Microscopic Particles in Two Fractions of Fresh Cerebrospinal Fluid in Twins with Schizophrenia or Bipolar Disorder and in Healthy Controls

Viktoria Johansson1, Rolf Nybom2, Lennart Wetterberg3*, Christina M. Hultman1, Tyrone D. Cannon4, Anette G. M. Johansson3, Carl Johan Ekman3, Mikael Landén1,5

1 Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden, 2 Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden, 3 Department of Clinical Neuroscience at St. Göran, Karolinska Institutet, Stockholm, Sweden, 4 Departments of Psychology and Psychiatry and Biobehavioral Sciences, University of California Los Angeles, Los Angeles, California, United States of America, 5 Institute of Neuroscience and Physiology, The Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden

Abstract

Background: Using scanning electron microscopy, microscopic structures have been identified in fresh cerebrospinal fluid (CSF) in patients with schizophrenia and bipolar disorder, but only rarely in control subjects. However, it has not been determined whether these microscopic particles represent state or trait markers, i.e. if their presence is related to clinical manifestations of the disease or if they also can be found in as yet asymptomatic individuals with a genetic liability. This question can be addressed by studying twins discordant or concordant for schizophrenia or bipolar disorder.

Methodology/Principal Findings: We investigated microscopic structures in CSF in 102 individuals: 21 monozygotic and 16 dizygotic twins affected or not affected with schizophrenia, schizoaffective disorder or bipolar disorder and in 65 healthy singleton controls. A first and a second fraction of CSF was freshly applied on filters and examined by scanning electron microscopy technique. Spherical particles with lipid appearance averaging between 0.1 to 8.0 μm in diameter were detected in the center of the filter as well as located in the margins of larger aggregates binding in a viscous state. Structures were found in 12 of 17 probands, 5 of 12 healthy co-twins and 3 of 73 healthy controls. Thus, a positive microscopic finding significantly increased the likelihood of belonging to the proband group (OR = 48, 95% CL: 8.2–550, p<0.0001) and the co-twin-group (OR = 16, 95% CL: 2.0–218, p = 0.006). Age, sex, history of alcohol abuse or anxiety syndrome, somatic disorder and markers of acute inflammatory activity did not account for group differences; nor did exposure to psychotropic medication.

Conclusion: Presence of microscopic particles in CSF may possibly reflect trait dependent genetic or environmental vulnerability in patients with schizophrenia, schizoaffective disorder or bipolar disorder.

Citation: Johansson V, Nybom R, Wetterberg L, Hultman CM, Cannon TD, et al. (2012) Microscopic Particles in Two Fractions of Fresh Cerebrospinal Fluid in Twins with Schizophrenia or Bipolar Disorder and in Healthy Controls. PLoS ONE 7(9): e45994. doi:10.1371/journal.pone.0045994

Editor: Colin Combs, University of North Dakota, United States of America

Received May 10, 2012; Accepted August 23, 2012; Published September 25, 2012

Copyright: © 2012 Johansson et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Financial support was provided through the regional agreement on medical training and clinical research between Stockholm County Council and the Karolinska Institutet (ALF 20100305; ALF 20090183); NIH T.D. Cannon RO1 MH52857; and through grants from the Swedish Medical Research Council (K2008-62X-14647-06-3, K2010-61X-21569-01-1, and K2010-61P-21568-01-4), and the Brain foundation (Hjärnfonden). There are no financial interests, personal relationships or affiliations, which could have influenced the work. There is no involvement of the sponsors in study design, analysis, and interpretation of the collected data nor in the reporting writing and in the choice of the journal.

Competing Interests: None of the funding sources of the authors were involved in the preparation of or decision to submit this manuscript. One of the authors (Rolf Nybom) is the inventor and patent owner of the special airtight device of the polycarbonate filters from Sempore®. Sweden used in this study, Patent No 9601247-1. The authors also confirm that this does not alter their adherence to all the PLOS ONE policies on sharing data and materials as detailed in the guide for authors. The patent is concerning 20 specifications which allow filtered material to concentrate to a small area in the middle of the filter. A figure of the filter device is included in the patent information: http://www.pvrs.se/spd/patent/?lang=en&n=hits-true&hitstart=0&tab=1&content=Rolf-Nybom&p2=57YKxsvXazY%3D%3D&p=1-Q5SGDSVkeNSd7eM2PNdVA%3D%3D&start=4.

* E-mail: lennart.wetterberg@ki.se

Introduction

Identifying biomarkers for psychiatric disorders would be an important milestone and a step towards developing tools for improved diagnostic procedures, identification of high-risk individuals, and personalized psychiatric treatment. Cerebrospinal fluid (CSF) is an especially suitable substrate to use in the quest for biomarkers in psychiatric disorder [1]. Because of its proximity to the central nervous system, CSF reflects the metabolic status of the brain and is analyzed for diagnostic purposes in neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease [2].

Schizophrenia is presumably a neurodevelopmental disorder characterized by hallucinations, delusions, and cognitive deficits. Bipolar disorder is an affective disorder that presents with episodic mood swings from high periods or mania to low periods of depression, but is also often accompanied by cognitive deficits and psychotic symptoms. Schizophrenia and bipolar disorder have a symptom overlap and in line with this reasoning, epidemiological
and genetic studies have suggested a partly shared genetic liability between bipolar disorder and schizophrenia [3], which suggests that these illnesses might share etiological and pathophysiological factors.

In 2002, micrometer-sized structures were identified in CSF from patients with schizophrenia using a scanning electron microscopic (SEM) technique. In 20 of 22 patients with schizophrenia but in only 2 of 38 non-schizophrenic controls micrometer-sized spherical particles were found in CSF [4]. One independent study using frozen CSF samples and different methodologies did not report an association between structures in CSF and schizophrenia [5]. However, in a more recent study of patients with bipolar disorder 45 of 56 patients had thread-like structures, spherical structures, or both vs. none of 20 controls without a psychiatric diagnosis [6]. In the bipolar study the SEM examination was carried out for both a first (the first 0.6 mL) and a second (the next 12 mL) fraction of CSF. Particularly noteworthy is that the structures were more often present in the first than in the second fraction. The microscopic structures were smaller than cells and larger than protein molecules. One hypothesis is that the particles may be related to childhood infections and the subsequent risk of psychotic illness as reported by Dalman et al. in a study of more than one million Swedish subjects [7]. The main finding of this study was that children who had been exposed to infections in utero were at a higher risk of developing schizophrenia than those who had not. The authors suggested that infections in utero could lead to the development of microglia, which are cells that play a role in the immune system and the brain.

The aim of the present study was to investigate the incidence of microscopic particles in two fractions of fresh cerebrospinal fluid in twin subjects discordant or concordant for schizophrenia or bipolar disorder, unaffected twin pairs, and healthy individuals without major psychiatric disorders. A secondary aim was to examine a large cohort of healthy controls using identical CSF sampling techniques that were used in the study of probands with bipolar disorder [6] to rule out confounding factors due to the collection procedure. We hypothesized that probands with schizophrenia and bipolar disorder, as well as their as yet healthy co-twins at genetic risk for these disorders, would show microscopic particles in CSF more often compared with healthy controls.

Materials and Methods

Ethics

The ethical review board of Karolinska Institutet, Stockholm, Sweden (#2007/779) approved the study of the twins, which was performed in compliance with the Helsinki Declaration. The collection of the healthy singleton subjects was part of an ongoing study approved by the Regional Ethics Committee in Stockholm (#2009/1221-32) and conducted in accordance with the latest Helsinki Protocol. All enrolled patients and control subjects consented orally and in writing to participate in the study. There were no adverse effects in the twins or the singletons, which may be explained by fixed routine and that the same neurologist performed the lumbar punctures.

Selection of twin subjects

Nineteen twin pairs were recruited for the study. The twin pairs were recruited from a nationwide cohort of Swedish-born, same-sex twins with schizophrenia, bipolar disorder, and healthy control-pairs ascertained through the Swedish Twin Registry. In total, 38 twins were included in the study where one or both twins in a pair had an ongoing or past history of bipolar disorder, schizophrenia, or schizoaffective disorder. In four of the included twin-pairs none was affected by bipolar disorder, schizophrenia or schizoaffective disorder. The sibling of one of the 38 recruited twins did not agree to participate in the lumbar puncture. None of the participants was hospitalized at the time of examination. All 37 twins and 65 healthy controls, who received a small remuneration for their participation, consented orally and in writing to take part in the study. The regional ethical review board of Karolinska Institutet, Stockholm, Sweden, approved the study, which was performed in compliance with the Helsinki Declaration.

Zygosity determination

The zygosity of the twins, mono- or dizygotic, was validated using a robust panel of 47 highly multiplexed singe nucleotide polymorphisms (SNPs) that provide reliable and high quality data on a range of different DNA templates [12]. Of the 19 twin pairs recruited for the study, 11 were monozygotic and 8 dizygotic.

Assessment of twin subjects

All twin individuals were interviewed with The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) [13] and for DSM-IV Axis II Disorders (SCID-II) [14]. Information about socioeconomic status, smoking habits, and current medication was collected. Psychiatric symptoms were rated using the following scales: Scale for Assessment of Negative Symptoms (SANS) [15], Scale for Assessment of Positive Symptoms (SAPS) [16], Hamilton Depression Rating Scale (HDRS) [17], and Young Mania Rating Scale (YMRS) [18]. The Global Assessment Function (GAF) [19] was used to assess DSM-IV Axis V. Because collection of the CSF in the twin participants was performed two months or more after the psychiatric assessment described above, a psychiatrist performed a complementary psychiatric assessment adjacent to the CSF-collection (LW or VJ) to update information about psychiatric status, current medication and somatic status. All information about heredity, age of onset, insight, period of active symptoms and lifetime somatic diagnosis was collected and psychiatric symptoms were rated using self-report questionnaires of YMRS [18] and Montgomery Asberg Depression Rating Scale (MADRS) [20]. Finally two clinically experienced researchers (CH and VJ) with access to information of the diagnostic assessments,
medical records and a full history of lifetime psychiatric diagnostic codes of the Hospital Discharge Registry (1973-2009), but with no access to the CSF-results, decided on a final consensus diagnosis.

Selection of singleton control subjects
Healthy volunteers were selected randomly for the study by Statistics Sweden (Swedish government agency that produces official statistics of Sweden, www.scb.se) from the National Population Register in the Stockholm area. The participants were matched on sex and age with patients with bipolar disorder enrolled in a prospective study (the St. Goran bipolar project) as previously described [21]. Exclusion criteria applied to the controls were any on-going psychiatric disorder, including bipolar disorder, schizophrenia and schizoaffective disorder, current treatment with any psychotropic drugs, a first-degree relative with schizophrenia, schizoaffective disorder or bipolar disorder. A further exclusion criterion was any condition that precluded magnetic resonance imaging of the brain (e.g., metal implants, shrapnel and certain heart operations). One of the 66 recruited controls was excluded because of a diagnosis of dementia that was revealed during the interview.

Assessment of singleton control subjects
A research nurse with psychiatric training conducted telephone interviews aimed at screening the participants, whereupon those who fulfilled our screening criteria were scheduled for a visit. At the visit, a psychiatrist interviewed eligible controls in a semi-structured manner (CJE and AJ), using a Swedish modified version of the Affective Disorder Evaluation assessment tool [22]. This interview included screening for bipolar illness as well as 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. The Mini International Neuropsychiatric interview [MINI] was used to screen for psychiatric disorders other than bipolar illness including psychosis and depression [23]. The Global Assessment Function (GAF) [19] was used to assess Axis V. The participants also completed four self-report questionnaires: YMRS [20], MADRS [21], the Alcohol Use Disorders Identification Test (AUDIT) [24] and the Drug Use Disorders Identification Test (DUDIT) [25].

Blood sampling and CSF/serum albumin ratio and BMI
The blood and CSF investigations for the twins lasted from March 2008 to September 2011 and for the controls from November 2009 to December 2010. Blood samples were collected with the subjects in a fasting state at 08 h under sterile conditions before the lumbar puncture. The integrity of the blood-CSF barrier of the twin and control samples was assessed by the albumin ratio, expressed as CSF albumin (mg/L)/serum albumin (g/L). Blood-CSF barrier dysfunction was defined as an albumin ratio >6.8 in individuals ≤44 years and >10.2 in individuals ≥45 years according to the reference limits presented by Blennow and coworkers [26]. Acute infection or inflammation was defined as high sensitivity C-reactive protein (HS-CRP) >3 mg/L. High level of white blood cell count (WBC) was defined as WBC >8.8×10⁹/L (reference of the hospital laboratory). Height and weight were recorded on the same day as blood and CSF sampling for calculation of the body mass index (BMI) as a heuristic proxy for body fat of the participants.

Collection of cerebrospinal fluid
The same neurologist performed the 102 lumbar punctures with all subjects in a sitting position. Sixteen twin pairs were examined on the same day and the remaining two pairs within the same month. One of two types of fine disposable needles was used; either Becton Dickinson (BD) 22 GA 3.00 IN, 0.70×75 mm (Quincke needle used in 26 twins and 17 control subjects) or BD Whitaker Needle 25 GA 3.50 IN, 0.50×90 mm (Sprotte needle used in 12 twins and 48 control subjects). The skin in the lumbar region was washed with sterile cotton swabs and chlorhexidine 5 mg/mL (Presenius Kabi) before puncture. The needles were inserted in vertebral interspace L3 to L4, or L4 to L5, and the very first 12 drops of CSF, approximately 0.6 mL, were collected in a sterile test tube for microscopic examination. The following 12 mL of CSF were allowed to drip spontaneously, or by suction six times using a 2 mL syringe due to slow flow, in a second test tube, which was gently inverted 10 times to secure homogeneous mixing of the components to avoid gradient effects. An aliquot of 0.6 mL of the mixed 12 mL was transferred into a third sterile test tube for immediate filtration and gold coating before microscopic examination. The first and the second fraction of CSF were handled in a similar way for SEM. The last mL of CSF for the control subjects was sent within 30 min after CSF withdrawal to the hospital laboratory for visual inspection and cell counting.

Filtering of cerebrospinal fluid and filter coating techniques
Of each 0.6 mL fresh 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 polycarbonate filter was specially prepared by GP Plastic AB (Gislaved, Sweden) and supplied by Sempore AB (Stockholm, Sweden). The filter was fitted to an airtight device designed with flow channels that allows CSF to stream to the center of the filter when vacuum suction is applied from below. This design does not allow particles with sizes larger than 0.6 μm to drip through the filter. The remaining structures in the CSF were thus concentrated in the center of the filter. Once the filter was dried it was coated in a JEOL, JFC-1200 Fine Coater (JEOL Tokyo, Japan) during two minutes with ionized gold to a thickness of 40 Å.

Scanning electron microscopy
The 204 filters in the present study were analyzed in a SEM 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 (RN). The peripheral area outside the center was mostly free of structures. To standardize the procedure, SEM images of the central areas of the filters were enlarged 50, 500 and 2000 times and saved for further reference. A second researcher (LW) rated the same images with similar results. The microscopic quantity of the morphological structures on each filter was rated in the following four categories: 0 = no, 1 = few, 2 = several and 3 = many structures (Fig. 1) as described previously by Bäve et al with good interrater consistency [6].

Particles larger than 0.6 μm and some with smaller diameters (down to 0.1 μm) were revealed (Fig. 1 and Fig. 2).

Blood sampling and CSF/serum albumin ratio
Blood samples were collected under sterile conditions before the lumbar puncture. The integrity of the blood-CSF barrier of the twin and control samples was assessed by the albumin ratio, expressed as CSF albumin (mg/L)/serum albumin (g/L). Blood-

PLOS ONE | www.plosone.org 3 September 2012 | Volume 7 | Issue 9 | e45994
CSF barrier dysfunction was defined as an albumin ratio >6.8 in individuals ≤44 years and >10.2 in individuals ≥45 years according to the reference limits presented by Blennow and coworkers [26]. Acute infection or inflammation was defined as high sensitivity C-reactive protein (HS-CRP) >3 mg/L. High level of white blood cell count (WBC) was defined as WBC >8.8×10^6/L (reference of the hospital laboratory).

Diagnostic categories

The results of the clinical assessment of the 37 twins are presented in Table 1. All patients with bipolar disorder type I had a history of one or more psychotic episodes confirmed by ICD-diagnosis information in register data. The cases diagnosed with schizophrenia, schizoaffective disorder or bipolar disorder are referred to as “proband”, whereas the healthy co-twins from the discordant twin pairs are referred to as “co-twin”. The twins and controls were categorized into three subgroups for statistical analysis (Table 2).

Statistical analysis

Descriptive statistics are expressed as means (standard deviations) or medians (minimum and maximum values) for continuous variables and frequencies (percentages) for categorical variables. Group differences in our data were examined using the exact conditional logistic regression model [Proc Logistic, EXACT-statement, Statistical Analysis Software (SAS 9.3)] [27] and non-independency within twin-pairs was controlled for in a separate analysis by removing either twin 1 or twin 2 from the disease concordant pairs in the proband-group and from the healthy twin-pairs in the control-group. The results were expressed as odds ratios (ORs) and Wald’s confidence limits (CLs) and a two-sided p-value < 0.05 was considered to be statistically significant. Group status entered the model as a dependent variable and any quantity of structures in the first or second fraction of CSF as an independent variable. Age, sex, BMI, lifetime alcohol abuse or dependence, lifetime anxiety syndrome, any medical disorder and smoking status entered the model as covariates. Albumin ratio was categorized as normal or as an indicator of blood-CSF barrier dysfunction.
dysfunction if the ratio exceeded the reference limit and entered the model as a covariate. Likewise HS-CRP and WBC were categorized as normal or above the reference limit and entered the model as covariates. The effect of psychotropic medication was assessed using logistic regression models with the CSF finding as the dependent variable and medication (along with age and sex) as the independent variable. Subgroups of psychotropic medications were tested as follows: neuroleptics, lithium, anticonvulsants and antidepressants. Fisher’s exact test was used to examine any association between a positive CSF finding and zygosity.

Results

Structures in CSF identified by SEM

Structures of the CSF were examined on two filters from each participant, one from the first and one from the second sample of fresh CSF. Spherical particles were noted with the appearance of small lipid bodies averaging between 0.1 to 8.0 μm. Filters with a negative finding did not present any structures (Fig. 1 A). Particles with sizes down to a diameter of 0.1 μm were found both separately on the filter (Fig. 1 B) and in the margin of larger aggregates adhering together in a viscous state (Fig. 1 C) or as separate aggregates between 0.2 to 5.0 μm (Fig. 1 D). Microscopic pictures of representative structures categorized as containing ‘no particles’, ‘few particles’, ‘several particles’ and ‘many particles’ in the first or second fraction of CSF are depicted in Fig. 1 a–d. In total 12 of 17 probands, 5 of 12 co-twins and 3 of 73 healthy controls presented particles in their CSF.

Association between CSF-structures and group-status

Demographic data are presented in Table 3. The distribution of positive CSF findings in the first or the second fraction in probands, co-twins, and controls are seen in Table 4 along with the logistic regression analysis with the control group as a reference, and age and sex as covariates. The results showed that a positive microscopic finding in CSF significantly increased the

Table 1. Diagnostic classification in 37 twins participating in the CSF study.

| Diagnostic status      | Twin subjects | Twins from concordant pair* | Twins from discordant pair | Twins from control pair |
|------------------------|---------------|-----------------------------|-----------------------------|-------------------------|
|                        | MZ twins      | DZ twins                   |                             |                         |
| Schizophrenia          | 4             | 3                           | 5                           | 2                       |
| Schizoaffective disorder| 1             | 1                           | 0                           | 2                       |
| Bipolar disorder type 1| 4             | 1                           | 1                           | 4                       |
| Bipolar disorder type 2| 1             | 2                           | 0                           | 3                       |
| Life-time depression   | 6             | 3                           | 0                           | 5                       |
| Not affected           | 5             | 6                           | 0                           | 7                       |
| Total                  | 21            | 16                          | 6                           | 23                      |

*Classified as concordant when both individuals in the pair were affected by schizophrenia, schizoaffective disorder or bipolar disorder.

doi:10.1371/journal.pone.0045994.t001
probability of being a proband or a co-twin compared to the rate observed in the control group. When analyzing the results of the first and the second CSF-fraction separately, the results were still significant when analyzing only the first fraction. Seven individuals displayed particles in the second fraction of CSF; six of them belonged to the proband group, one to the co-twin group, and all 7 individuals displayed a positive finding also in the first CSF fraction. The results of quantification of morphological structures in the categories ‘no’, ‘few’, ‘several’ and ‘many’ in the first CSF fraction (Table 5) and the second CSF fraction (Table 6) are presented in the diagnostic categories. For detailed information on all participants see Table S1.

A series of analyses were conducted in order to determine whether a history of alcohol abuse or anxiety syndrome, somatic disorder, smoking, blood-CSF-barrier dysfunction (high albumin ratio), BMI, type of needle used in the lumbar puncture procedure, acute infection or inflammation and use of psychotropic medica-
tions could account for the above associations. A previous or ongoing history of alcohol abuse or dependency strongly predicted the risk of being a proband (OR = 89.8, CL 3.1-999, p = 0.009). However, when we tested the effect of a positive CSF finding among the probands and controls with no history of alcohol abuse or dependency (probands n = 10 and controls n = 72), microscopic particles in the CSF continued to be associated with proband status (OR = 131, CL 9.0-999, p<0.0001). Anxiety syndrome, somatic disorder, smoking and type of needle at lumbar puncture (Quincke vs. Sprotte needle) did not predict the risk of being a proband or a co-twin. Including high vs. low levels of albumin ratio in the model did not change the significance of the associations between CSF particles and proband or co-twin status. When individuals (n = 7) with a high albumin ratio - indicating a defect blood-CSF-barrier - were removed from the analysis, the association between proband/co-twin status and a positive CSF finding remained significant.

### Table 2. Subgroups for analysis of CSF data and the distribution of the diagnostic categories.

| Group status                      | Schizophrenia | Schizoaffective disorder | Bipolar disorder | Total |
|-----------------------------------|---------------|--------------------------|------------------|-------|
| Probands                          | 7             | 2                        | 8                | 17    |
| Co-twins                          | 3*            | 2*                       | 7*               | 12    |
| Healthy controls and unaffected twins** | -             | -                        | -                | 73    |

*Indicates the disease status of the proband, 11 complete discordant twin pairs in total. **In total 65 singletons and 4 complete twin pairs.
doi:10.1371/journal.pone.0045994.t002

### Table 3. Sample demographics and laboratory data of 102 subjects included in the study.

| Demographic data                                      | Probands, N = 17 | Co-twins, N = 12 | Control-twins/singletons, N = 73 |
|-------------------------------------------------------|------------------|------------------|----------------------------------|
| Age (years, mean ± SD)                                | 49.5±12.1        | 51.1±10.6        | 39.7±14.3                       |
| Sex (female %)                                        | 58.8             | 66.7             | 57.5                            |
| Cohabitation status (%)                               | 23.5             | 41.7             | 64.4                            |
| Higher education (%)                                  | 35.3             | 50.0             | 45.2                            |
| Occupation status (%)                                 | 29.4             | 75.0             | 89.0                            |
| Current medication (%)                                | 82.4             | 41.7             | 16.4                            |
| Current psychotropic medication (%)                   | 76.5             | 25.0             | 2.7*                            |
| Previous/current somatic illness (%)                  | 41.2             | 50.0             | 20.6                            |
| Previous alcohol abuse/dependence (%)                 | 41.1             | 8.3              | 1.4                             |
| Previous history of anxiety syndrome (%)              | 41.1             | 8.3              | 11.0                            |
| Smoking (%)                                           | 47.1             | 8.3              | 15.1                            |
| Snuff user (%)                                        | 29.4             | 8.3              | 11.0                            |
| Body mass index (mean ± SD)                           | 28.0±7.8         | 27.5±6.0         | 24.2±3.6                        |
| Global Assessment of Functioning (median (max-min))   | 55 (35–80)       | 75 (50–100)      | 80 (70–92)                      |

| Laboratory data                                       |                  |                  |                                 |
|-------------------------------------------------------|------------------|------------------|----------------------------------|
| Albumin ratio (mean ± SD)                              | 5.8±1.5          | 4.6±1.9          | 5.1±2.3                         |
| White blood cell count (mean ± SD)                     | 6.5±2.0          | 5.9±1.3          | 5.7±1.7                         |
| C-reactive protein (mean ± SD)                         | 2.3±3.0          | 4.1±5.6          | 2.6±4.8 (n = 66)                |

a) Defined as living with partner/family. 
b) Defined as studies on University level. 
c) Defined as employment in the open labor. 
d) Defined as prescribed medication taken daily. 
*A twin pair unaffected by schizophrenia or bipolar disorder were on antidepressants at the time of lumbar puncture.
doi:10.1371/journal.pone.0045994.t003
Indicators of infection and inflammation

Since acute or chronic infection may be a cause of the structures in CSF, we investigated measures of acute and chronic infection or inflammation. HS-CRP and WBC were used as indicators of acute infection. Excluding subjects from the analysis with HS-CRP or WBC above the reference limit did not change the significance of the associations of CSF particles with the proband or co-twin status. The effect of a previous infection, chronic rheumatic disorder, or gastro-inflammatory disorder were included separately in the model but did not affect the association between proband or co-twin status and microscopic particles.

Psychotropic medication and statins

The effect of psychotropic medication on the microscopic CSF findings was tested in the proband group, since the non-proband subjects had not received psychotropic medications with the exception of three co-twins and two control-twins treated with antidepressants at the time of lumbar puncture. Neither “any psychotropic medication” (OR = 5.43, CL 0.51–infinity, p = 0.24), nor any of the separately tested psychotropic medications [neuroleptics (OR = 4.0, CL 0.4–120 p = 0.32), lithium (OR = 0.39, CL 0.07–7.3 p = 0.56), anticonvulsants (OR = 0.27, CL 0.03–3.73 p = 0.40)] had a significant effect on the presence of CSF structures. The effect of antidepressants was tested on the individuals with a diagnosis of schizophrenia, schizaffective disorder, bipolar disorder, and depression (OR = 1.1, CL 0.14–8.36, p =1).

Six individuals, five twins and one healthy singleton were prescribed statins (cholesterol-lowering drugs) of whom three displayed microscopic CSF particles.

Association between CSF findings and zygosity

To explore a possible genetic contribution to the CSF results, we tested the effect of zygosity in the co-twin group. There was a clear tendency for a positive CSF finding to be more frequent in the MZ co-twins (57%) than in the DZ co-twins (20%), but this difference did not reach significance in this limited sample of twins (Fisher’s Exact Test: F = 3, p = 0.22).

Association between CSF-structures and different diagnostic categories

Table 4. Distribution of scanning electron microscopic findings in cerebrospinal fluid (CSF) and results from the logistic regression analysis.

| Group status and N       | Positive CSF findings (%) | Odds ratio* | Confidence limits | 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 and unaffected twins (N=73) | 3 (4.1)                  | Reference   | -                | -       |
| Total (N = 102)          | 20 (19.6)                  |             |                  |         |

*Logistic regression analysis with age and sex as covariates.
doi:10.1371/journal.pone.0045994.t004

Table 5. First fraction of cerebrospinal fluid (0.2 mL of 0.6 mL) examined with scanning electron microscope in twins and singleton controls.

| Diagnostic groups                | Amount of particles in the first cerebrospinal fluid fraction |
|----------------------------------|-------------------------------------------------------------|
|                                  | No  | Few | Several | Many |
| Schizophrenia                    | 0   | 4   | 1       | 2    |
| Schizoaffective disorder         | 1   | 0   | 1       | 0    |
| Bipolar disorder type I          | 1   | 3   | 1       | 0    |
| Bipolar disorder type II         | 3   | 0   | 0       | 0    |
| Depression                       | 11  | 3   | 1       | 0    |
| Unaffected                       | 66  | 4   | 0       | 0    |
| Total                            | 82  | 14  | 4       | 2    |

doi:10.1371/journal.pone.0045994.t005
spherical particles in his CSF (Fig. 3 B).

L), a slightly increased level of HS-CRP (3.5 mg/L) and displayed

The first twin was affected with Parkinson's disease, had a normal

(14.5 and 12.0) and a medical history of atrial fibrillation (Fig. 4).

antidepressive treatment, increased CSF/serum albumin ratio

participants had a previous history of depression, ongoing

displayed particles in the first fraction of CSF (Fig. 3 A, B). Both

The association between CSF-particles and group-status when excluding the subjects with a history of lifetime depression in the control-group increased in the proband group (OR = 163.1, CL 13.4--999, p < 0.0001, probands n = 17 and controls n = 61) as well as in co-twins (OR = 51.5, CL 3.6--999, p < 0.0009 co-twins n = 12 and controls n = 61).

Association between CSF-structures and group-status considering non-independency within twin-pairs

We performed a separate analysis to compensate for non-independency within twin-pairs by removing either twin 1 or twin 2 from the disease concordant pairs in the proband group and from the healthy twin-pairs in the control-group. When we tested the effect in probands (n = 14) and co-twins (n = 12) compared to controls (n = 69) particles in CSF continued to be associated with proband status (OR 51.9 CL 6.7--877.3, p < 0.0001) and co-twin status (OR 23.3 CL 2.4--399.7, p = 0.003). We got the same results when removing twin 1 as when removing twin 2.

Description of control subjects with a positive finding in CSF

Three individuals assigned to the healthy controls and unaffected twins had structures in their CSF (Table 4). One female singleton control under the age of 45 years displayed particles in her CSF and was the only one with a positive CSF finding from the 65 singleton controls. She was apparently healthy with no medication, had no family history of psychiatric disorders and her CSF/serum albumin ratio was within the normal reference range. The SEM picture of her first CSF fraction reveals a chord extending towards a larger cluster of spherical particles with a diameter of 0.1–0.2 μm located in the center of the filter (Fig. 2). Two male participants from a monzygotic twin pair with a history of depression and anti-depressive treatment displayed particles in the first fraction of CSF (Fig. 3 A, B). Both participants had a previous history of depression, ongoing antidepressive treatment, increased CSF/serum albumin ratio (14.5 and 12.0) and a medical history of atrial fibrillation (Fig. 4). The first twin was affected with Parkinson’s disease, had a normal WBC (3.9×10⁷/L), an increased level of HS-CRP (16.9 mg/L) and protein-like aggregates were observed in his CSF sample (Fig. 3 A). The second twin had a normal level of WBC (5.7×10⁷/L), a slightly increased level of HS-CRP (3.5 mg/L) and displayed spherical particles in his CSF (Fig. 3 B).

Discussion

We analyzed CSF in 37 twins and 65 controls using the SEM technique to identify potential genetic and environmental effects influencing accumulation of particles in CSF. The main findings from this report are as follows.

First, the structures in CSF were strongly associated with schizophrenia and bipolar disorder. These results confirm previous findings in schizophrenia [4] and bipolar disorder [6].

Second, the structures were rare in the healthy controls, implying that structures detected with this method are exceptional in the normal population. The CSF of the twins and controls were sampled following identical procedures, which were also used in a previous study investigating bipolar disorder [6], which rules out the possibility that the findings were due to different sampling techniques.

Third, we found an association between structures in the CSF and co-twin status, which indicates a genetic or environmental effect. Control for potential confounding factors in the logistic regression models suggests that the CSF structures do not seem to be explained by medication, alcohol abuse, anxiety disorder, somatic disorder, BMI or type of needle used during lumbar puncture.

Finally, we studied the distribution of CSF structures in the monozygotic and dizygotic co-twins and observed a clear trend towards a higher level of positive CSF findings in the monozygotic co-twins. These results did not reach the statistical threshold, probably because of a lack of power due to small sample size. Taken together the findings are consistent with a genetic influence on the CSF findings.

CSF/serum albumin ratio has been suggested as a marker of blood-CSF barrier dysfunction [26,28]. Earlier studies have reported on increased ratio in some patients with schizophrenia [29,30]. However, none of the participants with schizophrenia or bipolar disorder in our sample had an increased albumin ratio. Thus, when included in the model blood-CSF barrier dysfunction, did not have any effect on the main results. We also included markers for acute inflammatory activity, current medication and information about somatic disorders, [e.g., inflammatory-related disorders and neurological disorders] in the analysis. The results support the assumption that the presence of CSF structures is not sensitive to ongoing infection or inflammation. Investigating the presence of previous infections, we decided not to include reports of unspecified virus infections in that they are very common and unspecific. The effect of psychotropic medication (antipsychotics, lithium, anticonvulsants and antidepressants), examined in the

Table 6. Second fraction of cerebrospinal fluid (0.2 mL of 12 mL) examined with scanning electron microscope in twins and singleton controls.

| Diagnostic groups               | Amount of particles in the second cerebrospinal fluid fraction |
|--------------------------------|---------------------------------------------------------------|
|                                | No  | Few | Several | Many |
| Schizophrenia                  | 3   | 2   | 0       | 2    |
| Schizoaffective disorder       | 1   | 1   | 0       | 0    |
| Bipolar disorder type I        | 4   | 1   | 0       | 0    |
| Bipolar disorder type II       | 3   | 0   | 0       | 0    |
| Depression                     | 14  | 1   | 0       | 0    |
| Unaffected                     | 70  | 0   | 0       | 0    |
| Total                          | 95  | 5   | 0       | 2    |

doi:10.1371/journal.pone.0045994.t006
proband group only did not show any effect on the presence of CSF particles when tested separately or combined. This finding contradicts the view that CSF structures are related to psychotrophic medication.

When analyzing the probands with schizophrenia and schizoaffective disorder and their co-twins separately from the probands with bipolar disorder and their co-twins we found a stronger association between schizophrenia and schizoaffective disorder and particles in CSF compared to bipolar disorder. There was no significant association between the co-twins discordant for bipolar disorder and particles. However when we analyzed without the patients with bipolar disorder type II and their co-twins the association between group-status and particles increased compared to our main results indicating that the particles in CSF may be associated with psychotic symptoms. This finding is in line with the tendency reported in the study by Båve et al. in which patients with bipolar disorder type I were more likely to display CSF structures than patients with bipolar II disorder [6].

In 13 of the 20 individuals with a positive CSF-finding, structures were identified in the first 0.6 ml of CSF but not the second fraction. A consensus guideline for neurological research from 2009 prescribes that the very first mL of CSF should be discarded [31]. Interestingly following this recommendation would only have identified individuals with CSF structures in the second fraction. One hypothesis is that a low level of neuroinflammation or neurodegeneration in schizophrenia and bipolar disorder gives rise to a smaller amount of apoptotic products in the CSF. The individuals who also display structures in the second CSF fraction may have numerous particles in their CSF, indicating that they have a less effective cleansing function of CSF or a higher level of neurodegeneration in the brain [8,32]. The possibility that the structures are unspecific artifacts does not accord with the negative findings in the controls.

Only 3 controls presented structures in their CSF. One female singleton control presented spherical particles in her CSF of a similar type found in patients with schizophrenia and bipolar disorder (Fig. 2). Particles of similar size and form were found in the second CSF fraction of an unaffected, un-medicated male co-twin of a brother with schizoaffective disorder (Fig. 1 B). Further, a monozygotic twin pair that served as controls presented particles in their CSF (Fig. 3). The structures observed in the first twin are similar to protein-like aggregates, possibly related to the increased albumin ratio in CSF (Fig. 3 A). The structures in the second twin

![Figure 3. Scanning electron microscopy (SEM) picture of cerebrospinal fluid (CSF) from monozygotic twins unaffected by psychotic disorder. SEM pictures of a polycarbonate filter with the first fraction of CSF from a monozygotic twin pair unaffected by psychotic illness, but with a medical history of episodic depressions and atrial fibrillation. Both twins displayed the highest CSF albumin concentration in the study. The twin in figure A) also had a history of Parkinson’s disease treated with L-dopa. Figure B) contains subcellular structures with spherical shapes. doi:10.1371/journal.pone.0045994.g003](http://www.plosone.org/)

![Figure 4. Albumin ratio CSF/serum versus blood leukocytes in 37 twin subjects. A nonparametric bivariate density graphic plot of the albumin ratio expressed as cerebrospinal fluid (CSF) albumin (mg/L)/serum albumin (g/L) on the ordinate vs. the leukocyte number \( \times 10^9/L \) on the abscissa in 37 twins. To the right are two outliers with a high albumin ratio, indicating that they have a defect in their blood-CSF barrier function (for individual albumin ratio see Table S1 in Supporting Information). doi:10.1371/journal.pone.0045994.g004](http://www.plosone.org/)
were similar to the spherical particles observed in the proband sample (Fig. 3B). The findings in both twins may be related to a blood-CSF barrier dysfunction. They may also be latent disease carriers for bipolar disorder or schizophrenia.

Furthermore, spherical CSF-particles may also occur in other disorders involving the central nervous system as recently reported in amyotrophic lateral sclerosis by Zachau et al. [33]. In this study the CSF of an ALS patient displayed 100 times more phosphatidylserine-positive microparticles and 400 times more cell-derived microparticles of leukocyte origin compared to healthy controls although the leukocyte count in CSF was normal. Similar studies of microparticles in CSF in schizophrenia and bipolar disorders are called for.

Strengths and limitations

Strengths of the present study include the careful lifetime assessment of the psychiatric diagnosis, controlling for potential confounding factors in the analysis and an identical collection procedure of the CSF in all participants included in the study. Moreover, the control group is large and population-based. However, several limitations of the study also need to be addressed. The possible confounding effects of perinatal and childhood infections were not clarified because of lack of information. The small sample size of disease discordant twin-pairs prevented further analysis of the genetic contribution of the CSF findings. In addition, psychotic symptoms of the probands were not investigated using a structured instrument such as SANS and SAPS [15,16] adjacent to the collection of CSF.

Further studies

Further research in larger twin samples of cases diagnosed with schizophrenia and bipolar disorder as well as high-risk individuals (e.g., first-degree relatives of patients with psychosis) may help to answer the question of whether structures of the CSF precede a manifest disease state and may serve as a trait marker. Further work is needed to explore the origin and the composition of the anatomical structures in both the first and second CSF fractions.

Conclusions

The main conclusion from this study is the strong statistical support for structures in CSF identified with the SEM technique in patients with bipolar disorder or schizophrenia. The results indicate that the CSF structures likely originate from trait-dependent factors.

Supporting Information

Table S1 Detailed information on the characteristics of 102 individuals included in the study.

(DOC)

Acknowledgments

We thank the project manager Lennart Martinsson, research nurses Marina Wennberg, Agneta Carlswald-Kjellin and Stina Stadler and Drs. Yuigne Hallstrom, Ulvi Bave and Biostatistician Cecilia Lundholm for their professional help.

Author Contributions

Conceived and designed the experiments: VJ RN LW CMH TC AGJ CJE ML. Performed the experiments: VJ RN LW CMH TC JE ML. Analyzed the data: VJ TC CMH LW ML. Contributed reagents/materials/analysis tools: RN LW ML. Wrote the paper: VJ TC CMH LW ML.

References

1. Schwarz E, Bahn S (2008) Cerebrospinal fluid: identification of diagnostic markers for schizophrenia. Expert review of molecular diagnostics 8: 209–216.
2. Mattsson N (2011) CSF biomarkers in neurodegenerative diseases. Clinical chemistry and laboratory medicine : CCLM/FESCC 49: 345–352.
3. Lichtenstein P, Yin H, Bjork C, Pawitan Y, Cannon TD, et al. (2009) Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. Lancet 373: 234–239.
4. Wetterberg L, Nybom R, Bratlid T, Fladby T, Olsson B, et al. (2002) Micrometer-sized particles in cerebrospinal fluid (CSF) in patients with schizophrenia. Neuroscience letters 329: 91–95.
5. Eklof J, Wahbeek B, Niskanen T, Burtt N, Ntalla I, et al. (2003) No association between micrometer-sized particles in human cerebrospinal fluid and schizophrenia. Neurology 48: 60–64.
6. Bäckman A, Nybom R, Landén M, Wetterberg L. (2006) Micrometer-sized thread-like and/or spherical particles in the first fraction of cerebrospinal fluid in patients with bipolar disorder. Bipolar disorders 12: 284–294.
7. Dahman C, Allebeck P, Gunnell D, Harrison G, Kristensson K, et al. (2008) Infections in the CNS during childhood and the risk of subsequent psychotic illness: a cohort study of more than one million Swedish subjects. The American journal of psychiatry 163: 59–65.
8. Gupta S, Soelting PR, Grzybowski MA, Boesiger P, Bidlishine J, et al. (2010) Cerebrospinal fluid dynamics in the human cranial subarachnoid space: an overlooked mediator of cerebral disease. 1. Computational model. Journal of the Royal Society, Interface/the Royal Society 7: 1195–1204.
9. Lawrie SM, Olabi B, Hall J, McIntosh AM (2011) Do we have any solid evidence of clinical utility about the pathophysiology of schizophrenia? World psychiatry : official journal of the World Psychiatric Association 10: 19–31.
10. Anderson C, Thunell S, Flodgren Y, Forsell C, Landin G, et al. (1995) Diagnosis of acute intermittent porphyria in northern Sweden: an evaluation of mutation analysis and biochemical methods. Journal of internal medicine 237: 301–304.
11. Torjesen S, Onstad S, Skre I, Edvarden J, Kringlen E (1993) “True” schizotypal personality disorder: a study of co-twins and relatives of schizophrenic probands. The American journal of psychiatry 150: 1661–1667.
12. Hasegawa U, Gerlach M, Makela TV, Lindstedt A, Zachelli ML, et al. (2004) Large-scale zygosity testing using single nucleotide polymorphisms. Twin research and human genetics : the official journal of the International Society for Twin Studies 10: 604–625.
26. Blennow K, Fredman P, Wallin A, Gottfries CG, Karlsson I, et al. (1993) Protein analysis in cerebrospinal fluid. II. Reference values derived from healthy individuals 18–88 years of age. European neurology 33: 129–133.

27. Derr RE (2000) Performing Exact Logistic Regression with the SAS System. SAS Institute Inc., Cary, NC.

28. Reiber H, Felgenhauer K (1987) Protein transfer at the blood cerebrospinal fluid barrier and the quantitation of the humoral immune response within the central nervous system. Clinica chimica acta; international journal of clinical chemistry 163: 319–328.

29. Bauer K, Kornhuber J (1987) Blood-cerebrospinal fluid barrier in schizophrenic patients. European archives of psychiatry and neurological sciences 256: 257–259.

30. Kirch DG, Alexander RC, Sudhalth RL, Papadopoulos NM, Kaufmann CA, et al. (1992) Blood-CSF barrier permeability and central nervous system immunoglobulin G in schizophrenia. Journal of neural transmission General section 89: 219–232.

31. Teunissen CE, Petzold A, Bennett JL, Berven FS, Brundin L, et al. (2009) A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology 73: 1914–1922.

32. Bechter K (2011) The peripheral cerebrospinal fluid outflow pathway - physiology and pathophysiology of CSF recirculation: A review and hypothesis. Neurology Psychiatry and Brain Research 17: 51–66.

33. Zachau AC, Landén M, Mobarrez F, Nybom R, Wallén H, et al. (2012) Leukocyte-derived microparticles and scanning electron microscopic structures in two fractions of fresh cerebrospinal fluid in amyotrophic lateral sclerosis; a case report. Journal of Medical Case Reports 6: 274 doi:10.1186/1752-1947-6-274.