

# Increased levels of IL-6 in the cerebrospinal fluid of patients with chronic schizophrenia — significance for activation of the kynurenine pathway

Lilly Schwieler, PhD\*; Markus K. Larsson, MSc\*; Elisabeth Skogh, MD; Magdalena E. Kegel, MSc; Funda Orhan, MSc; Sally Abdelmoaty, PhD; Anja Finn, MSc; Maria Bhat, BSc; Martin Samuelsson, MD, PhD; Kristina Lundberg, MD; Marja-Liisa Dahl, MD, PhD; Carl Sellgren, MD, PhD; Ina Schuppe-Koistinen, PhD; Camilla I. Svensson, PhD; Sophie Erhardt, PhD; Göran Engberg, PhD

**Background:** Accumulating evidence indicates that schizophrenia is associated with brain immune activation. While a number of reports suggest increased cytokine levels in patients with schizophrenia, many of these studies have been limited by their focus on peripheral cytokines or confounded by various antipsychotic treatments. Here, well-characterized patients with schizophrenia, all receiving olanzapine treatment, and healthy volunteers were analyzed with regard to cerebrospinal fluid (CSF) levels of cytokines. We correlated the CSF cytokine levels to previously analyzed metabolites of the kynurenine (KYN) pathway. **Methods:** We analyzed the CSF from patients and controls using electrochemiluminescence detection with regard to cytokines. Cell culture media from human cortical astrocytes were analyzed for KYN and kynurenic acid (KYNA) using high-pressure liquid chromatography or liquid chromatography/mass spectrometry. **Results:** We included 23 patients and 37 controls in our study. Patients with schizophrenia had increased CSF levels of interleukin (IL)-6 compared with healthy volunteers. In patients, we also observed a positive correlation between IL-6 and the tryptophan:KYNA ratio, indicating that IL-6 activates the KYN pathway. In line with this, application of IL-6 to cultured human astrocytes increased cell medium concentration of KYNA. **Limitations:** The CSF samples had been frozen and thawed twice before analysis of cytokines. Median age differed between patients and controls. When appropriate, all present analyses were adjusted for age. **Conclusion:** We have shown that IL-6, KYN and KYNA are elevated in patients with chronic schizophrenia, strengthening the idea of brain immune activation in patients with this disease. Our concurrent cell culture and clinical findings suggest that IL-6 induces the KYN pathway, leading to increased production of the *N*-methyl-D-aspartate receptor antagonist KYNA in patients with schizophrenia.

## Introduction

The role of immune activation in schizophrenia has received increasing interest in recent years. Epidemiological studies have shown that infections and autoimmune diseases are clinically important risk factors for the development of schizophrenia.<sup>1–3</sup> Furthermore, a recent study integrating results from a meta-analysis of genome-wide association studies in schizophrenia found that the most significant changes were observed in genetic loci related to the immune system.<sup>4</sup> Also,

patients with schizophrenia show microglial activation, as revealed by positron emission tomography.<sup>5,6</sup> Further, postmortem studies using messenger RNA (mRNA) expression or immunohistochemical detection have shown increased levels of immune-related compounds.<sup>7–9</sup> A number of investigations have focused on cytokines, but these studies have not yielded a unanimous picture, possibly because of a number of confounding factors, such as smoking and dietary habits, body mass index (BMI), type and duration of antipsychotic treatment and drug abuse — all factors potentially affecting the

**Correspondence to:** G. Engberg, Department of Physiology and Pharmacology, Karolinska Institutet SE-171 77, Stockholm, Sweden; goran.engberg@ki.se

\*These authors contributed equally to this work.

*J Psychiatry Neurosci* 2015;40(2):126–33.

Submitted May 15, 2014; Revised Aug. 13, 2014; Accepted Sept. 3, 2014; Early-released Dec. 2, 2014.

DOI: 10.1503/jpn.140126

©2015 8872147 Canada Inc.

immune system.<sup>10</sup> However, according to recent meta-analyses,<sup>11,12</sup> some cytokines are significantly associated with schizophrenia (e.g., interleukin [IL]-6). Still, in the majority of these studies, only peripheral cytokines were investigated, and reports of brain cytokine levels in patients with schizophrenia are relatively sparse and limited by the lack of a control group of healthy volunteers or by a low sensitivity of the assay. Recently though, cerebrospinal fluid (CSF) IL-1 $\beta$  was shown to be markedly elevated in first-episode patients compared with healthy individuals.<sup>13</sup> A few studies have also shown that CSF IL-6 is elevated in patients with chronic schizophrenia.<sup>14,15</sup> Although evidence of immune activation in schizophrenia is emerging, its functional relationship to aberrant brain neurotransmission and behaviour has been unclear. Activation of the kynurenine (KYN) pathway has been suggested as a mechanism serving to transfer information from the immune system of the brain to neurons.<sup>16-18</sup> Tryptophan degradation along this pathway occurs in various cells, including monocyte-derived macrophages and microglia, and produces several neuroactive compounds, such as quinolinic acid, an *N*-methyl-D-aspartate (NMDA) receptor agonist and kynurenic acid (KYNA), a blocker of the NMDA receptor and the  $\alpha 7$  nicotinic receptor.<sup>18</sup> Indeed, in patients with schizophrenia and those with bipolar disorder with psychosis, elevated levels of KYNA and its precursor KYN in the CSF or in the postmortem brain have been consistently reported.<sup>19-26</sup>

The aim of the present study, using a well-characterized cohort of olanzapine-treated patients with chronic schizophrenia, was to investigate CSF levels of cytokines and correlate those to previously measured levels of tryptophan metabolites of the KYN pathway in the same patients. Further, since an association between CSF IL-6 and the production of KYNA has been found in patients, as reflected by the tryptophan:KYNA ratio, we investigated a putative interplay between this cytokine and the production of KYNA in human astrocyte cultures.

## Methods

### Patients

We collected CSF from patients with DSM-IV-verified schizophrenia or schizoaffective disorder from January 2005 through December 2007. All patients were outpatients with no history of drug abuse and were medicated with olanzapine (2.5–25 mg/d) as the only antipsychotic drug. Full details of the study design and patient characteristics, including serum and CSF concentrations of olanzapine (analyzed by liquid chromatography and tandem mass spectrometry, as described previously<sup>27</sup>) have been published elsewhere.<sup>28</sup> All patients included in the study were somatically healthy according to routine laboratory analyses and physical examination. Patients who received medical treatment for infectious diseases, cancer, diabetes type 1 or 2 with a blood glucose level higher than 15 mmol/L, a history of myocardial infarction, hypertension (systolic blood pressure > 160 mm Hg, diastolic blood pressure > 110 mm Hg) or medicated with drugs affecting platelet function were not included in the study. The only concomitant

drugs accepted were zopiclone and lithium. Smoking was permitted. Symptom severity was rated in all patients using the Brief Psychiatric Rating Scale (BPRS) and Global Assessment of Functioning (GAF) scale. Plasma glucose, insulin, triglycerides and cholesterol were analyzed at the Department of Clinical Chemistry, Linköping University Hospital, Sweden, as part of routine clinical analyses.

### Healthy volunteers

Healthy controls were mainly recruited among medical students, hospital staff members and their relatives from January 2005 through December 2008. They were all in good physical health, as confirmed by a medical check-up, including routine laboratory tests and a physical examination. All volunteers were free from medication for at least 1 month and free from any form of substance abuse, except for smoking. Volunteers were subjected to a semi-structured interview, using the Structured Clinical Interview of DSM-IV disorders<sup>29</sup> and a questionnaire for personality disorders,<sup>30</sup> or interviewed by a psychiatrist to exclude mental illness. None of them had a family history of major psychosis or suicide in first- or second-degree relatives, and they were all found to be free from current signs of psychiatric morbidity or difficulties in social adjustment at the time of sampling.

### CSF sampling

Lumbar puncture was performed after a minimum of 8 hours of fasting (coffee not allowed). There were no restrictions on posture or rest. At about 8 am, a disposable needle (BD Whitacre Needle 0.7  $\times$  90 mm) was inserted at the L4–5 level with the participant in the right decubitus position. The CSF was allowed to drip into a plastic test tube. Two 6 mL fractions of CSF were collected and protected from light and centrifuged at 3500 rpm for 10 minutes within 30 minutes of the puncture. Each 6 mL sample was divided into 2 plastic tubes and stored at  $-70^{\circ}\text{C}$  until analysis. All CSF samples had been frozen and thawed twice before cytokine analysis.

The CSF levels of tryptophan, KYN and KYNA from a subsample of patients ( $n = 11$ , all men) and controls ( $n = 18$ , all men) have previously been published.<sup>25</sup>

### Policy and ethics

The work described in the present study was carried out in accordance with the Declaration of Helsinki. The Ethics Committee of the Medical Faculty of Linköping University, Sweden; the Swedish Medical Products Agency; and the Swedish Data Inspection Board approved the studies involving patients and healthy volunteers, respectively. All patients and healthy volunteers received verbal and written information about the study and provided written informed consent. At this time patients were stable owing to medication, they showed no acute psychosis, and they had a relatively high level of functioning. The same senior psychiatrist (E.S., K.L.) followed the patients for the duration of the study and was convinced that the patients understood the information and accepted to participate.

### Human astrocyte culture

Human embryonic primary cortical astrocytes were purchased from ScienCell Research Laboratories and cultured according to manufacturer's recommendations. All experiments were performed on cells in passage 4. Cells were serum starved (0.02% fetal bovine serum and 0.01% growth supplement mix) for 24 hours before the experiments and were then stimulated with IL-6 (10 ng/mL) for 24, 48 and 72 hours. Cell culture supernatants were collected and immediately frozen on dry ice and kept at  $-80^{\circ}\text{C}$  until analysis. Prior to analysis, residual protein was precipitated using the following procedure. Samples were centrifuged at 14000 rpm for 5 minutes, and an equal volume of perchloric acid (0.4 M) was added to the supernatants before additional centrifugation. Following addition of 70% perchloric acid, samples were centrifuged twice. All cell culture experiments were performed in triplicate and repeated twice.

### Cytokine analyses

We analyzed IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, tumour necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\alpha$ -2a and IFN- $\gamma$  in CSF using a customized Human Ultra-Sensitive 10-Plex Kit (MesoScale Discovery) in 2011. The assays were analyzed as per the manufacturers protocol ([www.mesoscale.com](http://www.mesoscale.com)), including a long primary incubation time (overnight at  $4^{\circ}\text{C}$ ). The sample volume was 50  $\mu\text{L}$ . The intra-assay coefficient of variation was below 20% for all analytes presented. In analogy with the study by Maier and colleagues,<sup>31</sup> particularly reliable detections of IL-6 and IL-8 was achieved. The limits of detection in our analysis were as follows: IL-1 $\beta$  (0.06 pg/mL), IL-2 (0.23 pg/mL), IL-4 (0.20 pg/mL), IL-6 (0.26 pg/mL), IL-8 (0.12 pg/mL), IL-10 (0.23 pg/mL), IL-18 (1.57 pg/mL), TNF- $\alpha$ , (0.18 pg/mL), IFN- $\alpha$ -2a (0.74 pg/mL) and IFN- $\gamma$  (0.24 pg/mL).

### Analysis of tryptophan metabolites

We analyzed tryptophan, KYN and KYNA using a reversed phase high-performance liquid chromatography (HPLC) system, as previously described;<sup>23,25</sup> 50  $\mu\text{L}$  samples were manually injected.

### Analysis of KYN in astrocyte media

Standard curves were prepared in the range of 0.0004 to 10  $\mu\text{mol/L}$  of KYN dissolved in Dulbecco phosphate-buffered saline (PBS) aliquoted and stored below  $-70^{\circ}\text{C}$ . Astrocyte media and standard samples (50  $\mu\text{L}$ ) were prepared using solid phase extraction (Oasis MAX). We used D4-KYN as the internal standard and added it to each standard and media sample to a final concentration of 0.5  $\mu\text{mol/L}$ . We purchased PBS from Gibco and KYN from Buchem.

After solid phase extraction the sample eluate was evaporated with nitrogen at  $50^{\circ}\text{C}$  and redissolved with 100  $\mu\text{L}$  0.1% formic acid in MilliQ water. We injected 7.5  $\mu\text{L}$  of the filtrate into an Acquity HPLC system (Waters Corporation) equipped with a HSST3 2.1  $\times$  100 mm, 1.8  $\mu\text{m}$  particle col-

umn. The detection was performed using a Waters Xevo TQ-S triple quadrupole mass spectrometer operating in positive ionization MS/MS configuration. The mobile phase was run at a flow rate of 300  $\mu\text{L}/\text{min}$  and consisted of 0.1% formic acid in MilliQ water (A phase) and 95% acetonitrile 0.1% formic acid (B phase) starting with 10% B for 2.5 minutes following gradient elution, with a total run time of 10 minutes. The formic acid and acetonitrile were purchased as MS-grade from Sigma-Aldrich. The mass spectrometer was tuned for KYN and set at capillary voltage of 2.0 kV, cone voltage of 4 V, source temperature of  $150^{\circ}\text{C}$ , desolvation temperature of  $600^{\circ}\text{C}$ , desolvation gas flow of 1000 L/hr and collision energy of 16 eV. Mass spectral transition was  $m/z$  209 > 146 for KYN and 213 > 150 for the internal standard.

Calibration was performed using standards covering the range of the media concentration. Eleven concentration points were used to establish a linear calibration curve and plotted using the ratio of analyte peak area over internal standard peak area after integration by Masslynx 4.1 software (Waters Corporation). The retention time for KYN was 1.8 minutes.

### Statistical analysis

Analyses of potential confounders were performed using a Mann-Whitney *U* test or Fisher exact test. Comparisons of CSF IL-6, IL-8, tryptophan, KYN, KYNA, tryptophan:KYN ratio, KYN:KYNA ratio and tryptophan:KYNA ratio between patients and controls were first analyzed using the Mann-Whitney *U* test. These analyses were then also performed using logistic regression analyses with age as a covariate and group (controls v. patients) as the dependent variable. We performed all correlation analyses using a Spearman rank correlation analysis. All values are reported as medians and interquartile ranges (IQR). We analyzed cell culture data using 1-way analysis of variance (ANOVA) with Bonferroni correction for comparisons within each time point. Differences in IL-6 between freeze/thaw cycles were analyzed using the Mann-Whitney *U* test. All values are reported as means and standard errors of the mean. We checked the assumptions of each test that we used. All reported *p* values are 2-sided. We performed our analyses using SPSS Statistics version 20.0 (IBM Inc.) or Prism version 6.0 (GraphPad Software Inc.).

## Results

### Participants

We included 23 patients (15 men, median age 37 [range 23–49] yr; 8 women, median age 35 [range 26–46] yr), 2 of whom had schizoaffective disorder, and 37 controls (23 men, median age 24 [range 21–51] yr; 14 women, median age 23 [range 19–32] yr) in the study. The median age was significantly higher in patients than controls (median 35.0, IQR 32.0–41.0 v. median 23.0, IQR 22.0–25.5,  $p < 0.001$ ). Sex distribution, the percentage of smokers and BMI did not differ significantly between the groups (Table 1). In addition to olanzapine, 2 patients were taking zopiclone and the 2 patients with schizoaffective disorder were taking lithium. Six patients and 3 controls were smokers.

### Analysis of potential confounders

No association was found between the CSF cytokines (IL-6 or IL-8) and age, plasma glucose, cholesterol, triglycerides, serum insulin or CSF/serum olanzapine. We found that CSF IL-8, but not IL-6, correlated with BMI in patients after age correction (Table 2).

### Detection of cytokines in CSF

We detected IL-6 in the CSF in all 37 controls and in 21 patients, and IL-8 was detected in the CSF of 36 controls and 22 patients. The other cytokines analyzed were undetectable (IFN- $\gamma$ , IL-1 $\beta$  and IL-4) or were found to be very close to the detection limit of the assay, hereby only detected in a limited number of samples (IL-2, IL-10, IL-18, TNF- $\alpha$ , and IFN- $\alpha$ -2a).

### CSF IL-6 in patients versus controls

The CSF IL-6 concentration was elevated in patients with chronic schizophrenia compared with controls (2.68 pg/mL, IQR 1.79–3.99 pg/mL,  $n = 21$  v. 1.50 pg/mL, IQR 1.01–2.25 pg/mL,  $n = 37$ ,  $p = 0.001$ ; Fig. 1A). When stratifying the analysis by sex, IL-6 was significantly increased both in men (patients: 2.68 pg/mL, IQR 1.67–3.88 pg/mL,  $n = 15$ ; controls: 1.12 pg/mL, IQR 0.85–1.68 pg/mL,  $n = 23$ ,  $p = 0.006$ ) and women (patients: 3.25 pg/mL, IQR 1.92–5.83 pg/mL,  $n = 6$ ; controls: 1.76 pg/mL, IQR 1.41–2.59 pg/mL,  $n = 14$ ,  $p = 0.026$ ). No correlation between age and CSF IL-6 was observed in either patients ( $r = 0.062$ ,  $p = 0.79$ ) or controls ( $r = 0.018$ ,  $p = 0.92$ ). In line with this, IL-6 was significantly higher in patients than controls after adjusting for age ( $p = 0.002$ ).

**Table 1: Demographic characteristics of patients with schizophrenia and controls**

Characteristic	Group; median (IQR)*		$p$ value†
	Control, $n = 37$	Schizophrenia, $n = 23$	
Age, yr	23.0 (22.0–25.5)	35.0 (32.0–41.0)	< 0.001‡
Male sex, %	62.2	65.2	> 0.99§
Smokers, %	8.1	26.1	0.07§
BMI	23.0 (22.0–26.0)	26.2 (22.1–27.2)	0.19‡

BMI = body mass index; IQR = interquartile range.  
 \*Unless otherwise indicated.  
 †All reported  $p$  values are 2-sided.  
 ‡Mann–Whitney  $U$  test.  
 §Fisher exact test.

**Table 2: Clinical characteristics and their association with IL-6 and IL-8**

Characteristic	Median (IQR)	IL-6, $n = 21$		IL-8, $n = 22$	
		$r$	$p$ value	$r$	$p$ value
<b>Olanzapine treatment</b>					
Years of treatment	4.5 (0.9–8.5)	–0.138	0.55	0.196	0.38
Daily dose, mg	10 (7.5–20)	0.074	0.75	–0.283	0.20
Hours from last dose to sampling	12 (10.25–12.75)	0.37	0.10	–0.064	0.78
Olanzapine, CSF, nM	10.9 (8.1–19.3)	–0.069	0.77	–0.078	0.73
Olanzapine, serum, nM	101 (71–171)	0.032	0.89	–0.139	0.54
<b>Metabolic variables</b>					
BMI	26.2 (22.1–27.2)	0.025	0.92	–0.472	0.027
Plasma glucose, mg/dL	5.1 (4.9–5.4)	0.038	0.87	0.094	0.68
Plasma insulin, $\mu$ IU/mL	45 (32–88)	–0.249	0.28	0.054	0.81
Plasma cholesterol, mg/dL	4.7 (4.4–5.5)	–0.065	0.78	0.043	0.85
Plasma triglycerides, mg/dL	1.3 (0.84–2.0)	–0.261	0.25	0.012	0.96
<b>Other variables</b>					
Age, yr	35 (32–41)	0.062	0.79	0.002	0.99
GAF	58.0 (49–63)	0.15	0.52	0.235	0.29
BPRS	33.0 (27.0–35)	–0.122	0.60	–0.32	0.15
Age at first episode, yr	24 (20–28)	0.03	0.90	0.09	0.69
Duration of illness, yr	11 (5–15.25)	0.018	0.94	–0.04	0.86

BMI = body mass index; BPRS = Brief Psychiatric Rating Scale; CSF = cerebrospinal fluid; GAF = Global Assessment of Functioning scale; IL = interleukin; IQR = interquartile range.

### CSF IL-8 in patients versus controls

The CSF IL-8 levels did not differ between patients with schizophrenia and controls (11.15 pg/mL, IQR 9.80–15.24 pg/mL,  $n = 22$  v. 10.5 pg/mL, IQR 8.55–12.99 pg/mL,  $n = 36$ ,  $p = 0.16$ , and  $p = 0.74$ , adjusted for age and BMI; Fig. 1B).

### Correlations between IL-6 and KYN and KYNA

The CSF levels of KYN were increased in patients with schizophrenia compared with controls (patients: 57.9 nM, IQR 48.0–71.3,  $n = 23$ ; controls: 32.2 nM, IQR 26.2–49.0,  $n = 37$ ,  $p < 0.001$  and  $p = 0.007$ , adjusted for age). The CSF levels of KYNA were also increased in patients compared with controls (patients: 1.87 nM, IQR 1.63–2.29,  $n = 23$ ; controls: 1.50 nM, IQR 1.14–1.92,  $n = 37$ ,  $p = 0.006$  and  $p = 0.040$ , adjusted for age). There were no significant differences in CSF tryptophan between patients with schizophrenia and controls (1.71 nM, IQR 1.61–1.87,  $n = 23$  v. 1.77 nM, IQR 1.47–1.94,  $n = 37$ ,  $p = 0.83$  and  $p = 0.72$ , adjusted for age).

The tryptophan:KYN and the tryptophan:KYNA ratios were also significantly lower in patients than controls (patients:  $p < 0.001$  and  $p = 0.005$ , adjusted for age; controls:  $p = 0.034$  and  $p = 0.023$ , adjusted for age), whereas the KYN:KYNA ratio was close to significantly decreased in patients ( $p = 0.05$  and  $p = 0.10$ , adjusted for age). We detected a correlation between IL-6 and the production of KYNA in patients with schizophrenia ( $r = -0.49$ ;  $p = 0.024$ ), as reflected by the tryptophan:KYNA ratio.

### The effects of IL-6 on KYN and KYNA formation in human cortical astrocytes cells

To investigate a putative association between the elevated levels of IL-6 and KYNA observed in patients, we exposed

cultured fetal human cortical astrocytes to recombinant human IL-6 (10 ng/mL). Increased levels of KYNA were detected in the cell medium after 48 hours ( $1.55 \pm 0.097$  nM v.  $1.29 \pm 0.054$  nM,  $p = 0.038$ ) and 72 hours ( $1.83 \pm 0.070$  nM v.  $1.56 \pm 0.053$  nM,  $p = 0.045$ ) of IL-6 stimulation (Fig. 2A). The KYN levels were not affected by IL-6 treatment at any time point (Fig. 2B).

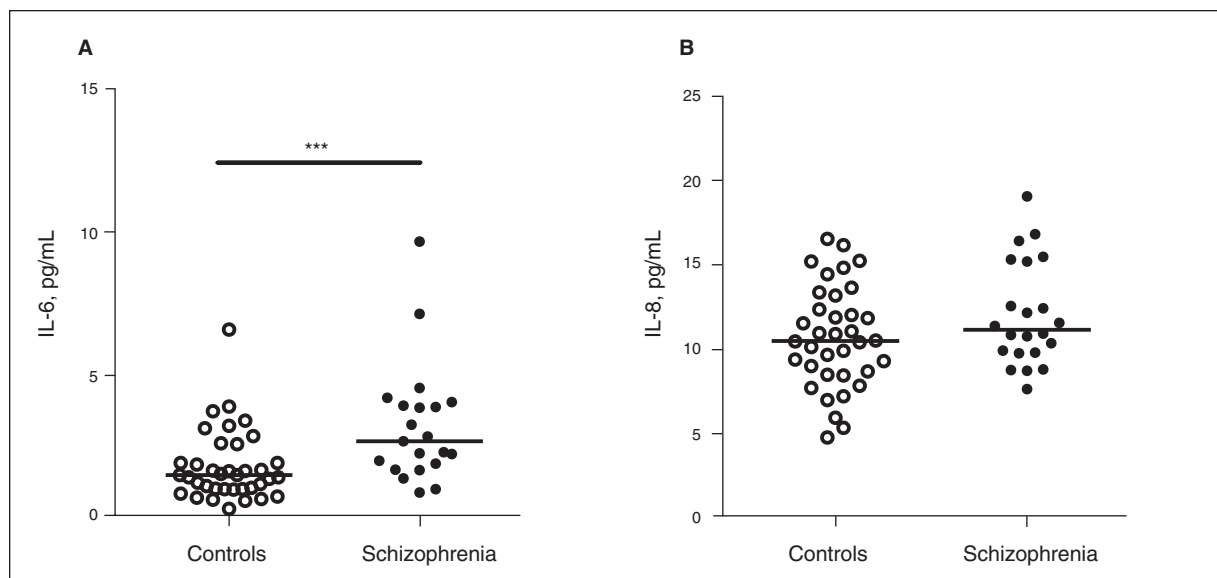
### Effects of repeated thawing/freezing cycles on the stability of IL-6

No significant difference in IL-6 concentrations was observed between CSF samples that were thawed once from  $-70^{\circ}\text{C}$  ( $1.03 \pm 0.29$  pg/mL,  $n = 5$ ) and samples after 2 thawing/freezing cycles from  $-70^{\circ}\text{C}$  ( $1.01 \pm 0.28$  pg/mL,  $n = 5$ ,  $p = 0.84$ ).

## Discussion

In the present study, CSF inflammatory markers were analyzed in a well-characterized cohort of olanzapine-treated outpatients with chronic schizophrenia. Among the 10 different cytokines analyzed, we found increased levels of IL-6 in patients compared with controls. Levels of other cytokines were unchanged (IL-8) or were found to be under the detection limit of the assay. Elevated levels of CSF IL-6 were not associated with age, sex, BMI or smoking habits or with any of the metabolic parameters measured, such as plasma levels of glucose, insulin, cholesterol and triglycerides. Furthermore, in line with previous studies,<sup>32,33</sup> treatment with olanzapine appeared not to influence IL-6, as CSF or serum levels of olanzapine did not correlate with CSF IL-6.

Levels of CSF IL-6 have previously been studied in other psychiatric conditions. In major depression, CSF IL-6 was reported not to differ from controls.<sup>34,35</sup> Further, in euthymic



**Fig. 1:** (A) Interleukin (IL)-6 and (B) IL-8 in the cerebrospinal fluid (CSF) of controls and patients with schizophrenia. Each point represents the concentration of a single CSF sample, and the horizontal lines represent the median for each group. \*\*\* $p < 0.001$  (Mann–Whitney  $U$  test).



patients with bipolar disorder or in suicide attempters with depression this cytokine is reduced or elevated, respectively.<sup>36,37</sup> The presently observed increase in CSF IL-6 in patients with chronic schizophrenia is in agreement with results of previous studies analyzing this cytokine in plasma or CSF.<sup>11,12,14,15,38</sup> However, in a recent study from our laboratory, CSF IL-6 levels were not altered in patients with first-episode schizophrenia compared with healthy volunteers;<sup>13</sup> rather, in those patients CSF IL-1 $\beta$  was increased. In the present study, IL-1 $\beta$  was undetectable both in patients and controls. The discrepancy between the results of our previous study and the present results may be related to the use of different platforms for the analysis of cytokines (Luminex vs. MesoScale) or to the fact that all CSF samples in the present study had been stored for 3–6 years and frozen and thawed twice. Both storage time and multiple freezing/thawing cycles are reported to affect the concentration of cytokines, including IL-1 $\beta$ , in human samples.<sup>39</sup> However, both of our investigations and another study<sup>39</sup> suggest that IL-6 may be an exception in this regard. The reason for the discrepancy with regard to CSF IL-6 levels in patients with first-episode versus chronic schizophrenia is unclear, but may be related to the chronic progress of the disease.

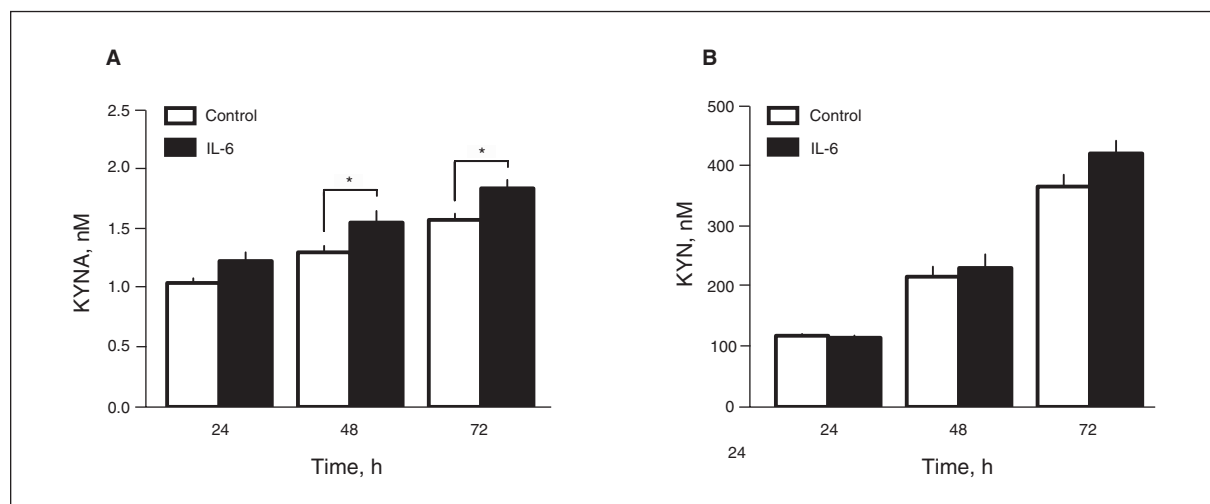
In the present study we confirm that CSF levels of KYN and KYNA are increased in patients with schizophrenia. We also discovered a positive correlation between CSF IL-6 and the tryptophan:KYNA ratio. Proinflammatory cytokines, such as IFN- $\gamma$ , TNF- $\alpha$  or IL-1 $\beta$  are known to induce tryptophan 2,3-dioxygenase (TDO) and/or indolamine 2,3-dioxygenase (IDO), rate-limiting enzymes of the KYN pathway.<sup>40,41</sup> Further, injection of IL-6 into the rat hippocampus has been found to be associated with the induction of IDO.<sup>42</sup> A decreased tryptophan:KYNA ratio, tentatively reflecting the activity of IDO/TDO, would explain the elevation in CSF KYN and KYNA consistently found in patients with schizophrenia. Indeed, increased TDO activity in the postmortem brains of patients with schizophrenia has been reported.<sup>22,43</sup>

In line with this, the present study shows that fetal human cortical astrocytes respond to application of IL-6 with an increased KYNA synthesis, suggesting an intimate interplay between IL-6 and the KYN pathway.

Increased levels of brain KYNA may be the link between immune activation and aberrant neurotransmission and behaviour in patients with schizophrenia. Thus, experimental studies demonstrate that elevated KYNA affects brain glutamatergic/dopaminergic neurotransmission, hereby implicating activation of the KYN pathway in established models of schizophrenia.<sup>18,43,44</sup> Moreover, elevated KYNA induces schizophrenia-like behaviour, such as disrupted prepulse inhibition<sup>45</sup> and auditory sensory gating<sup>46</sup> as well as impaired contextual discriminations,<sup>47</sup> spatial working memory<sup>48,49</sup> and attentional set-shifting,<sup>50</sup> in rodents. Notably, a specific inhibitor of KYN aminotransferase II, which reduces brain KYNA levels, prevents ketamine-induced working memory impairments and tends to attenuate hallucinatory-like behaviours in primates.<sup>51</sup> Together, these findings strongly support the hypothesis that increased brain KYNA constitutes a major trigger for cognitive and psychotic symptoms and should encourage the development of high-quality biomarkers and novel treatment approaches based on the KYN pathway.

#### Limitations

The major limitation of the present study was the use of CSF that had been frozen and thawed twice. Although KYNA is a stable compound and not affected by repeated freezing and thawing,<sup>52</sup> some cytokines are considerably more sensitive in this regard. Also, the use of a multiplex assay to quantify cytokines may yield less sensitivity than a singleplex procedure. The median age differed significantly between patients and controls; however, we controlled for this confounder in the statistical analysis. Finally, given the circadian rhythmicity of CSF IL-6,<sup>53</sup> lumbar puncture was performed only once in each individual.



**Fig. 2:** (A) Kynurenic acid (KYNA) and (B) kynurenine (KYN) in human cortical astrocytes exposed to interleukin (IL)-6 for 24, 48 and 72 hours. Bars represent means  $\pm$  standard errors of the mean \* $p < 0.05$  versus control (1-way analysis of variance).

## Conclusion

Our data support previous findings that brain IL-6, KYN and KYNA are elevated in patients with chronic schizophrenia and strengthen the idea of brain immune activation in people with the disease. The increased production of KYNA in fetal human astrocytes following exposure of IL-6 shows that this cytokine is able to induce the activity of the KYN pathway. No correlations between CSF IL-6 and psychiatric symptom scores (GAF and BPRS) were found, possibly owing to the fact that all patients were well controlled, stable and under chronic treatment with olanzapine. Future studies may reveal whether elevation in brain IL-6 contributes to psychiatric symptoms in patients with schizophrenia.

**Acknowledgements:** This work was supported by grants from the Swedish Medical Research Council awarded to G. Engberg (2009–3068, 2011–4789), S. Erhardt (2009–7053, 2013–2838), C. Svensson (2009–3068) and M.-L. Dahl (2008–2578, 2013–2592); the Swedish Brain foundation, Petrus och Augusta Hedlunds Stiftelse, Östergötland County Council; the AstraZeneca-Karolinska Institutet Joint Research Program in Translational Science, the Karolinska Institutet (KID). The authors thank the patients and healthy volunteers for their participation and the health professionals who facilitated the work.

**Affiliations:** Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden (Schwieler, Larsson, Kegel, Orhan, Abdelmoaty, Finn, Svensson, Erhardt, Engberg); Department of Clinical and Experimental Medicine, Section of Psychiatry, Faculty of Health Sciences, Linköping University, Linköping, Sweden (Skogh, Samuelsson, Lundberg); AstraZeneca, Research & Development, Innovative Medicines, Personalised Healthcare & Biomarkers, Science for Life Laboratory, Solna, Sweden (Bhat, Schuppe-Koistinen); Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden (Bhat, Schuppe-Koistinen); Department of Laboratory Medicine, Division of Clinical Pharmacology, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden (Dahl); Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden (Sellgren).

**Competing interests and funding:** None declared. The clinical research study was sponsored by Linköping University Hospital with an in-kind contribution to the sample analysis from AstraZeneca. AstraZeneca neither influenced nor sponsored the clinical research performed at Linköping University. These data arise from a sample collection, not from a prospective trial and are therefore not recorded in any clinical trial registry.

**Contributors:** L. Schwieler, M. Larsson, E. Skogh, C. Svensson, S. Erhardt and G. Engberg designed the study. L. Schwieler, M. Larsson, E. Skogh, M. Kegel, S. Abdelmoaty, M. Bhat, M. Samuelsson, M.-L. Dahl and S. Erhardt acquired the data, which L. Schwieler, M. Larsson, F. Orhan, A. Finn, C. Sellgren, I. Shuppe-Koistinen, C. Svensson, S. Erhardt and G. Engberg analyzed. L. Schwieler, M. Larsson, S. Erhardt and G. Engberg wrote the article, which all authors reviewed and approved for publication.

## References

1. Yolken RH, Torrey EF. Are some cases of psychosis caused by microbial agents? A review of the evidence. *Mol Psychiatry* 2008;13:470-9.
2. Dalman C, Allebeck P, Gunnell D, et al. Infections in the CNS during childhood and the risk of subsequent psychotic illness: a cohort study of more than one million Swedish subjects. *Am J Psychiatry* 2008;165:59-65.

3. Benros ME, Nielsen PR, Nordentoft M, et al. Autoimmune diseases and severe infections as risk factors for schizophrenia: a 30-year population-based register study. *Am J Psychiatry* 2011;168:1303-10.
4. Aberg KA, Liu Y, Bukszár J, et al. A comprehensive family-based replication study of schizophrenia genes. *JAMA Psychiatry* 2013;70:573-81.
5. Doorduyn J, de Vries EF, Willemsen AT, et al. Neuroinflammation in schizophrenia-related psychosis: a PET study. *J Nucl Med* 2009;50:1801-7.
6. van Berckel BN, Bossong MG, Boellaard R, et al. Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C] PK11195 positron emission tomography study. *Biol Psychiatry* 2008;64:820-2.
7. Radewicz K, Garey LJ, Gentleman SM, et al. Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics. *J Neuropathol Exp Neurol* 2000;59:137-50.
8. Busse S, Busse M, Schiltz K, et al. Different distribution patterns of lymphocytes and microglia in the hippocampus of patients with residual versus paranoid schizophrenia: Further evidence for disease course-related immune alterations? *Brain Behav Immun* 2012;26:1273-9.
9. Fillman SG, Cloonan N, Catts VS, et al. Increased inflammatory markers identified in the dorsolateral prefrontal cortex of individuals with schizophrenia. *Mol Psychiatry* 2013;18:206-14.
10. O'Connor MF, Bower JE, Cho HJ, et al. To assess, to control, to exclude: effects of biobehavioral factors on circulating inflammatory markers. *Brain Behav Immun* 2009;23:887-97.
11. Potvin S, Stip E, Sepehry AA, et al. Inflammatory cytokine alterations in schizophrenia: a systematic quantitative review. *Biol Psychiatry* 2008;63:801-8.
12. Miller BJ, Buckley P, Seabolt W, et al. Meta-analysis of cytokine alterations in schizophrenia: clinical status and antipsychotic effects. *Biol Psychiatry* 2011;70:663-71.
13. Söderlund J, Schröder J, Nordin C, et al. Activation of brain interleukin-1 $\beta$  in schizophrenia. *Mol Psychiatry* 2009;14:1069-71.
14. Garver DL, Tamas RL, Holcomb JA. Elevated interleukin-6 in the cerebrospinal fluid of a previously delineated schizophrenia subtype. *Neuropsychopharmacology* 2003;28:1515-20.
15. Sasayama D, Hattori K, Wakabayashi C, et al. Increased cerebrospinal fluid interleukin-6 levels in patients with schizophrenia and those with major depressive disorder. *J Psychiatr Res* 2013;47:401-6.
16. Asp L, Holtze M, Powell SB, et al. Neonatal infection with neurotropic influenza A virus induces the kynurenine pathway in early life and disrupts sensorimotor gating in adult Tap1 $^{-/-}$  mice. *Int J Neuropsychopharmacol* 2010;13:475-85.
17. Liu X-C, Holtze M, Powell SB, et al. Behavioral disturbances in adult mice following neonatal virus infection or kynurenine treatment — role of brain kynurenic acid. *Brain Behav Immun* 2014;36:80-9.
18. Schwarcz R, Bruno JP, Muchowski PJ, et al. Kynurenines in the mammalian brain: when physiology meets pathology. *Nat Rev Neurosci* 2012;13:465-77.
19. Erhardt S, Blennow K, Nordin C, et al. Kynurenic acid levels are elevated in the cerebrospinal fluid of patients with schizophrenia. *Neurosci Lett* 2001;313:96-8.
20. Schwarcz R, Rassoulpour A, Wu HQ, et al. Increased cortical kynurenate content in schizophrenia. *Biol Psychiatry* 2001;50:521-30.
21. Nilsson LK, Linderholm KR, Engberg G, et al. Elevated levels of kynurenic acid in the cerebrospinal fluid of male patients with schizophrenia. *Schizophr Res* 2005;80:315-22.
22. Miller CL, Llenos IC, Dulay JR, et al. Upregulation of the initiating step of the kynurenine pathway in postmortem anterior cingulate cortex from individuals with schizophrenia and bipolar disorder. *Brain Res* 2006;1073-1074:25-37.

23. Olsson SK, Samuelsson M, Saetre P, et al. Elevated levels of kynurenic acid in the cerebrospinal fluid of patients with bipolar disorder. *J Psychiatry Neurosci* 2010;35:195-9.
24. Sathyaikumar KV, Stachowski EK, Wonodi I, et al. Impaired kynurenic pathway metabolism in the prefrontal cortex of individuals with schizophrenia. *Schizophr Bull* 2011;37:1147-56.
25. Linderholm KR, Skogh E, Olsson SK, et al. Increased levels of kynurenic acid and kynurenic acid in the CSF of patients with schizophrenia. *Schizophr Bull* 2012;38:426-32.
26. Olsson SK, Sellgren C, Engberg G, et al. Cerebrospinal fluid kynurenic acid is associated with manic and psychotic features in patients with bipolar I disorder. *Bipolar Disord* 2012;14:719-26.
27. Josefsson M, Roman M, Skogh E, et al. Liquid chromatography/tandem mass spectrometry method for determination of olanzapine and N-desmethyloanzapine in human serum and cerebrospinal fluid. *J Pharm Biomed Anal* 2010;53:576-82.
28. Skogh E, Sjödin I, Josefsson M, et al. High correlation between serum and cerebrospinal fluid olanzapine concentrations in patients with schizophrenia or schizoaffective disorder medicating with oral olanzapine as the only antipsychotic drug. *J Clin Psychopharmacol* 2011;31:4-9.
29. First MB, Spitzer RI, Gibbon M, et al. *Structured Clinical Interview for DSM-IV Axis I Disorders, Clinician Version (SCID-CV)*. Washington (DC): American Psychiatric Press; 1996.
30. First MB, Spitzer RI, Gibbon M, et al. *Structured Clinical Interview for DSM-IV Axis II Personality Disorders (SCID-II)*. Washington (DC): American Psychiatric Press; 1997.
31. Maier B, Laurer H-L, Rose S, et al. Physiological levels of pro- and anti-inflammatory mediators in cerebrospinal fluid and plasma: a normative study. *J Neurotrauma* 2005;22:822-35.
32. Hori H, Yoshimura R, Yamada Y, et al. Effects of olanzapine on plasma levels of catecholamine metabolites, cytokines, and brain-derived neurotrophic factor in schizophrenic patients. *Int Clin Psychopharmacol* 2007;22:21-7.
33. Kluge M, Schuld A, Schacht A, et al. Effects of clozapine and olanzapine on cytokine systems are closely linked to weight gain and drug-induced fever. *Psychoneuroendocrinology* 2009;34:118-28.
34. Carpenter LL, Heninger GR, Malison RT, et al. Cerebrospinal fluid interleukin (IL)-6 in unipolar major depression. *J Affect Disord* 2004;79:285-9.
35. Martinez JM, Garakani A, Yehuda R, et al. Proinflammatory and "resiliency" proteins in the CSF of patients with major depression. *Depress Anxiety* 2012;29:32-8.
36. Söderlund J, Olsson SK, Samuelsson M, et al. Elevation of cerebrospinal fluid interleukin-1 $\beta$  in bipolar disorder. *J Psychiatry Neurosci* 2011;36:114-8.
37. Lindqvist D, Janelidze S, Hagell P, et al. Interleukin-6 is elevated in the cerebrospinal fluid of suicide attempters and related to symptom severity. *Biol Psychiatry* 2009;66:287-92.
38. McAllister-Sistilli CG, van Kammen DP, Kelley ME, et al. Elevated interleukin-6 in schizophrenia. *Psychiatry Res* 1999;87:129-36.
39. de Jager W, Bourcier K, Rijkers GT, et al. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol* 2009;10:52.
40. Kegel ME, Svensson CI, Erhardt S. IL-1 $\beta$  induces IDO and TDO transcription and provokes the release of kynurenic acid from human astrocytes in vitro. *Soc Neurosci Abstr* 240.19/E7; 2011.
41. Mándi Y, Vécsei L. The kynurenic system and immunoregulation. *J Neural Transm* 2012;119:197-209.
42. Kim H, Chen L, Lim G, et al. Brain indoleamine 2,3-dioxygenase contributes to the comorbidity of pain and depression. *J Clin Invest* 2012;122:2940-54.
43. Miller CL, Llenos IC, Dulay JR, et al. Expression of the kynurenic pathway enzyme tryptophan 2,3-dioxygenase is increased in the frontal cortex of individuals with schizophrenia. *Neurobiol Dis* 2004;15:618-29.
44. Erhardt S, Olsson SK, Engberg G. Pharmacological manipulation of kynurenic acid: potential in the treatment of psychiatric disorders. *CNS Drugs* 2009;23:91-101.
45. Erhardt S, Schwieler L, Emanuelsson C, et al. Endogenous kynurenic acid disrupts prepulse inhibition. *Biol Psychiatry* 2004;56:255-60.
46. Shepard PD, Joy B, Clerkin L, et al. Micromolar brain levels of kynurenic acid are associated with a disruption of auditory sensory gating in the rat. *Neuropsychopharmacology* 2003;28:1454-62.
47. Chess AC, Landers AM, Bucci DJ. L-kynurenic treatment alters contextual fear conditioning and context discrimination but not cue-specific fear conditioning. *Behav Brain Res* 2009;201:325-31.
48. Chess AC, Simoni MK, Alling TE, et al. Elevations of endogenous kynurenic acid produce spatial working memory deficits. *Schizophr Bull* 2007;33:797-804.
49. Pocivavsek A, Wu HQ, Potter MC, et al. Fluctuations in endogenous kynurenic acid control hippocampal glutamate and memory. *Neuropsychopharmacology* 2011;36:2357-67.
50. Alexander KS, Wu HQ, Schwarcz R, et al. Acute elevations of brain kynurenic acid impair cognitive flexibility: normalization by the  $\alpha$ 7 positive modulator galantamine. *Psychopharmacology (Berl)* 2012;220:627-37.
51. Kozak R, Campbell BM, Strick CA, et al. Reduction of brain kynurenic acid improves cognitive function. *J Neurosci* 2014;34:10592-602.
52. Swartz KJ, Matson WR, MacGarvey U, et al. Measurement of kynurenic acid in mammalian brain extracts and cerebrospinal fluid by high-performance liquid chromatography with fluorometric and coulometric electrode array detection. *Anal Biochem* 1990;185:363-76.
53. Agorastos A, Hauger RL, Barkauskas DA, et al. Circadian rhythmicity, variability and correlation of interleukin-6 levels in plasma and cerebrospinal fluid of healthy men. *Psychoneuroendocrinology* 2014;44:71-82.