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Article

Evaluation of Salivary Level of IFN-γ and IL-10 in Autism Spectrum Disorder

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Abstract: Background: Autism is a neurological disorder marked by difficulties in social and communicative abilities , as well as repetitive actions of behaviors. Despite more than five decades of investigations, the etiology of autism remains unclear, and no specific immune biomarkers have been identified yet as being associated with autism. Several studies have presented that the level of cytokines in autistic subjects differ from those of healthy children; this study aims to test the role that interferon gamma and interleukin-10 may play in the pathophysiology of autism and the severity of the disease. Materials and methods: The study group consisted of 40 Iraqi autistic children ranging in age from 3 to 12 years. According to the severity of the condition, children were grouped into three categories mild, moderate, and severe. The control group consisted of 40 children who were age and gender matched to the study group. Saliva samples were taken from all subjects. ELISA assay was carried out to estimate salivary levels of interferon- gamma and interleukin-10. Results: The study showed a significant elevation in interferon- gamma levels in autistic patients compared to controls, as well as a decrease in interleukin-10 levels, there was also a significant increase in interferon gamma / interleukin-10 ratio in autistic patients in compartion to controls. Conclusion: These findings indicated that alteration in cytokines level may reflect dysfunctional immune response in autistic patients and confirms the major role of immunity and neuroinflammation in the etiology of autism.

Keywords: Autism, Neuroinflammation, cytokines, proinflammatory, immune response.

1. Introduction

Autism spectrum is a complex neurodevelopmental disorder of early onset that is different in its clinical presentation, classically characterized by impaired social communications, limited patterns of behavior and selective attentions [1] . According to World Health Organization WHO, one in every 160 children worldwide has an ASD [2,3]. Although the causes of autism in the majority of patients are unknown, several lines of research support the notion that autism susceptibility is clearly attributed to both genetic and environmental factors that influence the development of abnormal cortical circuitry that underlie autistic cognitive processes, social impairment, and other behaviors [4]. Recent research suggests that inflammatory pathways have a role in autism [5] .

One of the most significant advances in ASD research in the last ten years has been evidence that active neuroinflammation, including chronically activated microglia, is a substantial component of ASD [6]. Microglia is the CNS's primary resident immune cells; they serve as key mediators of inflammation, as well as immune surveillance and synaptic pruning during normal neurodevelopment. Chronic microglial activation may contribute to the development and progression of neurodegenerative diseases [7]. Furthermore, some postmortem studies have reported that the structural and morphological characteristics of microglia are aberrant in the brains of people with ASD [8] .

While there is general agreement that cytokines and chemokines are part of the altered immune environment seen in autism, there is some disagreement about how such alterations manifest, specifically whether the pro-inflammatory Th1 or anti-inflammatory Th2 cellular response predominates. Numerous studies have found a correlation between cytokine levels and ASD status [9,10.] Cytokines have been shown to impact host responses to infection, injury, inflammation, and illnesses of unknown etiology. They have also been shown to affect brain tissue growth and cell proliferation [11] . A pleiotropic cytokine with antiviral, anticancer, and immunomodulatory properties is inter-

feron-gamma IFN-γ. As a result, it is crucial for synchronizing innate and adaptive immune responses [12,13,14]. IFN-γ a protein produced by immune cells, affects how different immune cells behave throughout the body. Through the activation of CD4 Th1 cells, CD8 cytotoxic T lymphocytes CTL, NK cells, DCs, and macrophages, IFN-γ in particular enhances antigen presentation. Contrarily, IFN-γ blocks the formation and functions of Th2 and Th17 cells, regulatory T Treg cells [15.] The proinflammatory reactions of both innate and adaptive immune cells are inhibited by the powerful anti-inflammatory cytokine interleukin-10 IL-10 [16.] Human IL-10 was formerly known as cytokine synthesis inhibitory factor CSIF because to its capacity to prevent different cell types from producing pro-inflammatory cytokines such as interferon IFN, interleukin-2 IL-2, and tumor necrosis factor alpha TNF-a [17,18,19.] While IL-10's primary function is to reduce inflammation, it also has immunostimulatory qualities for some cell types, such as cytotoxic T cells and NK cells.

Different cytokines' dynamic expression can change how well the immune system functions [20] . For instance, rising levels of IFN-γ and IL-12 can induce inflammation, whereas rising levels of Transforming growth factor-beta TGF-β can inhibit inflammation [21] . Numerous research have revealed that autistic individuals have different amounts of inflammatory cytokines, which might affect their ability to fight infection in the central nervous system and increase the activity of microglia in their brains [7, 22.] Similar to this, alterations in cytokine production or levels may indicate to an issue with cellular immunity. The autistic group exhibited significantly higher levels of both Th2 and Th1 cytokines, according to a study that assessed the production of several cytokines in peripheral blood mononuclear cells [23] . A relationship between higher levels of various Th1 pro-inflammatory cytokines and the severity of disease, especially aberrant behaviors and poor communications was observed by Ashwood et al. 2011 when cytokine levels in children with ASD were examined. The purpose of this study is to investigate the potential contribution of IFN-γ and IL-10 to the pathophysiology and severity of autism.

2. Materials and Methods

- **Subjects:** The study group included 40 children diagnosed with autism, with an age range of 3-12 years of both genders. Children were examined at specialized centers for autistic disorder in Baghdad City/Iraq, and they had a confirmed diagnosis of autism based on international criteria done by specialized medical staff. And they were categorized into three subgroups based on how severe the disease was mild, moderate, and severe. The control group included 40 children, matching in age and gender with the study groups. The study started in December 2021 and lasted for two months.

  Exclusion Criteria: Children with a diagnosis of metabolic diseases, such as diabetes, or another neurological or systemic chronic disease. Children who had used antibiotics and anti-inflammatory medications during the preceding two months as well as those with unauthorized parental permission were excluded from the research.

- **Saliva sample collection**: Three milliliters of non-stimulated saliva were collected through drooling; children were given clear, simple, and direct instructions to drool the saliva by tilting the head and placing the plane tube near the lips to facilitate saliva flow [25] . The saliva collection tubes were then numbered for each individual and transported to the laboratory in an ice box for centrifugation at 3000g for 10-15 minutes at 2.8°C. The 1-2 ml of supernatant was transferred to Eppendorf tubes, numbered, and frozen at -20°C for the biomarkers detection procedure.

- **Measurement of IFN-γ:** Detection of the levels of salivary IFN-γ were determined by the commercially-available ELISA kit and done according to the guidelines present in the attached leaflet Shanghai/China. In brief, This assay uses a quantitative sandwich enzyme immunoassay method to evaluate human IFN-γ in less than five hours. There is a pre-coated 96-well microplate with a IFN-γ-specific monoclonal antibody on it. The streptavidin-peroxidase combination detects IFN-γ in samples and standards sandwiched by the first immobilized antibody in the well and a biotinylated antibody specific for IFN-γ. A peroxidase enzyme substrate is then administered after any unbound material has been washed away. Within 10 minutes of applying the stop solution, the optical density OD value of each well was calculated using a microplate reader set to 450 nm.
- **Measurement of IL-10:** same procedure used in the measurement of IFN-γ

- **Statistical Analysis**: The Statistical Package for social science was used to describe, analyze, and present the data SPSS version -21 [26]. The significance of differences in mean was determined using the student's t-test. IFN-γ and IL-10 levels in the saliva were represented as mean and SD. P-values under 0.05 were used to determine if an analysis was statistically significant.

3. Results

3.1 Detection of Salivary IFN-γ Levels

The level of IFN-γ in patients and control groups are shown in the table 1 and figure 1, which illustrates that mean level of salivary IFN-γ in the patient group was higher 178.79±66.60 than the control group 51.36±14.88 with statistically significant differences between them P<0.05.

Table 1: Case Control Difference in Salivary Levels of IFN-γ ng/ml

Groups	Minimum	Maximum	Mean±SD	T test	P value
patient	83.481	347.780	178.790±66.605	11.809	0.000**
Control	23.837	88.650	51.362±14.881		

**=*significant at p<0.05 ; SD: Standard Deviation*

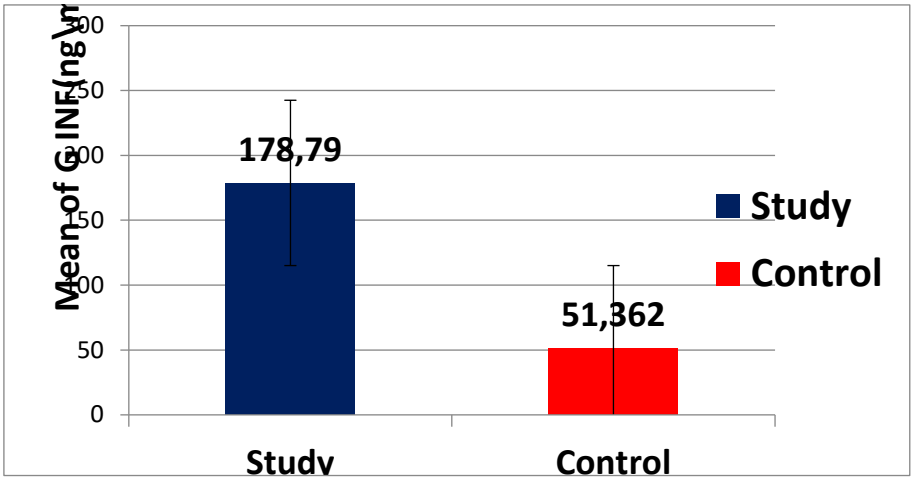

Figure 1: Levels of IFN-γ ng/ml in study and control group

3.1.1: IFN-γ level according to disease severity

With increasing autism severity, salivary IFN-γ levels rise significantly. Those children with mild autism had 127.49 ± 32.01, those with moderate had 155.43 ± 54.78, and those children with severe autism had 223.53 ± 61.51. The results revealed a highly significant difference p<0.05 between three groups of patients, as seen in the table 2 and figure 2.

Table 2: IFN-γ Level in Patient group according to Disease Severity.

Severity	Minimum	Maximum	Mean ± SD	F	P value
Mild	83.481	186.176	127.49 ± 32.01	10.666	0.000**
Moderate	90.595	281.450	155.43±54.78		
Severe	117.461	347.780	223.53±61.51		

**= =significant at p<0.05; SD: Standard Deviation

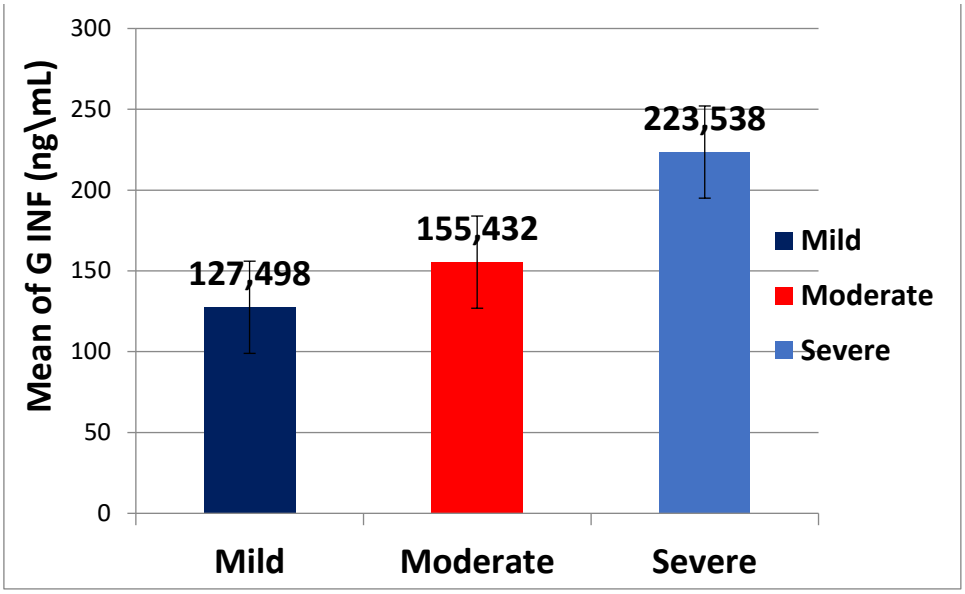

Figure 2 : Levels of IFN-γ according to disease severity

3.2 Detection of Salivary IL-10 Levels

This study found that the patients' salivary mean level of IL-10 was lower than the healthy group, with statistically significant differences. The mean level of the study group was 120.80±18.67 whereas the control group means level was 289.40 ±58.11, as shown in table 3 and figure 3.

Table 3: Case Control Difference in Salivary Levels of IL10 ng/ml.

Groups	Minimum	Maximum	Mean±SD	T test	p value
patient	67.178	158.607	120.801±18.678	17.469	0.000**
Control	157.519	405.217	289.404±58.116		

**= significant at p<0.05; SD: Standard Deviation

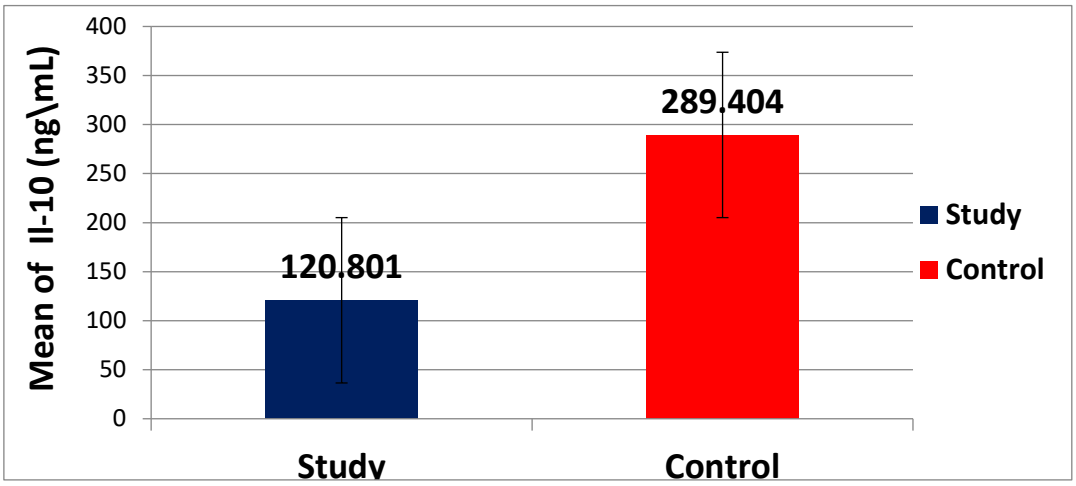

Figure 3: Levels of IL-10 ng/ml in study and control group

3.2.1: IL-10 level according to disease severity

The current results found that there was a non-significant difference P>0.05 in the salivary level of IL-10 level among three groups of patients, However, IL-10 levels increase somewhat as severity

increases. Children with mild autism had 117.10±23.49, those with moderate had 120.75±17.95, and those with severe autism had 122.58±17.79, as demonstrated in table 4 and figure 4.

Table 4: IL-10 Level in Patient Group According to Disease Severity.

Severity	Minimum	Maximum	Mean±SD	F	P-value
Mild	67.178	137.822	117.109±23.494	0.224	0.800^{NS}
Moderate	100.133	152.890	120.753±17.958		
Severe	97.932	158.607	122.580±17.792		

NS= Non significant; SD: Standard Deviation

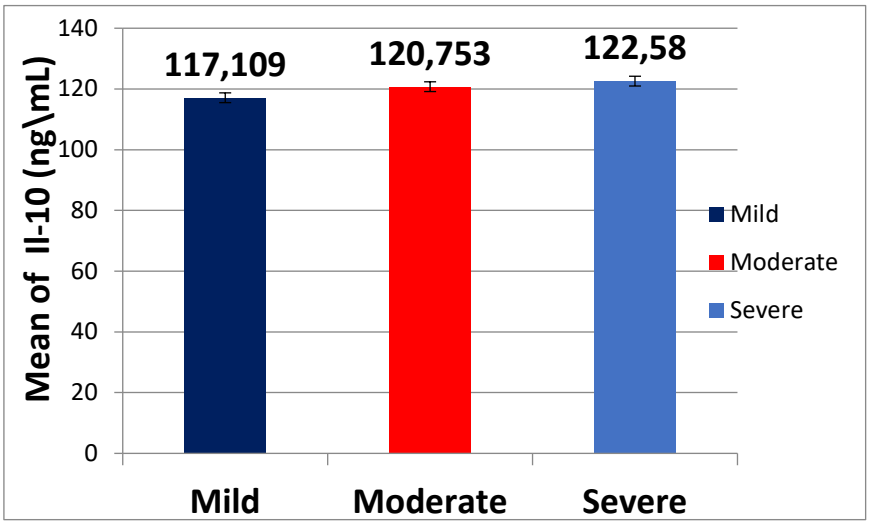

Figure 4: Levels of IL-10 according to disease severity

3.3 Estimation the Ratio of IFN-γ / IL10

The current study's measurement of the IFN-γ/IL10 ratio indicated a significant difference between the two study groups. According to table 5 and figure 5, the mean IFN-γ/IL10 ratio in autistic individuals 1.52±0.67 was significantly higher $p<0.05$ than the ratio in healthy controls 0.18±0.07.

Table 5: Ratio of IFN-γ: IL-10 levels

Statistics	Groups	
	patient	Control
Minimum	0.743	0.081
Maximum	3.219	0.378
Mean	1.528	0.186
±SD	0.671	0.073
±SE	0.106	0.011
T test	12.571	
P value	0.000**	

**= highly significant at $p<0.05$.

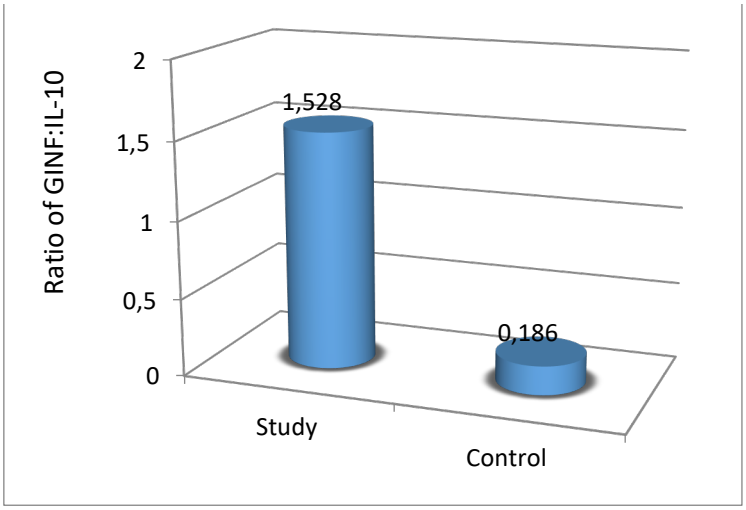

Figure 5: Ratio IFN-γ/ IL10

4. Discussion

The results of the current study found that salivary IFN-γ concentration was significantly increased in autistic children compared to normal children. The increase in IFN-γ demonstrated in the present study may indicate antigenic stimulation of T-helper1. Elevated IFN-γ is supported by previous studies [27,28,29] , suggesting a potential direct role for IFN-γ in the pathophysiology of ASD. However, Sweeten, et al., 2004 failed to show any differences in the level of IFN-γ [30] . On other hand, Gupta et al. 1998 and Iraqi study by Halboot et al. 2015 reported a decrease in the level of IFN-γ in ASD [31,32] . Another study conducted by Tostes et al. in 2012, showed that plasma levels of IFN-γ were significantly higher in children with autism, compared to the healthy subjects [28] . Additionally, earlier research offered more proof that elevated IFN-γ may be linked to increased oxidative stress, a factor that plays a significant role in the genesis of autism [33] Given that several studies compare adult controls with ASD children, these discrepancies are likely caused by the characteristics of the patient group under investigation and/or age mismatches of cases and controls, as well as by the analytical methods, statistical analysis's power, and the various specimens used. The results of the current study and earlier research support the existence of a Th-1 immune response in autistic children. In addition, IFN-γ is one of the cytokines best known for causing autoimmune disorders, and Hamdan et al. 2014 revealed the presence of proinflammatory cytokines in Alzheimer's disease [34] which would also be consistent with autoimmune pathogenesis.

Surprisingly, the data presented in this study revealed a significant increase in IFN-γ level with increasing severity of the disease. El-Ansary and Al-Ayadhi 2012 go in agreement with the present result [27] . Furthermore, Sasayama et al. 2017 suggest that IFN-γ levels are negatively related to cognitive development in children with ASD [35] . An elevated level of IFN-γ in patients with severe symptoms suggests that autism may be accompanied by excessive activation of the macrophages; The increased production of pro-inflammatory cytokines may also be thought to contribute to the genesis of some autistic symptoms. Thus, increased IFN-γ production may be responsible for autistic disorder symptoms such as social withdrawal, reduction of exploratory activity, sleep difficulties, and changes in mood [36] .

Noteworthy, the present study revealed a significant decrease in IL-10 level in an autistic patient in compared to control. This result was consistent with results [20,27,37,38] who reported a decrease level of IL-10 among autistic patients. This is contrary to the findings of [39,40,41,42] who mentioned that there were no differences in the levels of IL-10 between patients and control. However, Halboot et al. 2015 found an increased level of IL-10 in autistic patients [32] . This variability could be attributed to several factors regarding the specimen tested and sample size of studied researches. As well as, Rija et al., 2021 reported a decrease in IL-10 in rheumatoid arthritis patient, This observation is remarkable since IL-10 is an immunomodulatory cytokine that may be produced to inhibit the effects of inflammatory cytokines and that its level decreases in a variety of autoimmune illnesses [43] .

The main characteristics of ASD and its related behaviors in children with ASD may be affected by defective immunological responses and/or activities, according to recent data. Another theory is that abnormalities in shared basic cellular processes, such as those involved in signaling, emerge as

aberrations in both the immunological and neural systems. With regard to disease severity, the study showed no significant differences in the salivary level of IL-10 among three groups of patients. This finding is consistent with other findings reported by Ashwood et al. which indicated that although there was a trend for associations between more impaired social interactions and IL-10 level, the result did not show any statistical significance [24] .

The mean IFN-γ/IL10 ratio in autistic patients was significantly higher compared to controls. Studies have supported the idea that ASD has a more pro-inflammatory environment [44,45] agreed with the current study, and other researches further supports a Th1-dominated response[46,47] suggesting that ASD patients showed an increased innate and adaptive immune response through the Th1 pathway, proposing that the Th1 arm is activated in autism. This is supported by the claim that localized brain inflammation and autoimmune disorders might be involved in the pathogenesis of ASD. Gupta et al. 1998, on the other hand, observed decreased proportions of Th1 - IFN-γ and increase Th2-IL10 indicating a shift in Th1/Th2 ratio [31] . Hamdan et al. 2014 also demonstrated this shift and attributed it to the role of IL-10 as an anti-inflammatory in reducing inflammation through suppression of pro-inflammatory cytokines [34]. In conclusion these findings indicated that alteration in cytokines level may reflect dysfunctional immune response in autistic patients and confirms the crtical role of neuroinflammation in the etiology of syndrome.

Ethical approval: All of the participants were given detailed information about the study and the procedures, and their informed consent was written on a form that had been approved by the ethics committee of the University of Baghdad's College of Dentistry.

Conflicting interests: The authors state that they have no competing interests.

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