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ORIGINAL ARTICLE

Transforming Growth Factor Beta 2 (TGF-β2), Heat Shock Protein 70 (HSP_{70}) And Interferon (IFN) γ As Biomarkers in Newly Diagnosed Autistic Children

[1]Magdy Karm El din, [1]Omar E. El Shourbagy, [2]Mohamed M. Amin, [3]Ibrahim H. Bayomy, [4]Abeer A. Tony, [5]Doaa O.A. Yosef

[1]Department of Medical Studies for Children FPCS, Ain Shams University
[2]Department of Microbiology and Immunology, Faculty of Medicine, Aswan University
[3]Department of Microbiology and Immunology, Faculty of Medicine, Al-Azhar University
[4]Department of Neuropsychiatry, Faculty of Medicine, Aswan University
[5]M.Sc. of Pediatrics, Ministry of Health

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Corresponding Author:
Mohamed M. Amin
Department of Microbiology and Immunology, Faculty of Medicine, Aswan University
Tel.: 01122044705
mohamed.mostafa@aswu.edu.eg

ABSTRACT

***Background**: Autism is a complex developmental condition that typically manifests in the first three years of life. **Objectives**: detection of plasma level of (TGF-β2), (HSP70) and (IFN) γ in children with Autism Spectrum Disorder (ASD) and to find out correlation between their plasma levels and severity of ASD. **Methodology**: 40 children newly diagnosed with ASD recruited from Child Outpatients Clinics, Faculty of Medicine, Ain Shams University. All children were subjected to developmental history, prenatal, Physical and neurological examination, Cognitive age using Stanford Bine intelligence scale to exclude other mental or neurobehavioral disorders and laboratory detection of plasma level of HSP70, TGF-β2 and INF-γ by an ELISA kit. **Results**: HSP70 was (15.901±2.638), IFNγ was (92.37±7.893) and TGFβ2 was (68.7±8.39), **conclusion**: There was a negative correlation between the level of TGFβ2 and CARS while there were a positive correlation between the level of HSP70 and IFN-γ with CARS.*

INTRODUCTION

Autism is a serious problem with the development of the body and mind of a person seen as troubles with social interaction and communication. Often there is also restricted and repeating behavior. Parents usually (see/hear/become aware of) signs in the first two or three years of their child's life [1]. These signs often develop slowly, though some children with autism reach their developmental (important things that are done or completed) at an (usual/ commonly and regular/ healthy) pace and then worsen [2]. Autism is a highly (number or thing that changes) neurodevelopmental sickness/problem that first appears during (very beginning stages) or (the time when a person is a child), and generally follows a steady course without (temporarily free of disease) [3]

Childhood Autism Rating Scale (CARS) is an (instance of watching, noticing, or making a statement) test/list of questions that was developed to identify children with autism in comparison with other children with developmental disabilities and to decide/figure out the extreme harshness of signs of sickness. CARS is used for children over age of two years or more without upper limit [5]. Autism is one of the five serious problems with the development of the body and the mind (PDD), which are seen as (existing all over a large area) of social interactions and communication, and very much restricted interests and highly repeating behavior [4]. Unlike with autism, people with Asperger disease have no big delay in the development of language [6].

Cytokines may affect the behavior through the effects on brain chemical function, neurogenesis, neuroendocrine activity, and changes to brain circuitry. Cytokines increase the release and decrease reuptake of glutamate which is excitatory brain chemical, which can result in the disease-related process of excitotoxicity [7]

A model for some types of ASD; claims an increased excitation/inhibition ratio in key nerve-related/brain-related systems, such as sensory, memory-helper, social, and emotional systems. An alternate communication pathway has (not very long ago) been proposed based on the new and exciting work by *Louveau* and *Fellow*[8] workers who identified functional lymphatic tubes (in the body) in the CNS that carry fluid and unable to be harmed cells from the brain and spinal cord fluid, and in doing so discovered a pathway for unable to be harmed cells to exit the CNS

Cytokine profiles at birth, including made higher IL-1β and IL4, are related to an ASD (identification of a disease or problem, or its cause) later in (the time when a person is a child) and change/differ with ASD sign of sickness (seriousness/ level). Elevation of IL-1β and IL-4 may reflect a (before a baby's birth) unable to be harmed challenge, and an association with both ASD risk and thinking-related developmental results suggests the possibility of a worldwide hit/effect of early cytokine dysregulation [9]

Changed cytokine profiles have been regularly (all the time) linked to ASD in children during this period. In high functioning male children with ASD, the plasma levels of IL-1β, IL-1 receptor, (IL-1RA), IL-5, IL-8, IL12(p70), IL-13, and IL-17 are high/higher relative to matched controls[10], IL-1b, a pro-insulting/swelling cytokine, activates neutrophils and macrophages to phagocytose. IL-5 and IL-13 stimulate B cells to (release fluid) immunoglobulins including IgE, which is a (person who tries to settle an argument) of (likely to have a strong, bad body reaction) swelling. IL-12(p70) is a pro-insulting/swelling cytokine that improves Th1 and NK cell responses [11]

Further example of unable to be harmed in the (after the birth of a child) period involves (stimulation of action/making active and effective) of the monocytic and Th1 arm of the unable to be harmed response, via increased IL-1RA and increased IFN- ϒ (match up each pair of items in order), and this has been found in children with ASD. Unable to be harmed-helped settle (an argument) (machines/methods/ways) have also been guessed as reflecting a permanent state of clearly stated/particular cytokine (stimulation of action/making active and effective). Immunocytochemical studies have identified marked (stimulation of action/making active and effective) of microglia and astroglia connected with the increased production of two cytokines by neuroglia, macrophage chemoattractant protein (MCP)-1, and TGF-β2 Also, a (like nothing else in the world) profile of pro-insulting/swelling cytokines has been identified in brain and spinal cord fluid [12]

The role of (outside of a cell) 70 kDa heat shock protein 70 (HSP70) in central nervous system swelling is hugely understudied, (even though there is the existence of) (event(s) or object(s) that prove something) supporting its ability to drive a pro-insulting/swelling state. Heat shock proteins (HSPs) are caused in response to many injuries including stroke, (related to the breakdown of nerve function) disease, epilepsy and (serious physical or emotional harm).

The overexpression of HSP70 serves a (serving or acting to prevent harm) role in (more than two, but not a lot of) different models of nervous system injury, but has also been linked to a harmful role in some sicknesses [13]

METHODOLOGY

Subjects:

This study was carried on two groups; group I included 40 patients newly with AD and group II included 40 normal children matches with age, sex and recruited from the Child Outpatients Clinics at Faculty of Medicine, Ain Shams University, in the period from March, 2016 to Jan, 2018. Consent was taken from parents for their siblings to be enrolled in the study.

Inclusion criteria:

Including Age range of 3–13 years, all Children diagnosed by using Diagnostic and Statistical Manual of Mental Disorders fourth edition text revised (DSM-IV) (TR) and (CARS).

Exclusion criteria:

Other mental or neurobehavioral disorders, chronic diseases or infections, medications modulating immunity and immune deficiency states,

Methods:

All children in this study were subjected to full history taking especially developmental history and prenatal complications, physical examination especially neurological examination, Cognitive age (mental age) using Stanford Bine intelligence scale (1960). And (DSM IV) criteria for diagnosis of autistic disorder [14]

Qualitative impairment in social interaction or qualitative impairments in communication.

Restricted, repetitive and stereotyped patterns of behavior, interests and activities.

Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: Social interaction, language as used in social communication, or symbolic or imaginative play.

The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

Child Autistic Rating Scale (CARS) [15]: The Childhood Autism Rating Scale (CARS) is a 15-item behavioral rating scale developed to identify children with autism and to categorize these behaviors from mild to moderate to severe. The total CARS score may range from a low of 15 (obtained when the child¹s behavior is rated as falling within normal limits on all 15 scales) to a high of 60 (obtained when the child's behavior is rated as severely abnormal on all 15 scales).

Laboratory Tests:

Laboratory detection of plasma level of HSP70, TGF-β2 and INF-γ by an ELISA kits, products of Bioassay Technology laboratory of SHANGHAI KORIAN BIOTECH CO.

Peripheral blood sample collection:

Three cubic milliliters of blood were drawn by venipuncture of peripheral vein into a tube containing K-EDTA as anticoagulant (1 mg/ml), mixed for 10-20 mins and centrifugated for 20 min at the speed of 2000-3000 r.p.m. the supernatant was removed, and Centrifugated again, if precipitation appeared. Solid

phase capture sandwich ELISA assay was done using a micro-well format.

Statistical analysis:

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation, analysis of variance [ANOVA] test and chi-square test, linear correlation coefficient by SPSS V.25. P-value < 0.05*: was considered significant.

Ethical Considerations:

The protocol of this thesis was approved by the Ethical Committee of the Faculty of Postgraduate Childhood Studies, Ain Shams University.

RESULTS

In the present work, in group I, 82.5% of children were males and 17.5% were females, while in Group II 75% of children of were males and 25% were females, with No significant statistical difference between the two groups.

As regards prenatal history, in group I, 18 children (45%) had positive prenatal history while in group II only 6 children (15%) had positive prenatal history with high statistically significant difference between the two groups.

According to delayed motor development, in the group I, 5 children (12.5%) had positive history of delayed motor development, while in group II, 2 children (5%) had positive history of delayed motor development, with no statistically significant difference between the two groups.

As regards family history, in the group I, 14 children (35%) had positive family history, while in group II, 2 children (5%) had positive family history, with high statistically significant difference between the two groups. The results are shown in table (1)

Table 1: The difference between the two groups regarding gender, prenatal history, family history and delayed motor speech

	Group I	Group II	P value
Gender			
Male	82.5 %	75 %	0.293
female	17.5 %	25 %	
Prenatal history			
Positive	45%	15%	<0.01
Negative	55%	85%	
delayed motor development			
Positive	12.5%	5%	
Negative	87.5%	95%	0.216
Family history			
Positive	35%	5%	<0.01
Negative	65%	95%	

The range and mean of age among the studied sample, in group I, the range was (3.1 - 5.5) years (mean ±SD: 3.99±0.683), while the range of group II was (3 - 5) years (mean ± SD: 4.07±0.684), with no statistically significant difference

As regards to maternal age, in group I, the range of maternal age was (23 – 39) years (mean ±SD: 30.925±4.6871), while in group II, the range was (22 – 25) years (mean ± SD: 23.450±1.0849), with highly statistical significant difference between the two groups.

The body mass index, in group I, the range of children's BMI was (17.1 – 19.0) (mean ±SD: 18.3±0.37), while in group II, the range was (17.2 – 19.1) (mean ± SD: 18.29±0.48), with no statistical significant difference.

For plasma HSP70 level in ng\ml, in group I, the range of HSP70 was (12.95 – 22.64) ng/ml (mean ±SD: 15.901±2.638), while in group II, the range was (7.5 – 12.1) ng/ml (mean ± SD: 10.143±1.199), with high statistical significant difference between the two groups. (P value = <0.01)

On the other hand, the plasma IFN-γ in ng/ml, in group 1, the range of IFN-γ was (76.26 – 110.23) ng/ml (mean ±SD: 92.37±7.893), while in group II, the range was (42.12 – 57.23) ng/ml (mean ± SD: 51.53±4.252), with high statistical significant difference between the two groups (P value = < 0.01)

For TGF-β2 in ng/ml In ASD group 1, the range of TGF-β2 was (52.55 – 78.76) ng/ml (mean ±SD: 68.7±8.39), while in group II, the range was (92.05 – 115.78) ng/ml (mean ± SD: 108.4±6.32), with high statistical significant difference between the two groups (P value = <0.01). The results are shown in table (2)

Table 2: the differences between group I and group II regarding children age, maternal age, BMI, and the plasma level of HSP70, IFN-γ and TGF-β2

	Group I		Group II		
	Range	**Mean**	**Range**	**Mean**	**P value**
Children age	3.1- 5.5	3.99	3- 5	4.07	0.602
Maternal age	23 - 39	30.925	22 - 25	23.450	<0.01
BMI	17.1- 19	18.3	17.2- 19.1	18.29	0.656
HSP70	12.95 - 22.64	15.901	7.5- 12.1	10.143	< 0.01*
IFN-γ	76.27- 110.23	92.37	42.12- 57.23	51.53	< 0.01*
TGF-β2	52.55- 78.76	68.7	92.05- 115.78	108.4	<0.01*

Cases were classified according to CARS into: Mild n = 12, Moderate n= 24 and Severe n = 4 cases (fig 1)

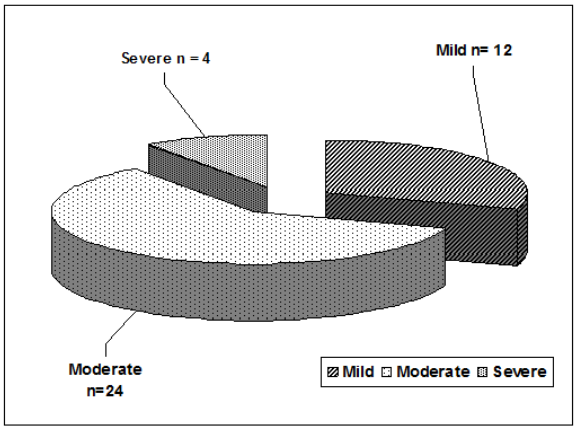

Fig 1: CARS classification of severity of Autism to mild moderate and severe

When we compared the severity of autism with the plasma levels of HSP 70 ng/ml, in mild cases, the range was (12.95 –13.98) ng/ml(, (mean ± SD: 13.54 ± 0.352), in moderate cases, the range was (14.02 – 19.22 ng/ml) (mean ±SD: 16.12±1.695), while in severe cases, the range was (20.52- 22.64 ng/ml) (mean ±SD: 1.66 ±0.999) with high statistical significant difference between the groups (P value = < 0.01) and strong correlation (r= 0.870). (fig-2)

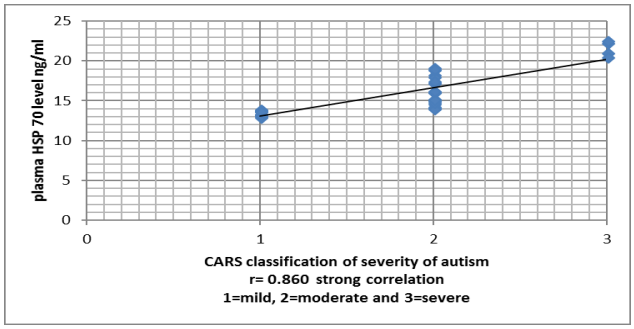

Fig 2: Correlation between severity of Autism and plasma level of HSP70

When we compared the severity of autism with the plasma levels of IFN- γ ng/ml, In mild cases, the range was (76.27 –90.89 ng/ml (, (mean ±SD: 83.139 ± 5.141), in moderate cases, the range was (92.82 – 96.1 ng/ml) (mean ± SD: 94.38±1.143), while in severe cases, the range was (104.9 – 110.2 ng/ml) (mean ±SD: 107.95 ± 2.463) with high statistical significant difference between the groups. (P value = < 0.01) and strong correlation (r=0.909) (fig-3)

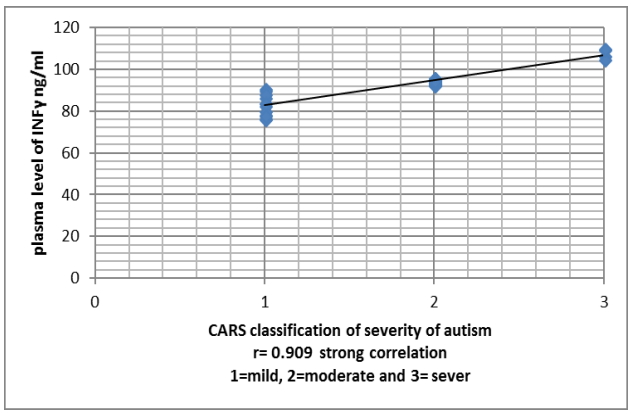

Fig 3: Correlation between severity of Autism and plasma level of IFN-Y

When we compared the severity of autism With the plasma levels of TGF-β2 ng/ml, In mild cases, the range was (76.94 –78.76 ng/ml (, (mean ±SD: 77.89 ± 0.66), in moderate cases, the range was (59.34 – 76.46 ng/ml) (mean ± SD: 66.62±5.52), while in severe cases, the range was (52.55 – 54.78 ng/ml) (mean ±SD: 53.52 ± 1.02) with high statistical significant difference between the groups (P value = < 0.01) and negative correlation (r= -0.841) (fig-4)

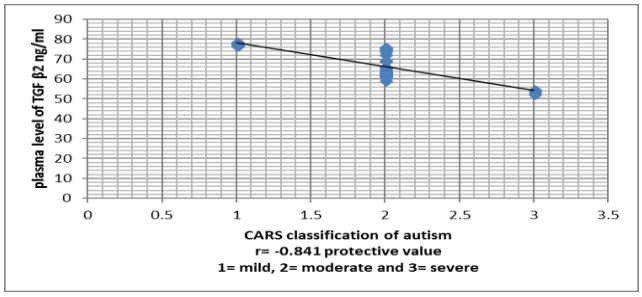

Fig 4: Correlation between severity of Autism and plasma level of TGF-β2

DISCUSSION

The present study was conducted on 40 autistic children and 40 controls, their age ranged from 3 to 5.5 years with a mean age 4.1 year. In the current study 82.5% of patients were male and 17.5% were female in a ratio 4.7:1. These findings agree with other studies that ensure that males are predominantly affected with autism than females [16]. In the UK, the male: female ratio was 6.5:1; and it was 5:1 in Canada[18].

The mean maternal age at the time of conception was significantly higher in children with autism 30.92 ± 4.69 years than in normal children 23.45 ± 1.1 years. These findings agreed with *tarek et al.,2015* , in which the mean maternal age at the time of conception was 29.33 ± 4.61 years in a group of patients. These findings are in accordance with many studies which reported an association of ASD with advanced maternal age [20, 21].

El-Baz et al., 2011, in their study about ASDs in Arab countries which recruited a total of 37 boys and 23 girls from three Arab countries (Egypt, Saudi Arabia, Jordan). High maternal age (mother, >35 years) at birth was found in 23% of autistic children.

The present study showed that 45% of the autistic children have a positive history of prenatal complication, while only 15% of normal children and more in sever autistic children (75%) than in moderate (58.3%) and mild autistic children (8.3%). These findings are similar to the results of *Tarek et al., 2015*, in which 50% of the autistic children have a history of prenatal complication. Such findings go hand in hand with the work of *Glasson et al., 2004* who showed that fetal/infant characteristics such as low APGAR scores, breech presentation, and fetal distress have been observed in autism. Also that postnatal factors such as history of hypoxia, resuscitation and history of jaundice were considered significant risk factors for autism[22].

In the current study there are no statically significant between autistic and normal children in the occurrence of natal complications. These findings agree with a case control study in which Fetal distress, Birth injury and neonatal nursery admission are not related to occurrence of autism [24]

In the current study 35% of autistic children have positive family history while only 5% of normal children. These findings are in the agreement with *Farida et al.,2012*. study in which Positive family history was found to be statistically significantly associated with the risk of autism (16% of cases versus 1% of control) and a population-based cohort of all Swedish children in which the risk of autism is increased 10-fold if a full sibling has the diagnosis and about 2-fold if a cousin has the diagnosis. These findings may inform counseling families with affected children [26]

In the present study there is no statistically significant difference between autistic and normal children as regard weight, height and BMI. This finding agrees with *Tarek et al., 2015*study[19].

In our study the delay in spoken language was the character of autistic children (90% of autistic and no one in normal children). Deficits in language development have been widely studied, as they are often considered a defining characteristic of autism [27]

TGF-β2 has been linked to ASD in multiple studies [28, 29]. Children with ASD had significantly lower plasma TGF-β2 levels (68.7 ± 8.39) ng/ml compared with normal controls with the mean of (108.4 ± 6.32) ng/ml with P **<0.01**. This agrees with previous researchers. [28,29] Altered TGF-β2 levels in brain specimens of subjects with autism; *Okada et al., 2007* in adult patients with ASD that the serum levels of TGF-β2 were significantly lower than those of normal controls. *Ashwood et al., 2008*, found that plasma levels were significantly lower in children with ASD compared with typically developing general population controls. There was a negative correlation between the level of TGF-β2 and CARS which is a diagnostic tool for autism and reflects the severity of autism in the current study. The lower the level of TGFβ2 the higher the score with CARS which indicates more severity. Such findings agreed with *Tostes et al., 2012* who found that decreasing levels of TGFβ2 in plasma correlated with worse behavioral scores on the ABC (aberrant behavior checklist).

Heat shock proteins (HSPs) play a central role in preventing protein misfolding and inhibiting apoptotic activity and represent a class of proteins potentially involved in neurological disorders [31]. There was a positive correlation between the level of HSP70 and CARS which is a diagnostic tool for autism and reflects the severity of autism in the current study. The higher the level of HSP70 the higher the score with CARS which indicates more severity. The significant increase in HSP70 reported in our study could be easily related to oxidative stress as the most important mechanism involved in the etiology of autism. These findings agree with record of Saudi autistic patients have remarkably higher plasma HSP70 compared to age and gender-matched controls[32].

In the present study there is increase of IFN-γ in autistic children (92.37 + 7.893) than normal control (51.53 + 4.252) with highly statistically difference and there was a positive correlation between the level of IFN-γ and CARS which is a diagnostic tool for autism and reflects the severity of autism in the current study. The reported elevation of IFN- γ could support the previous work showing that Peripheral blood mononuclear cell (PBMNC) of autistic children produce remarkably high levels of Il-12 and IFN-γ, or express higher than normal levels of mRNA for IFN-γ [33] and the work reported that plasma levels of vasoactive intestinal peptide (VIP), IFN- γ and NO were significantly higher in children with autism, compared to the healthy subjects and that a positive correlation between plasma levels of NO and IFN-γ exists[34].

CONCLUSION

Autism is one of five disorders that falls under the umbrella of Pervasive Developmental Disorders (PDD), a category of neurological disorders characterized by ‘‘severe and pervasive impairment in several areas of development.’’

The present study confirms the role of neuroinflammation mechanisms in the etiology of autism together with the possibility of the use of HSP70, TGF-β2, and INF-γ as predictive biomarkers.

There is reduction in TGFβ2 level in plasma of children who have ASD. There is increase in HSP70 and INF-γ levels in plasma of children who have ASD. There is a negative correlation between TGFβ2 plasma level and the severity of the disease; the lower the level of TGFβ2, the higher the scores of CARS indicating more severity. There is a positive correlation between HSP70 and INF-γ levels and the severity of the disease; the lower the level of TGF-β2, the higher the scores of CARS indicating more severity.

Recommendations:

The use of HSP70, TGF-β2, and INF-γ as a serological marker in children who have recently been diagnosed with ASD as well as their use as biological markers to monitor the efficacy of therapy.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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