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Role of Macrophage Migration Inhibitory Factor and Some Immunoglobulins in Autistic Patients

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{قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ
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الْحَكِيمُ}

Dedications

To ...

My Dears: father, mother, brothers and sisters.

For their support and encouragement, I hope this thesis serves to repay some of their contributions.

Ali sabry

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ALI SABRY

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairment in social interactions and communication deficits. Over the last decades the prevalence of this disorder has dramatically increased. A potential role for immune dysfunction has been suggested in ASD.

The aim of the present study is to investigate the association of some Immunological markers such as Macrophage migration inhibitory factor (MIF) and immunoglobulins (IgG and, IgM) among Iraqi autistic children.

A case-control study was conducted on 60 autistic children compared 3 to 12 years (48 males and 12 female) with male to female sex ratio of 4:1, whose Mean age ± SD (6.01 ± 2.45) years old which present in AL- Imam AL-Hussein Institute for care of autistic children in AL- Najaf city and Ruqayah center for Hearing and Speech in AL-Diwaniaya city compared with equal number of healthy control group participated during the period from 1st of December 2015 to 30th of March 2016.

They were interviewed by using socio demographic questionnaire, A Semi-structured interview schedule base on (ISAA) Indian Scale for Assessment of Autism. The association of some Immunological markers such as Macrophage migration inhibitory factor (MIF) and immunoglobulins (IgG and, IgM) among Iraqi autistic children were assessed by using enzyme linked immunosorbent assay (ELISA).

The results revealed a significant increase ($P < 0.001$) in autistic patients serum levels of MIF(mean 13.31 ng/ml,ranged from 2.55 - 18.73 ng/ml) compared with healthy control (mean 5.61ng/ml,ranged from 2.42 - 10.74 ng/ml). Where there is a significant decrease ($P < 0.001$) in the

autistic patients serum levels of total IgG (mean 20.66 µg/ml, ranged from 10.55 – 34.52 µg/ml) compared with healthy control(mean 38.01µg/ml,ranged from 26.89 – 50.24 µg/ml), and decrease in the autistic patients serum levels of total IgM (mean 208.18 mg/dl,ranged from 163.41– 245.75 mg/dl) compared with healthy control (mean 245.53 mg/dl,ranged from 200.96- 271.02mg/dl).

The results of study revealed that sleep problems and seizure are present in 13 (21.7 %) and 7(11.7 %) respectively, which are important clinical features of autism. Also the results showed 52 (86.67%) of the autistic children were from urban area ($p < 0.001$). No significant association was found between birth order and autism ($P > 0.05$), and anxiety and autism ($P > 0.05$).

while the results revealed significant correlation between sleep problem and severity of autistic behavior ($p < 0.05$), but non-significant correlation between child's age, child's gender and seizure with severity of autistic behavior ($P > 0.05$).

In conclusion the study revealed the important role of MIF in the pathogenesis of Iraqi autistic children, it showed higher level of serum MIF and lower levels of serum IgG and IgM in autistic patient compared with healthy controls.

List of contents

List of contents

List of Tables

List of Figures

List of Abbreviation

Abbreviation	Meaning
µg	Micro Gram
µl	Micro liter
AAP	American Academy of Pediatrics
ABO	Blood Groups
ADDM	Autism and Developmental Disabilities Monitoring
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
BBB	Blood Brain Barrier
CARS	Childhood Autism Rating Scale
CC	Corpus Callosum
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDC	Centers for Disease Control and Prevention
CMA	Chromosomal Micro Array Analysis
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT scan	Computerized Tomography Scan
DD	Developmental Delay
DD	Developmental Disorders
DL	Deciliters
DSM-III	Diagnostic and Statistical Manual of Mental Disorders-III
DTI	Diffusion Tensor Imaging
EEG	Electroencephalogram
ELISA	Enzyme-Linked Immunosorbent Assay Technique
fMRI	Functional Magnetic Resonance Imaging
FPE	Female Protective Effect’

GI	**Gastrointestinal**
HRP	**Horseradish Peroxidase**
IgG	**Immunoglobulin G**
IgM	**Immunoglobulin M**
IL-1β	**Interleukin-1 β**
IL-2	**Interleukin-2**
IL-6	**Interleukin-6**
IQ	**Intelligence Quotient**
ISAA	**Indian Scale for Assessment of Autism**
KSA	**Saudi Arabia Kingdom**
MECP2 gene	**Methyl-CpG-binding Protein-2**
Mg	**Milligrams**
MIF	**Macrophage Migration Inhibitory Factor**
Ml	**Milliliters**
MRI	**Magnetic Resonance Imaging**
N	**Number**
Ng	**Nano grams**
NHSR	**National Health Statistics Reports**
NIMH	**National Institute of Mental Health**
NINDS	**National Institute of Neurological Disorders and Stroke**
Nm	**Nano Meter**
NS	**No Significant**
OD	**Optical Density**
PBMCs	**Peripheral Blood Mononuclear Cells**
PDD-NOS	**Pervasive Developmental Disorder Not Otherwise Specified**
PTEN gene	**Phosphatase and Tensin Homolog Gene**
r.p.m.	**Round Per Minute**
Rh	**Rhesus Factor**
S	**Significant**

SD	**Standard Deviation**
SE	**Standard Error**
SMR	**Standardized Mortality Ratio**
SNPs	**Single-Nucleotide Polymorphisms**
SPSS	**Statistical Package for Social Sciences**
TGF-b	**Transforming Growth Factor β**
Th1	**T helper 1**
Th2	**T helper 2**
TMB substrate	**Tetra methyl benzidine**
TSC	**Tuberous Sclerosis**
U.S.A	**United States of America**
UAE	**United Arab Emirates**
UK	**United Kingdom**

1- Introduction and Literatures Review:

1-1 Introduction:

Autism spectrum disorder (ASD) involves a complex interplay of both genetic and environmental risk factors, with immune alterations and synaptic connection deficiency in early life. Immunological imbalance (including autoimmunity) has been proposed as a major etiological component in ASD. Also, epidemiological studies have established a correlation of ASD with family history of autoimmune diseases; associations with major histocompatibility complex haplotypes and abnormal levels of immunological markers in the blood (Gottfried *et al*.,2015). It is a heterogeneous group of behaviorally defined disorders that are widely considered to be genetic in origin on account of the high rates of heritability (Miles., 2011).

It is a neurodevelopmental disorder characterized by impaired social interaction, verbal and non-verbal communication, and restricted and repetitive behavior (Kohane *et al*., 2012). Parents usually notice signs in the first two years of their child's life (Myers and Johnson., 2007). It affects boys four times more frequently than girls suggesting involvement of the sex chromosomes (Nygren *et al*., 2012). When females are affected, they usually exhibit severe mental retardation (Karen *et al.,* 2011). Over the past decades the prevalence of this disorder has dramatically increased. Although the reason for this increase is still up for debate, In 2012, the Autism and Developmental Disabilities Monitoring (ADDM) Network published data from 14 sites for the 2008 surveillance year, reporting a combined ASD prevalence of 11.3 per 1,000 children aged 8 years (or one in 88) children (CDC.,

2012). Comparison of the 2008 findings with those for previous surveillance years showed an increase in ASD prevalence of approximately 23% compared with the 2006 estimates and 78% compared with 2002.

While the etiology and pathogenesis of autism are poorly understood, there is evidence that immune system abnormalities are associated with symptoms in a substantial number of affected individuals (Onore *et al*., 2012). Immune dysfunction plays a major role in the pathophysiology of ASD(Abdallah *et al* ., 2012). Inflammatory changes in the central nervous system (CNS)(Pardo-Villamizar., 2008), and the peripheral immune system (Ashwood and Van de Water., 2004), have been repeatedly reported in different biologic samples of individuals with ASD. Interestingly, such dysfunctional immune profiles have been reported during pregnancy, after birth and post mortem which may indicate an ongoing immune dysfunctional profile in individuals with ASD (Abdallah *et al*., 2011).

Aim of study

The aim of present study is to investigate the association of some Immunological markers such as Macrophage migration inhibitory factor (MIF), and immunoglobulin (IgG and, IgM) among Iraqi autistic patients by the following objectives:

A- Study of the some subjects characteristics like age, sex, duration of the disease, behavior and family history.

B- Determining the levels of Macrophage migration inhibitory factor (MIF), and immunoglobulin (IgG and IgM) in those patients compared to the normal population by using an enzyme-linked immunosorbent assay (ELISA) technique.

C- Evaluating the relationship between these levels and the severity of the disease and comparing them with normal persons.

1.2 Literature Review.

1.2.1 Overview of Autism.

Autism spectrum disorders are a complex group of severe neurodevelopmental disorders that affect over 1% of children in the United States (CDC.,2009). Typical symptoms of autism include impairments in social interaction, deficits in verbal and nonverbal communication, repetitive behaviors and restricted interests (Abrahams and Geschwind,. 2008). In the majority of cases, the etiology of ASD is not known and likely involves complex interactions between genetic, epigenetic and environmental factors. Papers have described links between genes that encode for immune-related proteins and ASD, suggesting that abnormalities in the immune system may influence aspects of brain development and synaptic functions that negatively impact clinical outcomes relevant to ASD (Enstrom *et al*., 2009). Taken together with well-established reports of cytokine-mediated influences on neuronal function, differentiation, migration, proliferation, and behavioral impairments in animal models, there is an emerging view of synergistic relationships between immune dysfunction and genetic predisposition that contribute to a subset of ASD cases (Ashwood *et al.*, 2011).

1.2.2 History and Classification of Autism

The first use of the term “autistic” was in 1911, by the Swiss psychiatrist Eugen Bleuler (1857–1939), referring to the limitation of human relationships and the loss of contact with reality presented by patients with schizophrenia (Ashok *et al.*, 2012). The term was then adopted by the Austrian pediatrician Hans Asperger (1906–1980)

working at the University Children's Hospital-Vienna, referring to "autistic psychopaths." Asperger was investigating a form of autism spectrum disorder (ASD) now known as Asperger syndrome and not widely recognized as a separate diagnosis until 1981. In 1943, the Austrian-American psychiatrist Leo Kanner (1894–1981) used the term "autistic disturbances of affective contact" to describe 11 children with behavior marked by difficulties in establishing affective and interpersonal contact (Kanner.,1943). He reported a form distinct from other diseases, such as schizophrenia, and that seemed to affect patients from the beginning of their lives. In the following year, Hans Asperger described cases exhibiting some characteristics similar to autism, which included difficulty in social communication, but without cognitive loss (Frith., 1991). In 1980, the term "autism" was first inserted in the third edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-III). In 1994, the fourth edition of the DSM included new criteria due to the need to identify subgroups of individuals with autism, for both practical purposes and research, considering the subdivisions: typical autism, pervasive developmental disorder not otherwise specified (PDD-NOS), and Asperger syndrome (Louveau *et al.*, 2015).

The fifth edition, DSM considered instead of three domains of autism symptoms (social impairment, language/ communication impairment, and repetitive/restricted behaviors), only two categories: (1) social communication impairment and (2) restricted interest/repetitive behaviors. Also, the new classification eliminated the previously separate subcategories into the broad term ASD (Giovannoni *et al.*, 1998 ; APA.; 2013). As a developmental disorder, ASD includes different degrees of severity and males are more affected than females by a ratio 5:1 approximately (Forrester *et al.*, 2008) .

1.2.3 Epidemiology of Autism

The 2012 review of global prevalence estimates of autism spectrum disorders found that the median of the disease affect 62 cases per 10,000 people (Elsabbagh *et al.*, 2012), with 4:1 male-to-female ratio (Ali and Haitham., 2008). In Iraq, there are some studies that have shown the prevalence of the autistic children in Iraq but it is still uncertain because its lack of inclusiveness , because there are a lot of private centers for special needs and autistic children in Iraq shall not be subjected to the control or not supervised by any official specialists (Sakr and Amal., 2014). International studies conducted about the autism in Iraq as well.One of these in (2011) was prepared by the Autism Research Center at Cambridge University, the study showed that after the war in Iraq at 2003, autism cases recorded higher levels than before the war. According to this study, 75 child are affected by autism out of 10,000 child with age range between 5 to 10 years old in 2012, an article published on the website of the University of Guilford which estimated that 5,000 Iraqi child are affected by autism (Sakr and Amal., 2014). Also in Saudi Arabia and according to report conducted by Jada Center for Autistic Children in 2011 there is 1 autistic child in every 90 or 100 births, this is equivalent to 1% of the population (Aal., 2011), In Oman, estimated an overall prevalence of 1.4 ASD cases per 10,000 children (Al-Farsi and Al-Sharbati., 2011). The prevalence of ASD was 29 per 10,000 children in UAE (Eapen *et al.*, 2007), and 4.3 per 10,000 in Bahrain (Al-Ansari and Ahmed., 2013).

The prevalence of ASD among children with developmental disorders in Egypt and Tunisia was documented as 33.6% and 11.5% respectively (Seif Eldin *et al.*, 2008).

The U.S. Department of Health and Human Services and the U.S. Centers for Disease Control and Prevention (CDC), in 2011 and 2012 reported that 1 from 50 U.S. children aged 6 to 17 years had been diagnosed with autism (200 per 10,000) (NHSR., 2013). This prevalence was significantly higher than that which reported in 2007 (1 in 88) for children in the same age group (CDC. 2014). In the United Kingdom, currently reported autism prevalence is 1 in 64 children (157 per 10,000) (Baron-Cohen *et al*., 2009).

1.2.4 Etiology and Risk Factors of Autism

The etiology of ASD has been a widely debated issue for several decades. However, the etiology of ASD remains poorly understood (Rossignol and Frye., 2011a).Twin studies provide evidence that susceptibility to ASD may have significant environmental components, in addition to genetic heritability (Ronald and Hoekstra., 2011 ; Hallmayer *et al*., 2011). Others have proposed that it may be a combination of three factors-genetic , environmental and neurological development (King, 2015). This wide range of factors has caused uncertainty among parents and family members and so have led some to deduce their own etiology.

Some parents believe that ASD is caused by a combination of biological and environmental factors (Dardennes *et al*., 2011 and Mercer, *et al*., 2006), while many others hold the opinion that there is a significant connection between vaccines and autism spectrum disorder (Bazzano *et al*., 2012 ; Russell *et al*., 2009). Although many studies around the world have shown that there is no relationship between vaccines and ASD, many parents adamantly hold the view to the contrary (Bazzano *et al*., 2012 ; Gerber and Offit., 2009).

1.2.4.1 Gender and Genetic Factor

Sex act as a risk factor of ASD. The prevalence of ASD is higher in male than female. The reason for 4/1 male to female ratio in ASD is not very well understood but it is very important. Recent studies implicate some epigenetic phenomena such as sex-specific effects of Y-linked genes, balanced, as well as skewed X-inactivation, escaping X-inactivation, and parent-of-origin allelic gene among others in the etiology of ASD (Schaafsma and Pfaff ., 2014) , and heterogeneity in gene regulation at allelic level as well as total gene expression (Ben-David *et al*., 2014). These sex differences may be due to genetic and hormonal differences that could be initiated during early times of development due to differences in responses to and interactions with various environmental factors such as diet, stress, infection, and drugs. Due to the involvement of many X-linked genes involved in placenta formation and placenta-specific epigenetic processes, placenta plays an important role in sex-specific responses to environmental factors and disease states later in life (Gabory *et al*., 2013). Early maternal immune activation may cause prenatal stress, affecting boys more severely due to a vulnerable genotype (Schaafsma and Pfaff., 2014).

Genetic factors contribute significantly to disease expression and severity. The genetic cause has long been implicated to be a strong evidence-based etiology (Silver and Rapin., 2012), in cases of some co-occurring or associated conditions with ASD such as tuberous sclerosis, fragile X syndrome, Rett syndrome (Betancur *et al*., 2009) and some other. Siblings of autistic offspring have a higher incidence of autism than the general population (White., 2003) and twin studies have also indicated that dizygotic twins share much higher rates of autism concordance compared to non-twin siblings indicates a role for shared environmental risk factors in elevating autism risk (Hallmayer *et al.*,

2011). There is a wide range of phenotype but more genetically homogeneous ASD patients present with less phenotypic heterogeneity (Bruining *et al*., 2010). In addition, human genetic investigations and animal models of ASD detected de novo copy number mutations and rare variant mutations resulting in abnormal alleles in the person or close ancestry that influence neuroanatomical and behavioral traits (Shinoda *et al*., 2013; Ronemus *et al*., 2014; Malhotra *et al*., 2012). These studies have shown dysregulations in genes involved in synapse function (Zoghbi and Bear., 2012). A comprehensive and informative review of several genetic studies (Banerjee *et al*., 2014) show abnormal assembly or structure of several transmembrane and scaffolding proteins involved in synaptogenesis and its maintenance, as well as dysregulation of genes involved in the signal transduction mechanism of synapse formation, are among the major genetic abnormalities of ASD. With the discovery of several genes as well as interactions of multiple genes in one individual, epigenetic factors, and effects of environmental modifiers on these genes in ASD, genetic causes including the diagnosable medical conditions, single-gene defect, and cytogenetic problems comprise 25% of the ASD patients so far (Miles., 2011).

1.2.4.2 Environmental Factors

a. Prenatal and Perinatal Factors

A meta-analysis of prenatal factors, limited to pregnancy-related factors, identified few significant risk factors(Gardener *et al*., 2009)**.** The main factors are maternal gestational diabetes, maternal bleeding during pregnancy, and maternal medication. More interestingly, maternal viral infection in the first trimester and maternal bacterial infection in the second trimester were found to be associated with ASDs in the offspring (Atladóttir *et al.,* 2010).

Moreover, exposure to intrauterine infections was associated with a significant increase in the risk for autism. It seems that gestational viral infections trigger a maternal immune response, which can perturb fetal brain development, at least in part through interleukin-6. The same authors identified several potential risk factors such as, the main being fetal presentation, umbilical cord complications, fetal distress, birth injury or trauma, multiple births, maternal hemorrhage, summer birth, low birth weight, small for gestational age, low 5minute Apgar score, meconium aspiration, neonatal anemia, ABO or Rh incompatibility, and hyperbilirubinemia (Smith *et al*., 2007). Visser *et al*,. (2013) found that the exposure to smoking during pregnancy and suboptimal conditions following the birth of newborns were related to increased likelihood of ASD.

b. Drugs and Toxic Exposure

Exposure to medication during pregnancy was found to increase autism risk in the most meta-analyses studies (Gardener *et al*., 2009). Prenatal exposure to valproate is a recognized risk factor for ASD, especially in the first trimester of pregnancy. Children exposed in utero to valproate have 8-fold increased risk to have ASD (Rasalam *et al*., 2005). Moreover, one of the major concerns regarding medication exposure during pregnancy concerns the use of antidepressants, since selective serotonin reuptake inhibitor medication during pregnancy increased from 1.5% in 1996 to 6.4% in 2004 and 6.2% in 2005 (Andrade *et al*., 2008). It was suggested that antidepressant exposure during pregnancy modestly increases the risk of ASD, especially in the first semester (Croen *et al*., 2011). Exposure in utero to an organophosphate insecticide, chlorpyrifos, was found to increase ASD risk and it was suggested that synthetic chemicals should be far more explored

(Landrigan.,2010). Lastly, mercury is a ubiquitous environmental contaminant, that is, transformed into the volatile neurotoxins methylmercury and ethyl mercury. Exposure to high levels of environmental mercury and pollutants during pregnancy are also linked to increased risk of ASD (Leslie and Koger., 2011). Another study observed that there was a significantly increased risk for the incidence of ASD after thimerosal-containing vaccine in comparison to the thimerosal-free vaccine (Geier *et al.,* 2013). Large-scale epidemiological surveys have disputed a causal link between ASD and thimerosal exposure (Clements and McIntyre., 2006).

c. Maternal Immune Activation :

A number of studies show a link between ASD and a family history of autoimmune diseases or those families with altered inflammatory cytokines or other immune problems (Gesundheit *et al.*, 2013).

When antibodies developed in immune-mediated disorders were introduced to pregnant monkeys, the offspring showed behavioral changes and CNS pathology (Libbey and Fujinam., 2010). Perinatal exposure to infection has been implicated in the pathogenesis of ASD and schizophrenia (Meyer *et al.,* 2011). Activation of the immune system in pregnant mice leads to the activation of macrophages in the offspring (Onore *et al.*, 2014).

A study investigating the role of maternal autoimmune disease, asthma, and allergy on developmental disorders looked at 560 ASD patients and 168 cases of developmental delay without autism (DD) has found a significant modest increase in both the ASD and DD combined (the ASD alone data was not significant) in the children of sick mothers during pregnancy. Anti- phospholipid antibodies have been linked with

psychological problems such as cognitive malfunction, repetitive behavior and anxiety. Increased levels of anticardiolipin, β 2-glycoprotein 1, and anti-phospholipid antibodies were found in the blood plasma of the ASD children compared to their age matched typically developing children and the DD children(Careaga *et al.*, 2013).

Results of the animal studies indicate that the behavioral and maternal immune activation are different among different mice species referring to the possibility that a subpopulation of human might be more vulnerable to particular environmental agents(Schwartzer *et al.*, 2013).

Maternal immune activation due to infections, inflammatory diseases and autoimmune diseases can have a deleterious effect on the fetus by affecting fetal tissue and its consequences during postnatal period. This area deserves more focus. Animal models of ASD especially the infection models can be very informative.

1.2.5 Pathomechanisms of Autism

Deficits in synaptic maturation, which are characterized by weak functional connectivity across brain regions, may play a role in the pathomechanisms of neurodevelopmental disorders, including autism (Courchesne and Pierce , 2005).

1.2.5.1 Neuropathological Changes in ASD.

Autism involves early brain overgrowth and dysfunction, which is most strongly evident in the prefrontal cortex (Hazlett *et al.*, 2011). Increased brain size occurs without concomitant signs of edema (Casanova., 2007). An excess amount of neurons in the prefrontal cortex signals a disturbance in prenatal development and may be associated with an abnormal cell type and laminar development (Stoner *et al*., 2014). In postmortem and neuroimaging studies of ASD individuals exhibit

numerous abnormalities including larger gray matter (Hazlett *et al.*, 2006 ; Ecker *et al.*, 2013), white matter (Hazlett *et al.*, 2006), amygdala (Bellani *et al.*, 2013a), hippocampus volumes (Groen *et al.*, 2010), smaller corpus callosum volumes (Bellani *et al.*, 2013b), abnormal cortical thickness (Raznahan *et al.*, 2010 ; Wallace *et al.*, 2010), and lower numbers of neurons have been reported in the fusiform gyrus of the temporal lobe and the cerebellum (Schumann *et al*., 2011). These findings have been interpreted as supporting evidence for different theories of ASD including, for example, the amygdala theory of autism (Baron-Cohen *et al.*, 2000) and the "under connectivity" theory of ASD (Just *et al.*, 2007) .

1.2.5.2. Immune system Imbalance in ASD:

Immunocytochemical studies have shown a marked activation of microglia and astroglia, and cytokine profiling has indicated that macrophage chemoattractant protein-1 and tumor growth factor-β1, which are derived from neuroglia, were the most prevalent cytokines in the brain tissues (Vargas *et al*., 2005). An immunohistochemical study in autopsy brains with autism and matched controls showed significantly increased densities of microglia in two functionally and anatomically disparate cortical areas, namely the front insular and visual cortices, suggesting the dense distribution of microglia throughout the cerebral cortex in brains with autism (Tetreault *et al*.,2012).

1.2.5.2. 1 Inflammatory Response In Autism

Other conditions in ASD such as inflammation, inflammatory response, and immune activation have long been implicated in the pathogenesis of ASD but studies so far were not conclusive (Jyonouchi *et al.*, 2012). A number of studies reveal abnormalities of the peripheral immune system supporting the ideas of immune involvement in ASD

however, immune abnormalities such as activation of microglial cells and innate neuroimmune system are also found in the brain and cerebrospinal fluid (CSF) of ASD patient (Pardo *et al.,* 2005).

Neuroimmune abnormalities have been recently reviewed elsewhere (Goyal and Miyan., 2014). Blood brain barrier (BBB) is an important regulator of the brain homeostasis (Samsam ., 2012). There are evidences that the BBB function is altered in ASD children due to neurological inflammation, immune dysregulation and increased inflammatory cytokines in the brain (Noriega and Savelkoul., 2014).

Significantly lower subpopulation of CD4+ and CD8+lymphocyte as well as imbalance between Th1 and Th2- like cytokines have been observed in autism (Gupta *et al.,* 1998 ; Molloy *et al.,* 2006) . Since the Th2 pathway produces more immune-suppressory cytokines compared to Th1 arm that favors more the pro-inflammatory cytokines, and both arms are reportedly activated with a predominant Th2 arm in ASD patients, it might help the body tolerate and not to react towards many antigens (possibly penetrated through GI tract), but these antigens can have deleterious effect on other tissues such as brain. More research is needed to understand the role of immune system in ASD. A number of other immune abnormalities have also been reported in ASD (Randolph-Gips and Srinivasan., 2012).

1.2.5.2. 2 Cytokines Imbalance in ASD:

Cytokines have been shown to regulate the growth and cell proliferation of neuronal tissue and to modulate host responses to infection, injury, inflammation, and diseases of uncertain etiology (Manzardo *et al.,* 2012). Cytokines are secreted proteins that control the intensity, duration, and characters of an immune response. Cytokines also interact with neural systems and are involved in neural development and

maintenance (Bauer *et al*., 2007). Cytokine profiles have been repeatedly linked to ASDs (Molloy *et al*., 2006), and cytokine levels are reportedly increased in pregnant mothers whose children later develop ASD (Goines *et al.*, 2011).

Transforming growth factor beta (TGF-β) has been linked to ASDs in multiple studies (El Gohary *et al*., 2015). TGF-β is involved in diverse aspects of development, cell migration, apoptosis, and regulation in both the immune system and central nervous system (CNS) (Gomes *et al*., 2005). Independent studies have described decreased levels of TGF-β in blood samples from individuals with ASDs. El Gohary *et al.*, 2015 reported their findings among a group of children with ASDs compared with age-matched controls. Similar data were reported by (Ashwood *et al*., 2008) in a large, thoroughly characterized group of children and found that lower TGF-β correlated with more severe behavioral scores in ASD children. In contrast to the observation of lower TGF-β in peripheral blood, TGF-β levels in postmortem brain and cerebrospinal fluid samples were higher in persons with ASDs than those in controls (Vargas *et al*., 2005). Although the relationship between CNS and peripheral TGF-β is unclear, these studies collectively suggest that TGF-β dysregulation may have a lifelong role in ASDs.

Another cytokine recently linked to ASDs is macrophage inhibitory factor (MIF) (Grigorenko *et al*., 2008). MIF is a pro-inflammatory immune regulator that is constitutively expressed in brain tissues and has important influences on neural and endocrine systems (Grigorenko *et al.*, 2008 ; Fingerle-Rowson and Bucala., 2001). Genotyping studies on more than 1000 families uncovered two polymorphisms in the promoter region of MIF that is associated with autism. Additionally, plasma levels of MIF were higher in individuals with autism than in typically developing controls. Finally, individuals with autism with the highest

levels of plasma MIF were found to have the most severe behavioral symptoms (Grigorenko *et al*., 2008).

Another cytokine linked to ASDs is Interleukin-1β. IL-1β is a pro-inflammatory cytokine produced by various sources, including monocytes, macrophages, dendritic cells, neutrophil leukocytes and endothelial cells (Netea *et al.,* 2010). Among previous reports studies that examined serum levels of selected cytokines, i.e., IL-1β (Emanuele *et al.,* 2010) in autistic subjects reported no change. However, two other studies using multiplex assay in ASD have demonstrated a significant increase in plasma IL-1β levels in 2- to 5-year-old children with ASD (Ashwood *et al.,* 2011) also in serum IL-1β levels in adults with Asperger's syndrome(Schwarz *et al.,* 2010). Given the wide variety of functions of IL-1β as an important mediator of inflammatory response, including cell proliferation, differentiation and apoptosis, it is not surprising that this cytokine can serve as a marker for abnormal response in subjects with ASD. On the other hand, IL-1RA binds to the cell surface IL-1 receptor, inhibits the activities of IL-1β, and modulates IL-1-related immune responses (Netea *et al.,* 2010).

Interleukin-2 (IL-2) is a well-known cytokine that plays an important role in multiple immune-regulatory functions related to T-cells in peripheral and CNS (De Araujo *et al.,* 2009). Singh et al. found that serum concentrations of soluble IL-2 were significantly higher in autistic children compared with normal controls. In addition, they showed that stimulated peripheral blood mononuclear cells (PBMCs) from children with autism secrete significantly higher amounts of IL-2, whereas soluble IL-2 receptor levels did not differ between autistic and control subjects (Singh, *et al*., 1991). However, other studies have reported that IL-2

levels did not differ significantly between subjects with autism and control groups (Ashwood, *et al.*, 2010).

Concerning Interleukin-6 (IL-6)**,** there is extensive evidence that IL-6 can alter neurodevelopment and function (Goines and Ashwood., 2013). Many independent studies show IL-6 dysregulation in individuals with autism. Children and adults with the disorder have higher circulating IL-6 levels compared to typical controls (Emanuele *et al.*, 2010; Ashwood, *et al.*, 2011). Further, cellular IL-6 production is increased with and without stimulation (Malik *et al.*, 2011). Increased IL-6 is also found in postmortem brain specimens from ASD subjects. Specifically, immune histochemical analysis of cerebellar sections showed significantly more IL-6 staining in autism postmortem brain specimens (Wei *et al.*, 2011). additional analyses of homogenates of the frontal cortex and anterior cingulate gyrus also showed higher IL-6 levels (Li *et al.*, 2009). Another studies demonstrated that IL6 over expression in granule cells caused impairments in granule cell adhesion and migration but had little effect on the formation of dendritic spines or granule cell apoptosis (Wei *et al.*, 2011).

1.2.5.2.3 Immunoglobulins Imbalance in ASD:

Immunoglobulins (Ig) are proteins produced by B cells that specifically target entities for destruction and removal. There are several classes of Ig, each with a specific role in immunological processes. Recently, decreased levels of total plasma IgG and IgM were described in a large group of individuals with autism compared with age-matched individuals without autism. The reduced levels also correlated with behavior, such that individuals with autism with the most severe behavioral symptom scores had the lowest IgG and IgM levels (Heuer *et al.*, 2008). Further characterization of IgG subclasses demonstrated that young children with autism have significantly higher levels of IgG4

compared with age-matched typically developing children (Enstrom *et al*., 2008) . Although the relationship between reduced total Ig and behavior is unclear, it is possible that a defect in a shared signaling pathway leads to both altered neurodevelopment and immune function. Studies are currently underway to examine this hypothesis.

1.2.6 Microbiota in ASD:

Microbiota is an emerging topic that has attracted several researchers to look for the possible connection between the GI microflora and behavioral abnormalities. Earlier report of deficient disaccharidase enzymatic activity in ASD children and GI symptom (Horvath *et al.*, 1999), prompted investigations looking for intestinal mucosal microbiota involved in carbohydrate metabolism. Abnormal carbohydrate digestion and transport and mucosal dysbiosis (imbalance in the intestinal microbial ecosystem) was reported in the ASD children (Williams *et al.*, 2011).

Gut dysbiosis was proposed to be involved in the pathogenesis of several diseases(Petrof *et al.,* 2013). Reduced level of fermenters has been found in the intestinal microflora of the ASD patients (Kang *et al.*,2013). The microbiota-gut-brain axis refers to the ability of gut microbiota to communicate with brain and regulate behavior (Montiel-Castro *et al.,* 2013). Fecal microbiota trans-plantation has been used in treating several GI disorders but increase knowledge and control trials are needed before it can be used broadly in clinic (Aroniadis and Brand., 2013).

Nevertheless, other studies didn't find a difference in GI microbiota of ASD children with and without GI disturbances (Gondalia *et al.*, 2012). Imbalance in gut microbiota population may render the intestinal mucosa susceptible to injuries ,infections, inflammation, abnormal

digestion, immune imbalance, immune reaction and cross reaction in other tissues including the brain. More research is emerging in this area.

1.2.7 Clinical Features of Autism

a. Sleep problems: Sleep disruption is estimated to affect from 44% to 83% of individuals with ASD, with delayed sleep onset and nighttime wakening being the most predominant symptoms (Krakowiak *et al*., 2008). Several studies have demonstrated that disruption in sleep patterns is associated with problem behaviors during the day, particularly in low-functioning ASD individuals (Cohen *et al*., 2014), and lower overall functioning in several measures of development including greater problems with language and communication (Taylor *et al*., 2012). Melatonin is a safe and effective treatment for sleep disturbance but is less effective for night time wakening (Rossignol and Frye., 2011b) and has been shown to improve daytime behavior and parenting stress (Malow *et al.,* 2012).

b. Seizures : One in four children with ASD has seizures, often starting either in early childhood or during the teen years (NIMH., 2013). Sometimes lack of sleep or a high fever can trigger a seizure. An electroencephalogram (EEG) a nonsurgical test that records electrical activity in the brain, can help confirm whether a child is having seizures. Recently, there have also been reports of high rates of epileptiform EEGs in children with autism without a history of seizures (Kim *et al.*, 2006)

C. Anxiety : Anxiety is very common in ASD (Vasa and Mazurek, 2015), particularly in high-functioning ASD children (Chandler *et al.*, 2015). Anxiety is related to aggressive behavior (Pugliese *et al.*, 2013), more severe repetitive behaviors and lower overall development (Magiati *et al.*, 2015) and sleep disruption (Mazurek and Petroski, 2015). A wide

variety of treatments for anxiety have been studied in individuals with ASD. The best studied treatments for anxiety in ASD include intranasal oxytocin (Hofmann, *et al.,* 2015) and cognitive-behavioral therapy (Ung *et al.*, 2015).

d. Comorbidity of Mental Disorders : Children with ASD can also develop mental disorders such as anxiety disorders, attention deficit hyperactivity disorder (ADHD), or depression. Research shows that people with ASD are at higher risk for some mental disorders than people without ASD (Leyfer *et al.,* 2006). Managing these co-occurring conditions with medications or behavioral therapy, which teaches children how to control their behavior, can reduce symptoms that appear to worsen a child's ASD symptoms. Controlling these conditions will allow children with ASD to focus more on managing the ASD (Simonoff *et al*., 2008).

e. Mortality : Shavelle et al. investigated the mortality rate of ASD in over 13,000 patients between 1983 and 1997 (Shavelle *et al*.,2001) and found it to be more than twice that of neurotypical peers. Standardized mortality ratio (SMR) was estimated as 2.4:1. Similar mortality rates have been reported in other studies (Gillberg *et al*., 2010 ; Mouridsen *et al*., 2008) with a consistent increased mortality rate for ASD and a substantially greater risk in female ASD patients. The elevated mortality risk associated with ASD appeared related to the presence of comorbid medical conditions and intellectual disability rather than ASD itself suggesting the importance of coordinated medical care for this high risk sub-population of individuals with ASD (Bilder *et al.*, 2013)

1.2.8 Diagnosis of Autism

The number of children who are diagnosed with autism spectrum disorders (ASD) has increased. The Centers for Disease Control (CDC., 2012) now estimates that 1 in 88 children have ASD (1 in 54 boys and 1 in 252 girls). This represents a 23% increase from data collected two years previously (CDC., 2009). This increased prevalence suggests that there is a growing need for screening and further referral, when indicated, for a diagnostic evaluation for children suspected of having ASD. To receive appropriate diagnostic services, a child must be able to obtain a comprehensive evaluation conducted by competent and qualified personnel using a protocol of acceptable tools and procedures. This is especially critical since early diagnosis of ASD is needed to help children and their families to realize the positive outcomes that can be achieved by participating in appropriate intervention services at the earliest point (Volkmar *et al.*, 2011).

The diagnostic medical testing of children with ASD or An intellectual disability (formerly mental retardation) has focused on four main areas–genetic testing, neuro imaging, EEG, and metabolic screening. In recent years, there have been significant changes in the recommended diagnostic approach to evaluating children with ASD or intellectual disability. Some conventional tests have been deemed un necessary because of their very low diagnostic yield and others have been rendered‘ obsolete ’with the development of more sophisticated alternative.

1.2.8.1 Genetic Test :

Advances in molecular genetic testing have radically transformed the clinical approach to etiologic evaluation of children with autism and intellectual disability. Chromosomal karyotypes–routine G-banding or when testing for fragility of the X chromosome– have now been replaced by molecular genetic techniques that have a higher yield.

Chromosomal micro array analysis (CMA), other-wise known as comparative genomic hybridization, is the most robust test available to clinicians for identifying a genetic basis for ASD or intellectual disability **(**Shen *et al.,* 2010). Whereas G-banded karyotype would identify a genetic abnormality in fewer than 3% of cases, CMA–with its ability to detect clinically significant copy-number variants with100 times greater resolution than standard karyotyping–has identified clinically significant abnormalities in 8% or more of ASD cases. Although the American Academy of Pediatrics (AAP's) 2007 guidelines did not recommend routine CMA testing , a recent AAP publication detailed the advantages of CMA over karyotypes **(**Shen *et al.,* 2010). The AAP's most recent recommendations to physicians regarding the initial medical evaluation of a child with ASD are outlined in the newly revised Autism Toolkit. There, the AAP recommends in its Physician Fact Sheet 'that CMA be offered to all patients with ASD (AAP., 2012).

Many commercial laboratories offer CMA test batteries that focus on the genetic anomalies most commonly associated with ASD. Interestingly, researchers reported on the findings of a proprietary genetic test battery intended to predict ASDs. Focusing on the 237 genetic markers on 146 genes and related cellular pathways linked to ASDs, this test takes the novel approach of examining multiple genetic mutations and their additive contribution, while considering protective versus vulnerability single-nucleotide polymorphisms (SNPs) and the genetic differences between ethnicities. Investigators reported that the test correctly predicted ASDs in 72% of cases in two independent sets of central European-descending populations (Skafidas *et al.,* 2012). The outcome of ongoing evaluations among other ethnic groups will further determine the test's accuracy and specificity. Nonetheless, like some of the early screening parent questionnaires, this test may

aid early detection of ASDs in some cases.Though not yet clinically available through commercial laboratories, exome sequencing has recently been reported to identify de-novo genetic mutations in about 15% of children with severe intellectual disability, some of whom also had autism (Mefford., 2012). This investigational technique will likely be available in the not-too distant future. Ultimately, it is anticipated that whole-genome sequencing will become commercially available and will supplant all other genetic tests in the evaluation of children with severe developmental disability. A proof of concept study showed that whole-genome sequencing can be done in a clinical setting (Saunders *et al.*, 2012).

Even though chromosomal karyotypes should no longer be routinely ordered, a karyotype is indicated if a balanced translocation is suspected, as these will not be detected with CMA. Clinicians should suspect a balanced translocation if there is a history of more than two miscarriages. children with ASD and macrocephaly (head circumference >2 standard deviations above the mean), PTEN gene
(AAP., 2012).

1.2.8.2 Neuroimaging:

Neuroimaging technologies have likewise become more sophisticated, with functional magnetic resonance imaging (fMRI) emerging as an effective research tool for isolating frequently disrupted neural systems that may underlie ASDs (AAP., 2012). It is suspected that brain abnormalities found consistently among patients with ASDs are a product of underlying genetic mutations that influence the expression of key proteins in the brain, and thus result in inefficient neuronal migration, cortex organization, and overall neural circuitry (Sowell and Bookheimer., 2012).

Current studies are using neuroimaging to isolate these problem regions in the brains of people with and without ASDs, noting differences in gene expression, brain architecture, and displayed behaviors that characterize ASD (Anagnostou and Taylor., 2011). Careful analysis of the structural and volumetric measures derived from brain MRIs has failed to identify a consistent pattern of early brain development in children with ASDs. Some studies using diffusion tensor imaging DTI (a technique that measures water diffusion within a tissue) suggest that there is a distinct white-matter fiber tract maturation pattern discernable in high-risk infants who eventually develop an ASD or ASD-like symptom. It is hoped that identification of ASD brain biomarkers will soon allow earlier and enhanced ASD-risk detection and that, with time, neuroimaging will provide an objective diagnostic test. At this time, isolated, stable macrocephaly is not an indication for an MRI or CT scan. The AAP recommends that MRIs should only be considered in children with acute regression, microcephaly, midline facial defects, neurocutaneous lesions (with or without wood lamp),or abnormalities on neurologic examination (AAP., 2012).

1.2.8.3 Electroencephalogram (EEG):

Guidelines suggest that a sleep-deprived EEG with adequate sampling of slow-wave sleep should only be performed if there is a history of acute developmental regression, unexplained behavior change, clinical seizures, or suspicion of subclinical seizures(AAP., 2012).

1.2.8.4 Metabolic Testing:

Metabolic disorders, resulting from biological errors in amino acid, carbohydrate, purine, peptide, or mitochondrial metabolism, are the cause of an ASD in fewer than 5% of all cases. Metabolic testing should not be routinely performed. The AAP recommends that testing be limited to children with cyclic vomiting, hypotonia, lethargy (especially when associated with mild illnesses), poor growth, unusual odors, multiple organ involvement, ataxia or other movement disorder, or evidence of a storage disease (e.g., coarse features). Testing should include lactate, pyruvate, carnitine, acyl-carnitine profile, liver and renal function, amino acids including testing for phenyl ketonuria, and urine organic acids. Lead levels should be monitored in children with ASD and a history of pica, especially those living in a high-risk environment. Serum ferritin level may also be indicated to assess iron stores. According to the AAP, there is no evidence that hair analysis, micronutrient levels, intestinal permeability studies, stool analyses, urinary peptides, or mercury levels are helpful (Filipek *et al.*, 2000 ; AAP., 2012)

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2.Materials and Methods:

2.1. Materials

2.1.1. Equipments and Instruments:

The equipments and instruments used in this study are listed in table (2-1).

Table (2-1): Equipments & Instruments with their Remarks

Equipments/ Instruments	Company	Country
Centrifuge	Bioneer	Korea
Cylinder (100 ml)	AMSCO	Germany
Eppendorf tubes	Eppendrof	Korea
Incubator	Memmert	Germany
Micropipettes	CYAN	Belgium
Micro-plate washer.	BioTeK	(U.S.A)
Microtiter plate reader (450 nm filter)	Bio TeK	(U.S.A)
Microtiter plate shaker	KAHN	Italy
Multichannel micropipette reservoir	SIAMED	Germany
Plain test tube	Laiwu Yaohua	China
Printer	Brother	China
Rack	Sterellin Ltd.	UK.
Refrigerator	Sanyo medical	Japan
Sterile syringes		China
Tips	Sterellin Ltd.	UK.

2.1.2.Immunological Kits:

The immunological kits used in this study are listed in table (2.2)

Table (2-2):Immunological Kits with their Remarks

Immunological Kits	Company	Country
Human **MIF** ELISA Kit	**USCN**	**China**
Human **IgG** ELISA Kit		
Human **IgM** ELISA Kit		

2.1.2.1 Human MIF Enzyme –Linked Immunosorbent Assay kit

This ELISA kit is used for the quantitative determination of MIF concentration in serum. The contents with companies and countries of origin are listed in table (2-3).

Table (2-3):ELISA Kit Components for MIF Estimation

Components	Specifications	Storage
Micro ELISA Plate	1 copy	4℃
Plate Sealer	8 wells ×12 strips	
Standard	2 vial	4℃
Detection Reagent A	1vial 120μL	4℃
Detection Reagent B	1vial 120μL	4℃
Wash Buffer (30 × concentrate)	1vial 120μL	4℃
TMB Substrate	1vial 9mL	4℃
Manual	1 copy	

Standard Diluent	1vial 20mL	4℃
Assay Diluent A	1vial 12mL	4℃
Assay Diluent B	1vial 12mL	4℃
Stop Solution	1vial 6mL	4℃

2.1.2.2 Total IgG Enzyme–Linked Immunosorbent Assay kit

Total IgG ELISA kit for the quantitative determination of total IgG in human serum. Kit components, their volume and remarks are listed in Table (2-4).

Table (2-4):ELISA Kit Components for IgG **Estimation.**

Components	Specifications	Storage
Micro ELISA Plate	1 copy	4℃
Plate Sealer	8 wells × 12 strips	
Standard	2 vial	4℃
Detection Reagent A	1vial 120μL	4℃
Detection Reagent B	1vial 120μL	4℃
Wash Buffer (30 × concentrate)	1vial 120μL	4℃
TMB Substrate	1vial 9mL	4℃
Manual	1 copy	
Standard Diluent	1vial 20mL	4℃
Assay Diluent A	1vial 12mL	4℃
Assay Diluent B	1vial 12mL	4℃
Stop Solution	1vial 6mL	4℃

2.1.2.3 Total IgM Enzyme–Linked Immunosorbent Assay kit

Total IgM ELISA kit for the quantitative determination of total IgM in human serum. Kit components, their volume and remarks are listed in Table (2-5).

Table (2-5):ELISA Kit Components for IgM **Estimation.**

Components	Specifications	Storage
Micro ELISA Plate	1 copy	4℃
Plate Sealer	8 wells × 12 strips	
Standard	2 vial	4℃
Detection Reagent A	1vial 120μL	4℃
Detection Reagent B	1vial 120μL	4℃
Wash Buffer (30 × concentrate)	1vial 120μL	4℃
TMB Substrate	1vial 9mL	4℃
Manual	1 copy	
Standard Diluent	1vial 20mL	4℃
Assay Diluent A	1vial 12mL	4℃
Assay Diluent B	1vial 12mL	4℃
Stop Solution	1vial 6mL	4℃

2.2 Methods

2.2.1. Patients and control

2.2.1.1 Study design

A case-control study was conducted on the following study groups during the period from the first of December 2015 to the 30th of March 2016. In this study patients group composed of (60),(48) of them are males and (12) females with age range 3-12 years old .This study is carried out at Al-Imam AL- Hussein Institute for the care of autistic children in AL-Najaf and Ruqayah Center for Hearing and Speech in AL-Diwaniaya. The patients were diagnosed clinically by specialist Psychiatrist. Patients were interviewed directly by using an anonymous questionnaire form which covered age, sex, family size , birth order , residence, positive history of seizures , positive history of recurrent Infection , positive history of parental consanguinity and others (appendix 1). Another group consist of 60 apparently healthy individuals (40 male and 20 female) also included in this study as a control group. Verbal informed consent was obtained from all participants.
This study was done to assess MIF, IgG and IgM by ELISA technique.

2.2.1.2 Inclusion Criteria

2.2.1.2.1 Inclusion Criteria of Patients

- Male and female subjects were 3 to 12 years of age.
- Family History of autistic patients.
- Diagnosis of autism case was carried out by psychiatrist .
- No features of other diseases.
- Their parents and institutes administrators accept to participate in the study.

2.2.1.2.2 Inclusion Criteria of Control

- Male and female subjects were 3 to 12 years of age.
- No past or present diagnosis of autism and other neuropsychiatric disorder.
- No major medical issues .

2.2.1.3. Exclusion Criteria of Patient

- Immunocompromised patients.
- Non autistic children in Institutes.
- Patients with other neuropsychiatric disorder.
- Patients over 12 years of age .
- Patients not consenting.
- children who are difficult to deal and to take measurement for them.

2.2.1.4 Clinical Assessment of Patients

- Name, age and gender.
- Family history.
- Physical examination .
- Direct observation and assessment of social and communication skills and behavior.
- Evaluation of speech, language and communication skills.
- Assessment of comorbid conditions.

2.2.1.5 Indian Scale for Assessment of Autism (ISAA)

The ISAA was commissioned by the National Institute for the Mentally Handicapped (NIMH., 2008) as a suitable tool for identification and rating the severity of autism in developing countries as opposed to

the present tests that have mostly western parameters, like the Childhood Autism Rating Scale (CARS). Children were rated according to the ISAA, based on behavioral observation and interaction with the examiner and parents. The ISAA evaluation was completed by an independent, qualified psychiatrist, blinded to the DSM-IV-TR diagnosis. The scale has 40 items based on DSM-IV-TR criteria, divided into six domains: social relationship and reciprocity; emotional responsiveness; speech-language and communication; behavior patterns; sensory aspects and cognitive components . Participants' behavior was rated on a five point scale (rarely, sometimes, frequently, mostly and always). According to the ISAA manual, autism is defined by a score of 70 points. Total scores of 70 to 106 indicates mild autism, 107-153 moderate autism, and scores of 153 and above indicates severe autism. The criterion test validity of ISAA was determined by comparison of total scores obtained on the tool with those on CARS. Pearson Product moment correlation was computed and the resulting correlation r = 0. 77 ($p < 0.001$) reveals that ISAA has high degree of validity as that of CARS. The Arabic translation of the ISAA was conducted by the authors with inter-rater reliability and convergent validity similar to those of the original version (Amr *et al.*, 2011) .

2.2.1.6 Collection of Blood Samples:

Three milliliter of blood for each case and control groups were collected from vein puncture in sterile test tubes (Plain tube) and allow sample to clot for few minutes at room temperature then followed by separation of serum from the clot by centrifugation for 10 minutes at 2500 r.p.m. Then the serum was divided into three Eppendorf tubes, labeled and stored at -20^{o}C one for MIF ELISA assay procedure , one for IgG ELISA assay procedure and other for IgM ELISA assay procedure.

2.2.2 Immunological Study

The reagents preparation and assay procedure were carried out according to manufacturer's description.

2.2.2.1 Enzyme–Linked Immunosorbent Assay of MIF

2.2.2.1.1 Principle of the Test

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Macrophage Migration Inhibitory Factor (MIF). Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to MIF. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain MIF, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of MIF in the samples is then determined by comparing the O.D. of the samples to the standard curve.

2.2.2.1.2 Preparation of Reagents

- Washing solution**:** solution was diluted with distilled water 1:9 before used.
- All reagents and samples were brought to room temperature before used.

- Centrifugation to all samples.

2.2.2.1.3 Assay Steps

1. The reagents, samples and standards were prepared before used.

2. A volume of 100µl of standard or sample were added to each well and incubated for 2 hours at 37^{o}C.

3. The wells were aspirated.

4. A volume of 100µl of prepared detection reagent A added, then incubated for 1 hour at 37^{o}C.

5. The wells were aspirated and washed 3 times.

6. A volume of 100µl of prepared detection reagent B added, then incubated for 30 minutes at 37^{o}C.

7. The wells were aspirated and washed 5 times.

8. Nineteen µl of substrate solution added, then incubated for 20 minutes at 37^{o}C.

9. A volume of 50µl of stop solution was added.

10. The absorbance was read at 450 nm.

11. The results were calculated.

2.2.2.1.4 Standard Curve for MIF Human ELISA.

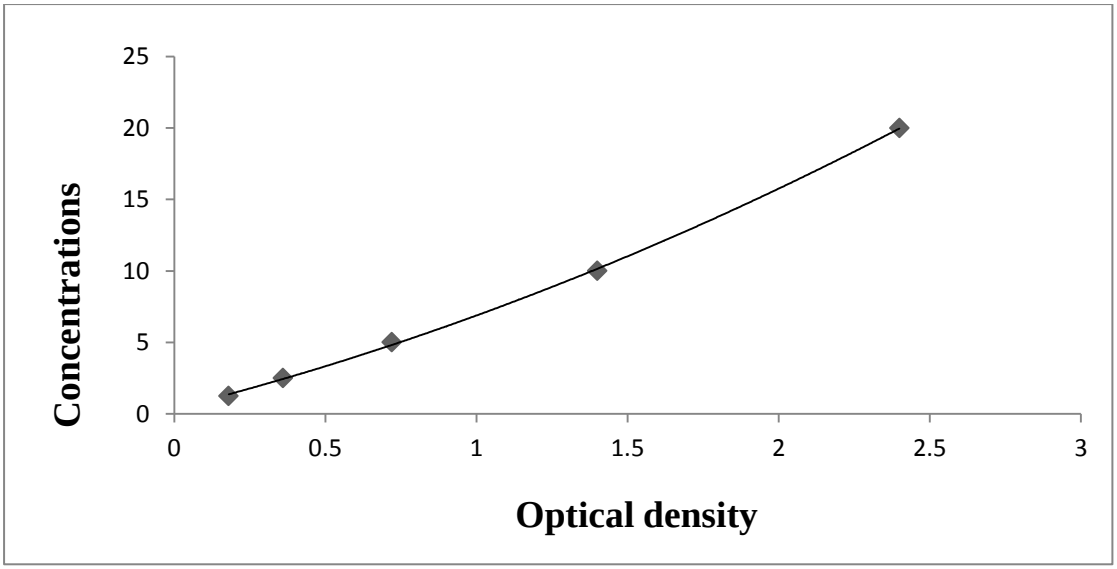

Figure (2-1): Standard curve for MIF Human ELISA

2.2.2.2 Enzyme–Linked Immunosorbent Assay of IgG

2.2.2.2.1 Principle of the Test

This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to Immunoglobulin G (IgG) has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled IgG and unlabeled IgG (Standards or samples) with the pre-coated antibody specific to IgG. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of IgG in the sample. After addition of the substrate solution, the intensity of color developed is inversely proportional to the concentration of IgG in the sample.

2.2.2.2.2 Preparation of Reagents

- Washing solution**:** solution was diluted with distilled water 1:9 before used.

- All reagents and samples were brought to room temperature before used.
- Centrifugation to all samples.

2.2.2.2.3 Assay Procedure

1. The reagents, samples and standards were prepared before used.

2. A volume of 50µl of standard or sample and 50µL of detection reagent A were added to each well. Then mixed and Incubated for 1 hour at $37^{o}C$.

3. The wells were aspirated and washed 3 times.

4. A volume of 100µl of prepared detection reagent B added, then incubated for 30 minutes at $37^{o}C$.

5. The wells were aspirated and washed 5 times.

6. Nineteen µl of substrate solution added, then incubated for 20 minutes at $37^{o}C$.

7. A volume of 50µl of stop solution was added.

8. The absorbance was read at 450 nm.

9. The results were calculated.

2.2.2.2.4 Standard Curve for IgG Human ELISA

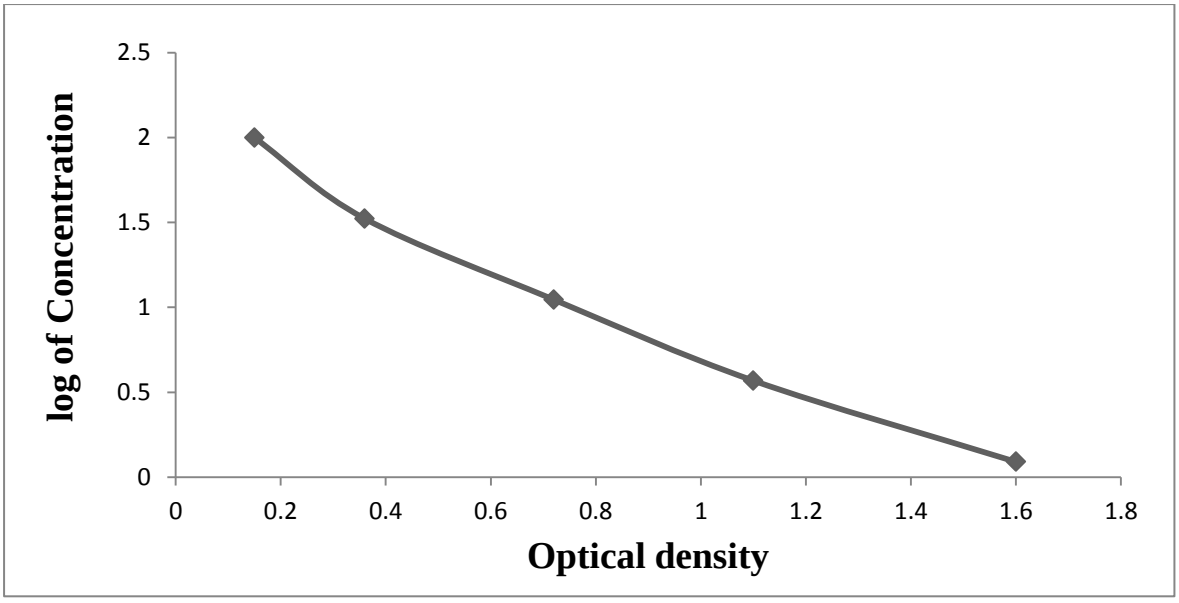

Figure (2-2): Standard curve IgG Human ELISA

2.2.2.3 Enzyme–linked immunosorbent assay of IgM

2.2.2.3.1 Principle of the Test

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Immunoglobulin M (IgM). Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to IgM. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain IgM, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of IgM in the samples is then determined by comparing the O.D. of the samples to the standard curve.

2.2.2.3.2 Preparation of Reagents

- Washing solution**:** solution was diluted with distilled water 1:9 before used.
- All reagents and samples were brought to room temperature before used.
- Centrifugation to all samples.

2.2.2.3.3 Assay Procedure

1. The reagents, samples and standards were prepared before used.
2. A volume of 100µl of standard or sample were added to each well and incubated for 2 hours at 37^{o}C.
3. The wells were aspirated.
4. A volume of 100µl of prepared detection reagent A added then incubated for 1 hour at 37^{o}C.
5. The wells were aspirated and washed 3 times.
6. A volume of 100µl of prepared detection reagent B added, then incubated for 30 minutes at 37^{o}C.
7. The wells were aspirated and washed 5 times.
8. Nineteen µl of substrate solution added, then incubated for 20 minutes at 37^{o}C.
9. A volume of 50µl of stop solution was added.
10. The absorbance was read at 450 nm.
11. The results were calculated.

2.2.2.3.4 Standard Curve for IgM Human ELISA

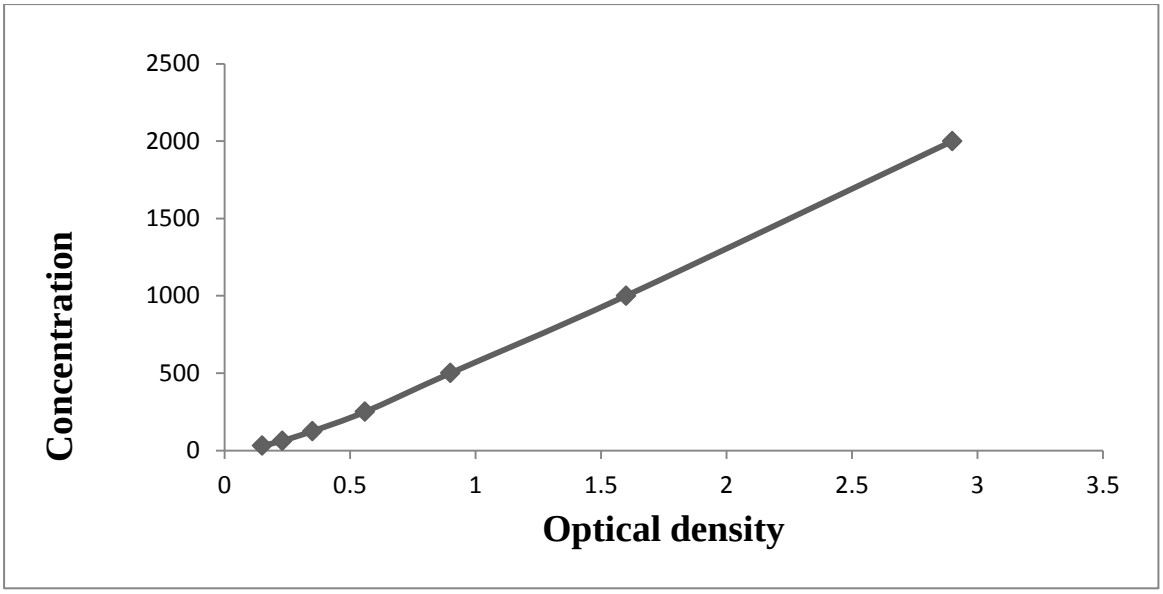

Figure (2-3): Standard Curve for IgM Human ELISA

2.2.3 Statistical Analysis

Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using SPSS version 20 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2010.

Some of the outcome quantitative variables were non-normally distributed. These variables are: Serum MIF, Serum IgG and Serum IgM. The quantitative variables were normally distributed and were thus conveniently described by mean, SD (standard deviation) and SE (standard error), and the parametric statistical tests of significance were used. The independent samples t-test was used to test the statistical significance of difference in mean between 2 groups. Chi-square test was used for the assessment of association between the variables studied. The p-value of less than 0.05 was statistically significant, and highly significant for p-value of less than 0.001.

3. Results and Discussion.

3.1 Subjects Demographic Characteristics.

This is a case-control study include 60 autistic patients (48 males and 12 females), the age of the patients ranged from 3-12 years with a mean age of 6.01 years (SD ± 2.45), compared with 60 (40 males and 20 females) apparently healthy subjects with age ranged from 3 to 12 years, and mean age of 5.81 years (SD ± 2.61) as a control group (table 3-1 and 3-2).

Table (3-1): Distribution of autistic patients according to age and gender

Age (years) Groups	Males NO.	Females NO.	Total NO.(%)
3-6	25	4	29 (48.3%)
7-10	18	7	25 (41.7%)
>10	5	1	6 (10%)
Total	48	12	60(100%)

Table(3-2): Mean age of population study

Population study comparison			
	Healthy controls	Cases (Autism)	P-value
Age (years)			0.194[NS]
Range	(3 - 12)	(3 - 12)	
Mean	5.81	6.01	
SD	2.61	2.45	
SE	0.65	0.31	
NO.	60	60	

❖ NS= No Significant (p > 0.05), SD= Standard Deviation, SE= Standard Error, NO= Number

The age and gender characteristic of patients who have autism in table (3-1), show the highest frequency of autism among children particularly in the age from 3-6 years old (48.3%), followed by the age group of 7-10 years old (41.7 %**)** and decrease with age for both sexes**.** These results agree with the results of Malow., (2006), which indicate that the majority of autistic children ages are preschool age children.

These results also agree with the results of Abbas., (2013), who studied 80 cases of autism at age 3-14 years, from specialized institutes for care of autistic children in Baghdad (Rami Institute ,Rahman Institute and Noor Institute), which found high prevalence of autistic patients in the age (3-6) years, followed by age (7-10) years and decrease with age. Also, these results are nearly similar to study done by Lazam and Al- Hemiary., (2013), which showed that 66% of autism patients were reported in the age group 1–5 years old.

Table (3-2) shows the mean age of patients are 6.01 ± 2.45 and 5.81 ± 2.61 for control group (P = 0.194), this is in consistence with Rashid *et al*.,(2012),who studied 31 children (29 males and 2 females) who were diagnosed as autistic children, their ages range between 2-13 years and found the mean age of autistic patients was (5.9 ± 3.4), and these results also agree with Ismael and jacoub., (2012) and Dawood., (2013) who found the mean age of autistic patients (6.12 ± 2.72 and 5.78 ± 2.54 respectively). But the current study disagrees in mean age with studies of Napolioni *et al*., (2013), who found mean age for autistic patients was 8.11± 3.65 and 7.44 ± 3.12 for control (p=0.218) and Mostafa *et al.* (2012) who found that the children age ranging between 4 and 11 years, with a mean 8.2 ± 2. Our results also disagree with results of Tostes *et al*., (2013) who found mean age was (7.4 ± 2.9) for

24 patients. This differences may be due to the selection criteria for each study (Sipos *et al*., 2012).

The present study revealed higher prevalence of autism in male children (80%) than female (20%),with a ratio of 4:1, which is statistically significant (P-value <0.001), table (3-3), indicate that males were more affected by autism, because the females are under diagnosed, these results agree with the results of Schaafsma and Pfaf.,(2014). The female with ASD have been reported to have an elevated mortality risk compared with male, these results agree with the results of Bilder *et al*., (2013). Other studies showed higher prevalence of autism in male than female because the females with ASD are protected against some of the symptoms of ASD (often called the 'female protective effect' or FPE) (Robinson *et al.,* 2013).

Table (3-3): Gender Distribution in Population study.

Population study comparison					
	Healthy controls		Cases (Autism)		P
Gender	NO.	%	NO.	%	<0.001
Male	40	66.7	48	80	
Female	20	33.3	12	20	
Total	60	100.0	60	100.0	

❖ **S= significant association (p <0.05).**

These results agree with most studies that regarded sex as a risk factors for autism. From these , Karmeet *et al*., (2015),who studied 40 cases of autism at age 3-10 years, from specialized institutes, which observed the higher prevalence of autism in male than female with 4:1 ratio. Wtwt and Farhood *et al*.,(2014),also studied 70 cases of autism, from specialized institutes (Al Rehma Institute of Autism and Babil

Specialized Institute of Autism in Babylon city and Al Imam Al Hussein Institute of Autism In Karbala city), observed the higher prevalence of autism in child male than female with 4:1 ratio. Shao *et al.,* (2002) and Liu *et al.,* (2001) reported that the males have more prevalence of autism than female suggested that autism is an X-linked disorder, this may explain male predominant of autism, but other study, Hallmayer *et al.,* (1996) found cases of male-to-male transmission of autism in multiplex families.

Table (3-4) revealed that 52 (86.67%) of the children with autism were from urban area ,and these results indicate high significant correlation between residence and autism($p < 0.001$).

Table (3-4): Distribution of Autistic Patients and Control Group According to Some Variables

S= significant association (p <0.05).* NS= No Significant (p > 0.05) fisher exact test

Population study comparison					
Variable	**Healthy controls**		**Cases (Autism)**		**P**
Residence	**N.**	**%**	**N.**	**%**	**<0.001**
Urban	**33**	**55**	**52**	**86.7**	
Rural	**27**	**45**	**8**	**13.3**	
Total	**60**	**100.0**	**60**	**100.0**	
Birth order					**0.131 NS**
1st baby	**26**	**43.3**	**18**	**30**	
2nd &more	**34**	**56.7**	**42**	**70**	
Total	**60**	**100.0**	**60**	**100.0**	

This might be due to remote site of institutes from rural area, lack of awareness of autism signs by parents and even doctor (Levy *et al.,*

2003) other reason was reported as higher neonatal environmental microbial exposures in urban areas (Becker., 2010). These results agree with the results of Wtwt and Farhood., (2014), who studied 70 cases with autism collected from 3 specialized institutes for mental handicapped found that 59 (84.3%) were from urban area. Our results also agree with the results of Lai *et al.*,(2012), which indicated an association between ASD and urbanicity (i.e. higher risk of autism in urban versus rural districts). Our results disagree with the results of Malek *et al.*, (2015), which showed that there is no relationship between the urban/rural residence and autism.

Also table (3-4) showed that there are 18 (30%) of children with autism were first baby, and these results showed no-significant association between birth order and autism, (P> 0.05). Based on these findings, birth order is probably not a direct and decisive determining factor for autism. Our results are in agreement with that of Wtwt and Farhood., (2014); Brimacombe *et al*., (2007) which showed that there is no association between birth order and autism. But our results disagree with the results of Schrieken *et al.*, (2013) and Sasanfar *et al.*, (2010) which reported that the first birth order is a risk factor for autism. The present study also disagree with the results of Hasnain and Akter., (2014) which indicate significant association between birth order and autism, where 58% of children of autism were first born.

Concerning levels of education the father and mothers of autistic children have higher level of education (53.33 % and 43.3% respectively) which is statistically significant (P <0.05), table (3-5).

Table (3-5):Association Between autistic patients and Parents Demographic Factors

Variable	Father		P value	Mother		P value
Education level	**N.**	**%**	**<0.001**	**N.**	**%**	**0.001**
Illiterate	**6**	**10**		**15**	**25**	
Primary	**9**	**15**		**8**	**13.33**	
Secondary	**13**	**21.7**		**11**	**18.33**	
Higher education	**32**	**53.3**		**26**	**43.33**	
Total	**60**	**100.0**		**60**	**100.0**	
Occupation	**N.**	**%**	**<0.001**	**N.**	**%**	**0.019**
Employee	**46**	**76.7**		**33**	**55**	
Not employee	**14**	**23.3**		**27**	**45**	
Total	**60**	**100.0**		**60**	**100.0**	

S= significant association (p <0.05).

These results are consistent with the results of Ou *et al.,* (2015), which showed highest frequency of autism with higher education of parents which is statistically significant (P< 0.05).

Our results revealed significant association between occupation of father and autistic children (p <0.05), The results show that 46 (76.7%) of autistic children had employed fathers, while 14 (23.3%) of them, their fathers are not employed, table (3-5). These results also

revealed significant association between occupation of mother and autistic children ($p < 0.05$), The results show that 33(55%) of autistic children had employed mothers, while 27 (45%) of them, their mothers are not employed. The high percent of employed parents among participants is explained by good economic status of the family allowing their children attending specialized centers of autism. Our results is approximately similar to the results of Wtwt and Farhood., (2014), which indicate high percent of autistic children have employed parents. Our results agree with results of Dardas and Ahmad., (2014) for occupation of father which indicated that high percent of autistic children have employed father (84.3%), but disagree for occupation of mother which indicated low percent of autistic children have employed mother (19.3%).

3.2 The Severity of Autistic Behavior

Autism children were rated according to the Indian Scale for Assessment of Autism (ISAA), based on behavioral observation and interaction with the examiner and parents.

The study throw light on the possible effects of child's age, child's gender, seizure and sleep problems on the severity autistic behavior, table (Table 3-6).

Table (3-6), shows there is no relationship between the severity of autistic behavior and child's age ($p > 0.05$). These results are similar to study done by Dawood., (2013) which indicated that there is no relationship between the severity of autistic behavior and child's age. The result of the present study was consistent with Venker *et al.*, (2014) who reported that age had no bearing on the severity of autistic

symptoms. These results also agree with results of Kern *et al.,* (2014) which showed that there was no significant relationship between age and the severity.

The current study detects there is no relationship between the severity of autistic behavior and child's gender ($p > 0.05$) table (3-6). These results are similar to study done by Dawood., (2013) which indicated that there is no relationship between the severity of autistic behavior and child's gender. These results agree with study of Venker *et al.,* (2014) who reported that gender had no bearing on the severity of autistic symptoms. These results agree with study of Kern *et al.,* (2014) which showed that there was no significant relationship between gender and severity. But our results disagree with results of Dworzynski *et al.,* (2012), which indicate that ASD-diagnosed girls had a higher mean total problem score (hyperactivity, anxiety, and conduct, peer, and prosocial problems) than male.

Although our results indicated that individuals with autism and seizures are more apt to have moderate to severe autistic behavior, but these results shows there is no significant association between the severity of autistic behavior and seizure ($p > 0.05$). Our results disagree with study of Hartley-McAndrew and Weinstock., (2010) which indicate ASD children with seizures revealed significantly worse behaviors as compared to those without seizures.

The current study shows 8 (13.33%) of autistic children with severe behavior have sleep problems, table (3-6), and there is association between the severity of autistic behavior and sleep problems ($p <0.05$). Where increase sleep problems, will increase the severity of autistic behavior. These results agree with study of Park *et al .,* (2012); and Tudor *et al.,* (2012), which show that insufficient sleep exacerbates

the severity of ASD symptoms (e.g., repetitive behaviors, social and communication difficulties). Our results also agree with results of Richdale and Schreck., (2009); Kozlowski *et al.*, (2012); Matson *et al.*, (2008), which indicated fewer hours of sleep have been shown to associate with ASD severity such as social skill deficits compared to children with ASD who do not experience sleep difficulties**.** Our results also agree with results of Schreck *et al.*, (2004) which indicate fewer hours of sleep per night predicted ASD severity score, social skill deficit, and stereotypic behavior**.** And results of Mayes and Calhoun., (2009), which indicate sleep problems increased with severity of ASD symptoms.

Table (3-6): Association Between the Severity of Autistic Behavior and Some Variables

	Severity of Autistic Behavior (ISAA Score)						
Variables	**Mild behavior (70 -106 Score)**		**Moderate behavior (107-153 Score)**		**Severe behavior (>153 Score)**		**P value**
child's age	**N.**	**%**	**N.**	**%**	**N.**	**%**	**0.994**
3-6	**6**	**10**	**14**	**23.33**	**9**	**15**	
7-10	**6**	**10**	**11**	**18.33**	**8**	**13.33**	
>10	**1**	**1.7**	**3**	**5**	**2**	**3.33**	
Total	**13**	**21.7**	**28**	**46.7**	**19**	**31.7**	
child's gender	**N.**	**%**	**N.**	**%**	**N.**	**%**	**0.311**
Male	**11**	**18.33**	**24**	**40**	**13**	**21.7**	
Female	**2**	**3.33**	**4**	**6.7**	**6**	**10**	
Total	**13**	**21.7**	**28**	**46.7**	**19**	**31.7**	
Seizure	**N.**	**%**	**N.**	**%**	**N.**	**%**	**0.304**

Present	**1**	**1.7**	**2**	**3.33**	**4**	**6.7**	
Absents	**12**	**20**	**26**	**43.3**	**15**	**25**	
Total	**13**	**21.7**	**28**	**46.7**	**19**	**31.7**	
Sleep problems	**N.**	**%**	**N.**	**%**	**N.**	**%**	**0.014**
Present	**0**	**0**	**5**	**8.33**	**8**	**13.33**	
Absents	**13**	**21.7**	**23**	**38.33**	**11**	**18.33**	
Total	**13**	**21.7**	**28**	**46.7**	**19**	**31.7**	

S= significant association (p <0.05). * NS= No Significant (p > 0.05)

3.3: Clinical Features of Autism

Table 3-7 show the distribution of some clinical features among autistic patients, there was significant association between seizure, sleep problems and autistic children (p <0.05). Concerning anxiety these is non-significant association with autistic children (p > 0.05).

The current study showed significant development of some clinical features in autistic patients which present as sleep problems and seizure, in our study there was (21.7 %) and (11.67 %) of cases presented with sleep problems and seizure respectively.

Table (3-7): Distribution of Some Clinical Features Among autistic patients

***S= significant association (p <0.05). * NS= No Significant (p > 0.05)**

Clinical Features		Cases (Autism)		P value
		N.	%	
Seizure	Positive	7	11.7	0.007
	Negative	53	88.3	
	Total	60	100.0	
Sleep problems	Positive	13	21.7	<0.001
	Negative	47	78.3	
	Total	60	100.0	
Anxiety	Positive	3	5	0.083
	Negative	57	95	
	Total	60	100.0	

Mechanism for seizure in autism may be explained by abnormal formation of synapses that occur where disrupted synaptic development may also contribute to seizure, and this may explain why the two conditions are associated (Tuchman *et al.*, 2008). This result which is consistent with results of AL- Shimery *et al* ., (2011) showed that seizure was found in 9% of children with autism. Pavone *et al.*, (2004) found the prevalence of seizures is only 6% to 8% in children with autism after excluding other factors that cause seizures, also this results agree with results of Gelder *et al*., (2007) and Pickett *et al.*, (2009), which showed seizure disorder in 25% of autistic children.

Authors has shown that sleep deprivation can often facilitate seizures, and conversely, seizures adversely affect sleep architecture (Malow., 2004). These results approximately agree with the results of AL- Shimery *et al* ., (2011), who studied 33 cases of autism at age 2-11 years, found the sleep problems presented in about 21% of child patients. While these results disagree with the results of Richdale *et al.*, (2009), that reported about two-thirds of individuals with autism are affected by sleep problems. Our results also disagree with the results of Vignoli *et al.*, (2015) and Dawood., (2013), that reported (41.2 % and 38.5% respectively) of individuals with autism are presented with sleep problems. In a Norwegian study, sleep problems in children with autism were reported to be more than ten times higher compared to controls. The authors also found that the sleep problems were more persistent over time, implying a need for increased awareness of these problems in children with autism (Sivertsen *et al.*, 2012).

But our study showed non-significant association between anxiety and autistic children where anxiety presented in about 5% of children with autism ($p > 0.05$), table (3-7). These results agree with the results of AL- Shimery *et al* ., (2011), which found the anxiety presented in about 6.1% of children patients. While these results disagree with the results of Dunsaed., (2014) which indicate significant relationship between ASD and generalized anxiety disorder where generalized anxiety disorder presented in about 17.3% of children with ASD.

These significant relationship between ASD and generalized anxiety disorder can be partially accounted by the mediating role of sleep problems in children with ASD (Dunsaed ., 2014).

3.4 Autistic Children with Family History of Autism and Other Psychiatric Disorders:

The presence of family history is an important contributory factor in autism. This study showed 8(13.3 %) of autistic patients have positive family history of autism and other psychiatric disorders, Figure (3-1).

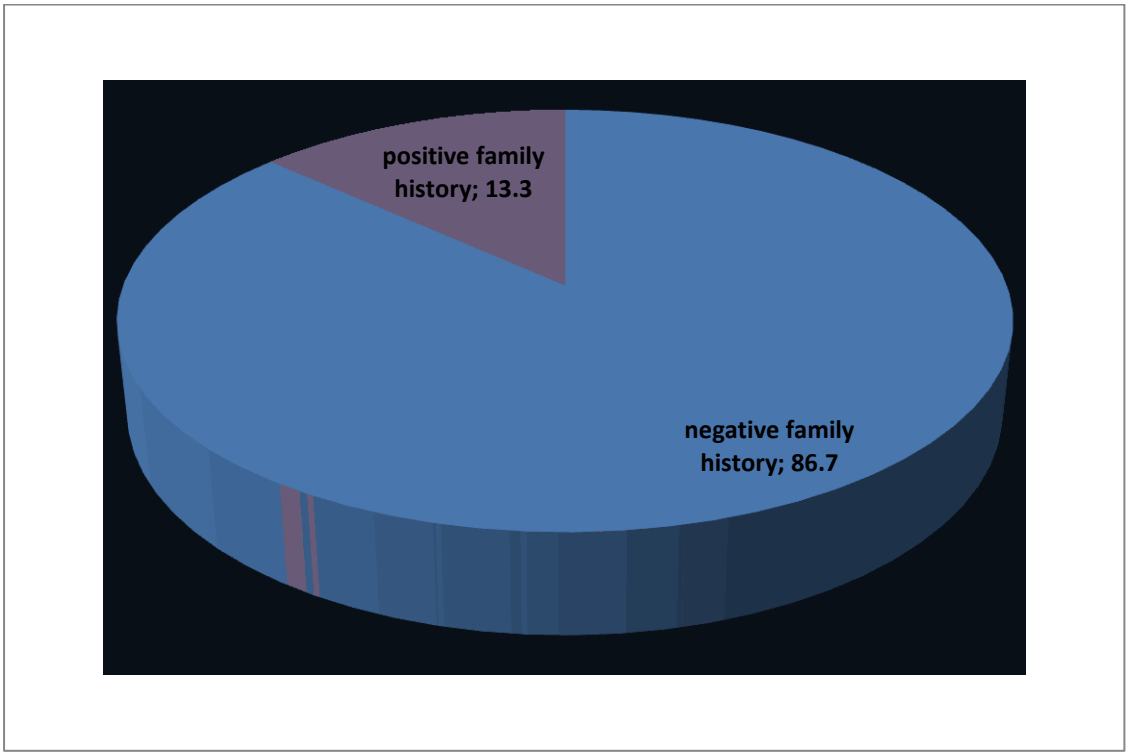

Figure (3-1): The association between history of family and cases of Autism

The presence of positive family history of autism is an important contributory factor, and may explain the genetic role for autism. These results agree with the results of Hussein *et al*,. (2011), who found that the family history was significantly associated with the risk of autism (16% of cases). These results also agree with the results of AL- Shimery *et al* ., (2011),which indicate that the positive family history of autism and other psychiatric disorders found in (15.2% of cases). Our results also agree

with results of El-Baza *et al.,* (2011),who studied 100 cases of autism their age ranged from 2 to 13 years, revealed that the positive family history was significantly associated with the risk of autism (16% of cases versus 1% of control). While this results disagree with results of Tomoum and Hassan., (2009), who studied 21cases diagnosed with autism with a mean age of 6.9 ± 2.9 years, which found that there was no family history in the studied cases.

3.5 Subjects Immunological Assessment

The mean serum concentration of MIF were significantly increased among cases with autism 13.31 ± 4.00 ng/ml as compared to healthy controls 5.61± 2.26ng/ml, while the mean serum concentration of IgG and IgM were significantly decreased among cases with autism 20.66 ± 6.13ug/ml and 208.18 ± 22.43mg/dl respectively compared to healthy controls 38.01 ± 7.21ug/ml and 245.53 ± 15.5mg/dl respectively, table (3-8).

Table(3-8): Population study Difference in Mean Serum Concentrations MIF, IgG and IgM

	Population study comparison		
	Healthy controls	**Cases (Autism)**	**P**
Serum MIF conc.ng/ml			**< 0.001**
Range	**(2.42 - 10.74)**	**(2.55 - 18.73)**	
Mean	**5.61**	**13.31**	
SD	**2.26**	**4.00**	
SE	**0.51**	**0.49**	
N	**60**	**60**	
Serum IgG conc.ug/ml			**< 0.001**
Range	**(26.89 – 50.24)**	**(10.55 – 34.52)**	
Mean	**38.01**	**20.66**	
SD	**7.21**	**6.14**	
SE	**1.61**	**0.74**	
N	**60**	**60**	

Serum IgM conc.mg/dl)			**< 0.001**
Range	**(200.96- 271.02)**	**(163.41– 245.75)**	
Mean	**245.53**	**208.18**	
SD	**15.5**	**22.43**	
SE	**3.46**	**2.72**	
N	**60**	**60**	

These results revealed a significant association between the concentration of MIF and the autism (P <0.001), table (3-8). Higher serum concentration of MIF may be due to its important role in pathophysiology of autism. MIF is a pro-inflammatory immune regulator that is constitutively expressed in brain tissues and has important influences on neural and endocrine systems (Grigorenko *et al.*, 2008).

The present results are similar to results of Grigorenko *et al.*, (2008), which revealed that the mean MIF value for cases (13.12 + 9.18 ng/mL) is higher than those of control group (6.87 + 2.75 ng/mL) and this difference was statistically significant (P <0.05), but these result disagree with result of Tomoum and Hassan., (2009) which reported that the levels of MIF was lower in the patients (3.73± 3.9); and there was no significant difference from those levels among the control group (4.1 ± 3.8).

Our study also shows a significant association between serum level of IgG and the autism (P<0.001), these lower serum concentration of IgG in autism suggests an underlying defect in immune function contributing in the development of autism. The present results are similar to results of Heuer *et al.*, (2012), who revealed that the total IgG was lower in the autistic patients than that of healthy controls

(P < 0.001). Chaudhry *et al.*, (2015) found that the mean IgG value for the cases was (1263 ±490.2) lower than those of control group (1565 ±488.4) and this difference was statistically significant (P <0.05). These results also agree with results of Grether *et al*., (2010), who reported that the serum IgG levels were significantly lower in autistic children as compared to controls. The results of our study disagree with results of Spiroski., (2015); Trajkovski *et al.*, (2004) which reported that there was increases in the total IgG level in autistic patients when was compare to healthy controls.

The current study revealed a significant association between serum level of IgM and autism (P<0.001), table (3-8). These lower serum concentration of IgM also suggests an underlying defect in immune function contributing in the development of autism. The present results are similar to results of Heuer *et al*., (2008), who revealed that the total IgM was lower in the autistic patients than that of healthy controls (P < 0.001). Chaudhry *et al.*, (2015) found the mean IgM value for cases (214.9 ± 95.1) lower than those of control group (221.4 ± 94.1) and this difference was statistically significant (P <0.05). El-Aziz and El-Din., (2012) conducted a study that there was immunoglobulin deficiencies when compare between autistic children and healthy controls. There was significant difference in immunoglobulin levels between two groups. Spiroski.,(2015) and Trajkovski *et al.*, (2004) disagree with our result when reported that there was an increase in IgM level in autistic children as compared to control.

3.5.1 Correlations Between the Severity of Autistic Behavior and Some Immunological Markers.

3.5.1.1 Correlations Between the Severity of Autistic Behavior and MIF Levels

The current study detects that the serum MIF levels is significantly correlated with behavioral symptoms of autistic children (P <0.001) table (3-9).

Table (3-9): Correlations Between the Severity of Autistic Behavior and MIF Levels

	severity of autistic behavior(ISAA Score)			
	Mild behavior (70 -106 Score)	**Moderate behavior (107-153 Score)**	**Severe behavior (>153 Score)**	**P value**
Serum MIF conc.ng/ml				**< 0.001**
Range	**(2.55 –18.73)**	**(5.95 –18.70)**	**(10.26 –18.39)**	
Mean	**10.15**	**13.16**	**15.74**	
SD	**4.56**	**3.61**	**2.95**	
SE	**1.26**	**0.68**	**0.68**	
N	**13**	**28**	**19**	
r= 0.492				

Serum MIF levels were plotted against total ISAA scores. It was found that the serum MIF levels shows a positive correlation to the severity of autism according to total ISAA Score. Thus, the higher the serum MIF level, the higher score of ISAA in autistic patients, which may be indicating that there was association between the increased level of MIF and the severity of behavioral symptoms in autism. These results agree with results of Grigorenko *et al*., (2008) which indicated that there is a statistically significant positive correlations between plasma MIF levels and behavior symptoms. But these result disagree with result of Tomoum and Hassan., (2009) which indicated the correlation of the MIF levels to the different behavioral parameters assessed by the CARS, revealed a negative correlation with the severity of behavior.

3.5.1.2 Correlations Between the Severity of Autistic Behavior and Immunoglobulin Levels

The current study detects that the serum Immunoglobulins levels are significantly negatively correlated with behavioral symptoms of autistic children (3-10).

Total IgG levels were plotted against total ISAA scores. Serum IgG levels show significant negative correlation with total ISAA Score ($r = -0.396$) ($P < 0.05$). Thus, the lower the serum IgG level, the higher the ISAA score, indicating increased severity of behavioral symptoms that are associated with autism. The same relationship was noted for IgM, although to a more degree of significance ($P < 0.001$). Although the relationship between reduced total Ig and behavior is unclear, it is possible that a defect in a shared signaling pathway leads to both altered neurodevelopment and Immune function (Goines and Van de Water., 2010).These results agree with results of Heuer *et al*., (2008) which indicated that there is a statistically significant negative correlations between plasma Immunoglobulin (IgG and IgM) levels and behavior symptoms. These results also agree with results of Heuer *et al*., (2012) which indicated that lower Immunoglobulins (IgG and IgM) levels is correlated with more severe scores on the aberrant behavior checklist (ABC).

Table (3-10): The Correlations Between the Severity of Autistic Behavior and Immunoglobulin Levels

	severity of autistic behavior(ISAA Score)			
	Mild behavior (70 -106 Score)	**Moderate behavior (107-153 Score)**	**Severe behavior (>153 Score)**	**P value**
Serum IgG conc.ug/ml				**< 0.05**
Range	**(16.80 – 34.52)**	**(12.65 – 30.45)**	**(10.55 – 33.28)**	
Mean	**24.09**	**21.71**	**17.36**	
SD	**6.97**	**5.6**	**5.64**	
SE	**1.93**	**1.06**	**1.29**	

N	**13**	**28**	**19**	
r = - 0.396				
Serum IgM conc.mg/dl				**< 0.001**
Range	**(208.19-244.30)**	**(171.35 - 245.75)**	**(163.41-225.52)**	
Mean	**232.63**	**209.37**	**187.94**	
SD	**10.21**	**18.49**	**16.21**	
SE	**2.83**	**3.49**	**3.72**	
N	**13**	**28**	**19**	
r = - 0.711				

Table (3-11): shows there was no association between serum MIF levels and the child's gender (P= 0.272). Our study also shows no significant association between serum level of IgG and the child's gender (P= 0.115), and the present study indicate no significant association between serum level of IgM and the child's gender (P= 0.175). Unfortunately no previous studies pay attention to study the association between serum MIF levels and the child's gender, but our results agree with results of Heuer *et al*., (2008) which indicated correction for age, sex, and allergy season did not alter significantly with immunoglobulin levels(IgG and IgM).

Table (3-11): Associations Between Immunological Parameters and Gender

	Population study comparison		
	Male	**Female**	**P**
Serum MIF conc.ng/ml			**0.272**

Range	**(2.55- 18.70)**	**(6.73- 18.73)**	
Mean	**13.03**	**14.50**	
SD	**4.18**	**3.78**	
SE	**0.60**	**1.09**	
N	**48**	**12**	
Serum IgG conc.ug/ml			**0.115**
Range	**(12.65– 34.52)**	**(10.55 – 33.28)**	
Mean	**21.5**	**18.25**	
SD	**6.39**	**5.79**	
SE	**0.92**	**1.67**	
N	**48**	**12**	
Serum IgM conc.mg/dl)			**0.175**
Range	**(163.41- 244.3)**	**(169.91– 245.75)**	
Mean	**209.64**	**199.58**	
SD	**21.47**	**27.23**	
SE	**3.1**	**7.86**	
N	**48**	**12**	

NS= No Significant (p > 0.05)

Conclusions:

This study showed**:**

1. That autism increases in children's (especially males) and frequently is found within the same family, also showed that autism is more in urban area.

2. The importance of MIF in pathogenesis of autistic population and association between MIF and autism susceptibility might be prospected.

3. Lower levels of serum IgG and IgM in autistic patient compared with healthy controls and these immunological parameters have significantly associated with the severity of autistic behavior and not associated with gender.

Recommendations:

1. Educate the people about psychiatric illnesses and encourage them for seeking advice when their children have abnormal behavior.

2. Pediatricians play an important role in early recognition of autism, they should have a strategy for assessing and carefully examine the patients who come with language disorders, hearing problems or mentally retarded to differentiate them from cases of autism and encourage specialist referral for the patients where early recognition of autistic children and referring them is important for both family and patient to provide appropriate interventions and supports to minimize stress and behavioral disturbance.

3. Interest of officials in autistic children and support those responsible for them and following them constantly as an important segment that cannot be overlooked.

4. A sequential studies for a class of autistic children and to create treatment programs and appropriate tests for the diagnosis and treatment.

5. Educational programs for parents to improve their coping strategies.

6. Study polymorphism of MIF in autistic patients.

7. Study other immunological parameters and other classes of immunoglobulins.

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الخلاصة :

اضطراب طيف التوحد (ASD) : هو اضطراب النمو العصبي الذي يتميز بضعف في التفاعل الاجتماعي وعجز في التواصل. هذا الاضطراب قد تزايد انتشاره بشكل ملحوظ على مدى العقود الماضية. ولقد اقترح الدور المحتمل للخلل المناعي في هذا الاضطراب.

الهدف من الدراسة الحالية هو لمعرفة ارتباط بعض المعلمات المناعية مثل العامل المثبط لهجرة الخلية البلعمية (MIF) وبعض الاجسام المضادة (IgG and, IgM) بين مرضى التوحد في العراق.

تضمنت هذه الدراسة جمع 60 عينة دم من اطفال مصابين بالتوحد تتراوح اعمارهم من 3 الى 12 سنة (48 ذكور و 12 اناث) حيث تقدر نسبة الذكور الى الاناث حوالي 1:4 ومتوسط اعمارهم (6.01 ± 2.45) متواجدين في معهد الامام الحسين لرعاية التوحد في محافظة النجف ومركز رقية للسمع والتخاطب في مدينة الديوانية وايضا تضمنت هذه الدراسة جمع عينات دم من اطفال غير مصابين بالتوحد (مجموعة سيطرة) مماثلين لمرضى التوحد بالعمر والجنس خلال الفترة الممتدة من كانون الاول / 2015 الى اذار / 2016 .

خلال هذه الدراسة تم استخدام استبيان الحالة الاجتماعية, جدول المقابلة المنظم على اساس المقياس الهندي لتقييم التوحد (ISAA) ثم تم الكشف عن مستوى ارتباط بعض المعلمات المناعية مثل العامل المثبط لهجرة الخلية البلعمية (MIF) وبعض الاجسام المضادة (IgM و IgG) بين مرضى التوحد ومجموعة السيطرة بواسطة استخدام تقنية تحليل الانزيم المرتبط المناعي (ELISA).

واظهرت النتائج زيادة معنوية ($P < 0.001$) لمستوى العامل المثبط لهجرة الخلية البلعمية (MIF) في مصل مرضى التوحد (المتوسط 13.3ng / ml بمدى يتراوح من 2.55 -18.73) عند المقارنة مع مجموعة السيطرة (المتوسط 5.61ng / ml بمدى يتراوح من (2.42 - 10.74) بينما اظهرت النتائج انخفاض معنوي ($p<0.001$) لمستوى IgG في مصل مرضى التوحد (المتوسط 20.66 ng /ml بمدى يتراوح من 10.55 - 34.52) عند المقارنة مع مجموعة السيطرة (المتوسط 38.01ng / ml بمدى يتراوح من 26.89 - 50.24) وانخفاض معنوي ($p<0.001$) لمستوى IgM في مصل مرضى التوحد (المتوسط 208.18 mg/dl بمدى يتراوح من 163.41- 245.75) عند المقارنة مع مجموعة السيطرة (المتوسط 245.53 mg/dl بمدى يتراوح من 200.96- 271.02).

اظهرت النتائج ايضا أن مشاكل النوم ونوبات الصرع توجد في 13(21%) و 7 (11%) من مرضى التوحد على التوالي, والتي هي ميزة مهمه سريريا للتوحد. واظهرت نتائج الدراسة ايضا (86.67 %) من اطفال التوحد يعيشون في المدن ($P < 0.001$) , ولا يوجد ارتباط معنوي بين ترتيب الطفل في العائلة واضطراب طيف التوحد ($P > 0.05$) ولا بين القلق والتوحد ($P > 0.05$) .

كشفت النتائج ايضا يوجد ارتباط معنوي بين مشاكل النوم وشدة سلوك التوحد ($p < 0.05$) و لا يوجد ارتباط معنوي بين عمر الطفل , جنسه و نوبة الصرع مع شدة سلوك التوحد ($P > 0.05$).

بالاعتماد على النتائج اعلاه تم الكشف عن الدور المهم للعامل المثبط لهجرة الخلية البلعمية (MIF) في إمراضيه الاطفال المصابين بالتوحد في العراق, زيادة معنوية لمستوى (MIF) و انخفاض معنوي لمستوى الاجسام المضادة (IgG and IgM) في مصل المرضى المصابين بالتوحد عند المقارنة مع مجموعة السيطرة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة القادسية /كلية الطب
فرع الاحياء المجهرية

دور العامل المثبط لهجرة الخلية البلعمية (MIF) وبعض الاجسام المضادة في مرضى التوحد

رسالة مقدمة الى
مجلس كلية الطب ـ جامعة القادسية
وهي جزء من متطلبات نيل درجة الماجستير علوم في
الأحياء المجهرية الطبية
تقدم بها

علي صبري شاكر
بكالوريوس علوم تحليلات مرضية _ جامعة الكوفة (2012)

بأشراف

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