Evaluation of Interleukin-6 and Tumor Necrosis Factor-Alpha as Inflammatory Biomarkers in Iraqi Children with Autism Spectrum Disorder

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Abstract: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social communication and repetitive behaviors. Studies have indicated that immune system dysregulation and chronic neuroinflammation play a significant role in the development of the disorder. This study aimed to investigate the potential use of inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), as biomarkers for predicting the diagnosis of ASD. The current study included 88 children, 50 of whom had ASD and 38 who were typically developing and healthy, all aged 3-13 years. Serum levels of IL-6 and TNF-α were measured in both groups, and statistical analyses were performed to assess differences and correlations between the two groups. Diagnostic accuracy was assessed using the ROC curve. Children with ASD showed higher levels of IL-6. (194.21 ± 81.89 pg/mL) and TNF-α (360.74 ± 134.54 pg/mL) compared with controls (75.60 ± 23.56 pg/mL and 103.80 ± 50.57 pg/mL; $p \leq 0.05$). A moderate positive correlation existed between IL-6 and TNF-α ($r = 0.395$, $p \leq 0.01$), and gestational age correlated strongly with birth weight ($r = 0.568$, $p \leq 0.001$). ROC curve analysis showed high performance: Interleukin-6 exhibited 90% sensitivity and 98% specificity at a concentration of 123.80 pg/ml, while tumor necrosis factor-alpha (TNF-α) showed 90% sensitivity and 100% specificity at a concentration of 228.92 pg/ml ($p \leq 0.001$). These results indicate a strong association between elevated levels of both interleukin-6 and TNF-α and autism spectrum disorder, supporting the role of inflammatory pathways and highlighting their potential use in the early detection and appropriate treatment of autism spectrum disorder.

1 INTRODUCTION

Autism Spectrum Disorder ASD is a group of complexes, multifactorial neurodevelopmental disorders characterized by a wide variety of symptoms. Behavioral, neurological, and psychological symptoms include difficulties in communication and social interaction, specific interests, and repetitive behaviors [1]. It is a heterogeneous disorder with a range of clinical manifestations that lead to social challenges [2]. The disorder presents three levels of impairment: mild, moderate, and severe [3]. Current diagnostic classification including the International Classification of diseases, Eleven Revision (ICD-11) Classify autism as a single spectrum disorder characterized by deficits in social interaction and social communication, accompanied by restricted and repetitive patterns of behavior, interests, or activities. The classification reflects the broad clinical heterogeneity of the disorder and recognize varying levels of support needs among individuals [4], [5]. Characteristics of this disorder are identified during early childhood, but a diagnosis of autism is often not made until later. The skills and needs of individuals with autism vary and can change over time. Some can live independently, while others face significant challenges and require lifelong support and care [5]. Variation in the prevalence of this disorder has been observed worldwide, and according to recent reports, the rate has ranged between 1% and 2%, with a continuous increase in the incidence rate, unaffected by geographical region or ethnic and cultural factors. Males are affected more than females, at a rate of two to five times [6]. As one of the diseases that affect children, this disorder has emerged as a major cause of disability among those with mental and neurological diseases or problems, making it a challenge to public health

and affecting quality of life [7]. Immune regulation abnormalities have been observed in individuals with autism spectrum disorder, particularly changes in cytokine levels. However, identifying a direct cause and link remains a challenge [8]. Studies and reviews have indicated that The role of cytokines in neuroinflammation and synaptic dysfunction, which in turn leads to the appearance of behavioral symptoms reflecting the disorder [9]. Interest in the disturbance of interleukins and cytokines levels has increased, given their ease of measurement, their ability to directly affect the central nervous system, and because they are a source of information about the immune system, they have been considered biomarkers [10], [11]. Pro-inflammatory and anti-inflammatory cytokines can interact with immune cells and neuroendocrine system transmitters such as neurotransmitters and hormones [12]. Pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor regulate the immune response and act as signaling molecules capable of influencing brain and behavior through neural and immune pathways [13]. IL-6 is a crucial link between innate and adaptive immunity, regulating lymphocyte activity, neurodevelopment, and the integrity of the blood-brain barrier [8], [14]. Disruption of interleukin-6 expression is associated with abnormal changes in the length and shape of neuronal dendrites and their distribution pattern. The increase of extra cells in the brain leads to an imbalance in neural circuits [15] Similarly, tumor necrosis factor-alpha (TNF-α) is another pro-inflammatory cytokine that plays a crucial role in regulating the inflammatory response, neurodevelopment, and synaptic dilation. Therefore, its disrupted secretion leads to impaired synaptic dilation, causing either hyperexcitability or hypoexcitability of neural circuits. This is believed to contribute to altered neural connectivity and impaired neuronal communication observed in autism spectrum disorder (ASD) [16], [17]. Both IL-6 and TNF-α influence brain development and inflammatory pathways. However, the mechanism by which they alter neurodevelopment remains unclear and requires further investigation [18]. Cytokine levels may be affected by different clinical and biological factors resulting in variations in their circulating levels among individuals [19]. Given that ASD presents a significant public health challenge, it is essential to elucidate the modifiable risk factors and physiological mechanisms of the disorder. This study aims to evaluate the relationship of pro-inflammatory cytokines (interleukin-6 and tumor necrosis factor-alpha) to autism spectrum disorder, focusing on their relationship to gestational age at birth and birth weight, and whether they can be used as biomarkers to predict pregnancy complications such as the risk of premature birth, low birth weight, and the likelihood of the child developing autism spectrum disorder.

2 MATERIALS AND METHODS

2.1 Blood Sample Collection

The current study included 88 participants aged 3-13 years, collected between September 2025 and January 2026. They were divided into two groups. The first group consisted of 50 children diagnosed with autism spectrum disorder (40 males and 10 females) from autism centers in Diyala and another group from the National Autism Center/Child Protection Teaching Hospital at Baghdad Medical City. All patients had been diagnosed with autism spectrum disorder by neurologists and psychiatrists. The second group consisted of 38 typically developing children (20 males and 18 females) who served as the control group. Three milliliters of blood were collected from all participants and placed in test tubes designed for serum separation. The blood samples were allowed to coagulate at room temperature for 15 minutes and then centrifuged for 10 minutes at 3500 rpm to obtain the serum. The serum was collected in 1.5 milliliters Eppendorf tubes for use in the Measurement of interleukin-6 and tumor necrosis factor-alpha.

2.2 Cytokine Measurement

Interleukin-6 and tumor necrosis factor-alpha (TNF-α) levels were measured using sandwich ELISA (enzyme-linked immunosorbent assay) Were determined using commercial Elisa kits (Whan USCN Business co, china, catalog number: IL-6 USEA079Hu, TNF-a USEA133Hu The established steps for each kit were followed and concentration of each biomarker was measured.

2.3 Ethical Considerations and Informed Consent

Before blood samples were taken from all participants in this study, the parents of all participants were informed of the study's objectives and purpose. Written consent was then obtained from each parent before the child's participation. The

study adhered strictly to the ethical principles of scientific research to maintain the confidentiality of all participant information.

Data were collected using a questionnaire designed according to the study's methodology. The questionnaire comprised five sections: demographic information, perinatal history, developmental and behavioral assessment, family medical history, and environmental and lifestyle factors. The questionnaire was distributed to study participants through face-to-face interviews with their caregivers.

2.4 Statistical Analysis

Statistical analysis of the data from the current study was performed using both SPSS (version 22) and GraphPad Prism (version 6). Continuous variables were represented as mean ± standard deviation. For comparisons between the two study groups, the independent samples t-test was used, while one-way analysis of variance (ANOVA) was used for comparisons between variables involving more than two groups. Pearson's correlation coefficient was calculated to assess the strength and direction of the relationship between the study variables. Additionally, the diagnostic accuracy of the immunological markers was evaluated using receiver operating characteristic (ROC) curve analysis, which includes both sensitivity and specificity estimates.

3 RESULTS AND DISCUSSIONS

Accumulating evidence suggests that disruptions in immune system regulation may contribute to the pathogenesis of neurodevelopmental disorders such as autism spectrum disorder (ASD), potentially by affecting synaptic plasticity and neural connectivity [15], [20]. Additionally, the emergence and severity of autistic traits have been associated with abnormal or heightened immune responses [20]. although current findings remain largely correlative and do not establish causality. [21]. Therefore, detailed mechanistic studies are warranted to clarify how immune dysregulation may influence neurodevelopment in ASD. In line with these observations, our data (Table 1) reveal highly significant differences between children with ASD and age-matched healthy controls, with p-values below 0.001 for both measured parameters. These results highlight substantial statistical differences between groups and underscore a potential association between the investigated immunological markers and ASD.

The data presented in Table 1 reveal a significant elevation of circulating pro-inflammatory cytokines in children diagnosed with autism spectrum disorder (ASD). Specifically, serum concentrations of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were substantially higher in the ASD cohort (194.21 ± 81.89 pg/mL and 360.74 ± 134.54 pg/mL, respectively) relative to age-matched controls (75.60 ± 23.56 pg/mL and 103.80 ± 50.57 pg/mL, respectively). Elevated IL-6 levels indicate its important role in the inflammatory response. The secretion of this interleukin-6 increases in immune cells, such as T cells and phagocytes, in response to inflammatory signals during chronic inflammatory or autoimmune diseases. Increased secretion reflects an active inflammatory state. Several studies have reported elevated cytokine levels in the blood, cerebrospinal fluid, and brain [15]. A recent study suggests that elevated IL-6 levels in autistic patients may be related to this. It raises the level of inflammation, allowing peripheral cytokines to cross the blood-brain barrier and activate cells in the nervous system called microglia. When these cells malfunction, it negatively affects synapses, impacting nerve signal transmission and contributing to the development of a disorder characterized by an imbalance between inhibitory and excitatory responses [22].

Furthermore, interleukin-6 plays a pivotal role in neuronal development and synaptic plasticity in the brain. Increased expression of interleukin-6 in granulocytes can impair cell adhesion and migration, leading to disruptions in excitatory and inhibitory neural networks. The findings of this study are consistent with those of Ashwood et al., who demonstrated significantly higher plasma concentrations of interleukin-6 in individuals with autism spectrum disorder compared to healthy individuals [23].

The results in Table 1 show a significant increase in tumor necrosis factor-alpha (TNF-α) levels in the group of individuals with autism spectrum disorder (360.74 ± 134.54 pg/ml) compared to the healthy control group (103.80 ± 50.57 pg/ml), indicating a statistically significant difference ($p \leq 0.001$). These findings are consistent with previous studies that have shown elevated TNF-α plasma concentrations in individuals with autism spectrum disorder, with these levels positively correlated with symptom severity. These studies support the idea of an underlying neuroinflammatory condition and suggest possible glial cell dysfunction in individuals with autism spectrum disorder [24].

Table 1: Comparison of concentration levels of both the cytokinesis protein interleukin-6 and tumor necrosis factor-alpha between autism spectrum patients and healthy control groups.

Study Groups		N	Mean	Std. Deviation	P value
IL-6	Patient	50	194.21	81.89	p<0.001***
	Control	38	75.60	23.56	
TNF-a	Patient	50	360.74	134.54	p<0.001***
	Control	38	103.80	50.57	

Furthermore, some studies have shown elevated tumor necrosis factor-alpha (TNF-α) levels in individuals with autism spectrum disorder, along with a positive correlation with other measured biomarkers. This elevation is linked to the severity of autism symptoms, suggesting a role for neuroimmune signaling pathways in the physiology of autism [25]. Other studies have examined both markers (IL-6 and TNF-α), with some results consistent with our findings and others showing some inconsistencies. Some studies have attributed elevated TNF-α to the role of B cells in weakened immunity and immune dysregulation through the expression of pro- and anti-inflammatory cytokines [26]. This immune dysregulation causes excessive stimulation of microglia and astrocytes, particularly in the fetus, leading to abnormal synaptic pruning and resulting in abnormal neural plasticity and connections [27], [28]. A study conducted in Iraq indicated a significant increase in the concentration of tumor necrosis factor-alpha (TNF-α) in patients with autism spectrum disorder (ASD) without a similar increase in the level of interleukin-6 (IL-6). This confirms the pivotal role of microglia as a major source of TNF-α in neuroinflammatory processes [29]. The results of the current study are consistent with other findings indicating elevated levels of both markers in the plasma and serum of individuals with autism spectrum disorder compared to the control group. These studies have identified monocytes, neutrophils, and CD4+ T cells as potential sources of these elevated cytokines [30], [31]. Figure 1. compares serum levels of interleukin-6 and TNF-α in patients with ASD and healthy individuals.

The results of the current study showed a moderate positive correlation between interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) levels ($r = 0.395$, $p < 0.01$). Furthermore, a strong positive correlation was observed between gestational age at birth (in weeks) and birth weight ($r = 0.568$, $p < 0.001$), as shown in Table 2.

The data summarized in the table indicate that no statistically significant relationship was observed between circulating levels of interleukin-6 (IL-6) or tumor necrosis factor-alpha (TNF-α) and the timing of motor (walking) or language (speech) development in children. Therefore, a positive correlation was observed between mean interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) concentrations ($r = 0.395$, $p < 0.01$), indicating a systematic increase in the concentrations of these two cellular motility agents.

These findings are consistent with recent reports showing elevated IL-6 levels in male children with autism spectrum disorder, while TNF-α concentrations appear to be higher in younger children with the disorder, particularly those under five years of age, compared to older individuals and typically developing children.

It is well established that increased production of pro-inflammatory cytokines contributes to neuro-inflammatory pathways involved in both the initiation and development of autism spectrum disorder [32], [33]. In addition, maternal immune system activation is associated with increased levels of cytokines in the body, such as interleukin-6, tumor necrosis factor-alpha, and interleukin-17, which have the potential to cross the placental barrier and influence fetal brain development [34]. In this regard, Reisinger and colleagues demonstrated that maternal cellular mechanisms are translocated across the placenta during immune system activation and enter the fetal environment. Because the fetal immune system is poorly regulated, repeated exposure to inflammation can disrupt the balance of cellular mechanisms and negatively impact neurodevelopmental processes. Existing research also highlights the mechanistic role of perinatal inflammation in shaping neurodevelopmental outcomes [35]. In another study reported that increased inflammatory activity, leading to elevated levels of interleukin-6 and tumor necrosis factor-alpha, was more pronounced in infants with low birth weight, while preterm birth was associated with a higher likelihood of later neurodevelopmental disorders. These findings

suggest that systemic inflammation is a biological pathway during the perinatal period and may contribute to long-term neurodevelopmental changes, although it was not specific to autism spectrum disorder [36]. To corroborate this finding, another study examining inflammatory markers in mothers and newborns demonstrated that infants with low birth weight and those who were premature had elevated levels of interleukin-6 and tumor necrosis factor-alpha during the second and third trimesters of pregnancy and were at increased.

Risk of developmental delays at 24 months of age [37]. Maternal health during pregnancy plays a significant role in determining the child's developmental outcomes, supporting the maternal immune system activation hypothesis, which suggests that exposure to intrauterine infections may impair normal fetal brain development. A substantial body of research in humans suggests a link between maternal inflammation and an increased risk of neurodevelopmental disorders [38]. Multiple factors may contribute to maternal inflammation, including obesity, asthma, autoimmune diseases, infectious diseases, and psychosocial stressors, all of which may interact and influence the uterine environment. These factors, in combination, may increase the likelihood of adverse neurodevelopmental outcomes in offspring, including autism spectrum disorder. Furthermore, maternal inflammatory responses during pregnancy have been linked to structural and functional changes in certain brain regions, including those responsible for cognition and emotional regulation. Elevated levels of pro-inflammatory cytokines are thought to impair cortical maturation and neural network formation, negatively impacting brain development pathways [39].

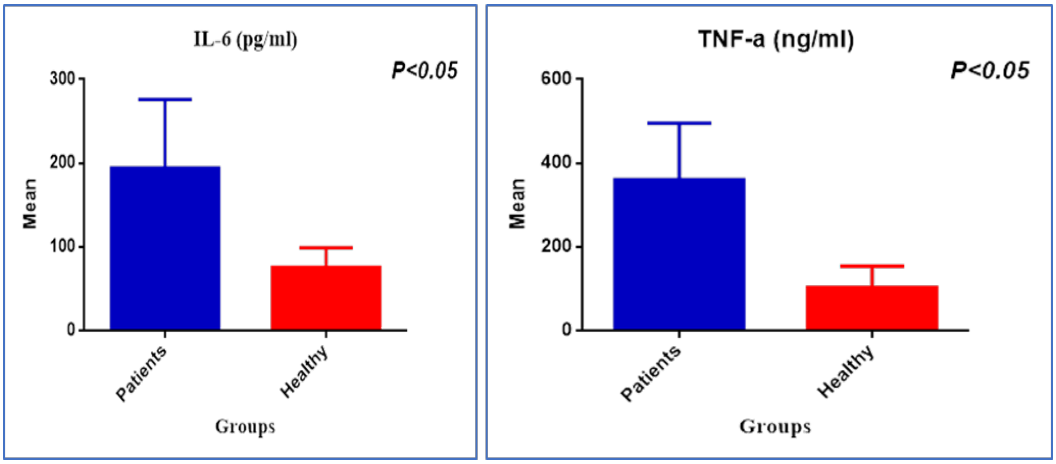

Figure 1: Comparison of serum cytokine levels of both interleukin-6 and tumor necrosis factor-alpha between autism spectrum patients and healthy individuals.

Table 2: Statistical analysis of the correlation between the study indicators.

		IL-6	Child's age	Gestational age at birth (weeks)	Child's age of speech onset in months	Child's age of walking onset in months
IL-6	Pearson coefficient	1	.109	.075	.048	.085
	p value		.452	.606	.739	.556
TNF-a	Pearson coefficient	.395**	.092	-.063	.157	-.155
	p value	<0.01	.524	.665	.275	.282
Maternal age at conception	Pearson coefficient	-.013	.095	.044	.219	.033
	p value	.926	.514	.762	.126	.823
Birth weight	Pearson coefficient	-.088	.097	.568**	.137	-.139
	p value	.544	.501	<0.001	.343	.335
Age of walking onset (months)	Pearson coefficient	.085	.246	-.079	.049	1
	p value	.556	.084	.584	.733	
**. Correlation is significant at the 0.01 level (2-tailed).						

Table 3: Analysis of the diagnostic efficiency of immune biomarkers (IL-6 and TNF-α) in autism spectrum disorder based on auc, threshold values, sensitivity, and specificity.

Variables	*AUC	Std. Error	P value	cut off	% Sensitivity	% Specificity
IL-6	917.	036.	***p<0.001	123.80	90%	98%
TNF-a	925.	035.	***p<0.001	228.92	90%	100%
AUC*= area under curve						

A successful pregnancy depends on a delicate balance between maternal immune tolerance and adequate fetal protection. Adverse delivery outcomes have been associated with disturbances in maternal inflammatory signaling during pregnancy, including low birth weight. For example, low birth weight has been associated with elevated levels of interleukin-6 (IL-6) [40]. Elevated levels of circulating cytokines such as interleukin-6 and tumor necrosis factor-alpha (TNF-α) can activate the maternal immune system, whether due to infectious agents or non-infectious conditions. These cytokines can cross the placental barrier and disrupt neurodevelopmental events, including neuronal migration, interneuron differentiation, and neural progenitor cell proliferation. These alterations may negatively affect neurogenesis and cortical organization, ultimately increasing susceptibility to neurodevelopmental disorders, including autism spectrum disorder [41]. Regarding the correlation between cytokine levels and perinatal parameters specifically, the results of the current study are inconsistent with those of some other studies that reported significant associations. Some studies that assessed the role of a wide range of inflammatory markers showed no statistically significant differences in most cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), while interleukin-4 (IL-4) showed variable levels in mothers during mid-pregnancy in pregnancies associated with autism spectrum disorder. These discrepancies underscore the multifactorial nature of the immune system's role in autism spectrum disorder and highlight the need for further research on these cytokines [40], [41]. Finally, receiver operating characteristic (ROC) curve analysis showed that interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) possess strong diagnostic power for autism spectrum disorder (ASD). At optimal borderline values of 123.80 and 228.92, respectively, both IL-6 and TNF-α achieved a sensitivity of 90%, while the specificity was 98% for IL-6 and 100% for TNF-α. These results indicate very strong diagnostic performance, supported by statistically significant differences ($p < 0.001$), as shown in Table 3.

4 CONCLUSIONS

The overproduction of pro-inflammatory cytokines disrupts the body's immune balance and leads to disease development, as these cytokines play a crucial role in regulating immune and inflammatory processes in humans. The results of this study showed a significant increase in serum concentrations of both interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in children with autism spectrum disorder compared to healthy children, suggesting an ongoing inflammatory state that may be linked to the clinical characteristics.

Furthermore, no statistically significant correlation was found between either interleukin-6 or tumor necrosis factor-alpha (TNF-α) and gestational age or birth weight (all $|r| \leq 0.097$, $p > 0.5$; Table 2), indicating that, within this cohort, circulating levels of these cytokines are not directly associated with perinatal growth parameters. In contrast, no statistically significant relationship was found between cytokine levels and the time to motor and language development.

The data from this study may be useful as future biomarkers for developing treatments or diagnostic methods for the early detection of signs of the disorder. However, the results cannot be generalized due to the small sample size, its limitation to a specific age group, and the broad scope of the sample without considering the cultural, economic, and environmental differences of the participants. The use of pro-inflammatory cytokines as biomarkers requires further longitudinal studies and necessitates conducting the study on a larger sample size.

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