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# Evaluation of fractalkine and interleukin-6 plasma levels in children with Autism Spectrum Disorder

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## Abstract

**Background:** the etiology of autism spectrum disorder (ASD) is multifactorial with a substantial immunological component. Children with ASD show abnormal levels of cytokines which significantly contribute to core features of the disorder such as behavioral and sensory impairments. Interleukin 6 (IL-6) represents a major neuroimmune mediator commonly implicated in ASD. Alternatively, fractalkine (FKN) is a key chemokine that regulates the homeostasis of cytokines and activation of microglia. However, its role in ASD pathophysiology is still understudied. In this pilot study, we evaluated the plasma levels of FKN and interleukin-6 (IL-6) in 40 Egyptian children diagnosed with ASD in comparison to 40 age and sex-matched controls using Enzyme-linked immunosorbent assay (ELISA). **Results:** both groups showed comparable circulating levels of FKN ($p$= 0.22) and IL-6 ($p$= 0.65). Receiver operating characteristic (ROC) curves showed poor diagnostic potential for FKN (AUC= 0.56, $p$= 0.33) and IL-6 (AUC= 0.53, $p$= 0.65) alike. Amongst the ASD group, FKN availability showed negligible associations with IL-6 levels, age, childhood autism rating scale, receptive expressive Arabic language scores and sensory profile. **Conclusion:** our pilot findings indicate that impact of FKN might affect ASD at the brain level with no changes to the peripheral concentrations. Further studies are warranted to evaluate the implication of both endogenous and circulating FKN in neuroinflammation among larger cohorts of children with ASD.



**Keywords:** Autism spectrum disorder, fractalkine, interleukin-6, neuroinflammation

## Background

Autism spectrum disorder (ASD) is a group of neurobehavioral conditions that affects about 1% of children worldwide [1]. It is mainly characterized by limited social communication skills, stereotypical interests and repetitive patterns. These symptoms are typically recognized by 3 years of age; whereas intervention programs are often initiated after 4 years. This delay in diagnosis and treatment may lead to less effective ASD management [2]. Therefore, many studies aimed to develop biological measurements for early ASD detection such as laboratory tests or radiographic scans. The combination of more than one biomarker can further enhance the discriminative power [3]. Hewitson and her colleagues suggested a panel of 12 blood-based markers for ASD prediction and targeted therapy. They include proteins that regulate various immunological pathways and cytokine production [4]. Previous studies reported abnormal cytokine profile among ASD cohorts with significant elevation of inflammatory mediators in their biological fluids. Among these cytokines, interleukin 6 (IL-6) showed consistent association with ASD traits [5].

Both prenatal and postnatal immunological factors are implicated in ASD pathology. The gestation period coincides with critical stages of the fetal neurodevelopment. Therefore, maternal immune activation (MIA) can mediate active and passive transport of cytokines in placenta which disturbs the signaling pathways. The excessive in utero exposure to inflammatory mediators leads to faulty immune programming that persists in postnatal life [6]. Maternal IL-6 unbalance during pregnancy accounts for impaired functional connectivity between different neonatal brain regions until 2 years of age [7]. The bidirectional diffusion of IL-6 between maternal and fetal circulation makes it a key player in MIA. It elicits prenatal stress in the form of increased hippocampal cytokine load and immunoreactive microglial cells in rodents [8]. MIA also affects the epigenetic memory of microglia, which act as the immune cells of the central nervous system (CNS), thus modifying their response to various inflammatory stimuli. The uncontrolled microglial activation induces neuroinflammation which underlies several brain diseases [9]. In ASD, microglial dysfunction and chronic neuroinflammation diminish the brain connectivity via synapse destruction [10]. Therefore, molecules that regulate microglial activation play key roles in ASD pathogenesis.

Humans have approximately 50 distinct chemotactic cytokines known as chemokines. These small proteins and their receptors control immune cell migration and inflammation [11]. A unique subclass of chemokine was identified in the late 1990s which included a single member called

fractalkine (FKN), or CX3C motif chemokine ligand 1 (CX3CL1). It exists either as a membrane-bound protein or a soluble 95 kDa glycoprotein. Unlike other chemokines, FKN is synthesized as a transmembrane polypeptide chain with 373 amino acids. It consists of N-terminal domain, mucin-rich stalk, transmembrane α helix and cytoplasmic tail [12]. The chemokine domain is located at the end of the membrane-anchored mucin stalk. This localization enables FKN to additionally act as a potent adhesion molecule for cells expressing CX3CR1. The soluble FKN form is enzymatically cleaved from the cell surface by metalloproteases either via constitutive or cytokine-induced shedding. Once the soluble FKN is released, it initiates chemotaxis for CX3CR1-bearing cells (for example: monocytes, microglia and natural killer cells) to regulate inflammatory response [13]. In microglia, the ligation of the exogenous CX3CL1, and not the membrane-bound isoform, allows for the internalization of the peptide to the cytoplasm. Therefore, the availability of soluble FKN is crucial to dampen microglial activation and provide neuroprotection [14]. In activated microglia, low concentrations of soluble FKN were sufficient to reduce the release of induced IL-6 in rat models. Similar studies reported the role of FKN signaling in downregulation of pro-inflammatory genes such as tumor necrosis factor-α, IL-1β and IL-6 [15].

Besides its anti-inflammatory role, FKN/CX3CR1 axis support neuronal maturation, microglial pruning and functional development of synapses during early postnatal life [16]. Therefore, a growing body of research investigates the therapeutic potential of FKN. For instance, the intravitreal injection of soluble FKN-expressing vectors reduces microgliosis, protects optic nerves and ultimately prevents diabetic retinopathy [17]. Similarly, administration of exogenous FKN promoted microglial transformation to M2 phenotype and partially restored spatial memory in mice with brain injury [18]. In human studies, various FKN and/or CX3CR1 abnormalities were reported in neurological disorders [19]. A specific CX3CR1 variant that interferes with FKN signaling was associated with both schizophrenia and ASD phenotypes [20]. Patients with schizophrenia also have diminished FKN levels in blood and prefrontal cortex [21, 22]. The circulating levels of the chemokine were lower in a group of individuals with attention deficit and hyperactivity disorder compared to their healthy family members [23]. Interestingly, postmortem brains of patients with Parkinson disease showed downregulation of neuronal FKN and increased microglial cell numbers in the substantia nigra which mostly indicates neuroinflammation [24].

The evident implication of IL-6 makes it a reliable candidate of pro-inflammation in ASD research. At the gene level, IL-6 polymorphisms have recently showed correlation with autistic features among Egyptian children [25]. On the other hand, the studies that evaluated FKN circulating levels among ASD populations to date are limited and mostly inconclusive [26, 27]. Also, it is not yet clear whether this chemokine modulates the secretion of key proinflammatory cytokines in ASD, particularly IL-6. Therefore, the present study evaluated the plasma levels of FKN in a cohort of Egyptian autistic children and its associations with IL-6 plasma concentrations and ASD clinical manifestations. It also assessed the validity of both immune molecules as blood-based biomarkers for ASD.

**Methods**

The study was approved by the ethics committee and written informed consents were obtained from the parents or legal guardians of all participants.

This pilot study was intended as a preliminary investigation. Therefore, no prior sample size calculations were done. Forty cases diagnosed with ASD were selected from patients who visit the outpatient clinic at the site of study. Their ages range from 5 to 12 years old. They were diagnosed according to the revised fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5-TR) [28]. The exclusion criteria omitted children with associated syndromes, other pervasive developmental disorders, history of traumatic brain injury, acute illness, significant sensory impairments or neurodegenerative disorders. For comparison, 40 age and sex-matched typically-developing children (TDC) were included as controls. All participants were free of immunotherapeutic drugs and antibiotics prior to the study.

Our subjects underwent standardized and detailed neurological examination neurological examination. All ASD participants underwent language assessment using Receptive Expressive Arabic Language (REAL) Scale [29]. REAL Scale is a tool to evaluate language skills among Arabic-speaking children aged 5-12 years 11 months old. The test was administered in approximately 90 minutes with 10 minutes break interval if needed. The raw receptive, expressive and total language scores were calculated and scaled. A percentile rank of $\geq$70 indicates acceptable Arabic language development. Meanwhile, 36 out of the total ASD cohort were further classified into mild-to-moderate and severe cases based on Childhood Autism Rating Scale (CARS) scores [30]. A score range between 30-36.5 implies mild or moderate autistic symptoms, whereas 37-60

indicates severe autism. Sensory profile 2 (SP-2) was also performed to identify impairments in the children's sensory processing [31]. The questionnaire was conducted and the raw scores were assigned to a quadrant to determine the sensory subtype of each child. The cut-off score of each subtype was as follows: seeker= 48, avoider= 47, sensor= 43 and bystander= 44. Additionally, six sensory modalities were assessed: auditory, visual, oral, touch, movement and body position.

Two milliliters of intravenous blood samples were collected from each subject in EDTA-coated vacutainers. To separate plasma, the samples were immediately centrifuged at 1000 x g at 2-8°C for 15 minutes. The plasma samples were stored as aliquots at –80°C until the day of assay.

Sandwich ELISA experiments were done using commercial 96-test kits (ELK Biotechnology, USA) specific for human CX3CL1 (Catalog number: ELK1422) and IL-6 (Catalog number: ELK1156). Intra and inter-assay precision of both kits were CV%< 8% and CV%< 10% respectively. The manufacturer's protocol was followed and optical density (OD) readings were measured at wavelength 450 nm using Multiskan$^{TM}$ FC microplate photometer (Thermo Scientific, Singapore). The unknown concentrations were interpolated from the resultant standard curves.

GraphPad Prism software (Version 6) was used for all statistical analyses. Shapiro-Wilk test was performed to check the distribution of quantitative datasets. Accordingly, unpaired student *t*-test and Mann-Whitney U were used to compare the parametric and non-parametric data respectively. Qualitative data were compared using chi-square test. Receiver operating characteristic (ROC) curve was plotted to evaluate the diagnostic accuracy of FKN and IL-6. The statistical significance was determined at level $p < 0.05$.

**Results**

Both study groups were matching with respect to age and sex percentage (Table 1). As shown in Table 2, about one third of children with ASD showed severe symptoms as indicated by the CARS scores. The REAL percentile rank of all ASD participants falls in the bottom quartile range <25 which reflects severely delayed Arabic language development. Over 55% of the cases showed either severe hyper- or hyper sensory sensitivity.

**Table 1. Demographic characteristics of all participants.** Numerical and categorical data are represented as mean± standard deviation and count (percentage) respectively.

| | TDC (n=40) | ASD (n= 40) | *P*-value |
|---|---|---|---|
| **Age (years)** | 7.67± 1.58 | 6.96± 2.28 | 0.1185 |
| **Gender** | | | |
| Males | 29 (72.5%) | 28 (70%) | 0.8049 |
| Females | 11 (27.5%) | 12 (30%) | |

**ASD:** autism spectrum disorder; TDC: typically-developing children; *p*-value: probability value.

**Table 2. Clinical characteristics of participants with ASD.**

| | Mean± SD or N (%) |
|---|---|
| **CARS (n= 36)** | |
| Mild to moderate | 23 (63.9%) |
| Severe | 13 (36.1%) |
| **REAL Scale (n=40)** | |
| Raw receptive language score | 91.43± 19.98 |
| Raw expressive language score | 112.48± 50.62 |
| Total language score | 203.9± 62.34 |
| **Sensory subtypes (n=36)** | |
| Seeker | 7 (19.4%) |
| Avoider | 9 (25%) |
| Sensor | 11 (30.6%) |
| Bystander | 9 (25%) |
| **Sensory modalities (n=36)** | |
| Auditory | 17.39± 7.40 |
| Visual | 17.08± 4.73 |
| Touch | 19.47± 6.27 |
| Movement | 22.50± 5.83 |
| Body position | 22.33± 8.93 |
| Oral | 28.33± 11.20 |

ASD: autism spectrum disorder; CARS: Childhood autism rating scale; N: number; REAL: Receptive Expressive Arabic Language; SD: standard deviation.

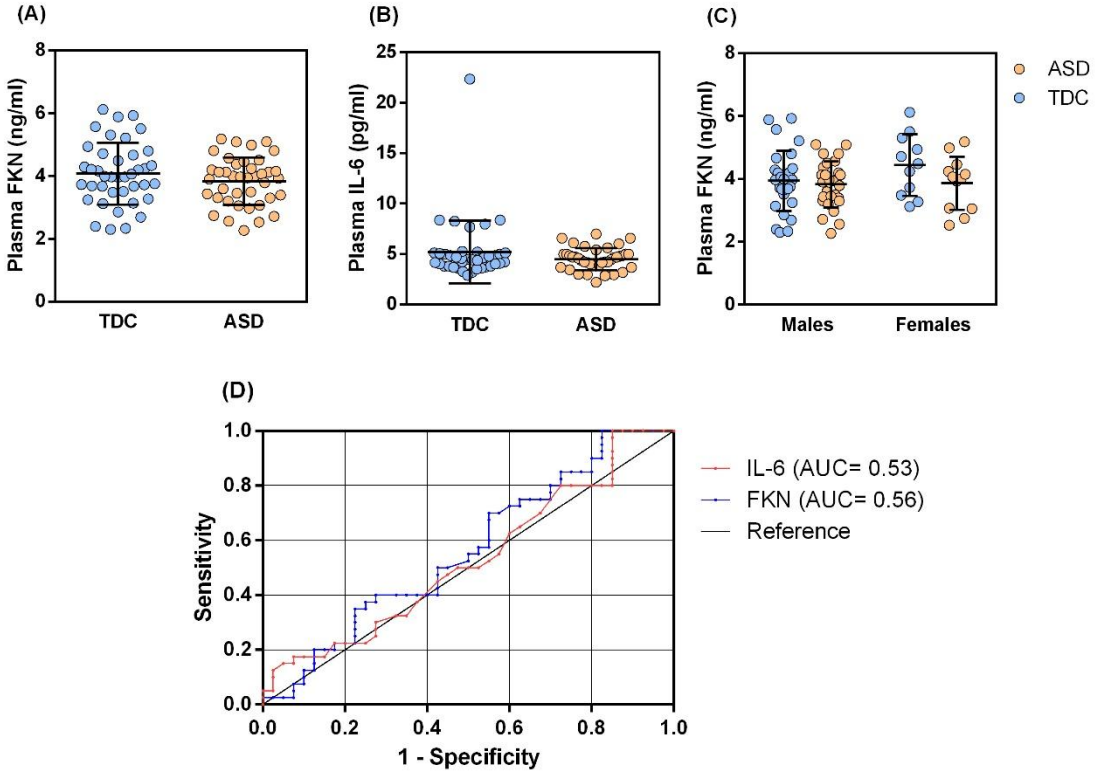



**Figure 1. (A)** the plasma concentration of FKN in ASD children (3.84± 0.76) and TDC (4.08± 0.98) showed negligible difference ($p$= 0.22). Similarly, **(B)** plasma IL-6 showed comparable levels ($p$= 0.65) in both ASD (4.50± 1.10) and control (5.21± 3.12) groups, **(C)** Sex stratification showed non-significant difference between TDC and ASD among males ($p$= 0.62) and females ($p$= 0.14) with respect to FKN circulating levels, **(D)** ROC curves of both FKN (AUC= 0.56, 95% CI= 0.44-0.69, $p$= 0.33) and IL-6 (AUC= 0.53, 95% CI= 0.40- 0.66, $p$= 0.65) showed poor and statistically non-significant discriminative potential. ASD: autism spectrum disorder; AUC: area under the curve, CI: confidence interval, FKN: fractalkine, IL-6: interleukin-6, TDC: typically developing children, ROC: receiver operating characteristic.

**Table 3. Spearman correlation of fractalkine plasma levels with the biochemical and clinical parameters of ASD.**

| | ***R*** | ***P*-value** |
|---|---|---|
| **IL-6** | -0.1901 | 0.2400 |
| **Age** | -0.0061 | 0.9719 |
| **CARS** | 0.1177 | 0.4941 |

| | | |
|---|---|---|
| **REAL** | | |
| Raw receptive language score | 0.1849 | 0.2534 |
| Raw expressive language score | 0.08928 | 0.5838 |
| Total language score | 0.1572 | 0.3327 |
| **Sensory profile** | | |
| Auditory | 0.2199 | 0.2043 |
| Visual | -0.1769 | 0.3095 |
| Touch | -0.1748 | 0.3152 |
| Movement | 0.0205 | 0.9069 |
| Body position | 0.1099 | 0.5296 |
| Oral | -0.1339 | 0.4433 |

ASD: autism spectrum disorder; CARS: Childhood autism rating scale; IL-6: interleukin-6; *p*-value: probability value; r: correlation coefficient; REAL: Receptive Expressive Arabic Language.

**Discussion**

ASD is a common neurodevelopmental condition with prevalence rates ranging from 0.7 to 3% across different studies [32]. In Egypt, 33.6% of children with developmental disabilities are diagnosed with ASD. Such expansion of ASD incidence necessitates the development of effective personalized intervention [33]. The identification of ASD biomarkers enables targeted management given the heterogeneity of the disorder. It also allows for ASD detection at an early age when behavioral-based diagnosis is still not possible [34]. A wide range of blood-based biomarkers have been suggested which involves immune and inflammation-associated proteins. These immunological markers can also improve our understanding of ASD etiology and neuropathology [35].

According to Metwally and her team, 3.3% of Egyptian children are at high risk of ASD. They reported 2.75 folds increased odds among children who were born to mothers with health problems during pregnancy [36]. Maternal immune diseases represent prominent ASD determinants. Different studies have linked the altered cytokine profile in maternal serum, amnion and neonatal blood samples to the likelihood of autism [37]. ASD diagnosis in 7-12% of affected children is attributed to maternal autoantibodies [38]. IL-6 plays a pivotal role in MIA-associated ASD. The

inhibition of IL-6 alleviated social deficits among mice offspring [39]. Animal studies also showed that MIA-derived placental IL-6 initiate downstream signaling and induce neuropathology in fetal hindbrain. Moreover, maternal microbiome dysbiosis during pregnancy can lead to microglial activation via the CX3CR1/FKN axis and ultimately induce autism-like behaviors among offspring [40]. Many lines of evidence correlate chemokines and chemokine receptors with ASD pathogenesis [11]. Therefore, this pilot study aimed to evaluate the plasma levels of FKN in 40 Egyptian children diagnosed with ASD in comparison to 40 typically-developing children. Additionally, the association between the soluble FKN availability and IL-6 circulating levels as well as ASD phenotypes was assessed.

The current study revealed that children with ASD and their neurotypical counterparts have comparable levels of plasma FKN even when the subjects were categorized by gender. In agreement with our finding, the plasma cytokine profile in a group of Chinese children showed similar results [41]. However, Manzardo and co-authors found lower circulating levels of FKN in plasma samples of autistic children compared to unrelated controls with familial ASD history. They also reported sex-specific differences, where males exhibited significantly decreased FKN levels relative to females [42]. Similarly, diminished serum FKN levels were reported during early childhood (24- 72 months) among a Turkish autistic cohort [43]. This suggests that FKN may contribute to ASD progression during critical stages of neurodevelopment. Therefore, peripheral FKN availability might vary based on age group. A previous study quantified several immune mediators, including FKN, in serum and found comparable levels among children with and without ASD. However, the same study reported lack of association between FKN levels in serum and cerebrospinal fluid (CSF) among ASD patients. This indicates that the peripheral levels of immune markers do not necessarily mirror the CNS milieu. Moreover, the longitudinal design of that study revealed intra-individual fluctuations of some cytokines over time. Therefore, a single measurement of immunological analytes is unreliable indicator of the inflammatory status [44].

The pro-inflammatory cytokine profile among different ASD populations showed that blood levels of IL-6 is commonly elevated [26]. However, we did not find significant difference between autistic and typically developing children with respect to peripheral IL-6 concentrations. Similarly, another group of Arab children with and without ASD (2-4 years old), which involved Egyptian participants, exhibited comparable levels of circulating IL-6. On the other hand, a recent study

reported reduced IL-6 levels in plasma of Korean children with ASD and average age 7.1 [45]. These inconsistent results suggest that cytokine levels are possibly influenced by many variables such as age, ethnicity and sample size. Also, the nature of the control group can alter the comparison [41]. For instance, a cohort of adult individuals with ASD showed elevated IL-6 levels in bloodstream when compared to healthy unrelated controls; whereas, they had similar IL-6 concentrations in comparison to their first-degree relatives [46]. Unlike IL-6, the diagnostic value of FKN for ASD has not been evaluated to date. Based on ROC curve, our study ruled out both FKN and IL-6 as ASD blood-based markers due to their poor AUC. However, this finding should be interpreted cautiously because the peripheral immune profile does not always match that of the CNS as mentioned earlier. The CSF acts both as a barrier that guards the brain environment and indicator of neuroinflammation. Therefore, the pathogenesis and progression of ASD can be better monitored through the endogenous neuro-immune system [47].

During postnatal stage through adulthood, FKN contributes to the excitatory synaptic maturation, plasticity and activity in healthy brain. It, therefore, influences learning, memory and other behavioral outcomes [48]. We found no significant correlation between circulating FKN abundance and ASD phenotype with respect to symptom severity, language performance and sensory profile. The FKN levels also showed no age-dependent variations. This again aligns with the findings of Hu and colleagues who examined Chinese children with ASD [41]. It also agrees with another study that found no association between FKN serum levels and ASD severity among Turkish children [43]. Since social behavior is regulated by FKN neuronal expression in specific forebrain regions [40], circulating FKN availability may not associate with ASD symptoms. We also found that FKN plasma levels were irrelevant to that of IL-6 among children with ASD. This can be attributed to the spatiotemporal dynamics of cytokines and chemokines. An animal experiment showed that FKN intracerebroventricular administration significantly controlled the overproduction of IL-6 in hypothalamus [49]. Such effect may indicate that the homeostatic role of FKN is predominantly evident in the CNS microenvironment. Also, IL-6 signaling and tuning is controlled by various factors that are not limited to chemokine production [50]. For instance, the presence of specific IL-6 gene polymorphisms in ASD children modulates the cytokine levels independent of other confounders [25].

**Conclusion**

To our knowledge, this study is the first to investigate FKN levels and its implications with ASD manifestations and immune dysregulation among Egyptian children. Overall, it does not refute the neuro-immune hypothesis of autism; but rather re-considers the adequacy of peripheral cytokine status as neuroinflammation indicator. However, it has two main limitations: small sample size and the cross-sectional design. Although the cohort size is acceptable for a pilot study, it only gives preliminary estimates and requires subsequent adequately powered studies. Also, the single measurement of immunological markers could not take their fluctuations over time into account. Therefore, large scale investigations with repeated evaluation of the immune profile are warranted to validate our results.

**List of abbreviations**

ASD: Autism spectrum disorder

AUC: Area under the curve

CARS: Childhood autism rating scale

CI: Confidence interval

CSF: Cerebrospinal fluid

CSN: Central nervous system

CV: Coefficient of variation

CX3CL1: CX3C motif chemokine ligand 1

CX3CR1: CX3C motif chemokine receptor 1

DSM-5-TR: Diagnostic and Statistical Manual of Mental Disorders, fifth edition, text revision

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

FKN: Fractalkine

IL-6: Interleukin-6

MIA: Maternal immune activation

N: Number

OD: Optical density

*P*-value: Probability value

R: Correlation coefficient

REAL: Receptive expressive Arabic language.

ROC: Receiver operating characteristic

SD: Standard deviation

SP-2: Sensory profile 2

TDC: Typically-developing children

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