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# Increased concentration of extracellular vesicles exposing aquaporin 4 in cerebrospinal fluid in patients with bipolar disorder

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## SIGNIFICANT OUTCOMES AND LIMITATIONS

- The first study on aquaporin-4 (APQ4) extracellular vesicles (EV) in cerebrospinal fluid (CSF) in patients with bipolar disorders
- All 24 bipolar patients had significantly higher concentrations of AQP4 EV in CSF than all 14 healthy controls
- Limitations: No patient was examined in depressed or manic stage.

**ABSTRACT**

**Objectives**

To examine a hypothetical dysfunction of the brain water channels in bipolar disorder by analyzing aquaporin-4 (AQP4) exposing extracellular vesicles in cerebrospinal fluid from patients with bipolar disorder and healthy controls.

**Methods**

We analyzed exposure of three different epitopes of AQP4 (epitope amino acids 273-291, AQP4 N-terminal and AQP4 C-terminal) on extracellular vesicles in cerebrospinal fluid by flow cytometry in 24 patients with bipolar disorder and in 14 healthy controls. The analysis was replicated at an independent laboratory. Scanning electron microscopy was used to study filtered cerebrospinal fluid images for spherical particles.

**Results**

The concentration of extracellular vesicles (EV) in cerebrospinal fluid expressing AQP4, detected by the antibody to epitope amino acids 271-293, were significantly higher for all bipolar disorder patients (mean ± SD 148 ± 22, compared with healthy controls 9 ± 6 in the original study and 151 ± 37 and 16 ±10 in a replication assay ($p<0.0001$). The AQP4 EV concentration for the N-terminal epitope was 150 ± 46 for the patients and 17 ± 11 for the controls and for the C-terminal 151 ± 41EV/µl for patients and 16±10 for controls. The microscopic images of the cerebrospinal fluid from patients with bipolar disorder exposed spherical structures compatible in size with aquaporin-4 exposing extracellular vesicles.

**Conclusions**

Patients with bipolar disorder had higher concentrations of aquaporin-4-positive ($AQP4^+$) extracellular vesicles in the cerebrospinal fluid than any of the healthy controls. The finding calls for further studies using more specific antibodies to different isoforms of aquaporin-4 EV, ideally sampled in cerebrospinal fluid during manic, euthymic and depressive phases in a number of the same patients with bipolar disorder.

*Key words*: aquaporin-4 (AQP4), cerebrospinal fluid (CSF), flow cytometry, spherical structures, scanning electron microscopy

# 1. INTRODUCTION

Bipolar disorder (BD) is clinically characterized by biphasic episodes of increased (mania or hypomania) or decreased mood state (depression). Although BD is considered closely related to genetic and environmental factors, its underlaying neuro pathophysiology remains largely unknown. We have tested the rational that astrocytopathy is associated with BD using a new biological marker.

## 1.1 A human brain contains 25 000 000 000 000 astrocytes (von Bartheld et al., 2016).

In a recent review of postmortem evidence of brain inflammatory markers in BD eight studies found a decrease of astrocytes (Giriharan et al. 2020). Astrocytes delimit a territory over which they can exert their influence of receptors, ion water channels, transporters, and enzymes of several kinds within the central nervous system (CNS). When the astrocytes are activated, they become hypertrophic, proliferate, and alter their gene expression. The normal functions of astrocytes involve synaptic remodelling and pruning, neuronal network communication, blood-brain barrier support, regulation of CNS blood flow and metabolism. Some astrocytes in the brain my even gain the ability to form new neurons by activation of neural stem cell transcription in parenchymal astrocytes (Magnusson et al. 2020).

## 1.2 Aquaporin-4 (AQP4),

a membrane-bound protein that regulates water permeability is a member of the aquaporin family of water channel proteins is expressed in the endfeet interface of astrocytes in the central nervous system (CNS) (Nagelhus and Ottersen, 2013). Peter Agre who was awarded the Nobel Prize in chemistry 2003 *"for the discovery of water channels"* considered the aquaporins as "*the plumbing system for cells*". *"Every cell is primarily water. But the water doesn't just sit in the cell, it moves through it in a very organized way. The process occurs rapidly in tissues that have these aquaporins or water channels. For many years, scientists assumed that water leaked through the cell membrane, and some water does. But the very rapid movement of water through some cells was not explained by this theory*".

AQP4 has been found to participate in the regulation of extracellular space volume, ion homeostasis, circulation of cerebrospinal fluid (CSF), interstitial fluid resorption, waste clearance, neuroinflammation, osmosensation and even shown to be of importance in the slow wave energy regulation in human non-rapid eye movement sleep (Ulv Larsen et al., 2020). The perivascular polarization of AQP4 is under circadian control and highest during the rest phase in animal studies (Hablitz et al. 2020).

Abnormalities of AQP4 has recently been reported in a magnetic resonance imaging (MRI) study of cerebellum in a cohort of 50 BD patients in an ultra-high b-values diffusion examination (Zaho et al. 2020). The AQP4 abnormalities were located to the posterior lobe of cerebellum that indicate that the AQP4 dysfunction might also be a biological MRI-marker for the BD pathogenesis.

## 1.3 Neuromyelitis optica spectrum disorder and AQP4

Specific autoantibodies to AQP4 with complement-mediated astrocytic damage have been shown to characterize the neuromyelitis optica spectrum disorder (NMOSD) (Mader and Brimberg 2019). AQP4 is thus the target antigen of serum immunoglobulin G (IgG) autoantibody in NMOSD, a group of inflammatory, demyelinating diseases of CNS (for graphic hypothetical description of the inflammatory process see Figure 1, illustrations nr 1-6). In a case report of a NMOSD patient with affective disorder symptomatology, we recently reported significant increase in AQP4 positive extracellular vesicles in cerebrospinal fluid (CSF) already two years before the presence of AQP4-specific autoantibodies appeared (Bejerot et al., 2019).

Gur et al. (2020) performed an AQP4 study, also based on a case report, this time of a treatment resistant depression patient diagnosed with NMOSD with positive serum AQP4 autoantibodies. These authors investigated, for the first time, the presence of serum AQP4-IgG, using a cell-based assay, in 25 patients with bipolar disorder, during an acute major depressive episode and in 30 healthy controls (Gur et al. 2020). Contrary to their hypothesis, AQP4 autoantibodies were nor detected in serum of any of the 25 BD patients. They did, however, consider that AQP4 might still play a role in the pathogenesis of mood disorders through different mechanism of action such as altered brain AQP4 expression.

Based on our reported finding of "AQP4 positive extracellular vesicles (EV) in cerebrospinal fluid (CSF) already two years before the presence of AQP4-specific autoantibodies appeared" we have taken the investigation of BD patients one step further by establishing and testing a validated flow cytometric method for AQP4 positive EV at two independent laboratories. This method allowed us to investigate the hypothesis that EV analysis of CSF may be a more sensitive tool to detect aberrant central nervous system AQP4 water channels than examination of AQP4 antibodies in serum or plasma. Moreover, the same protocol has previously been used in patients with traumatic brain injury, in order to detect "leaking" astrocyte derived EV expressing AQP4 in plasma, but not in CSF (Nekludov et al., 2017).

EV, in our case, are submicron extracellular vesicles with a diameter between 0.1 and 1 µm, previously named microparticles, which are larger than the intracellular vesicles called exosomes that have a diameter of less than 0.1 µm. EV are budding and released from their mother cells in defenses to repair various damages caused by e g inflammations, infections, toxins, and apoptosis. EV analysis in CSF also allows us to identify their cellular origin and even different isoforms depending on access of specific antibodies.

## 1.4 Different isoforms of AQP4

Bipolar patients may have a special metabolic switch between the long isoform of AQP4 – isoform M1 - and the 22 amino acid shorter isoform M23 of AQP4 according to Furman et al. (2003) and Ciappelloni et al. (2019). Different isoforms of AQP4 could possibly contribute to the clinical features, with sometimes rapid and sometimes slower cycling mood swings between manic and depressive phases in patients with bipolar disorder.

## 1.5 The aim of the present study

The aim of the present study was to investigate extracellular vesicles of the water channels AQP4 by flow cytometry in CSF in a well-documented cohort of bipolar patients compared with healthy controls, as a reflection of a hypothetical dysfunction of brain astrocytes possibly related to the mood swings in bipolar disorder. Our aim was also trying to differentiate between extracellular vesicles of the long isoform M1 and the 22 amino acid shorter isoform M23 of AQP4 in this pilot study.

# 2 PATIENTS AND METHODS

## 2.1 Ethics

All participating patients and control subjects consented orally and in writing to participate in the study. The project was approved by the Stockholm Regional Ethical Review Board and conducted in accordance with the latest version of the Helsinki Protocol (case no. 2005/554-31/3 and #2009/1221-32). The healthy subjects received remuneration for their participation.

## 2.2 Patients

A total of 24 patients (15 female) with bipolar disorder, aged 22 - 79 y, were included (Table 1). All patients were in stable mood at the time of lumbar puncture for CSF collection. Twenty-two patients were recruited from 17th of January to 9th of June 2013 as part of a seven-year follow-up in the St. Göran bipolar study. Two patients were later included in conjunction with their first onset of bipolar disorder. The study setting was the bipolar outpatient unit at the Northern Stockholm psychiatric clinic, previously reported as ´the St. Göran study´(Båve et al. 2010). In summary, outpatients referred for treatment and continuing patients were invited to participate in the St. Göran project provided that they were at least 18 years old and met the DSM-IV criteria for bipolar disorder.

The clinical diagnosis of bipolar disorder was established according to the Affective Disorder Evaluation, which has previously been employed in the STEP-BD project (Sachs, Thase, Otto et al., 2003). The Affective Disorder Evaluation started with a social case history, after which followed the affective module of the Structured Clinical Interview for DSM-IV (APA, 2004). The number of lifetime affective episodes and their characteristics were documented as well as alcohol and drug abuse, violent behavior, childhood, family, treatment, and reproductive history, as well as their history of somatic illnesses. In addition to the Affective Disorder Evaluation, the structured psychiatric interview Mini International Neuropsychiatry Interview (MINI) (Sheehan, Lecrubier, Sheehan et al., 1998) was completed at baseline in order to screen for other psychiatric diagnoses the bipolar disorder. These interviews were conducted by board-certified psychiatrists working at the tertiary bipolar outpatient unit, or residents in psychiatry completing their psychiatry training at this unit. The assessments were based on all sources of information available including patient records and if feasible interviews with next of kin. The above acquired information was presented at a diagnostic case conference at which the final diagnostic decision was made by a consensus panel of experienced board-certified psychiatrists specialized in bipolar disorder. This procedure

served to minimize inter-rater variability. Patients were hence diagnosed according to Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition (DSM-IV) criteria, and according to International Statistical Classification of Diseases (ICD-10, WHO 1994).

## 2.3 Controls

Population-based controls were randomly selected by Statistics Sweden and contacted by mail. A research nurse contacted persons that volunteered to participate, and a preliminary telephone screening was conducted to exclude mental health and neurological problems as well as substance abuse. Eligible persons were scheduled for a one-day comprehensive assessment at which control subjects underwent a psychiatric interview by experienced clinicians using the M.I.N.I. Neuropsychiatric interview to exclude psychiatric disorders. Other exclusion criteria were neurological conditions other than mild migraines, untreated endocrine disorders, pregnancy, dementia, and a family history of schizophrenia or bipolar disorder in first-degree relatives. For this study, CSF from 14 controls (8 females) sampled in the St. Göran project in Gothenburg, Sweden was included for the study of AQP4 extracellular vesicles.

## 2.4 Collections of CSF

Lumbar punctures were performed in a sitting position 09-10.30 AM. Fine disposable needles (Becton Dickinson 22 GA 3.00 IN 0.7 x 75 mm) and identical procedures were used for all individuals: The skin in the lumbar region was thoroughly washed with sterile cotton swabs and chlorhexidine 5mg/ml (Fresenius Kabi) before puncture. The needle was inserted in vertebral inter space L3 to L4, or L4 to L5. For the present analyses, after 14 mL initial CSF sampling for future research, the very last 6-12 drops (approximately 0.3 mL CSF) were collected in sterile test tubes from 24 patients and 14 controls. None of the samples were centrifuged to ensure that no wasted brain components in CSF were pelleted or lost via uneven distribution in the sample. The samples were stored in a freezer within less than 1 hr. following the lumbar punctures until time of the assay of the vesicles.

## 2.5 Blood examination and recording of body mass index

Blood samples were collected at about 0800 h, prior to the lumbar puncture, and with patients fasting. Serum albumin, high-sensitivity C-reactive protein (HS-CRP), and white blood cell count (WBC) were analyzed. Body height and body weight were recorded on the same day as blood and CSF sampling and used for calculation of the body mass index (BMI) as a heuristic proxy for body fat of the participants.

## 2.6 Analysis of blood–CSF barrier function

Albumin levels in both CSF and serum were measured by immunonephelometry on a Beckman Image Immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA), at the Clinical Neurochemistry Laboratory in Mölndal, Sweden. The Swedish Board accredited the method for Accreditation and Conformity Assessment (SWEDAC). Experienced and board-certified laboratory technicians who were blinded to clinical information performed all measurements. Intra- and interassay coefficients of

variation were below 10%. To assess the blood CSF barrier function, the ratio between albumin concentration in CSF (mg/L) and serum (g/L) was calculated.

## 2.7 Flow cytometric analyses of extracellular aquaporin-4 exposing vesicles in CSF

Non-centrifuged CSF samples were analyzed after having been stored about two years in -80°. The samples were thawed in a 37°C water bath for 5 min given that, according to current pre-analytic recommendations, they should not be thawed on ice (Mullier et al. 2013). The samples were then centrifuged at 2000g for 20 min at room temperature in order to remove any large debris/fragments. Subsequently, 20 µL of the supernatant was incubated for 20 minutes in dark with 5 µl of anti-AQP4 antibodies as described below (see Table 2 for company, clon and final concentrations). All samples were measured on a Beckman Gallios flow cytometer (Brea, CA, USA). The EV gate was determined using Megamix-plus FSC bead from BioCytex, Marseille, France, which consists of a mix of beads with diameters of 0.1 µm, 0.3 µm, 0.5 µm and 0.9 µm. Conjugate isotype-matched immunoglobulin (IgG1-FITC) with no reactivity against human antigens was used as a negative control to define the background noise in the cytometry analysis. The threshold was based on forward scatter and EV were defined as particles less than 0.9 µm in size and positive for AQP4 antibodies. Results are presented as EV/µl CSF, processed from the 20 µl sample prepared for the flow cytometric analysis. The intra- and interassay coefficients of the flow cytometric analysis were less than 9.0% respectively.

The measurement was performed twice at two different facilities; Karolinska Institutet (test 1) and Uppsala University (test 2). In the first experiment, the samples were labeled with anti-AQP4 which targets the aa273-291 region of AQP4. In the second experiment, the same antibody was used as a validation together with two new antibodies which targets the N-terminal and C-terminal of AQP-4. The aim was to differentiate between the long isoform M1 and the 22 amino acid shorter isoform M23 of AQP4 (Furman et al., 2003). Both experiments (Test 1 and 2) were performed with Beckman Gallios flow cytometer as presented in Table 2 and 3.

## 2.8 Filtering of cerebrospinal fluid and the gold coating techniques for microscopic examinations

Of 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 filters were specially prepared by GP Plastic AB (Gislaved, Sweden), supplied by Sempo*re, AB (Stockholm, Sweden),* which allowed CSF to stream to the center of the filter when vacuum suction was applied from below. This design allows fluids and single un-aggregated particles with sizes smaller than 0.6 µm to drip through the filter. When the filters were completely dried after about two minutes of vacuum suction at room temperature, they were subsequently coated in a JEOL JFC-1200 Fine Coater (JEOL DATUM, Tokyo, Japan) for two minutes with ionized gold to a thickness of 40 Å.

## 2.9 Scanning electron microscopy

Polycarbonate filters were analyzed with a scanning electron microscopy (SEM) microscope (Philips High Resolution SEM 515). The full area of the filter with a diameter of 1 cm was examined for size, shape, particles, and for the presence of any other structure of possible biological significance. Of previously examined CSF filters from 85 healthy controls 82 were free of similar structures (Johansson et al., 2012).

## 2.10 Statistical analysis

Wilcoxon rank Sum Test was used to identify differences between the 14 healthy controls and the 24 bipolar patients. In all statistical analyses, p values $< 0.05$ were taken to indicate statistical significance, and the SAS/STAT® software was used for all calculations.

# 3 RESULTS

## 3.1 Demographics

We included 24 patients with Bipolar disorder, with a mean age of 48 years (range 22 – 79) and a mean age of onset of 20 years (range 12 – 54). The 14 persons in the healthy control group had a mean age of 45 years (range 23 – 64) and they had never been treated for any episodes of depression or mania/hypomania. The characteristics of the patients and controls are presented in Table 1.

## 3.2 The aquaporin-4 extracellular vesicles in cerebrospinal fluid

The aquaporin-4+ EV were defined by size (forward scatter and side scatter) and expression of AQP4. Total counts and size distribution of EV is seen in a patient in red and in a healthy control in blue as well as the individual expression in a patient and in a control (Fig. 1, see subfigures 7, 8, 9, and 11). The number of extracellular EV expressing AQP4, detected by the antigen to epitope aa 271-293, was significantly higher for all bipolar disorder patients (mean ± SD 146 ± 23, and 160 ± 18 EV/µl for type I and II, respectively) compared with controls (9 ± 6 EV/µl, $p<0.0001$). This difference remained for all age groups and regardless of number of disease episodes, even including the two youngest patients with first onset of bipolar symptoms at age 22 and 28 years (Table 3). Intriguingly, the EV results showed no overlap between the groups for vesicles AQP4 as the maximal values for any control was less than the lowest value for any patient.

## 3.3 Other characteristics of the bipolar disorder cohort and control group

Table I shows that the BMI did not differ significantly between patients and controls ($p=0.40$). The global assessments of symptoms and function were lower for the patients than for the controls. The mean albumin ratio was numerically higher in patients than controls, but not statistically different ($p=0.16$). The blood leukocyte levels were all within the normal range 3.3-8.8 x $10^9$ for all 24 patients (Table 1).

### 3.4 Scanning electron microscopy (SEM) images

The photo of the SEM analysis of CSF from a patient with bipolar disorder showed several spherical vesicles (Fig.1, subfigure 10) that are compatible with the AQP4 EV displayed in Fig. 1, subfigure 9. Significantly less AQP4 EV were found in CSF in a healthy control (Fig. 1, subfigure 11) and were not detected in the SEM photo (Fig. 1 subfigure 12). The scale in the SEM-photos is indicated by the dark filter pores with a diameter of 0.6 µm seen in Fig. 1 (10 and 12).

## 4 DISCUSSION

We analyzed three isoforms of aquaporin-4 extracellular vesicles in CSF from 24 patients with bipolar disorder and 14 controls in order to investigate the putative role of water channels in the pathophysiology of bipolar disorder. We found significantly higher concentration of extracellular water channel AQP4 exposing vesicles in CSF from patients than healthy controls. Indeed, the mean number of AQP4 EV in the 24 bipolar patients was ten times higher than in the 14 healthy controls for all three AQP4 epitopes tested. The main result was replicated with a second instrument in a laboratory at another research university.

### Aquaporin-4

The properties of water channels are of particular interest given the abundance of AQP4 tetramers at the blood-brain interface. Interface is used as term here instead of barrier as the end-feet lie outside of the blood-brain barrier as traditionally defined. Transport of ions, gases, or signaling molecules through these pores may affect local blood flow and directly or indirectly influence exchange of substrates between blood and brain (Nagelhus and Ottersen 2013, Papadopoulos and Verkman AS 2013, Smith et al., 2014, Assentoft et al., 2016, Di Benedetto et al. 2016). The more than tenfold increase in mean number of AQP4 EV in the bipolar patients compared to controls raise the question if this could be caused by a genetic variation in the regulation of the AQP4 gene (Kitchen et al., 2015).

### AQP4 isoforms

Astrocytes adapt their morphology to support the neuron. This plasticity is partially, as earlier stated, mediated by ion and water flows. The synaptic responsiveness relies on the AQP4 distribution of two main isoforms expressed as heterotetramers of M1 and M23 (Furman et al., 2003, Fenton et al., 2010, Crane et al., 2011). The presence of M23-AQP4 promotes the formation of large macromolecular aggregates called orthogonal matrices (Smith et al., 2014) that showed that the AQP4 aggregation state determines its subcellular localization and cellular functions. M1-AQP4 was freely mobile in the plasma membrane and could diffuse to rapidly extending lamellipodial regions to support cell migration. In contrast, M23-AQP4 forms large matrices instead of stably bound adhesion complexes that were polarized to astrocytes in vivo. Co-expressed variable size M1 and M23 AQP4 combines segregated due to diffusion screening of small, mobile M1-AQP4 enriched matrices to lamellipodia. Thus, aggregation status-

dependent mechanism for segregation of plasma membrane protein complexes may confer specific functional roles to M1 and M23-AQP4 (Smith et al., 2014). There is also a functional and molecular interaction between aquaporin 4 and Na, K-ATPase possibly associated with the symptomatology of bipolar disorder (Illarionova et al., 2010) that need further clinical investigations as well the role of aquaporin-4 in synaptic plasticity, memory and disease as described by Hubbard et al., (2017). Recently Ciappelloni et al. (2019) showed that the AQP4-M1 small aggregates on the cell surface and the AQP4-M23 larger clusters located near glutamatergic synapses could promote glutamate synapse activity and that autoantibodies to AQP4 reduced NMDA receptor (NMDAR) synaptic activity. The detailed results indicated that the dynamics of AQP4-M23, but less of AQP4-M1, affected the NMDAR synaptic function directly. There is even a possible association between the robust glycogen shunt activity in astrocytes and the effects of glutamatergic agents as reported by Walls et al. (2009). A disturbed regulatory switching mechanism of the AQP4 isoforms at astrocyte end-feets near synapses could possibly contribute to the clinical features, sometimes rapid and sometimes slower cycling mood swings between manic and depressive phases in patients with bipolar disorder. Our two AQP4 epitopes for the N- and C-terminals are not specific enough to test for the M1 and M23 isoforms.

Bejerot et al. (2019) suggested that AQP4 EV may act as a precursor to AQP4 autoantibodies and thus may have a similar function to AQP4 antibodies in this regard. Our new results with increased AQP4 EV in bipolar disorder lead us to the following hypothesis for directed study of a possible contributing cause of mood swings seen in bipolar disorder a) if low AQP4-M23 isoform cluster occurs, this results in low NMDAR synaptic activity with symptoms of depression, b) if high AQP4-M23 isoform cluster occurs, this results in high NMDAR synaptic activity with symptoms of mania or hypomania.

## AQP4+ EV in CSF might derive from different brain locations in different diseases

An increase of AQP4 + EV in CSF could conceivable be formed in astrocytes localized to different specific brain regions or cortical layers and thus be associated with different neuropsychiatric diseases such as Alzheimer's, amyotrophic lateral sclerosis, autism, epilepsy, neuromyelitis optica, Parkinson's disease, schizophrenia and other disorders.

## The microscopic findings in CSF

The nature of the scanning electron microscopic structures in the SEM analysis of CSF from the patients with bipolar disorder showed spherical vesicles compatible with AQP4 EV as seen in Fig.1, subfigures 9 and 10 in bipolar patients, with significant less AQP4 EV in CSF healthy controls (Fig. 1, subfigures 11 and 12).

## Limitations of the study

No patient with bipolar disorder has so far been investigated during un-medicated or medicated episodes of both manic and depressed stages. The patients were examined in Stockholm and the controls in Gothenburg.

# CONCLUSIONS

We identified increased levels of brain-derived EV exposing AQP4 in CSF of patients with bipolar disorder, compared with healthy controls. Regulatory metabolic switching between different isoforms of AQP4 may hypothetically be compatible with the clinically observable mood swings that occur in bipolar disorder. Because bipolar disorder is a mental illness in which spontaneous self-healing to euthymic mood can occur even in untreated patients, increased knowledge of the origin of the cyclic course of the disease can lead to improved prophylaxis and treatment. More specific autoantibodies are needed to differentiate between the long isoform M1 and the 22 amino acid shorter isoform M23 of AQP4 in bipolar depression during both manic and depressed disease stages.

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*Table 1. Characteristics of bipolar disorder patients and healthy controls in the study of AQP4 extracellular vesicles in cerebrospinal fluid, mean ± SD and (Range)*

| **Contents** | **Bipolar patients *n* = 24** | **Healthy controls *n* = 14** | **p value Bipolar disorder versus Controls. Wilcoxon rank Sum Test was used.** |
|---|---|---|---|
| Sex, female | 15 | 8 | NS |
| Age years | 48.1 ± 18 (22 - 79) | 45.4 ± 15.3 (23 - 64) | NS |
| Smoke | 4 | 3 | |
| Snuff | 3 | 3 | |
| Body mass index (BMI) | 25.9 ± 4.0 (19.6 - 33.8) | 24.7 ± 3.4 (20.1 - 30.3) | NS |
| *Psychiatric data* | | | |
| Age of onset in years | 19.3 (12 - 54) | - - - | |
| GAF symptom | 67.8 ± 12.2 (35 - 90) | 88.7 ± 3.2 (85 - 91) | p<0.025 |
| GAF function | 68.2 ± 11.6 (45 - 90) | 88.7 ± 3.2 (85 - 91) | p<0.05 |
| *Laboratory data* | | | |
| CSF/serum albumin ratio | 5.7 ± 2.6 (3.1 - 12.5) | 4.5 ± 1.6 (3.1 - 8.5) | NS |
| Erythrocytes in CSF, nr of cells | < 5 | < 5 | |
| *Medication* | | | |
| Lithium | 18 | 0 | |
| Valproate | 3 | 0 | |
| Lamotrigine | 3 | 0 | |
| Carbamazepine | 3 | 0 | |
| Antidepressants | 8 | 0 | |
| Somatic medication | 10 | 4 | |

- *SD* standard deviation,
- *GAF* Global Assessment of Functioning,
- *CRP* C-reactive protein,
- *CSF* cerebrospinal fluid

*Table 2. Aquaporin 4 (AQP4) epitopes used in the study of extracellular vesicles in patients with bipolar disorder*

| AQP-4 Epitopes | Supplier | Cat. No. | Clonality | Final Concentration |
|---|---|---|---|---|
| aa 273-291 | LS-Bio | LS-C229824-100 | Polyclonal | 5 µg/ml |
| N-terminal | Antibodies-online.com | ABIN5648960 | Monoclonal | 5 µg/ml |
| C-terminal | Antibodies-online.com | ABIN863209 | Polyclonal | 5 µg/ml |

*Table 3. Concentration of extracellular vesicles (EV) exposing aquaporin 4 (AQP4) per µl cerebrospinal fluid as mean ± SD and (Range)*

| AQP-4 Epitopes | Bipolar patients (24, 15 F) | Healthy controls (14, 8 F) | p-value |
|---|---|---|---|
| aa 273-291 Test 1 | 148 ±22 EV/µl (101-187) | 9 ±6 EV/µl (1.3-17) | <0.0001 |
| aa 273-291 Test 2 | 151 ± 37 EV/µl (85-249) | 16 ± 10 EV/µl (3-36) | <0.0001 |
| N-terminal | 150 ±46 EV/µl (69-259) | 17 ± 11 EV/µl (4-33) | <0.0001 |
| C-terminal | 151 ± 41 EV/µl (75-239) | 16 ±10 EV/µl (3-33) | <0.0001 |

- SD Standard deviation,
- F females, Range is the difference between the smallest and largest values.
- P-value for difference between bipolar patients and healthy controls.

**Figure 1.**

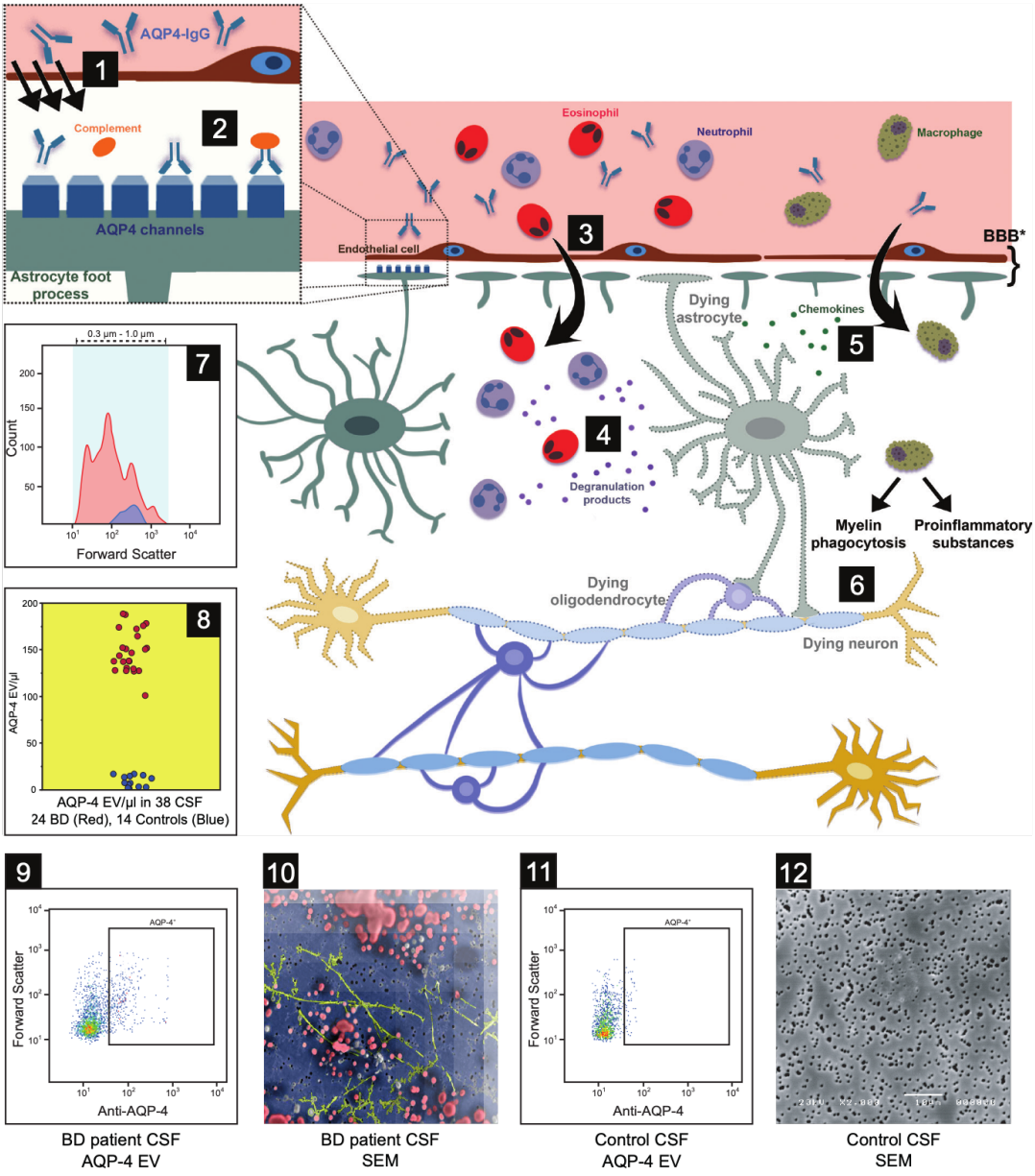
AQP4-IgG
1
Complement
2
AQP4 channels
Astrocyte foot process
Eosinophil
Neutrophil
Macrophage
Endothelial cell
3
BBB*
Dying astrocyte
Chemokines
5
4
Degranulation products
Myelin phagocytosis
Proinflammatory substances
6
Dying oligodendrocyte
Dying neuron
0.3 µm - 1.0 µm
7
Count
Forward Scatter
8
AQP-4 EV/µl
AQP-4 EV/µl in 38 CSF
24 BD (Red), 14 Controls (Blue)
9
Forward Scatter
Anti-AQP-4
BD patient CSF
AQP-4 EV
10
BD patient CSF
SEM
11
Forward Scatter
Anti-AQP-4
Control CSF
AQP-4 EV
12
Control CSF
SEM

**Figure legend to Fig 1**

A montage of hypothetical pathogenesis of extracellular aquaporin-4 vesicles (previously microparticles) in neuromyelitis optica spectrum disorder (number 1-6) and clinical findings in cerebrospinal fluid (CSF) in the present study of 24 euthymic patients diagnosed with bipolar disorder and 14 in CSF of healthy controls (number 7-12).

1. Aquaporin 4 (AQP4)-IgG accesses the CNS at areas of increased blood-brain barrier (BBB) permeability or injury or across endothelial cells by transcytosis. The antibody binds selectively to AQP4 antigen on astrocyte foot processes. The blood-brain barrier is formed by various components, some of which are illustrated: endothelial cells and astrocyte foot processes.

2. The antigen-antibody binding leads to complement activation and down regulation of the AQP4 water channel.

3. Activated complement increases blood-brain barrier permeability and leads to leukocyte infiltration, particularly neutrophils and eosinophils.

4. Degranulation products and vesicles (EV) in CSF indicate astrocyte damage.

5. Chemokines and vesicles are released from leukocytes and damaged astrocytes and attract macrophages.

6. Macrophages produce proinflammatory substances and phagocytose matter resulting in damage of oligodendrocytes and neurons.

7. Distribution of extracellular vesicles (EV) in CSF, regardless of expression and phenotype, in 24 bipolar patients (red) and in 14 healthy controls (blue). Forward scatter on the X-axis indicate the size of the particles, and count (number of events as detected by the flow cytometer) on the Y-axis.

8. A jitter plot of extracellular vesicles (EV) exposing aquaporin-4 (AQP4)/µl of cerebrospinal fluid (CSF) in 24 patients with bipolar disorders (red dots) and 14 healthy control individuals (blue dots). As seen, there is a clear delineation with no overlaps between patients and controls in number of AQP4 EV. To identify the individual dots in the diagram we used a jitter gram along the X-axis.

9. Representative flow cytometry plot demonstrating AQP4+ vesicles in CSF in a patient with bipolar disorder. Forward Scatter of EV on the Y-axis (i.e. size of particles) and Anti-AQP4 FITC binding on the X-axis.

10. Photo of scanning electron microscopy (SEM) of CSF on the polycarbonate filter (pore diameter 0.6 µm) displays spherical vesicles and other unidentified structures from the degranulation processes.

11. Representative flow cytometry plot demonstrating AQP4+ vesicles in CSF in a control subject. Forward Scatter of EV on the Y-axis (i.e. size of particles) and Anti-AQP4 FITC binding on the X-axis.

12. Photo of SEM of CSF from a healthy control did not reveal any vesicles on the polycarbonate filter. The many small black structures in the filter indicate open pores with a diameter of 0.6 µm. The cartoons 1-6 are adapted with permission from Dutra BG, da Rocha AJ, Nunes RH, et al. Radio-Graphics. 2018;38(1):169-193.