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# Aquaporin-4 positive extracellular vesicles and cytokines in cerebrospinal fluid in schizophrenia and obsessive-compulsive disorder, and associations with peripheral cytokines

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## ABSTRACT

Schizophrenia and obsessive-compulsive disorder (OCD) are complex neuropsychiatric disorders with emerging evidence implicating immune and neuroinflammatory mechanisms. This exploratory pilot study investigated aquaporin-4 positive (AQP4+) extracellular vesicles (EVs) in the cerebrospinal fluid (CSF) of 11 treatment-resistant patients with schizophrenia ( $n = 5$ ) or obsessive-compulsive disorder (OCD,  $n = 6$ ) receiving an add-on, single-infusion rituximab (1000 mg) treatment, a B-cell depleting therapy. CSF samples were collected pre-treatment and, for a subset, again five months post-treatment. AQP4+ EV levels in CSF were quantified using flow cytometry with antibodies targeting different regions of the AQP4 molecule. We also measured selected cytokines in CSF and plasma and cytokine gene expression in peripheral blood cells.

Patients with schizophrenia exhibited higher AQP4+ EV levels compared to those with OCD. In schizophrenia, AQP4+ EVs correlated positively with the inflammatory marker CXCL8/IL-8 but negatively with CSF-TNF- $\alpha$ . Plasma markers CXCL8/IL-8 and TGF- $\beta_1$  were positively associated with CSF-AQP4+ EVs in schizophrenia. Between 24- and 40-times higher concentrations of CXCL8/IL-8 in CSF than in plasma suggest intrathecal production of this chemokine in both disorders. Post-treatment, AQP4+ EV levels decreased in the patients who improved following rituximab but remained stable in non-responders.

These findings suggest that astrocyte-derived extracellular vesicles may play a role in neuroinflammatory processes linked to schizophrenia and possibly also to severe OCD. The observed relationships between AQP4+ EVs and cytokines support the hypothesis that astrocyte-derived EVs could modulate intrathecal immune responses and potentially also interact with peripheral immune mechanisms. Larger studies are warranted to validate these preliminary findings.

## 1. Introduction

Schizophrenia and obsessive-compulsive disorder (OCD) are complex psychiatric disorders with distinct yet overlapping neurobiological features. Despite extensive research efforts, the neurobiological mechanisms underlying these conditions remain incompletely understood. An increasing body of evidence suggests that neuroinflammation and immune dysregulation play significant roles in the pathophysiology of schizophrenia (Müller, 2018), while the evidence for OCD seems confined to certain subtypes (Endres et al., 2022). Prior studies of

schizophrenia have focussed on, e.g., microglial activation (Collste et al., 2017; Conen et al., 2021), cytokine elevations (Miller et al., 2011; Pillinger et al., 2019; Halstead et al., 2023), kynurenine metabolism (Erhardt et al., 2017) and complement activation (Sekar et al., 2016; Gracias et al., 2022) as contributing factors.

However, a relatively underexplored area is the role of astrocytes, central to, e.g. developmental synaptogenesis, homeostatic regulation of the neurons' extracellular environment and synaptic signalling (Verkhatsky et al., 2015; Kruyer et al., 2023). The involvement of astrocytes in neuroinflammatory processes has attracted increasing

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interest (Kim et al., 2018; Corsi-Zuelli and Deakin, 2021; Notter, 2021). It has been suggested that the developmental maturation of astrocytes and oligodendrocytes is disrupted in schizophrenia, thereby contributing to the neurodevelopmental aberrations leading to psychosis (Dietz et al., 2020; de Oliveira Figueiredo et al., 2022).

Astrocytes release extracellular vesicles (EVs, including microparticles and exosomes) in response to various cellular states, e.g. inflammatory activation (Gharbi et al., 2020). These EVs enable paracrine and endocrine cell-to-cell communication both within the central nervous system (CNS) and with peripheral systems by transporting proteins, lipids, and RNA (Wang et al., 2023; Filannino et al., 2024). Exosomes have been extracted from plasma in schizophrenia, finding e.g. altered levels of micro-RNAs (Kumar et al., 2024). One study reported elevated glial fibrillary acidic protein, suggesting astrocyte pathology (Ranganathan et al., 2022). To our knowledge, only one study has analysed exosomes in the cerebrospinal fluid (CSF) of patients with schizophrenia (Scheiber et al., 2024). The study focussed on the role of latent herpes simplex virus 1 infection in neuroinflammation and found elevated microRNA expression supporting this in CSF-exosomes from all six of their schizophrenia patients. However, previous studies have identified larger EVs (described as microparticles) in the CSF of schizophrenia patients (Wetterberg et al., 2002; Mobarrez et al., 2013). In a case with complex neuropsychiatric comorbidity (Bejerot et al., 2019), a significant subset of these particles was identified as expressing aquaporin-4 (AQP4), a water channel protein, essential in the glymphatic system and abundant on astrocyte endfeet. A recent magnetic resonance study showed potential disruption of the glymphatic system in schizophrenia (Abdolizadeh et al., 2024), AQP4 has been implicated in genetic studies of schizophrenia (Wu et al., 2020), and EV release is increased from activated astrocytes. This suggests that astrocyte-derived AQP4-positive (AQP4+) EVs may participate in neuroinflammatory mechanisms in schizophrenia.

Most research on neuroinflammation has analysed cytokines in plasma. In a meta-analysis of schizophrenia studies, seven cytokines were consistently elevated, among them interleukin (IL)-6, the chemokine CXC-motif ligand 8 (CXCL8, commonly referred to as IL-8) and tumour necrosis factor (TNF, also known as TNF- $\alpha$ ) (Halstead et al., 2023). In the CSF, only IL-6 and IL-8 reached meta-analytic significance in schizophrenia (Warren et al., 2024). Unfortunately, studies on the relationship between CSF- and plasma-cytokine levels are wanting (Wang and Miller, 2018; Gallego et al., 2018; Gigase et al., 2023), why notions of dynamic interactions between inflammation in the brain and in the periphery remain tentative.

In this study, we investigated AQP4+ EVs in the CSF of treatment-resistant patients with schizophrenia or OCD undergoing rituximab therapy, a B-cell-depleting treatment with emerging relevance in neuropsychiatry (Bejerot et al., 2023a). By comparing AQP4+ EV levels in schizophrenia and OCD and exploring their associations with CSF and peripheral cytokines, we aimed to elucidate the potential role of astrocyte-derived EVs in neuroinflammation and putative interactions between brain and periphery. Since this was a pilot treatment study, healthy controls were not included.

## 2. Materials and methods

### 2.1. Patients

The patients were recruited for a pilot study of rituximab treatment (Bejerot et al., 2023a), where lumbar puncture for investigation of CSF was an optional addition. For inclusion, age should be 18–40 years, with at least 2 year's duration of schizophrenia or OCD (diagnosed by DSM-5), presently treatment-resistant and rated as at least markedly ill and below 50 on the Global Assessment of Functioning (GAF) scale, and able to make an informed decision to consent to the trial. Participants should have been on a stable pharmacotherapy for schizophrenia or OCD for at least 1 month and were requested to keep it unchanged during the trial;

however, current clozapine treatment was not allowed, due to its immunomodulatory effects, possibly interacting with rituximab. Patients with relevant chronic or on-going infections, recent history of malignancy, previous or on-going immunosuppressive treatment or abnormal immunoglobulin levels were excluded; for details see Bejerot et al. (2023a).

Of the 19 rituximab-treated patients, five with schizophrenia and six with OCD consented to lumbar puncture before rituximab treatment and were included in the present work. Four patients (2 schizophrenia, 2 OCD) also consented to a second lumbar puncture, 5 months post-treatment.

### 2.2. Rating instruments

An extensive rating schedule for clinical information was applied (Bejerot et al., 2023a). In this report, the following rating instruments are included: for severity, Clinical global impression - Severity (CGI-S, Guy, 1976), for psychotic symptoms, Positive and negative syndrome scale (PANSS, Kay et al., 1987), for OCD symptoms, Yale-Brown obsessive-compulsive scale (Y-BOCS, Goodman et al., 1989) and National Institute of Mental Health global obsessive compulsive scale (NIMH-GOCS, Insel et al., 1983), and for global psychosocial functioning, Personal and social performance scale (PSP, Morosini et al., 2000).

### 2.3. Acquisition of plasma, peripheral blood cells (PBC) and cerebrospinal fluid

Phlebotomies for blood samples (fasting) were performed at baseline before the rituximab treatment and repeated 20 weeks after treatment. Samples were processed immediately for plasma and gene expression.

Lumbar punctures were performed in the L3/L4 or L4/L5 interspace following administration of local anaesthetic between 11 AM and 3 PM, median 7 days before the rituximab treatment. The median time interval between blood/plasma sampling and CSF sampling was 4 h. In a sub-sample, this was repeated a median of 142 days (range 130–152) after the rituximab treatment.

Aliquots of the samples were frozen within hours. Only one freezing and thawing cycle was performed.

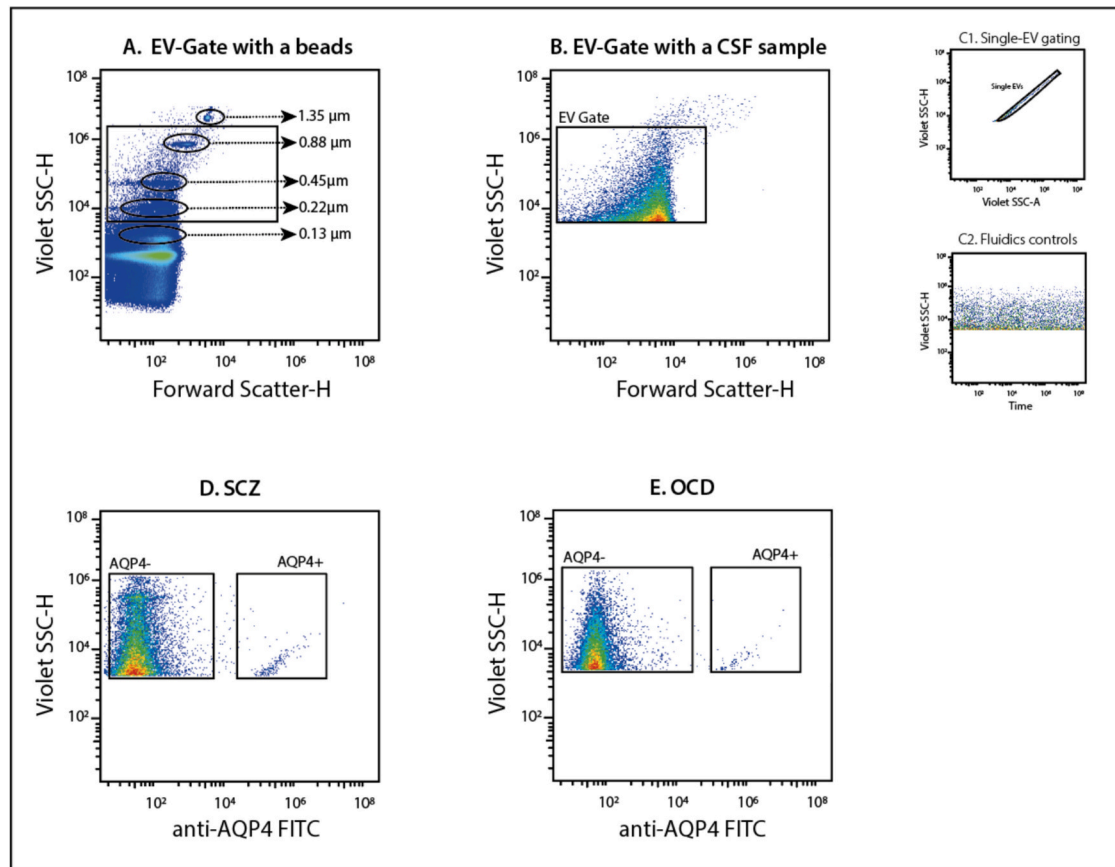
### 2.4. Flow cytometric analysis of AQP4-positive EVs in CSF

Non-centrifuged CSF samples were stored at  $-80^{\circ}\text{C}$  for approximately 2 years before analysis. For preparation, frozen CSF samples were thawed in a  $37^{\circ}\text{C}$  water bath for around 5 min. Initial centrifugation was conducted at  $2000 \times g$  for 20 min at room temperature to remove large debris, and the supernatant was carefully transferred to new tubes. A  $20 \mu\text{l}$  aliquot of this supernatant was then incubated in the dark for 20 min with  $5 \mu\text{l}$  of anti-AQP4 antibodies FITC (Antibodies Online) targeting either the C-terminal or N-terminal epitopes of the aquaporin molecule.

For flow cytometric analysis, the samples were diluted with  $120 \mu\text{l}$  of CytoFLEX Sheath Fluid (Beckman Coulter, Brea, CA, USA) and analysed using a CytoFLEX flow cytometer (Beckman Coulter). Gating was established with Nano fluorescent Yellow Particles sized at  $0.13 \mu\text{m}$ ,  $0.22 \mu\text{m}$ ,  $0.45 \mu\text{m}$ ,  $0.88 \mu\text{m}$ , and  $1.35 \mu\text{m}$  (Spherotech, Lake Forest, IL, USA), covering a detection range from approximately  $0.2 \mu\text{m}$  to  $1.0 \mu\text{m}$  (Fig. 1). The lower EV gate was set at  $0.2 \mu\text{m}$  to minimise background noise around  $0.13 \mu\text{m}$ , ensuring more accurate measurements.

The gating strategy was further validated using labelled and unlabelled EVs, accounting for differences in refractive indices between beads and EVs. Instrument calibration included unstained EVs isotype controls from (Beckman Coulter, Brea, CA, USA). The threshold was set to violet side scatter, and astrocyte-derived EVs were identified as those expressing AQP4.

Data analysis was performed with FlowJo (v10.10, Becton



**Fig. 1.** Representative flow cytometric analysis of aquaporin-4 positive extracellular vesicles (AQP4+ EVs) in cerebrospinal fluid (CSF) stained with anti-AQP4 FITC targeting the N-terminal epitope. Nano Fluorescent Yellow Particles (0.13–1.35  $\mu\text{m}$ ) were used to define the extracellular vesicle (EV) gate based on size and complexity (A). A CSF sample gated for EVs within this size range is shown (B). Single-EV gating was applied to exclude doublets (C1), and fluidics controls were included to ensure acquisition stability over time (C2). Representative contour plots of CSF samples from a schizophrenia (SCZ) patient (D) and an obsessive-compulsive disorder (OCD) patient (E) show differences in AQP4+ EVs, with a larger AQP4+ population detected in the schizophrenia sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dickinson, NJ, USA), and results are reported as the number of EVs per  $\mu\text{l}$  of CSF based on the 20  $\mu\text{l}$  supernatant. The intra- and inter-assay coefficients of variation for this flow cytometric analysis were maintained at less than 10 %.

## 2.5. CSF and plasma analyses of cytokines and chemokines

In the collected plasma and cerebrospinal fluid, quantification of soluble cytokines was performed using Multiplex bead technology (MILLIPLEX® MAP Kit, Human Cytokine/Chemokine Magnetic Bead Panels) according to the manufacturer's description (Merck Millipore, Burlington, MA, USA). All samples were analysed in duplicates on a Bio-Plex® 200 instrument (Bio-RAD, Hercules, CA, US), and Bio-Plex manager software version 6.2 were used to obtain concentrations. More specifically, results were obtained by comparing fluorescence in patient samples with a standard curve (5-parameter logistic curve fit) of known concentrations of each analyte.

The analysed cytokines included interferon (IFN)- $\gamma$ , interleukin (IL)-1RA, IL-6, IL-8 (=CXCL8), IL-10, IL-17A, IL-18, lymphotoxin- $\alpha$ , tumour necrosis factor (TNF, also known as TNF- $\alpha$ ), and transforming growth factor (TGF)- $\beta_1$ . Of these, IL-6, IL-8, TNF, and TGF- $\beta_1$  were reliably measured in 100 % of the CSF samples. Neither IFN- $\gamma$ , IL-1RA, IL-10, IL-17A, nor lymphotoxin- $\alpha$  were detected in any CSF sample, and IL-18 in only 4 of 15 samples. We limited our statistical evaluation to the four cytokines with complete CSF data: IL-6, IL-8, TNF and TGF- $\beta_1$ . For these, the detection limit (pg/ml) and mean assay coefficient of variation (%) were as follows for CSF analyses: IL-6 (0.7; 5.1 %), IL-8 (0.7; 3.5 %), TNF

(0.4; 8.3 %) and TGF- $\beta_1$  (2.7; 15.1 %); and for plasma analyses: IL-6 (0.6, 4.1 %), IL-8 (0.6; 4.4 %), TNF (0.5; 8.8 %) and TGF- $\beta_1$  (10.8; 3.7 %).

## 2.6. Determination of relative gene expression in peripheral blood cells

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesise cDNA, according to the manufacturer's instructions, using 100 ng RNA as template in each reaction. Real-time semi-quantitative PCR was performed using a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems) with fluorescent probes (Taq-Man Gene Expression Assays). Remaining material was also obtained from Applied Biosystems. Crossing threshold (Ct) values were calculated by QuantStudio 7 Flex Real-Time PCR system software using the second-derivative maximum method. All samples were analysed in duplicates, and the mean Ct values were used in further data analysis. Cycle threshold (Ct) cut-off value was set to 35, and all reactions had an efficiency between 90 and 110 %. Samples with a coefficient of variation (CV) >0.2 were reanalysed. TBP was used as a reference gene for normalisation, determined by using the NormFinder R package (MOMA, Aarhus University Hospital, Denmark). The untreated (week 0) samples were used as calibrators for calculating the relative expression values following treatment using the delta-delta method (2- $\Delta\Delta\text{Ct}$ ).

## 2.7. Statistics

The AQP4+ EVs and the IL-8 data exhibited a non-normal

distribution, necessitating the use of the Mann-Whitney *U* test for comparisons. For correlation analyses, the N-terminal and C-terminal measurements of AQP4+ EVs were examined both individually and as a mean value. Using the mean of C-terminal and N-terminal values provides a more balanced representation of AQP4+ EV levels, accounting for potential variability in antibody binding or epitope accessibility. Due to the small number of individuals and non-normal distribution of data, Spearman's correlation analysis was used. Given the exploratory nature of this study, corrections for multiple comparisons were not applied, and multivariate confounder analyses were not deemed feasible.

### 2.8. Ethics

The two studies from which the analysed samples were acquired were approved by the Swedish Ethical Review Agency (2019-00260 and 2019-00256) and the Swedish Medical Products Agency (Eu-nr 2018-004618-17 and 2018-004619-28). The studies were pre-registered on [ClinicalTrials.gov](https://www.clinicaltrials.gov): NCT03983031 and NCT03983018.

## 3. Results

### 3.1. Data on included patients

All patients, but one with OCD, were at least severely ill, according to the CGI-Severity. PSP was rated significantly lower (worse global functioning) in the schizophrenia group. All patients with schizophrenia were treated with antipsychotics, and most OCD patients with serotonin reuptake inhibitors. No differences between diagnoses were found on other measures (Table 1).

### 3.2. AQP4-positive EVs in CSF and clinical measures of symptoms and functions

The concentration of AQP4+ EVs in CSF was significantly higher in patients with schizophrenia than in those with OCD. This result was similar irrespective of whether the C-terminal antibody, the N-terminal or the mean of the two was used (Table 2, Fig. 2). The concentrations of EVs marked by the C-terminal and by the N-terminal domain of the AQP4 molecule were highly correlated with each other ( $\rho = 0.73$ ,  $p = 0.011$ ) and their correlations with other biomarkers were largely similar (Table S1A-C).

Higher C-terminal AQP4+ EVs were associated with lower global psychosocial functioning according to PSP ( $\rho = -0.63$ ,  $n = 11$ ,  $p = 0.040$ ) in the merged group.

In schizophrenia, the C-terminal AQP4+ EVs correlated positively with symptom severity (CGI-S,  $\rho = 0.71$ ,  $n = 5$ ,  $p = 0.18$ ) and negatively with functioning (PSP,  $\rho = -0.70$ ,  $n = 5$ ,  $p = 0.19$ ), although these associations did not achieve significance. Conversely, in the OCD group, the N-terminal AQP4+ EVs correlated negatively with severity (CGI-S,  $\rho = -0.62$ ,  $n = 6$ ,  $p = 0.19$ ) and positively with PSP ( $\rho = 0.99$ ,  $n = 6$ ,  $p < 0.001$ ), indicating that higher N-terminal AQP4+ EV levels were associated with a less severe clinical presentation.

No associations were found between the AQP4+ EVs and age, biological sex, body mass index, illness duration, nicotine use or drug treatment (when diagnosis was controlled for) with antipsychotics or serotonin reuptake inhibitors.

### 3.3. Correlations between AQP4+ EVs and cytokines

CSF-AQP4+ EVs and CSF-IL-8 were positively correlated ( $\rho = 0.61$ ,  $n = 11$ ,  $p = 0.047$ ) in the merged group. However, this correlation depended entirely on the schizophrenia sample ( $\rho = 1.0$ ,  $n = 5$ ,  $p < 0.01$ ). CSF-TNF, on the other hand, correlated negatively with AQP4+ EVs in schizophrenia ( $\rho = -1.0$ ,  $n = 5$ ,  $p < 0.01$ ) but not so in OCD ( $\rho = 0.58$ ,  $n = 6$ ,  $p = 0.23$ ), Fig. 3A-B. In schizophrenia, furthermore, CSF-AQP4+ EVs, as well as CSF-IL-8, correlated positively with peripheral

**Table 1**  
Demographics and baseline clinical descriptors of the included patients.

	SCZ, n = 5		OCD, n = 6		Statistics	
	Mean $\pm$ SD (Range)/Count				X <sup>2</sup>	MW/ X <sup>2</sup> p
Sex: males/females	3/2		3/3		0.11	0.74
Age at inclusion, years	26.6 $\pm$ 6.7 (19–34)		27.2 $\pm$ 6.1 (19–36)			0.79
Psychiatric illness duration, years	10.4 $\pm$ 6.8 (3–19)		13.3 $\pm$ 7.7 (5–24)			0.66
Height, m	1.72 $\pm$ 0.09 (1.65–1.83)		1.74 $\pm$ 0.11 (1.62–1.88)			0.93
Weight, kg	81.5 $\pm$ 18.4 (56–107)		74.1 $\pm$ 10.6 (62–84)			0.54
Body mass index, kg/m <sup>2</sup>	27.4 $\pm$ 6.2 (20.6–37.5)		24.4 $\pm$ 1.4 (23.1–26.3)			0.33
Any nicotine use	2/5		2/6		0.05	1.0
Number of concurrent psychiatric diagnoses <sup>a</sup>	4.6 $\pm$ 1.7 (2–6)		4.3 $\pm$ 2.1 (1–7)			0.79
CGI-Severity (1–7)	6.2 $\pm$ 0.45 (6–7)		5.7 $\pm$ 0.82 (5–7)			0.25
PSP total (0–100)	29.2 $\pm$ 7.6 (21–41)		44.5 $\pm$ 11.9 (30–66)			0.030
PANSS total (30–210)	110.2 $\pm$ 28.0 (77–137)					
PANSS positive (7–49)	22.8 $\pm$ 9.7 (8–31)					
PANSS negative (7–49)	32.4 $\pm$ 7.2 (26–42)					
Y-BOCS total (0–40)			26.5 $\pm$ 6.6 (15–33)			
NIMH-GOCS (1–15)			10.8 $\pm$ 2.3 (7–14)			
WAIS <sup>b</sup>	86 $\pm$ 31 (50–118)		110 $\pm$ 26 (85–140)			0.18
Number of psychotropic medications	2.2 $\pm$ 1.1 (1–4)		2.2 $\pm$ 1.7 (0–5)			0.92
D <sub>2</sub> -blocking antipsychotics	5/5		1/6		7.6	0.006
Chlorpromazine equivalent dose	330 $\pm$ 181 (180–600)		12.5 $\pm$ 30.6 (0–75)			0.004
SSRI/SNRI/clomipramine	1/5		5/6		4.4	0.036
C-reactive protein, plasma, mg/l	5.0 $\pm$ 5.9 (0.16–14.6)		0.63 $\pm$ 0.56 (0.21–1.55)			0.66

CGI = Clinical Global Impression, MW = Mann-Whitney *U* test, OCD = obsessive-compulsive disorder, NIMH-GOCS = National Institute of Mental Health Global Obsessive Compulsive Scale, PANSS = Positive and Negative Syndrome Scale, total, positive and negative subscales, PSP = Personal and Social Performance scale, SCZ = schizophrenia, SNRI = serotonin-norepinephrine reuptake inhibitor, SSRI = selective serotonin reuptake inhibitor, WAIS = Wechsler Adult Intelligence Scale, Y-BOCS = Yale-Brown Obsessive Compulsive Scale.

<sup>a</sup> Psychiatric comorbidity according to the Mini-International Neuropsychiatric Inventory (MINI).

<sup>b</sup> Based on four subscales.

TGF- $\beta$ <sub>1</sub> (plasma levels as well as PBC gene expression) and negatively with IL6 gene expression (Table 2, Table S1A-C).

### 3.4. Correlations between CSF-cytokines and clinical measures

In the merged group, lower global psychosocial functioning (PSP) was associated with higher CSF levels of IL-6 ( $\rho = -0.76$ ;  $n = 11$ ;  $p = 0.007$ ) and IL-8 ( $\rho = -0.75$ ;  $n = 11$ ;  $p = 0.008$ ), while CSF-TNF correlated positively with PSP ( $\rho = 0.69$ ;  $n = 11$ ;  $p = 0.019$ ) and negatively with CGI-S ( $\rho = -0.65$ ;  $n = 11$ ;  $p = 0.029$ ). In the schizophrenia group, higher CSF-IL-6 was associated with lower functioning (PSP,  $\rho = -0.90$ ;  $n = 5$ ;  $p = 0.037$ ).

### 3.5. Relationship between cytokines in CSF and peripheral blood

In the schizophrenia group, CSF-IL-8 correlated positively with

**Table 2**

Comparisons between diagnoses of AQP4+ extracellular vesicles (EVs), cytokines in cerebrospinal fluid (CSF), cytokines in plasma and the quotient of CSF/plasma cytokines (median (range)); and correlations between CSF and plasma measures (Spearman's  $\rho$ ,  $p$ ), all from baseline samples.

	SCZ (n = 5)	OCD (n = 6)	MW p	Cohen's $r^a$
CSF-AQP4+ extracellular vesicles, n per $\mu$ l				
C-terminal+	42.7 (33.0–75.1)	29.9 (22.0–34.1)	<b>0.011</b>	0.77
N-terminal+	40.2 (29.8–71.9)	27.6 (22.7–30.4)	<b>0.018</b>	0.72
Mean of C- and N-terminal+	41.5 (32.0–73.5)	30.0 (22.4–30.6)	<b>0.006</b>	0.83
CSF-cytokines, pg/ml				
IL-6	3.80 (2.03–7.19)	2.52 (1.52–4.84)	0.31	0.30
IL-8	35.2 (23.5–61.2)	26.5 (19.4–34.7)	<i>0.10</i>	0.50
TGF- $\beta_1$	112.9 (25.1–127.0)	74.2 (7.73–100.5)	<i>0.068</i>	0.55
TNF	3.27 (1.58–5.41)	4.36 (3.54–8.16)	<i>0.068</i>	0.55
Plasma-cytokines, pg/ml				
IL-6	0.84 (0.32–1.54)	0.32 (0.32–2.02)	0.43	0.24
IL-8	1.02 (0.32–1.41)	1.08 (0.76–2.64)	0.65	0.14
TGF- $\beta_1$	5563 (2901–40,137)	7143 (5443–58,895)	0.14	0.44
TNF	2.84 (1.85–10.33)	2.78 (2.65–10.40)	0.65	0.14
CSF-cytokines/plasma-cytokines quotient				
IL-6	4.67 (2.42–13.69)	6.17 (1.01–15.13)	0.72	0.11
IL-8	39.9 (27.9–73.4)	24.5 (7.4–45.7)	<i>0.068</i>	0.55
TGF- $\beta_1$	0.0195 (0.0032–0.0417)	0.0100 (0.0010–0.0160)	0.20	0.39
TNF	1.25 (0.15–1.90)	1.55 (0.38–2.30)	0.58	0.17
Correlations between CSF-AQP4+ EVs (mean of C- and N-) and CSF-cytokines				
CSF-IL-6	0.60, 0.29	–0.32, 0.54		
CSF-IL-8	<b>1.0, &lt;0.01</b>	–0.64, 0.17		
CSF-TGF- $\beta_1$	0.00, 1.0	–0.41, 0.43		
CSF-TNF	<b>–1.0, &lt;0.01</b>	0.58, 0.23		
Correlations between CSF-AQP4+ EVs (mean of C- and N-) and plasma-cytokines				
Plasma-IL-6	0.41, 0.49	<i>0.77, 0.072</i>		
Plasma-IL-8	<b>0.90, 0.037</b>	0.41, 0.43		
Plasma-TGF- $\beta_1$	<b>1.0, &lt;0.01</b>	0.32, 0.54		
Plasma-TNF	0.70, 0.19	0.32, 0.54		
Correlations between CSF-cytokines and plasma-cytokines				
IL-6	0.41, 0.49	–0.10, 0.85		
IL-8	<b>0.90, 0.037</b>	–0.54, 0.27		
TGF- $\beta_1$	0.00, 1.0	–0.31, 0.54		
TNF	–0.70, 0.19	–0.09, 0.87		

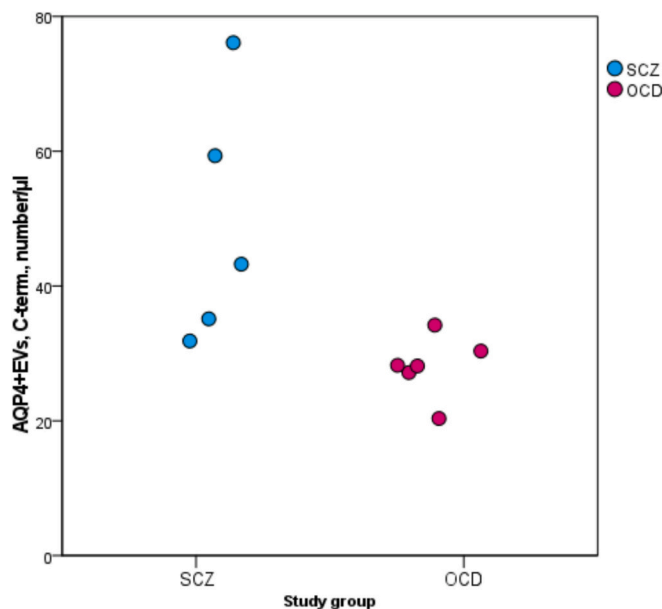
**Bold** numbers indicate significant p values ( $< 0.05$ ), *italics* denote trend significance ( $\leq 0.10$ ).

<sup>a</sup> Effect size for non-parametric tests, according to Fritz et al. 2012, J Exp Psychol.

plasma-IL-8 and plasma-TGF- $\beta_1$ , while CSF-TNF correlated negatively with plasma-IL-8 and plasma-TGF- $\beta_1$ . In OCD, no such correlations were found (Table S1B–C). The concentration of IL-8 in CSF compared to plasma was 39.9 times higher in the schizophrenia patients, while this quotient was 24.5 in the OCD group (Table 2).

### 3.6. Changes after rituximab treatment

Of the four patients with post-treatment CSF samples, two had responded clinically to rituximab, and their AQP4+ EVs decreased by 44 % (from 59.7 to 30.4 and from 27.0 to 17.4) while the two non-responders had negligible changes (from 32.3 to 29.8 and 30.6 to 30.1) (Fig. 4). In the CSF of the responder with schizophrenia, IL-6 and IL-8 decreased, while TNF and TGF- $\beta_1$  increased.



**Fig. 2.** Number of aquaporin-4-positive extracellular vesicles per  $\mu$ l of cerebrospinal fluid: distribution and comparison between patients with schizophrenia and patients with obsessive-compulsive disorder (MW  $p = 0.011$ ).

### 3.7. Other associations

Within the CSF, IL-8 and TNF showed a strong negative correlation in schizophrenia ( $\rho = -1.0$ ;  $n = 5$ ;  $p < 0.01$ ) but not in OCD. In schizophrenia, interestingly, CSF-TNF correlated negatively with plasma-IL-8, plasma-TGF- $\beta_1$  and TGF $\beta_1$  gene expression in PBCs (Table S1B–C).

## 4. Discussion

### 4.1. Astrocyte-derived EVs in psychiatric research

This exploratory, cross-sectional study provides the first quantitative analysis of AQP4+ EVs in the CSF of schizophrenia and OCD patients, identifying significantly elevated levels in schizophrenia and associations with immune markers. Despite the limited sample size, our results align with emerging literature highlighting the significance of astrocytic dysfunction in psychotic disorders (Dietz et al., 2020; Corsi-Zuelli and Deakin, 2021; Jeon et al., 2021; Ranganathan et al., 2022; Koskuvi et al., 2022; Kim et al., 2024) and suggest that astrocyte-derived EVs participate in CNS homeostasis and immune response (Zhang et al., 2023; Dai et al., 2023). The higher levels of AQP4+ EVs observed in schizophrenia may reflect increased astrocyte activation in response to putative inflammatory or neuroimmune signals, potentially contributing to the pathological mechanisms underlying schizophrenia. Hence, astrocyte-derived and other EVs may serve as diagnostic and prognostic biomarkers for psychiatric disorders (Ranganathan et al., 2022; Dai et al., 2023). Exosomes are routinely collected from plasma since they readily pass the blood-brain barrier, but this seems to be the case also for the bigger AQP4+ EVs, as shown in a study on patients with stress-induced exhaustion disorder (Wallensten et al., 2021). There, astrocyte-derived AQP4+ EVs were measured in plasma with a method like ours. However, larger and longitudinal studies are needed to validate these methods. If further validated in future studies and sampled by a non-invasive method, EVs have the potential to support clinicians in differential diagnostics and monitoring of personalised treatment.

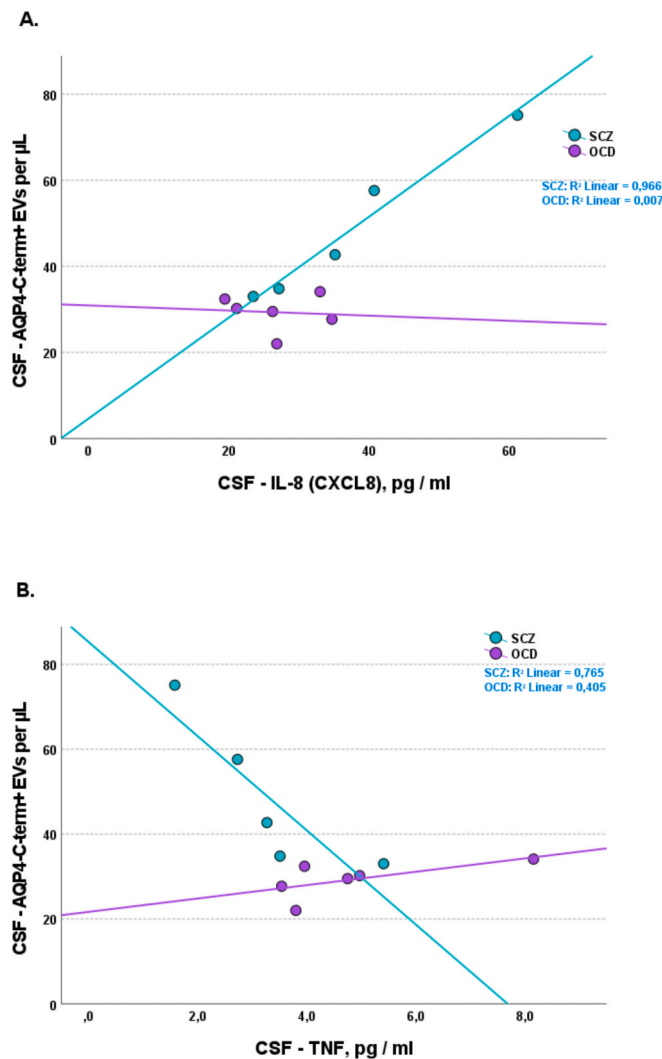


Fig. 3. Associations in the cerebrospinal fluid at baseline between AQP4+ extracellular vesicles and (A) Interleukin-8 (CXCL8), and (B) Tumour necrosis factor (TNF).

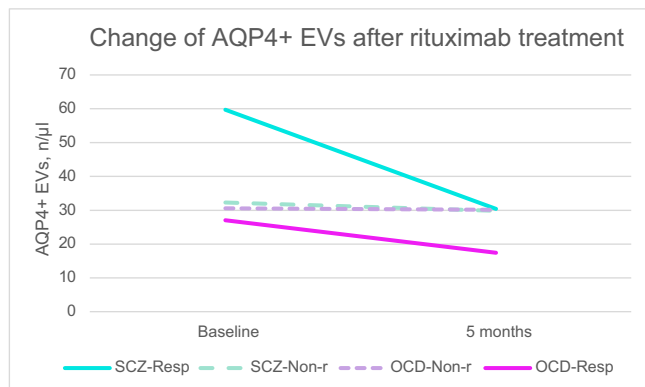


Fig. 4. AQP4+ extracellular vesicles in CSF before and 5 months after one treatment with 1000 mg rituximab,  $n = 4$ . Responders vs non-responders: MW  $p = .33$ .

#### 4.2. Is IL-8/CXCL8 mainly produced intrathecally in severe psychiatric disorders?

Two striking observations in the schizophrenia group were the strong correlations between CSF-AQP4+ EVs and IL-8 levels (in CSF and plasma, but not with gene expression) and the nearly 40-fold higher IL-8 concentrations in CSF compared to plasma. As the OCD patients had 25-fold higher CSF-IL-8, this suggests a predominantly intrathecal production of this chemokine in both disorders. This aligns with prior studies linking elevated CSF-IL-8 to schizophrenia (Gallego et al., 2018; Runge et al., 2021) and plasma-IL-8 to treatment-resistant and multiple-episode schizophrenia (Enache et al., 2021; Frydecka et al., 2018; Yan et al., 2024), indicating that CSF-IL-8 may serve as a marker of sustained intrathecal inflammation in severe psychiatric illness, with secondary leakage to peripheral blood (Maxeiner et al., 2014; Runge et al., 2021). However, few previous studies in schizophrenia have investigated the relationships between CSF and plasma levels of cytokines: Katila et al. (1994), Sasayama et al. (2013), and Gallego et al. (2024) reported no or low correlations (for IL-1 $\beta$ , IL-6 and TNF, respectively), while Coughlin et al. (2016) found a significant correlation for IL-6. To our knowledge, no previous study has reported on CSF/plasma quotients of cytokines.

IL-8, a chemokine associated with neutrophil recruitment, has gained attention for its role in neuroimmune signalling with potential roles in both neuroprotection and neuroinflammation (Tsai, 2021; Shkundin and Halaris, 2024), particularly in schizophrenia, with suggested involvement in maternal immune activation (Brown et al., 2004; Ellman et al., 2010) and oxidative stress (Wu et al., 2021). A possible association with IL-8 gene polymorphism (Ben Afia et al., 2020) and an omnigenic Mendelian randomisation (Liu et al., 2024) further support an etiological role of IL-8 in schizophrenia. Moreover, astrocytes, derived from human induced pluripotent stem cells (hiPSC) from patients with schizophrenia, secrete higher levels of IL-8 compared to controls (Trindade et al., 2023). In another hiPSC study (Szabo et al., 2025), lipopolysaccharide-stimulated schizophrenia-derived astrocytes produced elevated levels of IL-1 $\beta$ /IL-18 and a Th1/Th17 skewed priming of helper T lymphocytes, indicating that astrocytes may be bridging the innate and the adaptive immunity. The IL-8 expression of astrocytes is downregulated by the WNT/ $\beta$ -catenin pathway (Robinson et al., 2020), which may be dysregulated in schizophrenia (Vallée, 2022; Eren et al., 2023). Based on this and the strong association between IL-8 and AQP4+ EVs, we suggest that a significant proportion of intrathecally produced IL-8 in schizophrenia may be derived from astrocytes rather than microglia as previously presumed (Runge et al., 2021).

#### 4.3. Are astrocytes actively reducing the impact of TNF signals?

In schizophrenia, interestingly, AQP4+ EVs were negatively correlated with CSF-TNF and positively with plasma-TNF, while with IL-8, correlations were positive with both (Table S1B). TNF has pleiotropic roles in neuroinflammation, mediating both homeostatic and neurotoxic effects (Gonzalez Caldito, 2023). The inverse relationship between AQP4+ EVs and TNF in schizophrenia CSF suggests a possible “decoy” mechanism, wherein astrocyte-derived EVs bind to TNF, thereby modulating its inflammatory effects. This phenomenon has been described in Schwann cell EVs (Sadri et al., 2022) and may represent an adaptive mechanism to reduce TNF-mediated neurotoxicity. A related disease-modifying effect of astrocyte-derived EVs has recently been shown for neuromyelitis optica spectrum disorder (Jiang et al., 2024). Our finding that CSF-TNF in schizophrenia correlated in the opposite direction compared to other severity markers may also explain previous divergent findings concerning CSF-TNF (Gallego et al., 2024).

#### 4.4. Central vs. peripheral inflammatory interaction

The four cytokines we were able to measure in CSF were differently distributed among the compartments evaluated (Table 2). IL-8 displayed

a clear CSF dominance, while IL-6 and TNF were more evenly distributed. The anti-inflammatory cytokine TGF- $\beta_1$ , in contrast, showed a strong peripheral dominance, with markedly lower levels in the CSF. Intriguingly, in our schizophrenia patients, CSF-AQP4+ EVs and CSF-IL-8 correlated closely with peripheral TGF- $\beta_1$  (plasma protein levels as well as gene expression) but not at all with TGF- $\beta_1$  in the CSF. Consistent with this, TGF- $\beta_1$  was upregulated in peripheral mononuclear cells of schizophrenia patients (Amoli et al., 2019) and, in another study, both peripheral TGF- $\beta_1$  plasma levels and gene expression were elevated in schizophrenia and inversely correlated with cortical thickness and cognition (Pan et al., 2022), suggesting peripheral TGF- $\beta_1$  involvement in CNS pathogenesis. From these findings, a contribution of peripheral immunological mechanisms interacting with the CNS seems evident and needs to be addressed in future studies. IL-10, another anti-inflammatory cytokine, was below detection levels in all our CSF samples. The balance between IL-10 and TGF- $\beta$  is influenced by the interaction between astrocytes and microglia (Corsi-Zuelli and Deakin, 2021), why the very low levels of IL-10 in our study may be explained by a high activation of astrocytes, concurring with the severity of our patients' illness.

The absolute cytokine levels in CSF of our schizophrenia patients were higher than those reported in a prior study using a similar analytical method (Jeppesen et al., 2024). Compared to that and most other CSF studies (Warren et al., 2024), our schizophrenia patients were treatment-resistant, with PANSS scores in the higher severity range. This may explain the discrepancy in CSF levels, and that we could identify meaningful associations between measures.

#### 4.5. Treatment implications

The reduction of AQP4+ EVs in rituximab responders (2 out of 4 with repeated lumbar puncture) suggests that B-cell depletion indirectly modulates astrocyte function. This is in line with a study on multiple sclerosis; patients with active disease had significantly higher plasma levels of CNS-derived EVs compared to patients stabilised on ocrelizumab, another B-cell depleter (Mazzucco et al., 2022).

While rituximab primarily targets peripheral B-lymphocytes, its mode of action in inflammatory diseases is still a matter of debate. In research on multiple sclerosis, B-cell depletion is suggested to modulate B-cell/T-cell interaction, leading to decreased autoimmune activity. The mechanism may be similar in psychiatric diseases, since our preliminary results suggest that clinical improvement is not dependent on any reduction of pathological antibodies (Humble et al., 2025). Parallels with clozapine are noteworthy; long-term therapy with clozapine has been linked to a decline in class-switched memory B-cells (Ponsford et al., 2020) and reductions of immunoglobulins corresponding to clinical improvement (Griffiths et al., 2024). Clozapine's unique efficacy may be related to such immunological effects, which are further supported by a preclinical study (Larsson et al., 2015), where clozapine (but not haloperidol or olanzapine) significantly decreased IL-8 levels in the CSF. Our present study also raises the possibility that astrocyte-targeted interventions could play a future role in schizophrenia treatment, particularly in treatment-resistant patients with pronounced neuroinflammatory signatures. Currently, numerous methods targeting astrocytes therapeutically are explored in neurodegenerative disorders (Valori et al., 2021), but to our knowledge, none of them has been tested in psychiatric disorders.

#### 4.6. Methodological issues

How EVs are formed and released from the cell surface can impact the exact presentation of proteins like AQP4. Depending on how the EV is formed—whether by direct budding from the plasma membrane or through endosomal pathways—different portions of the AQP4 molecule may be exposed or expressed (van Niel et al., 2018). This variability in expression could affect detection and characterisation of AQP4+ EVs, as

some EVs may display only part of the protein or have varied epitope exposure. As we employed antibodies targeting both the N-terminal and C-terminal regions of the AQP4 protein, aiming for a more comprehensive characterisation, we address two key considerations: the potential variability in antibody binding or epitope exposure, which may impact detection accuracy, and the uncertainty regarding the exact expression pattern of AQP4 on EVs. By analysing both termini, we ensured a broad detection coverage, thereby improving the reliability of AQP4+ EV measurements.

#### 4.7. Limitations, strengths, and future directions

The primary limitation of this study is the small sample size, which limits generalizability and the strength of conclusions. However, the lack of associations between AQP4+ EVs and age, sex, body mass, illness duration and drug treatment argues for a link between concurrent pathology and levels of EVs.

Additionally, the lack of a healthy control group restricts the ability to determine whether observed levels of AQP4+ EVs and the differences between our clinical groups are disease-specific. However, a previous study of CSF-AQP4+ EVs in bipolar disorder demonstrated significantly lower levels among the healthy controls (Wetterberg et al., 2025). In our present study, the OCD patients may serve as a comparison group for schizophrenia, accounting for the effect of chronic illness or stress. Arguably, the use of patient controls instead of healthy controls may even strengthen the specificity of our findings for schizophrenia pathology.

A key strength is the inclusion of treatment-resistant, severely ill patients, which may explain the significant relationships found despite the small sample. Astrocytic pathology is likely to change throughout the course of the illness, as indicated by two recent studies utilising magnetic resonance spectroscopy, with myo-inositol and choline compounds as markers of astrocyte function in the anterior cingulate cortex. First-episode schizophrenia shows insufficient astrocyte function, normalising with successful treatment (Jeon et al., 2021), while treatment-resistant schizophrenia demonstrates signs of glia-related neuroinflammatory activation (Smucny et al., 2024). Furthermore, measuring cytokines across different compartments may help clarify dynamic interactions within the immune system.

While our findings highlight the potential of AQP4+ EVs as biomarkers of astrocytic activity, further research involving larger cohorts is essential to validate these results and their clinical significance. Such a study involving rituximab treatment for schizophrenia is currently ongoing (Bejerot et al., 2023b).

Future studies should examine the specific cargo (such as microRNA and proteins) of AQP4+ EVs to deepen understanding of their roles in schizophrenia pathogenesis, neuroimmune signalling and treatment effects. If EVs, derived from different cell populations within the CNS, could be identified by cell-specific markers, charted and characterised (e.g. by multiplexed immunofluorescence), this will inform us further on their roles in neuroinflammatory mechanisms and potentially elucidate the pathogenesis of schizophrenia.

#### 4.8. Conclusions

Our findings suggest that astrocyte-derived EVs participate in neuroinflammatory processes specific to schizophrenia, with possible implications for disease pathology and therapeutic strategies. The robust associations with intrathecal IL-8, interacting with peripheral immune signals, and the putative modulation of TNF response indicate an important role of astrocytes as mediators of neuroinflammation in psychiatric disorders. Further research is needed to elucidate the mechanisms underlying AQP4+ EV dynamics and their potential as biomarkers or therapeutic targets in psychotic disorders.

## CRedit authorship contribution statement

**Mats B. Humble:** Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis, Data curation, Conceptualization. **Fariborz Mobarrez:** Writing – review & editing, Visualization, Software, Resources, Methodology, Investigation, Data curation. **Daniel Eklund:** Writing – review & editing, Supervision, Resources, Methodology, Data curation, Conceptualization. **Susanne Bejerot:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Lennart Wetterberg:** Writing – review & editing, Funding acquisition, Conceptualization.

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## Declaration of competing interest

None for MH, SB, DE, FM or LW.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.schres.2025.08.006>.

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