Original Research Article

The Egyptian Journal of Immunology
Volume 30 (3), 2023: 13–22.
www.Ejimmunology.org

Assessment of the natural killer cell, IFN-γ and IL-10 blood levels in children with attention-deficit/hyperactivity disorder

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder, affects millions of youngsters and typically persists into adulthood. The pathophysiology of ADHD may be due to an impaired immune response, common genetics, and environmental linkages, as all have been suggested as potential underlying mechanisms for ADHD. During systemic inflammation, natural killer (NK) cells can produce pro-inflammatory cytokines like interferon (IFN- γ) and anti-inflammatory cytokines like interleukin (IL-10); this demonstrates the importance of both of their roles as regulators to counteract inflammation and prevent immune-mediated host damage. In this work we aimed to determine the role of inflammation in children with ADHD by measuring the level of NK cells in peripheral blood compared to typically developing children besides estimating the inflammatory cytokines INF-γ and IL -10 in both groups. This study included 50 children diagnosed with ADHD based on the Diagnostic and Statistical Manual of Mental Disorders-5th edition and 50 age and sex- matched normally developed children, as controls. The estimation of NK was done using flow cytometry, while the studied cytokines were measured using the ELISA technique. We found that children with ADHD had a significantly higher level of NK cells in peripheral blood compared to controls (p<0.001). Furthermore, increased IFN -γ, while decreased IL-10 serum levels were observed in children with ADHD compared to their control group. In conclusion our findings suggested that children with ADHD may have impaired immune responses, as NK cells were increased in peripheral blood compared to the control group. Also, the serum level of IFN –γ was higher, while the serum level of IL-10 was lower in ADHD children as compared to controls.

Keywords: ADHD, Children, Inflammatory cytokines, Natural killer cells.

Date received: 23 January 2023; **accepted:** 05 April 2023

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental condition marked by an inability to maintain attention, hyperactivity, and impulsive conduct In Egypt the prevalence of ADHD among the primary school children ranged between 6.5% and 7.9%.[1]

The diagnosis of ADHD is based on using the American Psychiatric Association Diagnostic and Statistical Manual (DSM) of Mental Disorders. 5th ed. (DSM-5) "Task Force criteria".[2] The DSM-5 states that children must meet at least six or more symptoms of attention deficit and/or six or more symptoms of hyperactivity-impulsivity for children, with onset of symptoms before age 12; a persistent pattern of inattention and/or hyperactivity-impulsivity that may interferes with their social life.[3]

Worldwide, the Conners' Rating Scales (CRS) are employed to support the diagnosis of ADHD as well as the most prevalent co-occurring difficulties as disruptive disorders and cognitive disabilities in children and adolescents.[4].The verbal and nonverbal subsets of Stanford Binet (SB) Intelligence Scale, Fifth Edition (SB-V) were used to evaluate the individuals' intellectual and cognitive functioning in ADHD children.[5]

The pathophysiology of ADHD is complex. However, recently there were growing evidence indicating that inflammation plays a role in neuropsychiatric illnesses.[6.] An impaired immune response, common genetics, and environmental linkages were all suggested as potential underlying mechanisms for ADHD, which could be confirmed by the co-occurrence of ADHD with inflammatory and autoimmune disorders.[7]

Several immunological mechanisms, such as the brain's primary immune cells; glial cells, which are essential for synaptic pruning throughout neurodevelopment, and numerous immune signaling molecules, including cytokines and complement proteins, were shown to regulate and alter neurogenesis.[8]

Microglia are the brain's primary resident immune cells. Microglial cells, when activated, emit pro-inflammatory cytokines and other substances including glutamate, which contribute to neuroinflammation.[9]

Inflammation in both peripheral blood and the brain can occur due to an interaction between peripheral immune cells and microglia.[9] In addition, cytokines were shown to play a vital role in dopaminergic pathways and the brain's tryptophan metabolism, both of which are linked to ADHD.[10]

Natural killer (NK) cells are large granular lymphocytes derived from common lymphoid progenitors. They are one of the newest innate lymphoid cell subsets, with the ability to influence both innate and adaptive immune responses. NK cells lack T and B cell receptors and are identified by their lack of CD3 expression.[11] Human NK cells are regarded as entirely mature when they exhibit high levels of cytolytic activity and can produce significant amounts of interferon gamma (IFN-γ).[12] The fact that NK cells can simultaneously produce pro-inflammatory (IFN) and anti-inflammatory interleukin 10 (IL-10) cytokines during systemic inflammation highlight the importance of their roles as regulators to counteract inflammation and prevent immune-mediated host damage.[12]

This work aimed to determine the role of inflammation in children with attention deficit hyperactive disorders by measuring the level of NK cells in peripheral blood compared to normally developed children besides measuring the inflammatory cytokines IFN-γ and IL-10 in included groups.

Subjects and Methods

This comparative study included 100 Egyptian children chosen randomly from the Pediatric Neuropsychiatric Clinic of Al-Azhar University hospital from August 2021 to May 2022. Of these, 50 children who matched the criteria of ADHD served as the case group. The other 50 children, typically developed, age and sex-matched were considered a control group. Children with comorbid psychiatric, neurological, or genetic disorders, patients with chronic systemic diseases, and patients with a history of psychotropic drug use were excluded from the study. All participants were assessed for clinical history, clinical examination, and psychometric tests during a hospital visit.

Diagnosis of ADHD

In this study, the diagnosis of ADHD depended on combining information from the clinical interview with data obtained from a tool for assessing ADHD and its prevalent comorbid conditions in children and adolescents, the Conners 3–P(S) [4] and clinical observations. Also, study subjects met the DSM-5. According to the DSM-5 criteria [2], presence of 6 or more symptoms of hyperactivity and impulsivity and/or six or more symptoms of inattention are required for diagnosis of ADHD that persist for at least 6 months. The SB Intelligence Scale [5] was done by testing the children individually in a quiet room in one session. The full test took approximately 45 minutes to administer by the psychologist in the Psychiatric Department.

For assessment of family's socioeconomic level, the Modified Fahmy & El-Sherbini scale was used[13]. The total score ranged from 5 to 19. Those with scores 15 or more were considered of high socioeconomic class; scores 11-14 were moderate socioeconomic class and <11 of low socioeconomic class.[13]

Laboratory tests

From each study participant a venous blood sample (4 ml) was collected, divided into two portions. The first portion (2 ml) was collected in an EDTA tube for complete blood counts (CBC) and flow cytometry analysis. The remaining part was evacuated in a serum-separator tube and centrifuged at 1370 g for 10 min. Serum was separated and kept frozen at -20°C until used for analysis of IFN-γ and IL10. They were assessed using commercial ELISA kits (LOT. No. 202112 and 202201, respectively, Sun Red Biotechnology Company, Shanghai, China) according to the manufacturer's instructions. The assay range was from 2 – 600 ng/ l and 10 – 3000 pg/ ml, respectively.

Flow cytometry analysis

Fluorescein isothiocyanate (FITC) labeled anti-CD3 and allophycocyanin (APC) labeled anti-CD56 monoclonal antibodies were used (lot no. 200084 & 200061, respectively, Beckman Coulter, France). FITC and APC negative isotype controls (CAT. NO.342409, BD Biosciences, San Joes, USA were used. Antibodies were titrated to determine the optimal concentrations used.

Four colors fluorescence-activated cell sorting instrument (BD Biosciences, San Joes, USA) with Cell Quest Pro software were used for data analysis. Compensation settings were done before acquiring the samples using color-calibrated beads (LOT.NO. 5093879, BD Biosciences San Joes, USA). The samples count was adjusted for (50,000 events) at acquisition using a phosphate-buffered saline (pH: 7.4). Unstained samples were acquired to detect the samples' autofluorescence.

Gating of lymphocytes was done first by using FSC⁄ SSC characteristics (Low forward and low side scatters). The estimation of NK was determined by using a dot plot graph and identified as $CD56^{+}/CD3^{-}$. NK cells levels are expressed as a percentage of both total leucocyte count and lymphocyte count.

Statistical analysis

The Statistical Program for Social Science (SPSS) version 20.0 was used for data analysis. Quantitative data with normal distributions were represented by means, standard deviations, and ranges, while non-normal distribution data shown as a median with an interquartile range (IQR). Additionally, the qualitative data were shown as percentages and numbers. Independent t-tests were used to compare two groups' quantitative data with normal distribution, whereas Mann-Whitney tests used for data with non-normal distribution. The Chi-square test was used to compare qualitative data between groups as well. To find correlations between quantitative variables in the same group, the Spearman correlation coefficient was utilized and the 95% of the confidence interval was chosen. The level of significance was set at $p<0.05$.

Results

Demographic data and characteristics of the studied children are illustrated in Table 1. Males are more likely to exhibit ADHD behaviors than females. A significantly higher number of children with ADHD was observed with non-

educated mothers and family with history of psychological disorders.

The Abbreviated CRS for ADHD symptoms was 17–26 in children with ADHD and 2–11 in controls. The 50 children diagnosed with ADHD were divided into three groups, 26 with combined symptoms, 16 with an inattentive type, and eight with the hyperactive type (Table 1).

Table 1. Comparison between the control group and ADHD group regarding demographic data and characteristics of the studied subjects.

		Control group	ADHD group		*p* value
		No.= 50	No.= 50		
Age(year)	Mean±SD	8.29 ± 2.08	8.34 ± 2.17		NS•
	Range	5 – 12	5 – 12		
Sex	Boy	39 (78.0%)	42 (84.0%)		NS
	Girl	11 (22.0%)	8 (16.0%)		
Residence:	Rural	23 (46.0%)	28 (56.0%)		NS
	Urban	27 (54.0%)	22 (44.0%)		
IQ %	90-100	37 (74.0%)	44 (88.0%)		NS
	100-110	13 (26.0%)	6 (12.0%)		
Abbreviated CRS Scale scores	Mean±SD	6.2±2.42	21.46±3.19		<0.0001
	Range	2-11	17-26		
Sex distribution across ADHD subtypes	ADHD subtype		Male, N (%)	Female, N (%)	NS
	Combined type (26)		23 (54.76%)	3 (37.5%)	
	Inattentive (16)		13 (30.95%)	3 (37. 5%)	
	Hyperactive (8)		6 (14.29%)	2 (25%)	
Mother age(year)	>30	18 (36.0%)	15 (30.0%)		NS
	30-40	21 (42.0%)	31 (62.0%)		
	<40	11 (22.0%)	4 (8.0%)		
Father age(year)	>30	0 (0.0%)	3 (6.0%)		NS
	30-40	14 (28.0%)	20 (40.0%)		
	<40	36 (72.0%)	27 (54.0%)		
Mother education	Not educated	3 (6.0%)	11 (22.0%)		0.044
	Primary	10 (20.0%)	10 (20.0%)		
	Preparat.	10 (20.0%)	2 (4.0%)		
	Secondary	8 (16.0%)	9 (18.0%)		
	Diploma	14 (28.0%)	16 (32.0%)		
	Faculty	5 (10.0%)	2 (4.0%)		
Father education	Not educated	3 (6.0%)	6 (12.0%)		NS
	Primary	5 (10.0%)	10 (20.0%)		
	Preparat.	0 (0.0%)	0 (0.0%)		
	Secondary	7 (14.0%)	7 (14.0%)		
	Diploma	25 (50.0%)	19 (38.0%)		
	Faculty	10 (20.0%)	8 (16.0%)		
Mother job	Housewife	25 (50.0%)	32 (64.0%)		NS
	Work	25 (50.0%)	18 (36.0%)		
Father job	Not work	4 (8.0%)	6 (12.0%)		NS
	Work	46 (92.0%)	44 (88.0%)		
Consanguinity	Negative	41 (82.0%)	40 (80.0%)		NS
	Positive	9 (18.0%)	10 (20.0%)		

Table 1. Continued.

		Control group	ADHD group	*p* value
		No.= 50	No.= 50	
Number of siblings	Less than 2	18 (36.0%)	10 (20.0%)	NS
	More than 2	32 (64.0%)	40 (80.0%)	
Family history of psychological disorders	No	48 (96.0%)	34 (68.0%)	<0.001
	Yes	2 (4.0%)	16 (32.0%)	
Socioeconomic status	Low	34 (68.0%)	36 (72.0%)	NS
	Middle	16 (32.0%)	14 (28.0%)	
Marital status	Married	46 (92.0%)	42 (84.0%)	NS
	Divorced	4 (8.0%)	8 (16.0%)	

P > 0.05 is not significant (NS). ; X^2: Chi-square test; •: Independent t-test; ADHD: Attention deficit hyperactivity disorder; IQ: intelligence quotient; CRS: Conner's Rating Scale.

There was no difference observed in total and differential blood count between the two studied groups (Table 2). However, there was a significantly increased NK cell level in children with ADHD compared to the control group. Also, there were significantly increased IFN –γ and decreased IL-10 serum levels in children with ADHD as compared to the control group (Table 3).

Flow cytometer results of the studied cases are illustrated in Figure 1. There was a negative correlation between the percentage of NK cell and serum IFN- γ levels (Figure 2).

Table 2. Comparison between the control group and the ADHD group regarding complete and differential blood count.

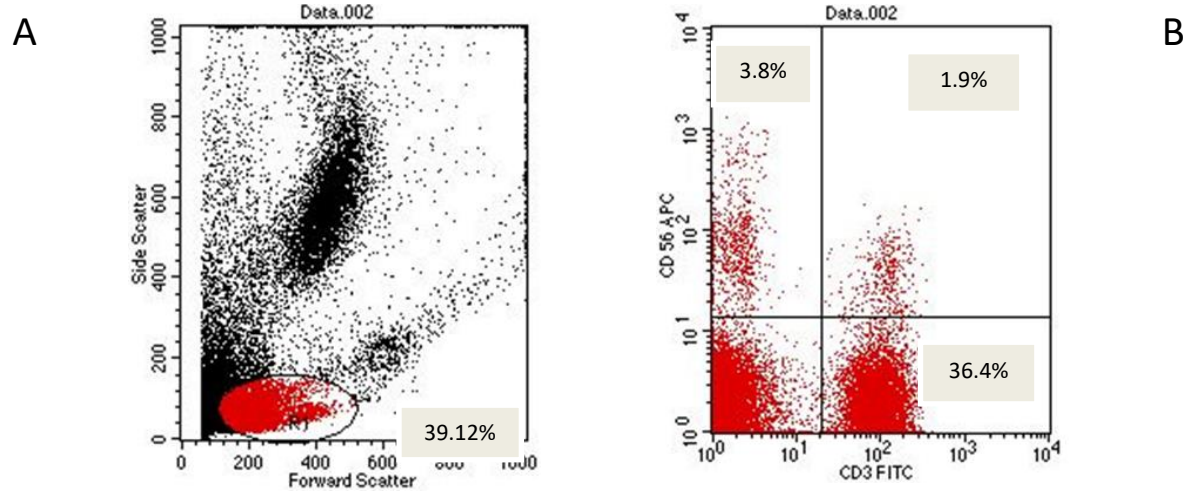
CBC		Control group	ADHD group	*p* value•
		No.= 50	No.= 50	
TLC (10^9/L)	Mean±SD	7.14 ±1.52	6.66 ± 1.89	NS
	Range	3.6 – 10.5	2.7 – 11.4	
LYMPH %	Mean±SD	44.76 ± 3.23	44.78 ± 9.81	NS
	Range	38.5 – 48.5	24.1 – 59	
RBC (10^{12}/L)	Mean±SD	4.71 ± 0.47	4.89 ± 0.42	0.043
	Range	3.9 – 5.87	4.1 – 5.8	
HB (gm/dl)	Mean±SD	12.09 ± 0.80	12.09 ± 0.95	NS
	Range	10.6 – 14.8	10.3 – 14.3	
MCV (fl)	Mean±SD	78.56 ± 4.65	79.00 ± 6.40	NS
	Range	65 – 90	64 – 90.7	
MCH (pg)	Mean±SD	25.60 ± 2.14	25.05 ± 2.72	NS
	Range	18 – 29	18 – 29	
PLT (10^9/L)	Mean±SD	244.36 ± 50.64	298.20 ± 60.76	<0.001
	Range	174 – 389	200 – 408	

P > 0.05 is not significant (NS). Chi-square test; •: Independent t-test; ADHD: attention deficit hyperactivity disorder; CBC: complete blood picture; TLC: total leucocyte count; LYMPH: lymphocyte; RBC: red blood cell; HB: hemoglobin; MCV: mean corpuscular volume; MCH: Mean corpuscular hemoglobin; PLT: platelet count.

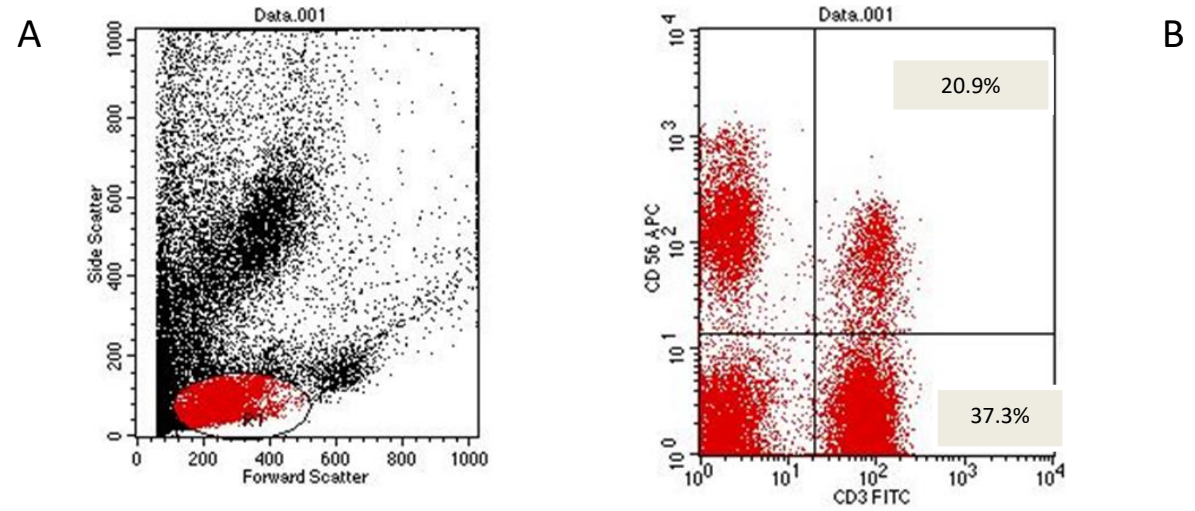
Table 3. Comparison between the control group and the ADHD group regarding NK cells level in peripheral blood (expressed as a percentage from total leukocyte count and lymphocytes), IFN and IL-10 serum levels.

Variables		Control group No.= 50	ADHD group No.= 50	•*p* value
% of NK cells relative to TLC	Mean±SD Range	1.72 ± 0.71 0.56 – 3.4	3.68 ± 1.72 0.5 – 7.82	<0.001
% of NK cells relative to lymph.	Mean±SD Range	5.67 ± 2.35 2.56 – 10.65	12.28 ± 3.97 3.8 – 20.9	<0.001
IFN-γ level (pg/ml)	Median (IQR) Range	38.65 (35.8 – 46.3) 25.6 – 67.8	156.6 (130.8 – 331) 49.9 – 394	<0.001
IL -10 level (pg/ml)	Median (IQR) Range	162.10 (141.3-225.3) 79.8 – 582.2	120.40 (101.4-169.2) 34.4 – 226.2	0.001

*$P \leq 0.05$ is significant. Chi-square test; •: Independent t-test; ADHD: attention deficit hyperactivity disorder; NK: natural killer; IFN-γ: interferon gamma; IL-10: interleukin 10; TLC: total leucocyte count; LYMPH: lymphocyte



1-Example of the results of control cases



2. Examples of the results of ADHD cases

Figure 1. Flow cytometric results: (A) Gating of lymphocytes by FSC/ SSC. (B) Gating of NK cells by (CD56+/CD3-). The percentage of NK cells in the ADHD case (20.9%) is higher than in control (3.8%).

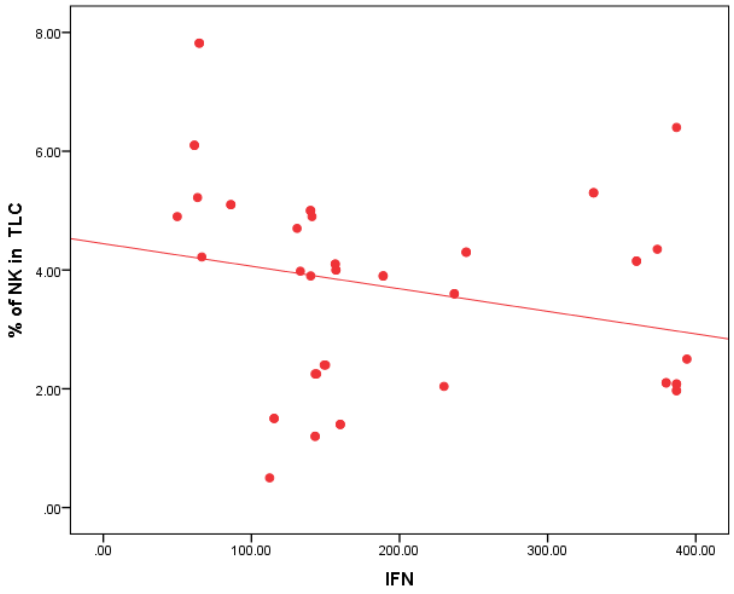


Figure 2 (a). Negative correlation between the percent of NK cells relative to leukocyte and IFN- γ.

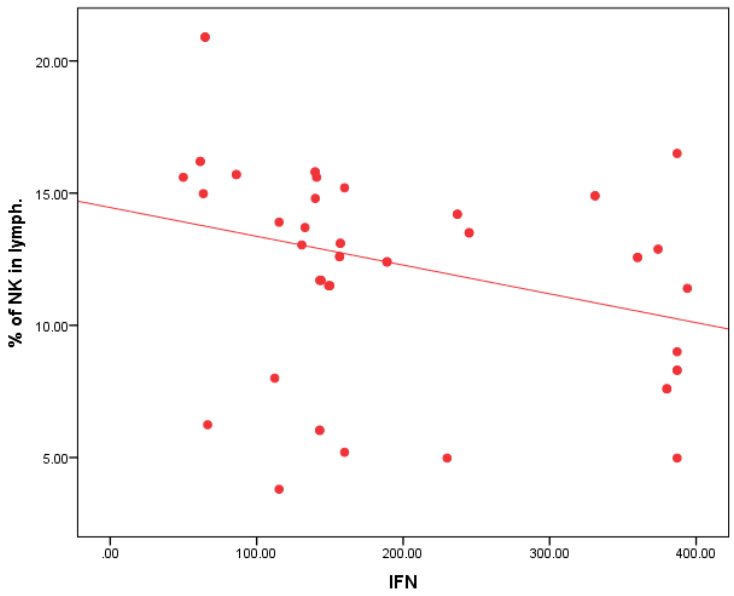


Figure 2 (b). Negative correlation between the percent of NK cells relative to lymphocyte and IFN-γ.

Discussion

Attention deficit hyperactivity disorder is a common neurodevelopmental illness that affects children's and adults' quality of life in social, academic, and occupational settings.[3] In this study, the inflammation status of children with ADHD was evaluated by measuring the NK cell level in peripheral blood using the flow cytometry technique. In addition, we estimated the inflammatory cytokines IFN-γ and IL-10 using the ELISA technique. A negative correlation between the level of NK cells and serum IFN-γ level was observed while no correlation between NK cell level and serum IL-10 was noticed.

In our study, males were more likely to exhibit ADHD behaviors than females as the sex distribution among our ADHD children was 84% males and 16 % females. Although for many years, gender has been considered a significant factor in ADHD research.[14] Differences in gender

among children with ADHD are not well understood, this may be due to boys are more likely to be referred, diagnosed, and treated for ADHD symptoms than girls.[15]

Non-educated mothers and family history of psychological disorders were more observed in the studied children with ADHD. Torvik et al., 2020[16] found a link between family problems and ADHD in school-aged boys. While Rowland et al., 2018[17] found that ADHD was more common in families with low parental education levels and poor socioeconomic status.

Most major psychiatric diseases, including bipolar disorder and depression, have been related to the immune system in some way [18]. ADHD has also been linked to immune system changes and inflammatory mechanisms, evident by a positive correlation with genetic factors related to inflammation, Therefore, the etiology of ADHD may be influenced by changes in pro-inflammatory and anti-inflammatory cytokines.[19]

In the present study, increased levels of NK cell in children with ADHD compared to the control group were observed by flow cytometry. There is currently general agreement that the interaction of neuro-anatomical and neurochemical systems has a role in the development of ADHD. This consensus is supported by research on the dopamine gene vector, twin and adoption studies, family genetics, neuroimaging, and neurotransmitter studies.[20] Many studies have shown that people with neuropsychiatric illnesses have impaired peripheral immune systems, and there is currently a lot of knowledge on the involvement of cytokines in brain processes.[21]

There was a significantly lower IL-10 blood level in children with ADHD compared to the normal controls. A previous study by Cortese et al., 2019,[22] showed a significant negative correlation between IL-10 and ADHD symptoms. While Donfrancesco et al., 2021[23] and Toto et al., 2015[24] showed a higher level of IL-10 in children with ADHD. The discrepancy in IL-10 results may be due to its dual role as anti-inflammatory and pro-inflammatory cytokines.

The present study showed a significantly higher IFN-γ blood level in children with ADHD compared to the normal controls. IFN-γ dynamic involvement in maintaining homeostasis and neuroprotection during active neuro-inflammation have also been documented.[25-27]

A previous study by Donfrancesco et al., 2021[23] showed higher IFN-γ levels in ADHD patients compared to the control. While Oades et al., 2010[28] found lower levels of IFN-γ in children receiving medication for ADHD. Also, Chang et al., 2022[29] found lower IFN-γ levels in ADHD without atopy compared to those with atopy. Also, decreased salivary IFN-γ in children with ADHD was observed by Yamaguchi et al., 2019.[30] This discrepancy in results may be explained by the different origins of IFN-γ from various immune cells and by the differences in the status of patients at the time of the study.

In conclusion, children with ADHD have increased levels of the NK cell in peripheral blood compared to the control group. The serum level of IFN-γ was higher, while the serum level of IL-10 is lower in ADHD children as compared to the control. These findings may suggest that elevated NK cell levels and IFN-γ may be involved in the inflammatory pathophysiology of childhood ADHD.

Acknowledgements

The authors would like to thank the patient's families for allowing their participation in this work.

Author Contributions

E.A.A made a substantial contribution to the concept and design of the work and draft of the manuscript. A.Y.S Contributed to the draft and interpretation of the article analysis. D.A.F and E.N.A contribute to the acquisition and the drafted manuscript. E. R. Y and I.H.F contribute to data analysis and interpretation. All authors approved the version to be published.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The protocol of the study was reviewed and approved by the Research Ethics Committee, Faculty of Medicine for Girls, Al-Azhar University.

Informed consent

At least one of the parents of each child signed a written informed consent form before the child was included in the study.

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