3 STUDY III: CEREBROSPINAL FLUID MICROGLIA AND AMYLOID BIOMARKERS IN TWINS CONCORDANT OR DISCORDANT FOR PSYCHOTIC DISORDERS

5.3.1 Results

The correlations of the biomarker levels within all twin-pairs (n=17) were estimated, with significant high correlations (r>0.7) being found in the MZ twin pairs of the biomarkers sCD14, YKL-40, Aβ-38, Aβ-40, sAPP-α, sAPP-β, T-tau, and P-tau. The correlation of sCD14 was especially high in the MZ twins (r=0.96, p<0.0001) but was also significant in the DZ twin pairs (r=0.73, p=0.028) (Figure 8A). No significant correlations were seen in the MZ twin pairs of MCP-1, Aβ-42, Aβ-1-42 and the albumin ratio. No other significant correlations were observed in the DZ pairs except for sCD14.

The co-twin control analysis of the MZ and DZ pairs discordant for schizophrenia or bipolar disorder (n=11) showed elevated levels of sCD14 in the probands compared with their non-affected co-twins (Figure 8B). When stratified by zygosity no general significant differences were noted within the MZ and DZ twins.

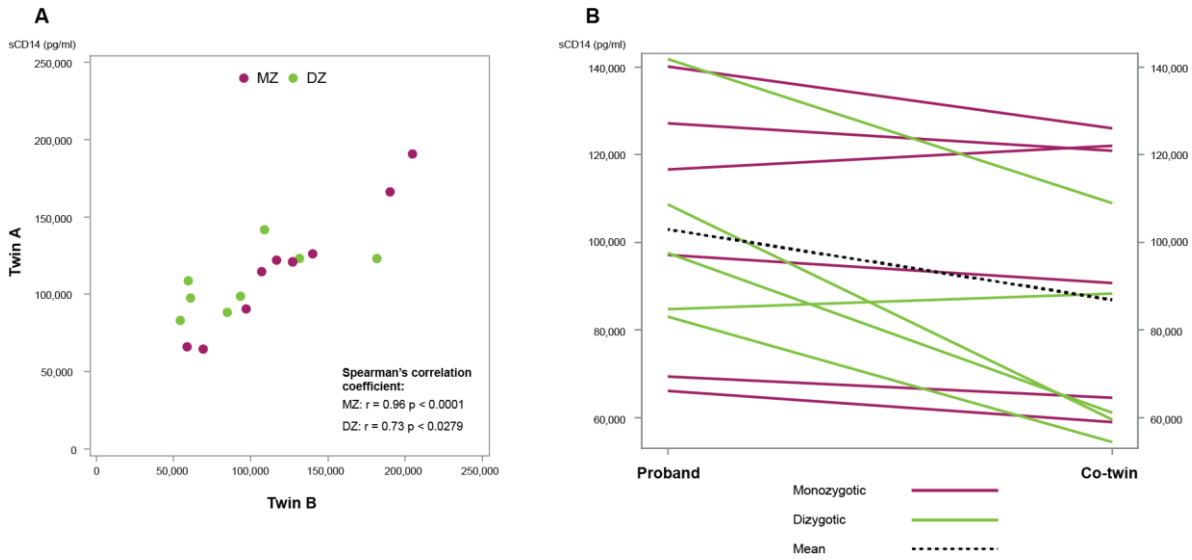

Figure 8 A: Scatterplot of the within twin pair correlations of sCD14 in the monozygotic (MZ) and the dizygotic (DZ) pairs (n=17). B: Profile plot of the within twin pair analysis of the disease discordant pairs (n=11) where each line illustrates the difference in sCD14 concentration between the proband and its co-twin.

We also analyzed the CSF biomarker levels in relation to psychometric assessment scales for psychosis: SANS, SAPS, SPQ-B and SCID-II screening (cluster A section). We found significant associations between sCD14 and the SANS scale measuring negative psychotic symptoms and the cluster A section of the SCID-II screening scale measuring paranoid, schizoid and schizotypal personality traits.

When we analyzed the influence of potential confounders on the biomarker levels, a strong association was detected between the albumin ratio and sCD14 [Estimate 0.5, 95% Confidence Interval (CI) 0.3-0.7, p<0.0001]. Increased albumin ratio levels indicate a disturbed function of the blood-CSF-barrier function. To further explore the relationship between sCD14 and the albumin ratio correlations of the intra-pair differences of sCD14 and

the albumin ratio were compared in the MZ and DZ twin pairs. The correlations were higher in the DZ pairs (r=0.81, p=0.015) compared with the MZ pairs (r=0.10, p=0.80) (Figure 9).

Finally, we analyzed associations between CSF biomarker levels and presence of microscopic particles presented in Study I. No significant associations were seen between microscopic particles and any of the CSF biomarkers.

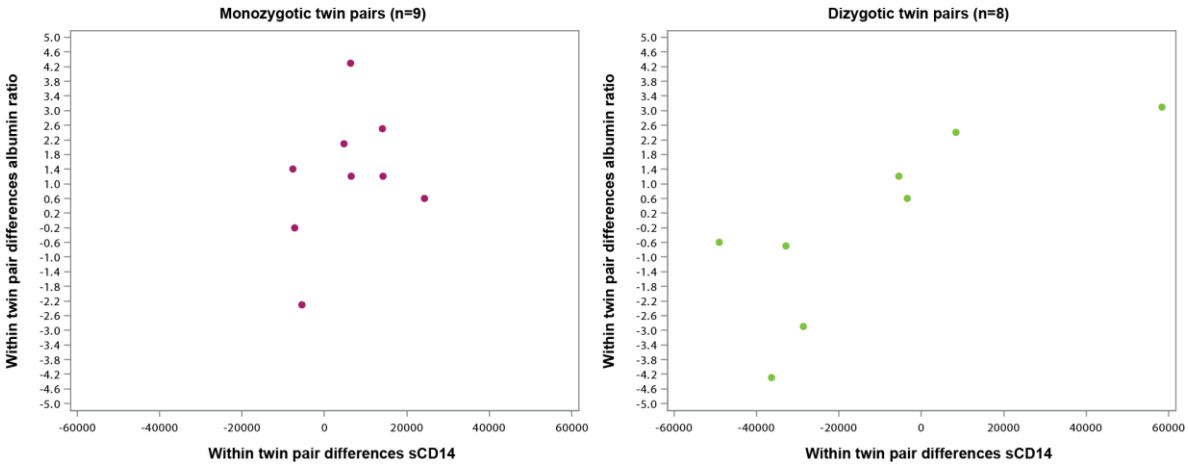

Figure 9. The intra-pair differences of the albumin ratio and sCD14 in the monozygotic and dizygotic twin pairs.

5.3.2 Comments

The high correlations within the MZ twin pairs of the immune markers (sCD14 and YKL-40), the amyloid proteins (Aβ-38, Aβ-40, sAPPα, sAPPβ) and the tau-proteins (t-tau, and p-tau) in CSF may indicate a substantial genetic influence. In the DZ twins a significant correlation was found of sCD14 only, but because of the wide CI it was not possible to exclude additional influences from a shared environment. By contrast, no significant correlations were found in the MZ or DZ twins for the amyloid proteins Aβ-42, Aβ-1-42, the chemokine MCP-1/CCL2, and the albumin ratio, indicating a lower degree of genetic influence.

In the co-twin control analysis elevated levels of sCD14 were found in the twins affected by psychotic disorders compared with their co-twins. This result precludes an effect due to a shared environment. Yet, no significant differences between proband and co-twin were seen in the MZ twin pairs alone and thus it was not possible to rule out genetic influences on the higher sCD14 levels in the patients. When analyzing the discordant twin pairs for schizophrenia, significant higher levels of sCD14 were seen using the paired t-test, but not in the bipolar twin pairs. This finding may together with the positive associations found between sCD14 and scales measuring negative psychotic symptoms and the SCID-II screening indicate that increased CSF sCD14 levels may be associated with psychosis.

An association between sCD14 and the albumin ratio has been reported in bipolar patients and healthy controls [85] and associations between elevated albumin ratio-levels (as an indicator of blood-CSF-barrier dysfunction) and psychotic disorders have also been found [75]. In this study the albumin ratio was significantly elevated in patients with schizophrenia and bipolar disorder compared with their non-affected co-twins. One explanation for the sCD14 elevation may be a consequence of a CSF barrier dysfunction (e.g., that increased

CSF sCD14 levels are caused by an influx of sCD14 from peripheral blood because of a general inflammatory process). Nevertheless, the study by Jakobsson and co-workers did not find any correlation between serum and CSF sCD14 levels in bipolar patients, which may speak against an influx of sCD14 from blood to CSF [85]. In the current study the correlation of the intra-pair differences of albumin ratio and sCD14 were higher in the DZ twins compared with the MZ twins (Figure 9). This result indicate that genetic confounding may play a role in the association between sCD14 and the albumin ratio which may not be in agreement with the explanation model of sCD14 influx from peripheral (Figure 9).

Thus, higher CSF sCD14 levels in schizophrenia and bipolar disorder may be due to an increased production by the macrophages of the CNS, microglia. The MZ intra-pair correlations indicate that regulation of the sCD14-levels is highly genetic. The CD14 gene is located on chromosome 5 and previous GWAS analyses have found associations between single nucleotide polymorphisms in the region and cardiovascular disease [81]. Yet, the GWAS study by the Psychiatric Genetic Consortium in 2013 [143] only found a single nucleotide polymorphisms association (chr5_140143664) approximately 150 base pairs away from the CD14 gene which is probably too far to be associated with the CD14 gene. Thus the functions and mechanisms underlying sCD14 remain elusive.

Biomarkers	**Spearman's correlation coefficient**	***p-value***
Aβ-42 and Aβ-1-42	r=0.94	p<0.0001
Aβ-40 and Aβ-1-42	r=0.94	p<0.0001
Total tau and P-tau	r=0.93	p<0.0001
Aβ-38 and Aβ-40	r=0.92	p<0.0001
sAPP-α and sAPP-β	r=0.92	p<0.0001
Aβ-38 and Aβ-42	r=0.91	p<0.0001
Aβ-40 and Aβ-42	r=0.90	p<0.0001
Aβ-38 and Aβ-1-42	r=0.85	p<0.0001
sAPP-beta and AB-40	r=0.71	p<0.0015

Table 4. Intra-pair correlations of all twin pairs (n=17) between biomarkers in which Spearman's coefficient was >0.7. *Aβ=Amyloid-β, P-tau= Phosphorylated tau, sAPP=soluble Amyloid Precursor Protein.*

When several biomarkers are tested simultaneously the problem of multiple testing arises. As we analyzed in total 12 different biomarkers including the albumin ratio and the significance level was set at α 0.05, then after Bonferroni correction we would arrive at a reference p-value of 0.0042 [144]. This means that none of the results from the co-twin control analysis would reach the significance level. But, several of the biomarkers are highly correlated within groups and this applies to the amyloid peptides (Aβ-38, Aβ-40, Aβ-42, Aβ-1-42), the tau-proteins (T-tau and P-tau) and sAPP-α and β (Table 4). If the amyloid peptides, the tau-proteins and the sAPPs are accounted for as three separate groups (instead of eight different biomarkers) then the reference p-value according to Bonferroni would arrive at 0.0071. This suggests that the significant results of the co-twin control analysis of sCD14 would remain significant.

5.4 STUDY IV: ASSOCIATION BETWEEN KYNURENIC ACID AND SCHIZOTYPAL PERSONALITY TRAITS IN TWIN PAIRS WITH PSYCHIATRIC MORBIDITY

5.4.1 Results

No differences for any of the tryptophan metabolites were found when comparing the probands and the co-twins. Nor were any significant within twin pair correlations found in the MZ pairs. The tryptophan metabolite results were analyzed against the results from the assessment scales measuring schizotypal and psychotic symptoms. Significant associations were found between kynurenic acid and the total scores of the SPQ-B and SCID-II (cluster A). A significant association was also found between quinolinic acid and the SPQ-B score (Table 5). No other kynurenines or cytokines were associated to any of the rating scales. Further analyses of the subscales within Cluster A revealed that kynurenic acid was associated to the subscale of schizotypal personality traits (p=0.013) and showed a trend towards significance of the paranoid subscale (p=0.062). No-significant results were found for the subscale of schizoid personality traits (p=0.103).

	SPQ-B *Estimate (95%CI)*	***P***	**SCID-II** *Estimate (95%CI)*	***P***	**Co-factor**
Kynurenic acid (nM)	1.048 (1.000-1.099)	0.049	1.082(1.015-1.153)	0.018	Smoking
Quinolinic acid (nM)	1.051 (1.006-1.098)	0.027	1.001 (0.928-1.079)	0.982	CSF Albumin
Tryptophan (μM)	1.007 (0.980-1.034)	0.606	1.008 (0.978-1.039)	0.588	None
IL-6 (pg/ml)	0.977 (0.925-1.031)	0.377	0.994 (0.925-1.070)	0.872	Snuff
IL-8 (pg/ml)	0.979 (0.956-1.002)	0.067	0.983 (0.956-1.011)	0.193	Neuroleptic
TNF-α (pg/ml)	0.985 (0.966-1.005)	0.127	0.985 (0.959-1.012)	0.241	Neuroleptic

	SANS *Estimate (95%CI)*	***P***	**SAPS** *Estimate (95%CI)*	***P***	**Co-factor**
Kynurenic acid (nM)	1.010 (0.998-1.023)	0.562	1.019 (0.998-1.042)	0.075	Smoking
Qunolinic acid (nM)	1.008 (0.995-1.021)	0.223	1.004 (0.984-1.025)	0.647	CSF Albumin
Tryptophan (μM)	0.997 (0.991-1.003)	0.346	0.996 (0.984-1.007)	0.455	None
IL-6 (pg/ml)	0.998 (0.984-1.012)	0.734	1.001 (0.986-1.015)	0.935	Snuff
IL-8 (pg/ml)	0.994 (0.986-1.003)	0.187	0.999 (0.987-1.012)	0.867	Neuroleptic
TNF-α (pg/ml)	0.996 (0.989-1.003)	0.278	0.992 (0.983-1.000)	0.057	Neuroleptic

Table 5. Association between tryptophan metabolites, cytokines and results from the psychometric assessment scales. *IL=Interleukin, TNF=Tumor necrosis factor*

5.4.2 Comments

Associations were found between kynurenic acid, quinolinic acid and schizotypal personality traits. Several studies have shown that schizotypal personality disorder symptoms are higher in patients with schizophrenia and their relatives than they are in the general population [145] and that SPQ-B total scores are stable over time in patients with schizophrenia and their relatives [146]. The association between kynurenic acid and the schizotypal traits in the SCID-II scale is also in line with these results. The result reinforces previous findings of an association between psychosis and an imbalance in the kynurenine metabolites of the tryptophan pathway. The within twin pair analyses did not show any significant differences and because of the small sample size it is risky to comment on potential genetic and environmental effects. The lack of correlations within the MZ twin pairs of all biomarkers investigated suggests that environmental factors have a greater influence on the tryptophan metabolites and the interleukins (Il-6 and Il-8) rather than genetic factors.

5.5 STUDY V: MULTIPLE SCLEROSIS AND PSYCHIATRIC DISORDERS: COMORBIDITY AND SIBLING RISK IN A NATIONWIDE SWEDISH COHORT

5.5.1 Results

Increased risk of MS was found in bipolar disorder, including bipolar disorder type I, and depression, but patients with schizophrenia were less likely to develop MS as shown in figure (Figure 10). No differences were seen in the mixed category. No differences were detected in the depression group when using one or two hospital admissions as criteria for depression. Corresponding results were seen in the reversed analysis assessing the risk of psychiatric disorders in patients affected by MS. No significant differences in MS risk were found in the siblings to patients with schizophrenia, bipolar disorder and depression. Similar results were seen when analyzing the data in the opposite direction.

In the comorbidity cohort the analysis was stratified by sex, which showed an increased risk of MS in males with bipolar type I compared to females (Figure 11). No temporal association was found between MS risk and date of onset of the psychiatric disorder, and conversely no temporal association was found between the risk of a psychiatric disorder and date of onset of MS.

5.5.2 Comments

As in previous studies, the risk of MS was increased in patients with bipolar disorder and depression. One possible explanation for these findings includes the psychological burden of being diagnosed with MS, which could increase the risk of depression. Another explanation is that MS-medications such as steroids and interferons trigger affective mood-swings that increase the probability of being diagnosed with depression or bipolar disorder in the MS patients. A temporal association could not be found, however, between MS and bipolar disorder and depression. For instance there were not any differences in the depression risk nearby the first MS diagnosis, compared to more than two years before or two years after the MS-onset. Instead the risk pattern was spread out over time and one possible explanation to this pattern may be that the CNS inflammation in MS decreases the threshold in the brain to be affected by changes in the mood as in depression or bipolar disorder. This increased susceptibility may start years before the patient is diagnosed with MS. Given the differences

between males and females, it is also possible that, for instance sex-hormones affect the risk in that estrogen has been found to be neuroprotective in animal models of MS [147].

The decreased MS risk in patients with schizophrenia was not seen in the siblings to the patients with schizophrenia. Nevertheless, in a large GWAS study carried out on patients with MS (n=27,148), schizophrenia (n=21,856) and bipolar disorder (n=16,731) MS risk alleles in the human leukocyte antigen complex were associated with a decreased risk of schizophrenia [148]. Further analysis showed that the alleles DRB1*03:01 and DQB1*02:01, reported to increase the risk of MS, decreased the risk of schizophrenia while no genetic overlap was found between MS and bipolar disorder. These results indicate that the sibling comparison in this study may not reveal certain genetic associations or that the size of the population analyzed in this study was not large enough.

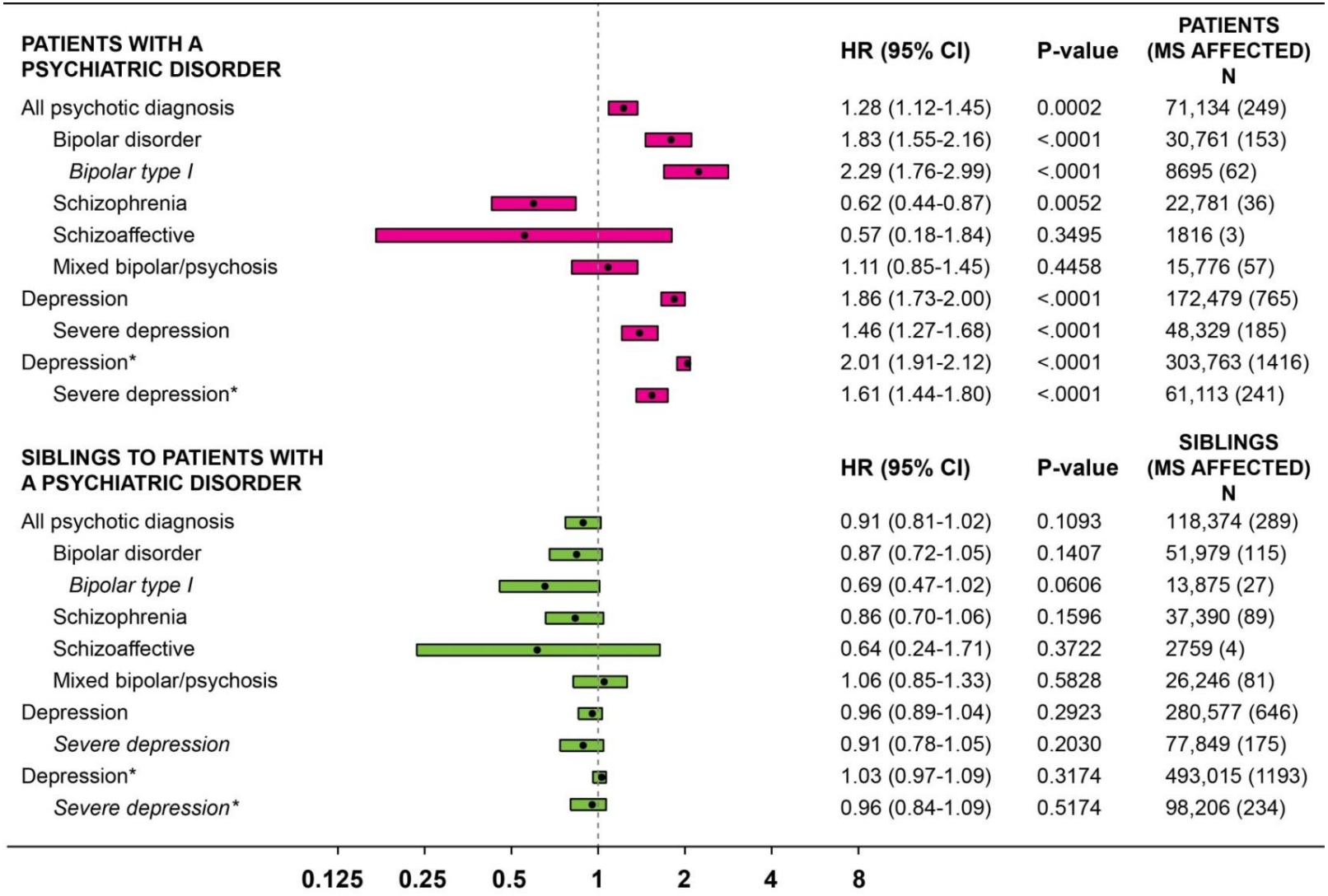

Figure 10. Risk of multiple sclerosis (MS) in patients with psychiatric disorders and their siblings. *The results from one and two depression diagnoses are included in the figure.*
*HR: Hazard ratio, CI: Confidence interval, * = Depression criteria of one hospital admission.*

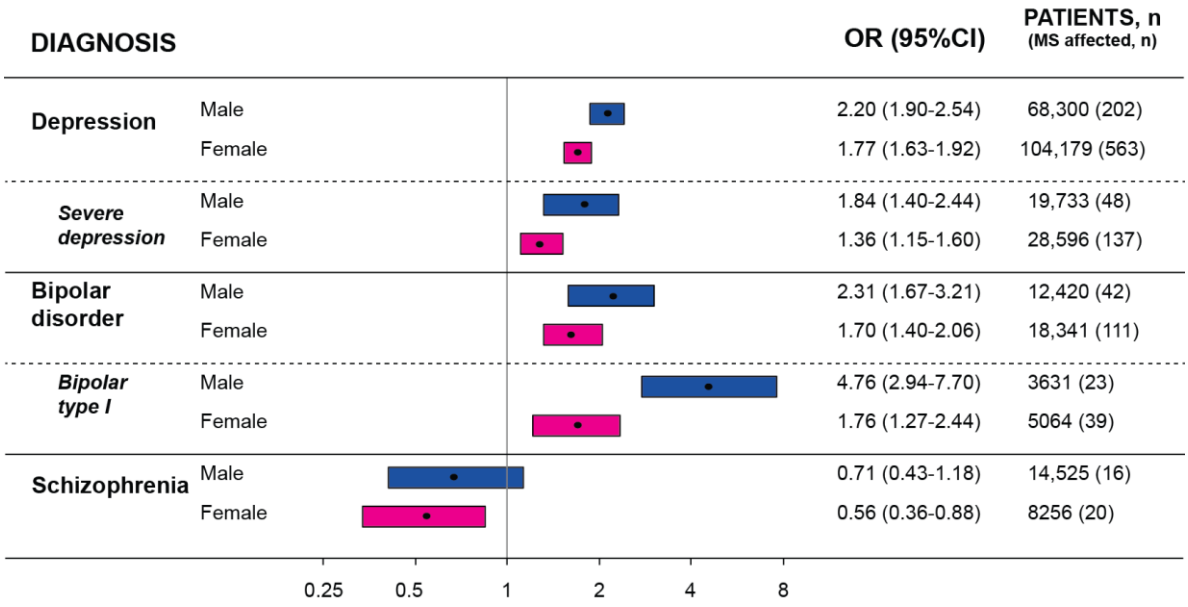

Figure 11. Risk of MS in various psychiatric disorders stratified by sex. *In comparison with females, MS risk was clearly increased in males with bipolar disorder and an episode of mania with psychosis (Bipolar type I).*

6 GENERAL DISCUSSION

6.1 CONCLUSIONS

Study I

- Microscopic structures were common in the CSF of twins with schizophrenia and bipolar disorder and were more frequent in their non-affected co-twins. This finding indicates that the precense of structures may be due to genetic or shared environmental factors.
- CSF structures were very unusual in healthy individuals, indicating that the structures are not likely to be found randomly in the healthy population.
- The structures in the CSF were also seen in patients with somatic disorders (e.g., instance Parkinson's disease) and may not be spcecific to psychiatric disorders.

Study II

- The CSF structures were stable over time according to a case-series study.
- In patients with schizophrenia microparticles (microvesicles) of leucocyte and endothelial origin were elevated, which may reflect raised immune activation.

Study III

- Several immune- and amyloid biomarkers were highly correlated within MZ twin pairs, suggesting that hereditary factors are of importance for its baseline levels.
- The biomarkers MCP-1, Aβ-42, Aβ-1-42 and the albumin ratio were not correlated in the MZ twins, indicating a higher degree of environmental influence.
- sCD14 was increased in twins with psychotic disorders as compared with their co-twins, indicating that disease-related factors may give rise to the elevated levels in addition to genetic factors.
- Higher levels of sCD14 were associated with a higher degree of negative psychotic symptoms and paranoid and schizotypal personality traits. This observation is in accordence with previous findings of elevated sCD14 levels in patients with schizophrenia and bipolar disorder.

Study IV

- Associations were found between higher degrees of schizotypal traits and increased levels of kynurenic acid and quinolinic acid.
- No significant correlations of any of the tryptophan metabolites or cytokines were found within the MZ twin pairs – indicating that environmental factors may be of importance for the biomarker levels in the tryptophan pathway.
- No changes in biomarker levels were seen when comparing the probands with the co-twins.
- An important limitation of this study was the limited sample of twins could lead to false negative results.

Study V

- A diagnosis of bipolar disorder or depression increased the risk of being affected by MS; conversly MS increases the risk of bipolar disorder and depression.
- Males with bipolar disorder type I had a greater risk of being affected by MS than females suggesting that sex-specific differences may impact disease liability.

- Schizophrenia patients had a decreased risk of being affected with MS, and conversly, MS decreased the risk of schizophrenia. This protective effect between schizophrenia and MS may partly be explained by pleiotrophic mechansims in the genome.
- No change in the risk of MS was found in siblings to patients with schizophrenia, bipolar disorder or depression. Conversly no increased psychopathology was seen in siblings to patients with MS. These findings indicate that there is no general genetically determined liability that cuases the observed comorbidities.
- There was no temporal association between the onset of MS and bipolar disorder, reducing the likelihood that the comorbidity is due to an effect of medication alone or a psychologically driven reaction by the time of disease onset.

6.2 METHODOLOGICAL CONSIDERATIONS

6.2.1 Strengths

The population-based recruitment of twins in Study I-IV is a general strength of this thesis, along with the detailed diagnostic characterization combined with information on hospital admissions over lifetime. Moreover, a thorough collection of demographic data and the measurement of symptoms using various assessment scales enabled us to integrate data from different domains. Because of the comprehensiveness of the Swedish registers, we were able to analyze in Study V comorbidities and sibling risks in the largest population-based study so far performed in our field of interest.

6.2.2 Small samples and twin designs

In Study I-IV potential CSF biomarkers of schizophrenia and bipolar disorder were studied in twins. By using a twin design, it is possible to disentangle genetic and environmental influences on a certain trait. However, it is a demanding task to collect enough CSF samples to reach sufficient power in the analysis. Twin pairs concordant or discordant for schizophrenia or bipolar disorder are rare and lumbar puncture is a troublesome procedure to endure as a study participant. In the recruitment procedure it is not unusual that the participant refuses to participate.

When using a small sample the risk of random errors is high and thus the precision is reduced. In Study I we tested the hypothesis that a positive

Testing the "Null hypothesis"

When performing an experiment, you set up a "null hypothesis", which usually means that there is no difference between the groups that you want to compare, and an "alternative hypothesis", which implies that the groups differ from each other. After a statistical test, you may reject the "null hypothesis" if you find a difference between the comparison groups. However, there is still a chance that the "null hypothesis" in reality is true; if so, the mistake that you make is called a "Type I error". Alternatively, you may not be able to reject the "null hypothesis". Then if the "alternative hypothesis" in reality is true, the mistake is called a "Type II error".

	"Null hypothesis" is true	**"Alternative hypothesis" is true**
Study result: "Null hypothesis" accepted	True null finding	Type II error (False negative result)
Study result: "Alternative hypothesis" accepted	Type I error (False positive result)	True finding

Table that summarizes the possible outcomes of hypothesis-testing

finding of CSF structures would increase the risk of psychosis or the heritability of psychosis. Before performing the analysis, a significance level of 0.05 was set. Thus, a p-value less than 0.05 implies that the results are inconsistent with the null hypothesis. To indicate precision of an estimate we use CIs and they are typically stated at 95% confidence level. It means that if the data were replicated many times, the true value should be within the CI 95% of the time. The CIs of the main results in Study I were wide, indicating a low precision of the results. A replication of the findings in a larger sample would increase the precision.

Systematic errors such as selection bias are a potential problem in our sample. Of the twins participating in the STAR study, only a small number (approximately 15% of the disease discordant pairs) agreed to participate in the CSF study. Selection bias may have arisen if those who agreed to participate in the CSF study differentiate from the whole twin population in a systematic way that we are not aware of. If that were the case, our results may not be generalizable to the population. Moreover, information bias may have arisen (e.g., by misclassification of the microscopic findings) in Study I and II during the scanning electron microscope investigation procedure. A way to overcome this problem is to have the gold-coated filters with CSF blindly re-examined by another independent researcher in the future.

Several biomarkers were analyzed simultaneously, in Study III and IV, which may introduce the problem of multiple comparisons. When the significance level is set at α 0.05, provided that the null hypothesis is true, the chance of a false positive result (Type I error) will be 1 out of 20. This result may be corrected for statistically by using the Bonferroni correction [144]. But the Bonferroni method is conservative and may instead increase the risk of false negatives (Type II error).

6.2.3 Large population-based samples

In Study V we used a matched cohort design in a large population-based sample. Using large sample sizes lowers the risk of random errors. This is because as random errors arise by chance, they can be corrected by increasing the sample size. Instead, systematic errors may arise if the measurements deviate from the true value in a systematic way. Three types of systematic bias are selection bias, information bias and confounding.

Selection bias occurs when the selection of participants is influenced by factors that may cause inaccurate results. For instance, we demanded two hospitalizations to be classified as schizophrenia, bipolar disorder or depression and therefore the less severe cases are at risk of not being included. We would miss, for example, the patients who were hospitalized only once and were followed-up in the out-patient department. This is because in the registers the out-patient clinics were included as late as 2001. The risk of selection bias applies especially to the depressive patients because most mild cases of depression are treated by family doctors and the diagnoses are not visible in the patient register. The decreased risk of MS in patients with schizophrenia could also be a result of ascertainment bias, which is a type of selection bias. As an example, if patients with schizophrenia have a tendency to be too disabled to seek health care when affected by neurological symptoms, the diagnosis of MS may go undetected in this patient group.

Information bias may result from measurement errors or misclassification. An example of misclassification bias in Study V is the risk of classifying schizophrenia as bipolar disorder, or conversely classifying bipolar disorder as schizophrenia. The risk of being affected by MS in schizophrenia and bipolar disorder has been shown to occur in the opposite directions. Too many misclassifications of schizophrenia and bipolar disorder would hypothetically lead to a null result. To overcome this problem we separated the groups of bipolar and schizophrenia patients carefully by not allowing any diagnostic overlap. This resulted in a larger group of unclear cases (n=15,776), with a mixture of schizophrenia, schizoaffective and bipolar diagnosis. Figure 12 depicts the number of patients with schizophrenia, schizoaffective and bipolar diagnosis.

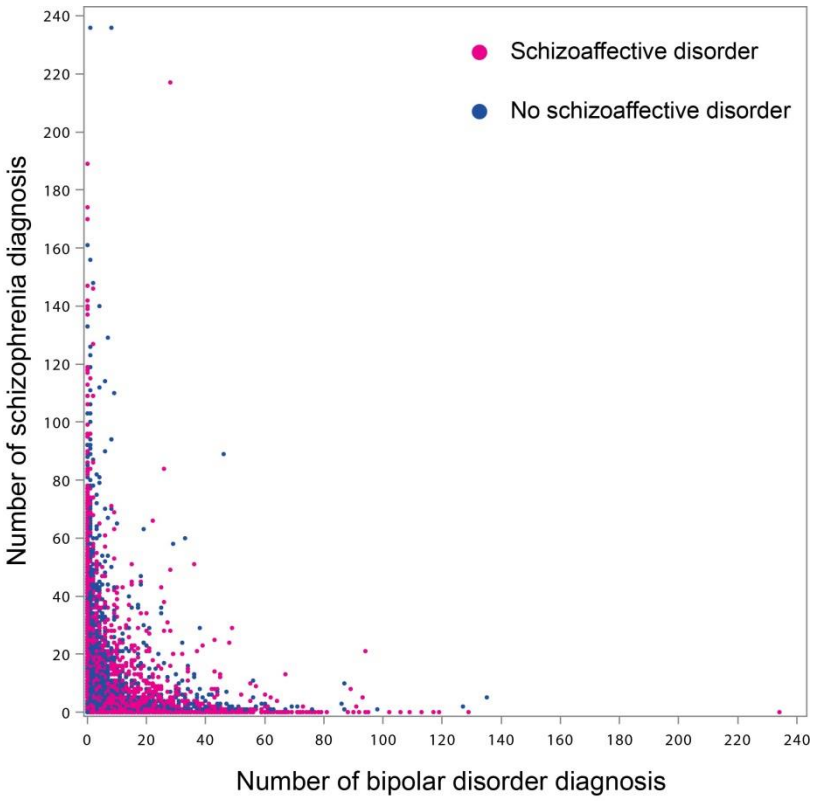

Figure 12. *Patients from the "Mixed bipolar/psychosis category with a combination of diagnosis of schizophrenia, bipolar disorder or schizoaffective disorder, n=13,333. The group of patients that had one diagnosis of schizophrenia, bipolar disorder or schizoaffective disorder in combination with one or several diagnosis of unclear bipolar disorder have been excluded from the diagram, n=2443.*

Another potential source of bias was the lack of a validation study of the depression diagnoses in the National Patient Register. High validity of a measurement means that it measures what it is supposed to measure. Thus, in our study there is an uncertainty regarding the depressive patients. The use of two discharge diagnoses for depression makes the specificity higher and decreases the risk of including false positive cases in the study; however, it may instead result in missed cases, i.e. lower sensitivity. On the other hand according to Figure 10 there were no significant differences in the risk of MS when applying the criteria of one hospitalization for depression compared with two hospitalizations. Nevertheless, with respect to depression, there is a risk that the results in Study 5 are underestimated in particular what regards to milder cases of depression that are more often diagnosed at Family doctors that are not providing data to the National Patient Register.

Confounding is a confusing of effects and is a form of systematic error. It must be associated with the disease and the exposure but must not be an effect of the exposure [149]. In Study V we used matching as a method to overcome the confounding effects of age and sex. We also selected the controls randomly. Still, we were not able to control for a number of known and unknown confounders (e.g., we did not have access to data on smoking status, which is a well-known risk factor for MS).

7 ETHICAL CONSIDERATIONS

All studies in this thesis were approved by the Regional Ethical Review Board in Stockholm. The studies were performed according to the Helsinki Declaration. In Study I-IV all enrolled patients and controls consented orally and in writing to participate in the study.

In Studies I-IV the study participants agreed to take part in a lumbar puncture as well as a blood sampling procedure. Lumbar puncture may result in side-effects, in which headache is the most common and may in some cases be very troublesome for the affected. Therefore, it is of importance that the potential risks the study participants are exposed to are balanced against the utility of the research performed. Schizophrenia and bipolar disorder are severe disorders causing a major distress for the affected individuals and their families. In this case the benefits of learning more about schizophrenia and bipolar disorder and ultimately providing a better life for these individuals outweigh the disadvantages. In Study V no interventions were made on the study participants and all the data used for the analyses were collected through the Swedish healthcare system and other population based registers in Sweden.

In Figure 2 (page 8) in the thesis summary a picture of a filter from a scanning electron microscopy examination is presented as an example. The CSF on the filter is from a patient included in another study that was approved by the ethical committee of the University of Umeå (#99-191).

8 THESIS IMPLICATIONS AND FUTURE RESEARCH

8.1 MICROSCOPIC STRUCTURES – A TRAIT MARKER OF PSYCHOSIS

In Study I we were able to replicate previous findings from bipolar and schizophrenia patients and found associations between micrometer-sized CSF structures and schizophrenia and bipolar disorder. We also observed an increased frequency of CSF structures in the non-affected twin siblings of the patients with psychosis. In Study II we found that the presence of CSF structures was stable over 3 years. In Study III we analyzed the relationship between CSF structures and immune and amyloid biomarkers but did not detect any associations.

The structures are very frequent, especially in the patients with schizophrenia and schizoaffective disorder where 8 out of 9 patients had a positive finding of CSF structures; on the other hand, the CSF structures were very rare in the healthy controls. Moreover, the structures seem to be stable over time and are more frequent in individuals with a susceptibility to schizophrenia and bipolar disorder. Even if our data indicate that the structures found in CSF may be potential biomarker candidates, the CSF structures are not yet suitable as biomarkers for several reasons. First, we found indications that similar structures occur in patients with other CNS disorders (e.g., Parkinson's disease and amyotrophic lateral sclerosis) [141] and may also be due to atrial fibrillation, i.e. they are not specific to psychotic disorders. Second, the technique to identify CSF structures depends on the assessment of a human and currently a safe technique to quantify the structures is lacking. Third, the scanning electron microscope technique is resource demanding and thus investigating larger samples is not feasible. Fourth, we have too little knowledge of the changes over time of the structures: for instance, we don't know if structures exist in the prodromal phase of psychosis. Finally, we don't know what the structures are composed of, how they are formed within the CSF and which role they play in the disease process.

Future research requires replications of the microscopic findings in other research groups using the same procedures. Standardizations of the measurements are essential to be able to evaluate and quantify the structures. Further studies are required where the microscopic findings are related to other measurements such as neuro-imaging data and neuropsychological testing.

8.2 NEUROINFLAMMATION AND THE TRYPTOPHAN METABOLISM – A COMPLEX NETWORK

In Study III and IV substances and metabolites linked to inflammation and the tryptophan metabolism were analyzed in a twin model. A growing number of studies in recent years have shown linkage of CNS disorders to neuroinflammatory processes and abnormalities in the tryptophan metabolism. In this thesis we found that elevated CSF sCD14 levels in schizophrenia and bipolar patients could be driven by disease-specific factors in combination with a genetic predisposition, although the limited number of twins in the analysis makes the conclusions premature. The associations between psychotic symptoms and higher CSF levels of sCD14, kynurenic acid and quinolinic acid strengthen previous findings of associations between psychosis and the biomarkers.

In the immune system a large number of signaling substances, metabolites and cells interact in a complex network that may be hard to overview. Single inflammatory markers may not be suitable as biomarkers alone because they are not specific to psychiatric disorders. One approach to overcome this problem would be to investigate a battery of biochemical markers alone or in combination with genetic, cognitive or neuroimaging biomarkers to determine

predictors of psychiatric disorders or symptoms in the complicated network of biochemical processes. The use of network analysis has shown to be promising even in smaller CSF samples in order to find pathways of significance [150]; however, larger sample sizes are essential to gain better precision in the analysis. sCD14 is increased in acute as well as chronic diseases and considering the high correlation of sCD14 in MZ twins, further analysis of the genetic properties would be one way to further explore its significance. In Study II, microparticles were analyzed for the first time in schizophrenia and the result showing microparticles of leucocyte and endothelial origin may be in agreement with the findings in Study III of an ongoing inflammatory process. Future studies on CSF in schizophrenia patients are desirable.

8.3 PSYCHIATRIC DISORDERS AND MULTIPLE SCLEROSIS – WHAT CAN WE LEARN?

On the surface, Study V does not seem to be related to the first four studies of this thesis. To achieve a better understanding of the mechanisms underlying a disease it is important to explore possible links between different illnesses in larger populations. Similar to schizophrenia and bipolar disorder, MS is an inflammatory disorder of the CNS. Through investigation of the comorbidity and sibling risk in relation to psychiatric disorders, hereditary and environmental relationships may be explored.

The interpretation of the co-occurrence of MS and bipolar disorder and depression indicates that neuroinflammation increases the risk for comorbidity of MS, as well as bipolar disorder and depression. A future study design would be to analyze whether the exposure for medications used for bipolar disorder (i.e. lithium), depression (i.e. antidepressants) or MS (i.e. interferon-γ or corticosteroids) would affect the prognosis and symptoms of the co-occurring disorder in comorbid cases. Further, the apparent protective effect of schizophrenia on MS is of major interest. The recent discovery of a pleiotropy in an immune-related locus that may explain this protective effect requires further investigation (e.g., by looking more closely at the actual gene and its products) [148]. A further possibility would be to characterize patients with schizophrenia and MS in combination. Differences in the risk of MS between males and females with bipolar disorder and an episode of mania should be of interest. Finally, possible androgen effects in relation the risk of MS in men and women need to be explored.

8.4 FUTURE BIOMARKER CHALLENGES

The development of biomarkers for psychiatric disorders is highly desirable. We believe that clinically useful biomarkers would improve psychiatric health care dramatically. Research in this area is intense and it is possible that biomarkers for clinical psychiatry will be available in the near future. It is also very likely that future progress within the field will change the psychiatric nomenclature, implying some major changes as well as minor adjustments.

A vision of the future for the clinician would be to have access to a combined battery of biomarkers from polygenic risk scores generated from GWAS, through laboratory tests, brain imaging investigations down to results from neuropsychological testing batteries and symptoms and signs presented by the patient. This thesis involves just a tiny part of an enormous puzzle involving many researchers throughout the world. It is our hope that every piece of information will be relevant.

9 APPENDIX

Appendix table. Descriptive data of all 37 twins of the CSF-cohort

Note: The twin numbers in the first column have been anonymized and are not comparable to the twin numbers of the supplement table of Study 1 (Table S1).

Twin	Zygosity	Sex	Diagnosis	Age of onset	Type of twin pair	Somatic disorder	Anti-psychotic medicine **
1A	MZ	Female	Schizophrenia	25	Concordant	Urinary calculus	Yes
1B	MZ	Female	Schizophrenia	18	Concordant	None	Yes
2A	MZ	Male	Schizophrenia	19	Concordant	None	No
2B	MZ	Male	Bipolar I	18	Concordant	None	No
3A	MZ	Female	Bipolar I	16	Discordant	None	Yes
3B	MZ	Female	None	.	Discordant	Vaginal bleeding	No
4A	MZ	Female	Schizophrenia	18	Discordant	Diabetes, Hypothyroidism	Yes
4B	MZ	Female	None		Discordant	Diabetes, Atrial fibrillation	No
5A*	MZ	Male	Depression	20	Discordant	None	No
6A	MZ	Female	Bipolar I	48	Discordant	Pain in shoulders	No
6B	MZ	Female	Depression	34	Discordant	Headache, Gastritis	No
7A	MZ	Female	Bipolar II	23	Discordant	Arthrosis	No
7B	MZ	Female	None	.	Discordant	None	No
8A	MZ	Male	Bipolar I	29	Discordant	Diabetes	No
8B	MZ	Male	None		Discordant	None	No
9A	MZ	Male	Depression	16	Discordant	None	No
9B	MZ	Male	Schizoaffective	16	Discordant	None	Yes
10A	MZ	Male	Depression	44	Control twin	None	No
10B	MZ	Male	None	.	Control twin	Chronic back pain	No
11A	MZ	Male	Depression	55	Control twin	Parkinson, Atrial fibrillation	No
11B	MZ	Male	Depression	51	Control twin	Atrial fibrillation	No
12A	DZ	Male	Schizophrenia	28	Concordant	None	No
12B	DZ	Male	Schizophrenia	31	Concordant	Visual disability	Yes
13A	DZ	Male	None	.	Discordant	None	No
13B	DZ	Male	Schizoaffective	27	Discordant	Heart disease	Yes
14A	DZ	Female	Bipolar II	19	Discordant	Overweight	Yes
14B	DZ	Female	Depression	29	Discordant	Tietze's syndrome	No
15A	DZ	Female	Depression	54	Discordant	None	No
15B	DZ	Female	Bipolar I	43	Discordant	None	No
16A	DZ	Female	None	.	Discordant	Malignant melanoma op.	No
16B	DZ	Female	Bipolar II	34	Discordant	None	No
17A	DZ	Female	None	.	Discordant	None	No
17B	DZ	Female	Schizophrenia	.	Discordant	None	Yes
18A	DZ	Male	Depression	27	Control twin	None	No
18B	DZ	Male	None	.	Control twin	Previous stroke	No
19A	DZ	Male	None	.	Control twin	None	No
19B	DZ	Male	None	.	Control twin	Slipped disc, Chronic pain	No

*The other twin in the pair declined participation
** Antipsychotic medication defined as taken daily.

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