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Certain Immunological, Biochemical and genetic aspects in a group of autistic patients

A Thesis

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By

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

وَالَّذِي هُوَ يُطْعِمُنِي وَيَسْقِينِ (٧٩) وَإِذَا مَرِضْتُ فَهُوَ يَشْفِينِ (٨٠) وَالَّذِي يُمِيتُنِي ثُمَّ يُحْيِينِ (٨١)

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Dedication

In the name of the Allah who shone with His splendid light alone I worship and to Him alone I prostrate humbled and thankful for His grace in completing this effort...

To... the owner of the highest paradise, the enlightening lamp of the nation, and its intercessor, the warner, the evangelist, our Prophet Muhammad (may God bless him and grant him peace) with pride and honor...

To the one who honored me by bearing his name, my father, may God Almighty have mercy on him...

Whoever made the most precious in order to reach a high scientific degree and left before he saw the fruit of his planting...

To the light of my eyes, the light of my path, and the joy of my life

My mother, then my mother, then my mother.. whose prayers and words were the companion of brilliance and excellence.. To the support, the arm and the arm, my brothers, I dedicate to you devotion with love, honor and dignity.

To the one who supported me and took my steps with me, my life partner, my dear husband.

To whom my eyes rejoice with his vision, and my heart rejoices with the joy of my dear son Abdullah.

To those who helped me and stood by my side in my research journey, my honorable supervisor, Dr. Ibrahim Hadi Mohammed

And Dr. Amer Dawod Majeed

To my homeland Iraq.

To everyone who helped me, my colleagues.

Researcher

Meeladlnbi Mudhafar Taha Saeed

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Researcher

Meeladlnbi Mudhafar Taha Saeed

Abstract

The current study aimed to evaluate the immune role and some biomarkers of autism patients. Blood were collected from Patients in Ibn Rushd Hospital in Baghdad, and the study was conducted from September 2020 to March 2021, where the immune role in autistic patients was evaluated for measuring the immune indicators. Cluster of differentiation (CD4), Interleukine-1α level (IL-1α) and Interferon gamma (IFN-γ) as well as Tumor necrosis factor-alpha (TNF α) and measured by enzyme-linked immunosorbent assay (ELISA) technique.

The biochemical indicators included Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Total Bilirubin (TBIL), and Creatinine) and hematological indicators (Platelets (PLT), Red blood cells (RBC), Hemoglobin (HGB), and white blood cells (WBC).

The study included 90 samples, including 60 samples for autistic patients (30 males and 30 females) with an age range ranging from (2 to 14 years), 30 samples were collected from healthy 15 males and 15 females within an age range (2 to 14 years), they were used as a control group that samples of autistic patients were collected before giving them treatment. After the patients consent.

The study showed that there is no effect of age and gender on autism. Was observed that the immunological indicators (CD4, IL_1α, IFN_γ, and TNF_α) were elevated in autistic patients (8.68 ± 3.06, 5.69 ± 2.50, 606.51 ± 286.62 and 84.07 ± 41.86) compared to healthy subjects (4.56 ± 1.99, 2.45 ± 1.11, 378.40 ± 126.35 and 29.80±13.76).

The results of the current study showed an increase in the chemical indicators (ALP, ALT, AST, and TBIL) in autistic patients compared to healthy controls, and also showed a low level of creatinine in autistic patients compared to healthy controls.

The results of the current study also showed a higher level of platelets (PLTs) in autistic patients compared to healthy controls, and a lower level of (RBC, HGB) in autistic patients compared to healthy controls.

The results of the current study showed that (ALP (95%), TBIL (95%), AST (80%), TNF-α (78%), Creatinine (76%), IFN-γ (75%), IL-1α (72%), and CD4(72%)) is more sensitive in diagnosing autism. It was also found that there significant correlations between the immune indicators ($P<0.05$).

The results of current study revealed no significant different in chromosomes between patients and controls.

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List of abbreviations

No.	Symbol	The meaning
1	4-AA	4-aminoantipyrin
2	4-NPP	4-nitrophenylphosphate
3	5-AIQ	5- aminoisoquinoline
4	ALP	Alkaline phosphatase
5	ALT	Alanine aminotransferase
6	AP	antisychotics
7	APA	American Psychiatric Association
8	AR-MetS	At risk of meeting MetS criteria
14	ASD	Autism spectrum disorder
16	AST	Aspartate aminotransferase
17	ATP	Adenosine triphosphate
18	AUC	Area under curve
19	CD4	Cluster of differentiation
20	CDC	Center for Disease Control and Prevention
21	CDS	Creatine deficiency syndrome
22	CNS	Central Nervous System
23	CNVs	Copy number variations
24	CR	creatine
25	CTD	Creatine transporter deficiency
26	CYFIP1	Cytoplasmic FMR1 interacting protein 1
27	DSM	Diagnostic and statistical manual of mental disorder
28	DSM-4	Diagnostic and statistical manual of mental disorder-4^{th} edition
28	EDTA	Ethylenediamine tetraacetic acid
30	ELISA	Enzyme –linked immunesorbent assay
31	FOXP1	Forkhead box P1
32	FOXP2	Forkhead box P2

33	GAA	guanidinoacetate
34	GABA	Gamma- amino butyric acid
35	GABRA5	Gamma-aminobutyric acid type A receptor subunit alpha5
36	GABRB3	Gamma- aminobutyric acid receptor subunit beta-3
37	GABRG3	Gamma-aminobutyric acid type A receptor subunit-gamma3
38	GGT	Gamma-glutamyl transferase
39	GM-CSF	Granulocyte-macrophage colony-stimulating factor
40	GOT	Glutamic- oxaloacetic transaminase
41	GPT	Glutamate-pyruvic transaminase
42	HCT	Haemato crit
43	HGB	Hemoglobin
44	Poly I:C	Polyinosinic: polycytidylic acid
45	IDA	Iron deficiency anemia
46	IDO	Indoleamine dioxygenase
47	IFCC's	International Federation of Clinical Chemistry and Laboratory Medicine
48	IFNG-AS1	Interferon gamma antisense RNA1
49	IFNGR1	Interferon gamma receptor1
50	IFNGR2	Interferon gamma receptor2
51	IFN-γ	Interferon gamma
52	IgA	Immunoglobulin A
53	IgG	Immunoglobulin G
54	IgM	Immunoglobulin M
55	IL-1Ra	Interleukin-1receptor antagonists
56	IL-1α	Interleukin-1alpha
57	IL-1β	Interleukin-1beta

58	IMMP2L	Inner mitochondrial membrane peptidase-like
59	ISCN	International system for human cytogenetic nomenclature
60	JAK-STAT	Janus kinase-signal transducer and activator of transcription
61	K2Cr2O7	Potassium dichromate
62	KCL	Potassium chloride
63	KH2PO4	Potassium dihydrogen phosphate
64	LDH	Lactate dehydrogenase
65	LPS	lipopolysaccharide
66	LTP	Long –term potentiation
67	MCH	Mean corpuscular hemoglobin
68	mCNV	Mosaic copy number variants
69	MCP	Monocyte chemoattractant protein-1
70	MCV	Mean corpuscular volume
71	MDH	Malate dehydrogenase
72	MeCP2	Gene that encodes the methyl-CpG-binding protein 2
73	MetS	Metabolic syndrome
74	MIA	maternal immune activation
75	MIF	Melanocyte- stimulating hormone inhibiting factor
76	MIP-1β	Macrophage inflammatory protein-1β
77	MR	Magnetic resonance
78	Na2HCO3	Sodium bicarbonate
79	Na2HPO4	Sodium phosphate mono Hydrogen
80	NaCL	Sodium chloride
81	NAD	Nicotinamide adenine dinucleotide
82	NADH	Nicotinamide adenine dinucleotide Phosphate
83	NF-*κ*B	Nuclear factor kappa B

84	NK	Natural killer
85	p	probability
86	PBS	Phosphate-Buffered saline
87	PdCNV	Potentially damaging copy number variation
88	PDD	Pervasive developmental disorder
89	PDD-NOS	Pervasive developmental disorder-not otherwise specified
90	pdSNV	Potentially damaging single nucleotide variation
91	PHA	Phytohaemaglutinin
92	PLTs	Platelets
93	POD	Peroxidase
94	PRRs	Pattern recognition receptors
95	PUFAs	Polyunsaturated fatty acids
96	r	Person correlation
97	RBC	Red blood cell
98	RELN	reelin
99	ROC	Receiver operating characteristic
100	RPMI	Ross well park memorial insttute
102	SNPs	Single nucleotide polymorphisms
103	ST7	Suppression of tumorigenicity7
104	Streptavidin-HRP	Streptavidin- Horseradish Peroxidase
105	TBIL	Total bilirubin
106	(TGF)β1	Transforming growth factor
107	Th1	T-helper-1
108	Th2	T-helper-2
109	TLR-agonists	Toll- like receptor agonists
110	TNFR1	Tumor necrosis factor receptor1
111	TNFR2	Tumor necrosis factor receptor2

112	TNF-α	Tumor necrosis factor-alpha
113	Tregs	Regulatory T cells
114	TSB	Total serum bilirubin
115	UBE3A	Ubiquitin protein ligase E3A
116	UBE3A	Ubiquitin-protein ligase E3A
117	VEGF	Vascular endothelial growth factor
118	VPA	Valproic acid
119	WBC	White blood cell

Introduction:

1-1 Autism Spectrum Disorders(ASD)

Autism is a neurodevelopmental disorder marked by difficulties with social communication and empathy, as well as restricted and stereotypical interests and atypical responses (Lochman *et al.*, 2018). ASDs diagnosed in psychology using the Autism Diagntostic Interview, Revised(ADI-R), which lists social impairments communication and interaction problems, repetitive habits, and narrowed interests are all symptoms of the condition. ASDs affect children under the age of three, and males are four to five times more likely than female (Fernell & Gillberg, 2010).

The term "autism" was coined by Leo Kanner to describe a group of children he was studying. The term "spectrum condition" was applied to the term "autism" because children may have a wide variety of symptoms or traits that affect them, ranging from mild to extreme (Willis, 2006).

Autism's etiology and pathophysiology are not fully understood. However, psychological, environmental, immunological, and genetic factors all play a role in the etiopathogenesis of this disease(chauhan & chauan 2006).

Effective treatment is normally delayed due to unreliable early diagnosis.Social reinforcement and behavioral modification are now the most effective approaches to ASD care. However, the efficacy of this therapy is contingent on starting care as soon as possible. The most effective approach is to diagnose and treat patients as soon as possible (Lord *et al.*, 2018).

There are five different types of autism spectrum disorders (ASDs). The autism spectrum disorders, Asperger syndrome, which is characterized by delays in cognitive growth and language, and pervasive developmental disorder, not otherwise defined (PDD-NOS), which is diagnosed when the complete range of criteria for autism or Asperger syndrome is not met, childhood developmental disorder, and Retts Syndrome (Gelder *et al.*, 2007).

The genes may interact with each other or with environmental factors, causing the emergence of autism, and the mutations that cause autism have not been definitively identified (Beaudct, 2007).

To comprehend the immune system's possible role in ASD, one must first become acquainted with the key actors in the immune system, which serves as the body's defense against intruders, is a complicated system that interacts with a variety of different bodily systems, including the endocrine and neurological systems(Carpentier & Palmer, 2009).

The innate immune response is a first-line defensive system that responds to infections in a non-specific manner. (Murphy, 2011).

The adaptive immune system is a highly specialized collection of cells that responds to infections via immunological memory to remove or avoid reoccurrence. Its capacity to detect and recall individual diseases is its strength (Murphy, 2011).

1-2 Aims of the study

1. Assessment the level of the immunological biomarkers (CD4, IL-1α, INF-γ, TNF-α) in addition to biochemical markers (ALP, ALT, AST, TBIL, Creatinine) in serum of autistic patients and comparing it with healthy children.
2. Measuring the level of (WBC, RBC, PLT, HGB) in the blood of autistic patients and comparing them with healthy children.
3. A study of the cytogenetic side of autistic patient**.**

Literature Review:

2-1 Autism Spectrum Disorder (ASD)

The concept of autism has gradually broadened since the time of Leo Kanner's first clinical descriptions in his 1943 seminal paper (Harris, 2018). Autism is a developmental condition that affects a person's ability to learn and process information. Since there are several different types of autism, the official title is Autism Spectrum Disorder. Some people with autism are high functioning, and their diagnosis is barely noticeable, while others are low functioning and may never be able to live independently. Asperger's syndrome, a subtype of autism, has been diagnosed in a large number of high-functioning people (National Institution of Neurological Disorders &Stroke, 2020).

Autism Spectrum Disorder (ASD) is one of the most common types of neurodevelopmental disorders, affecting 1-2% of the population, with a male to female ratio of 4-5:1, Social interaction and communication deficiencies, as well as repetitive and stereotyped behaviour , are all characteristics of ASD(Baio *et al*., 2018).

2-2 Historical Background

The " autistic " was coined in 1911 by Swiss psychiatrist Eugen Bleuler (1857-1939), to describe the limitations of human connections and patients with schizophrenia's loss of contact with reality (Ashok *et al*., 2012).

When American psychiatrist Leo Kanner reported 11 children with behavior characterized by difficulty in establishing affective and interpersonal connections in 1943, his study became the foundation for the contemporary diagnosis and diagnostic criteria (World health organization,

2010). The following year, Hans Asperger identified cases with some autism-like symptoms, such as trouble in social communication but no cognitive impairment (Frith, 1991). The fourth edition of the DSM (Diagnostic and statistical manual of mental disorder -4) was published in 1994, and it included new criteria in order to identify subgroups of people with autism for both practical and research purpose, taking into account the subdivision: typical autism, pervasive developmental disorder not otherwise specified (PDD-NOS), and Asperger syndrome (Louveau *et al*., 2015). Rutter proposed a more complete diagnosis of autism in 1978, which included four criteria: beginning before the age of 30, difficulty in social development, delay in language development, and insistence on similarity (Volkmar & Klin, 2005; Maston & Minshwai, 2006).

2-3 Signs and Symptoms

While ASD cannot be identified before a child is three years old, there are several symptoms that occur earlier in life, causing parents to note a difference in their child when compared to typically developing children. ASD is a widespread disorder marked by irregular brain development and severe behavioral symptoms (Lai *et al.*, 2019). ASD patients have atypical sensory reactivity, ritualized behavios, rigid adherence to schedules, severely limited desires, and repetitive motor movements or expression (echolalia) (Hazen *et al.*, 2014).

Social interaction and communication deficiencies are also common. Nonverbal contact, sustaining relationships, and reciprocating socio-emotional engagement are all areas where participants struggle (Pérez-Pereira & Conti-Ramsden, 2019). ASD is highly heterogeneous in terms of severity and related impairments, despite its central symptomology (Bennett *et al*., 2014).

Autism is lifelong disease that is often accompanied by comorbid conditions such as anexiety, insomnia, obsessive-compulsive disorder, and other mental illnesses (Hollocks *et al.,* 2019). Onset tests measuring verbal and nonverbal cognitive reasoning, lower functioning individuals often display below averagr intelligence (Song *et al*., 2019).

This subgroup of participants has even more delayed language learning and low verbal capacity (Bennette *et al*.,2014). These impairments may be caused by problems with mutual attention or atypical sensitivity to non-semantic information in speech. Furthermore, a lack of receptive and expressive language appears to be linked to poor social growth, especially in terms of understanding others' mental states and orienting to socially relevant stimuli (Bottema-Beutel *et al*., 2021).

2-4 Diagnosis of ASD

Although ASD can be diagnosed as early as 18 months of age (Hyman *et al.*, 2020), the latest review indicated that, globally, the mean age at ASD diagnosis ranges between 3 and 10 years (van't Hof *et al.*, 2021). Early detection of ASD can lead to early treatment , which has been shown to improve later language and cognitive abilities and ameliorate the core symptoms (Clark *et al.,* 2018). Although there is criticism of universal ASD screening due to insufficient evidence of its benefit , there is agreement that early identification and intervention is a public health priority and that universal screening is an essential tool for the early detection of ASD (Pierce *et al.,* 2016). The WHO (2014) states that the monitoring of child and adolescent development, in order to ensure timely detection and management of ASD in primary care, is a vital part of a national health system. ASD guidelines (and updates) and practice

parameters have recently been released in the United States (Hyman *et al.*, 2020), France (de Santé, 2018), and India (Dalwai *et al.*, 2017).

Issues related to autism are classified in the DSM-4 as part of a simple triad that involves impairments in social contact, social communication, and restricted behavior patterns (Wing *et al*., 2011). Children with autism spectrum disorder have significant communication difficulties (ASD). Since there is no medical examination for ASD, it is difficult to diagnosis. To make a diagnosis, doctors examine the child's behavior and growth (American Psychiatric Association, 2013).

Study results suggest the importance of earlier diagnosis in preventing secondary mental health problems in this population, particularly among those without cognitive delays (Hosozawa *et al.*, 2021).

2-5 Causes of Autism

2-5-1 Genetic Causes

A strong psycho-analytic tradition led to the growing belief that “refrigerator” mothers might be to blame. The first twin study of autism conducted by Folstein and Rutter was ground-breaking because it clearly showed a predominantly genetic contribution to autism (Folstein & Rutter 1977).

The most recent meta-analysis of all published twin studies of autism/autism spectrum disorder conducted by Tick and colleagues also yielded a large heritability estimate of 64–91% and no significant shared environmental contribution (Tick *et al.* 2016). These authors demonstrated that if the estimated prevalence rate of autism is incorrectly specified for the study population (1% instead of 5% which is the appropriate figure for a broader autism phenotype), this essentially results in an increased non

identical (dizygotic DZ) twin correlation but does not affect identical (monozygotic MZ) twin correlations, thereby resulting in a reduced heritability estimate and a stronger shared environmental contribution. Thus the shared environmental contribution observed in two outlying studies appeared to be explained by the assumption of prevalence and an overinclusion of concordant DZ twins (Frazier *et al.* 2014). The study by Tick and colleagues is also important in showing that if the autism broad phenotype is clinically recognised, then that ought to be taken into account by assessing different thresholds when fitting statistical models (Tick *et al.* 2016).

Analysing top variants and genes, authors demonstrated a role of the immune-related genes *RNF114*, *CDKN2A*, *KAZN*, *SPATA2* and *ZNF816A* in autistic-like traits. Brain-based genetic expression analyses further linked autistic-like traits to genes involved in immune functioning, and neuronal and synaptic signalling. Overall, authors findings highlight the potential of the autistic-like trait–based approach to address the challenges of genetic research in autism spectrum disorders (Arenella *et al.*, 2021).

Researchers showed that the *MTHFR C667T* variant, *SLC25A12* variation (rs2056202 and rs2292813) ,and *RELN* variants (rs362691 and rs736707) were a risk factor for the occurrence of ASD, while the *5-HTTLPR* was found with autism, but when subgroup analysis was performed according to ethnicity, the association was statistically significant (Wei *et al.*, 2021).

Investigators indicated that distinct categories of neurodevelopmental disorders (NDD) share some genetic risk factors with distinct phenotypic expression . The overlap at the genetic level was expected since patients diagnosed with different NDD also share comorbid disorders (Leblond *et*

al., 2021). Altogether it is now accepted that NDD is a wide continuum of genetic and phenotypic dimensions instead of restricted categories.

A more comprehensive list of ASD candidate genes can be found in Table 2-1.

Table 2-1: Several relevant ASD candidate genes (Masini *et al.*, 2020).

Gene Symbol	Gene Name	Alterations	Associated Syndromes
ANKRD11	Ankyrin repeat domain 11	Mutations; copy number loss	KBG syndrome; Cornelia de Lange syndrome
ARID1B	AT-rich interaction domain 1B	Mutations; copy number loss; copy number gain; translocation	Coffin–Siris syndrome
ASXL3	ASXL Transcriptional Regulator 3	Mutations	Bainbridge-Ropers syndrome
ATRX	ATRX Chromatin Remodeler	Mutations; copy number loss	
AUTS2	Autism susceptibility candidate 2	Mutations; copy number loss; copy number gain; inversion; translocation	
FOXG1	Forkhead box G1	Mutations; copy number loss; copy	Rett syndrome, FOXG1 syndrome,

		number gain; translocation	West syndrome,
FOXP1	Forkhead box P1	Mutations; copy number loss; inversion; translocation	
FOXP2	Forkhead box P2	Mutations; copy number loss; translocation	FOXP2-related speech and language disorder
MED13L	Mediator complex subunit 13-like	Mutations; copy number loss; copy number gain	

2-5-2 Epigenetic

The majority of recent studies have focused on genetic factors that play a role in the etiology of autism, as previously discussed, non-genetic factors are also likely to play a role in the etiology and pathogenesis of autism (Srancikova *et al.*, 2021). Different genetic features/pathways are thought to trigger different domains of autistic activity, but this has yet to be confirmed at the molecular level (Happe& Ronald, 2008). Autism is believed to be a genetically heterogeneous spectrum, with many genetic aberrations playing a role in reaching the autism phenotype threshold (Mahjani *et al.*, 2021).

As previously mentioned, genetic factors play a critical role in the etiology of ASD, and epigenetic factors cannot be ruled out. Many processes, such as cytosine methylation and post-translational modification of histones, are examples of epigenetic modifications that may serve as a

mechanism for regulating and controlling gene expression (Dall'Aglio *et al.,* 2018). Taken together, study findings provide initial evidence for *oxytocin receptor gene (OXTR)* hypermethylation in the intron area as a potential biomarker for adults with ASD with less severe developmental communication deficits, but with impairments in theory of mind and self-awareness. Also, *OXTR* methylation in the exon 1 area could be a potential biomarker of sociability sensitive to life experiences (Andari *et al.,* 2020).

Epigenetic modifications affecting DNA transcription and various pre-natal and post-natal exposure to a variety of environmental factors are also precipitating factors for the occurrence of ASD. All of these together cause dysregulation of glutamatergic signaling as well as imbalance in excitatory: inhibitory pathways resulting in glial cell activation and release of inflammatory mediators responsible for the aberrant social behavior which is observed in autistic patients (Bhandari *et al.,* 2020).

Recent study has shown that epigenetic factors, including DNA methylation, histone modifications and microRNAs (miRNAs), could play an important role in predisposition to autism (Masini *et al.,* 2020).

2-5-3 Environmental Causes

As mentioned above, several studies investigated the possible role of environmental factors in the etiology of ASD. According to recent studies, up to 40–50% of variance in ASD liability could be determined by environmental factors, such as drugs, toxic exposures, parental age, nutrition, fetal environment and many others (Deng *et al.,* 2015). However, while for some potential risk factors, there is strong evidence, supported by association studies but also by in vitro and in vivo studies, only weak associations have been described for many others. Below is reported an overview of the most-studied environmental factors (Bölte *et al.,* 2019).

Parental age is one of the most established environmental ASD risk factors. In fact, much evidence has correlated advanced paternal age (APA) with the development of bipolar disorder, schizophrenia, ADHD and ASD (Janecka *et al.*, 2017). A meta-analysis of 27 studies on the association between advanced parental age and ASD showed that a 10-year increase in maternal and paternal age is associated with a 20% higher risk of ASD in children (Wu *et al.*, 2017). A study has shown that age-related methylation changes observed in sperm could be related to an increased ASD risk in the offspring (Atsem *et al.*, 2016).

2-6 Clinical manifestations

ASD can be considered as a group of early-onset neuroevolutionary disorders which seem to be at the basis of alterations in brain connectivity, with cascading effects on many neuropsychological functions (Kana *et al.*, 2014). The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, APA 2013) gives the condition of autism the attribute of "spectrum" and uses criteria derived from diagnostic research assessment tools.

Individuals with ASD are characterized by persistent deficits in social communication and social interaction across multiple contexts and by restricted, repetitive patterns of behaviour, interests or activities. Deficiency in social communication and social interaction might appear in the form of deficits in social-emotional reciprocity, in nonverbal communicative behaviours used for social interaction and deficit in developing, maintaining and understanding age-appropriate relationships. As reported in the DSM-5, symptoms could be masked during early development and fully manifest only when social demands exceed limited capacities or may be hidden by learned strategies in later life (APA, 2013).

The DSM-5 proposes differentiations based on commorbidity with intellectual impairment, language impairment, another neurodevelopmental, mental, or behavioral disorder, genetic or medical condition or environmental factors. Furthermore, it is possible to differentiate between different levels of severity according to the level of support required to function in daily contexts. According to this description, it is clear that different clinical variants of ASD exist and should be taken into account for diagnosis and intervention (APA, 2013).

A distinction is made between a congenital form of ASD, representing a small percentage of cases in which the symptoms occur shortly after birth and in which the genetic fingerprint is prevalent, and a regressive or acquired form, in which the disorder appears after a period of typical development and it is not characterised by typical and constant genetic abnormalities, although several single nucleotide polymorphisms (SNPs) have been associated with the disease (Tamouza *et al.*, 2020). SNPs constitute variations of a single nucleotide in certain DNA traits. SNPs associated with ASD have been identified in genes encoding for proteins involved in different processes, including: cellular detoxification, some neuronal receptors and metabolism of several neurotransmitters and metabolites, in particular those of the metabolic circuits of methylation and transulfuration (Masini *et al.*, 2020).

2-7 Epidemiology

Autism Spectrum Disorder (ASD) is quickly becoming one of the world's most frequent developmental impairments (Boyle *et al.*, 2011). Males are four times as likely than girls to have ASD (Goldman, 2013). While the causes of this tendency are unknown, it has been suggested that

ASD is an extreme manifestation of male brain anatomy (Baron-Cohen *et al*., 2011).

The reported incidence of ASD in Iraq according to Ministry of Health (2017) ,the number of autism patients reached (1717) (The Iraqi Ministry of Health, 2017).

And in the statistics of the Iraqi Ministry of Health (2018), the number of autism patients reached (3092).

Also in the statistics of the Iraqi Ministry of Health (2019), the number of autism patients reached (4030) (The Iraqi Ministry of Health, 2019).

In addition to that the statistics of the Iraqi Ministry of Health (2020), the number of autism patients reached (2338) (The Iraqi Ministry of Health, 2020).

Autism has been considered relatively rare for many years, with a prevalence of less than 1 in 1000 children, while today, the estimated rate is 1 in 160, and it seems likely to increase in the coming years (WHO, 2019). In the last decade, the study of ASD genetics has proved to be crucial not only to interpret and explain its phenotypic heterogeneity but also to discover new diagnostic procedures and therapies. It is estimated to-date that hundreds of genes are involved in ASD, resulting in a unified spectrum of different phenotypes, including different language and social deficits with various associated sub-phenotypes (Iossifov *et al.*, 2014).

Genetic, environmental and developmental factors play a key role in the onset of autism spectrum disorders, as highlighted from many epidemiological studies (Elsabbagh *et al.*, 2012).

It is unlikely that a single condition or event plays a major role in the causality of ASD; based on research to date, rather, none of the risk factors identified is a necessary and sufficient condition for ASD. Even for syndromic or secondary autism, which refers to autism with a single defined cause, such as fragile X syndrome and tuberous sclerosis, none of these etiologies are specific to autism because each of them encompasses a variable proportion of individuals with and without autism. At present, ASD appears to have a multifactorial etiology to which developmental (in utero and early childhood), environmental and genetic aspects contribute, in as-yet unknown and different ways. Emerging methodologies in genomics and epigenomics research could be the key to elucidate the mysteries underlying the epidemiology of autism spectrum disorder (Masini *et al.*, 2020).

The number of reported cases of ASD has increased over the past 30 years and the current prevalence has been estimated to be at least 1.5% in developed countries (Whitely *et al.* 2021). Studies from different countries have suggested the prevalence of ASD, especially in combination with intellectual disability (ID), to be higher among children of immigrant than non-immigrant women (Abdullahi *et al.,* 2019). In contrast, evidence of lower prevalence was reported for children born to immigrant Hispanic women in the U.S, suggesting delayed services for these children (Baio *et al.* 2018).

2-8 Treatment

People with autism may benefit from early diagnosis, intervention, and treatment. Therapy has been shown to be very helpful in reducing the effects of autism when started at a young age. The aim of therapy is to teach the autistic children skills and behaviors that will help them to work within their disorder's confines (Mayo Foundation for Medical Education and Research) (MFMER, 2006).

The treatment and Education of Autistic and Associated Communication-handicapped Children is the most well-known model for special day classes for children with ASD (siegel, 2008). Speech and communication therapies improve language skills and vocabulary of children with autism. Adults with aphasia often experience severe deficits in communication similar to the deficits that young children with ASD experience. Often, conversations are limited because these clients cannot find the right words to communicate (MFMR, 2006).

Multimodal communication treatment is a relatively new treatment method used with moderate to severe aphasia that helps the patient to "make verbal and nonverbal representations of a concept more automatic, facilitating switching among modalities" (Wallace & Purdy, 2013).

2-9 Immune System and Cytokines In Autism

2-9-1 Immune system

The immune system is a complex set of defense mechanisms that are activated to defend an organism from pathogens that cause disease or illness, such as bacteria, viruses, fungi, and parasites. An antigen is a molecule that activates the immune system to generate antibodies that recognise, neutralize, or destroy an antigen that the host's body considers foreign. In a fully functioning immune system, which is made up of two interconnected systems: Innate and adaptive immunity, several interconnected organs, molecules, cells, and pathways play a role (Libby *et al.,* 2018).

These two mechanisms cooperate to keep the body safe from pathogens. Innate immunity refers to the host's first-line defense mechanisms that react to an infection within hours or minutes of the pathogen attacking the host. Physical barriers, such as the epithelial layers

of the skin and mucosal and glandular tissue surfaces attached to the body's openings, as well as chemical barriers, such as soluble antimicrobial proteins and peptides and an acidic pH, are used by the innate immune response(Labzin *et al.*, 2018). When pathogens cross these barriers, a pathogen-recognition mechanism involving a variety of cells with cell surface and intracellular receptors triggers cellular innate immune responses. Pattern recognition receptors expressed by several different immune cells identify pathogen- associated molecular patterns, which are recognized by groups of pathogens (Amarante-Mendes *et al.,* 2018).

Some cells are programmed to phagocytose and degrade the pathogen, which involves macrophages and neutrophils engulfing and killing extracellular microbes. Cell receptors can also be turned on, causing cells to develop antimicrobial substances that kill pathogens(Hume *et al.,* 2019). The development of cytokines and chemokines, which are proteins that recruit cells, molecules, and fluid to infection sites, is triggered by other cellular activation processes. Inflammation is a physiological alteration that occurs as a consequence of this treatment (Abdulkhaleq *et al.,* 2018).

When innate immunity is inadequate, such as when pathogen characteristic enable them to evade the innate immune system's defense mechanisms, the adaptive immune response is triggered. The adaptive response begins a few days after the pathogen is exposed and the physical or chemical barriers are breached. In comparison to the innate response, this response is more systematic and antigen specific. Pathogens can be recognized, eliminated, and remembered by the adaptive immune system (Yatim *et al.,* 2017). This memory portion is not thought to exist in the innate immune system. However, evidence of NK cell memory in viral

infections suggests that these cells have both innate and adaptive immunity properties (Vivier *et al.*, 2011).

Adaptive immune responses are divided into two categories, both of which are carried out by lymphocytes. The first form of response is an antibody response, which is carried out by B lymphocytes, or B cells, which secrete antibodies, also known as immunoglobulins, when triggered by an antigen. The bone marrow is where B cells are produced. A cell-mediated immune response is a form of adaptive immune response in which activated T cells recogize and neutralize or remove antigens. The thymus, a lymphoid organ of the immune system, is where T cells mature. Memory cells are B and T cells that remain after antigen exposure and are triggered by subsequent pathogen challenges (Evavold & Kagan, 2018).

The innate and adaptive immune responses described above are typical of a well-regulated immune system that contributes to homeostasis and prevents disturbances of normal body functions. An optimal balance of pro- and anti-inflammatory signaling is needed for a well-regulated immune system. Inflammation may not be a problem in and of itself, but it may become a problem when an unregulated or dysregulated immune system responds to physiological changes caused by pathogens. Upregulation of the inflammatory/ immune response or immune dysfunction may also be signs of a malfunctioning immune system, putting the host's defenses at risk. Immune deficiency is linked to allergies, asthma, and autoimmune disorders, among other things (Masi *et al*., 2017).

2-9-2 Cytokines

Cytokines are cell-signaling agents that help cells in the innate and adaptive immune systems communicate with one another. They are key regulators of inflammation, organizing the immune response to infection

and other threats, and are involved in a wide range of biological processes. Cytokines stimulate and modulate immune system function and induce their own synthesis as well as the synthesis of other cytokines as part of an interconnected network. While some remain cell-bound, they are usually soluble molecules. Adaptive immunity, pro-inflammatory signaling, and anti-inflammatory signaling are the three broad categories of cytokines based on the type of immune response they cause (Firestein *et al.*, 2020).

Cytokines are cell-produced proteins that act as molecular messengers between cells. Cytokines control a variety of inflammatory responses in arthritis. Cytokines are proteins that regulate the body's response to disease and infection and govern normal cellular activities. They are part of the immune system (Eustic & Ozeri, 2020).

Distinct types of immune cells and proteins perform different functions in the immune system. Among these proteins are cytokines. To understand inflammation, we must understand the functioning of cytokines. Cytokines are released by cells into the bloodstream or directly into tissues. The cytokines find and bind to the receptors of the immune cells they're meant to attack. The target cells are triggered or stimulated as a result of this contact (Eustic & Ozeri, 2020).

Biomarkers identified on the basis of association with clinical symptom severity in ASD may reflect effects rather than causes of autism. The search for biomarkers of pathogenesis may benefit from a greater focus on traits that predict autism recurrence, among both clinical and general populations (Constantino, 2021).

The term "cytokine" refers to a wide range of protein messengers, cytokines are given more particular names based on the type of cell that produces them or the action they have in the body:

- Lymphokines are proteins produced by lymphocytes that attract immune cells like macrophages.
- Monocytes produce cytokines, which attract neutrophils,
- Chemokines are linked to chemotactic effects.
- Interleukins are proteins produced by one leukocyte that act on other leukocytes, allowing cells to communicate with one another. Interleukins can have a big influence on cell-to-cell communication (Eustic &Ozeri, 2020).

2-9-2-1 Cluster of Differentiation (CD4+)

Cluster of differentiation 4 (CD4) coreceptor expressed in a subset of T cells, plays a role in differentiation, migration and cytokine expression (Zhen *et al.,* 2014). T cells involved in antigen recognition, CD4 stabilizes the ternary complex pMHC-TCR and CD4 recruits Lck kinase to phosphorylate ITAM and initiate intracellular signaling during activation of T cells induced by antigens. CD4 was originally described as an adhesion molecule that enhances contact between T cells and precenting cell antigens. In their pillar work, Doyle and Strominger found direct correlations of other specific T cells involved in interactions CD4 binds MHCII molecules with very low 3D affinity (Jönsson *et al.,* 2016).

T lymphocyte cells (CD4 +), known as helper T cells, are effector cells for cellmediated immunity. T lymphocytes (CD4 +) are naive and must be activated to start effector functions, this activation occurs through interactions with professional antigen- presenting cells (pro-APC) especially dendritic cells that lead to intracellular pathways that regulate T cell receptors (TCR) more specifically against antigens in T cells (Shen *et al.,* 2019).

TCR and its co-receptors, such as CD4, form complexes with class 2 MHC receptors and antigens. CD4 + lymphocyte cells are then activated and produce

cytokines to start the immune response of leukocyte cells or other immune cells of cell-mediated immunity and activate humoral immunity branches that depend on T cells, then CD4 + T cells recognize protein antigens and activate B cells to produce immunoglobulins in response to antigens (Bourne *et al.,* 2019).

In the ASD with gastrointestinal disorders , reporters found elevated frequencies of TH17 and TH17 1 populations, and coupled to this were decreased populations of IL-10 producing CD4 T cells, reductions in regulatory T cells, and decreased ratios of regulatory T cells compared to inflammatory TH17 subsets, Alterations in the ASD with no gastrointestinal disorders included elevated populations of CD4 T cells that could represent a shift to a TH2 response (Rose *et al.,* 2020).

Treatment of CD4 + T cells in *vitro* with IL-6 leads to much greater up regulation of *p*-STAT3, and IL-17A in ASD subjects than controls, and in finally, this treatment blockade of IL-6 mediated effects on CD4+ T cells (Nadeem *et al.,* 2020). Data results strongly support the lead induced CD4 + CD3 + T-cell dependent immune system activation and chronic inflammation as the key pathogenetic events in autism spectrum disorders. Moreover, data supports the CD4 + CD3 + T-cell dependence of mitochondrial dysfunction development in ASD patient reported in previous study (Harutyunyan *et al.,* 2021).

2-9-2-2 Interleukine-1α (IL-1α)

Members of the interleukin-1 (IL-1) family of cytokines are cardinal mediators of inflammation (Dinarello, 2018). Within this family, IL-1α and IL-1β are the archetypical pro-inflammatory cytokines. Following identification of these two cytokines in 1974, many studies explored the molecular biology and clinical effects of these molecules. However, IL-1α and IL-1β are not equally established players in the body of clinical medicine. At present, the inflammatory properties

of IL-1β are common knowledge in the biomedical field, and many are familiar with molecular mechanisms of IL-1β secretion (i.e. processing by the inflammasome). Conversely, surprisingly, the role of IL-1α in the pathogenesis of autoimmune and inflammatory diseases has remained relatively overlooked, perhaps because IL-1α is rarely observed in the circulation of patients with inflammatory diseases (Cavalli *et al.*, 2021).

IL-1α is constitutively present as a precursor in all healthy tissues of mesenchymal origin, in particular, cells rich in IL-1α constitute tissues with a barrier function, such as keratinocytes in the skin, type 2 epithelial cells in the lung, the epithelium of the entire gastrointestinal tract, endothelial cells in blood vessels, and astrocytes in the brain (Rider *et al.*, 2017). In addition to constitutive presence in barrier epithelia, production of IL-1α precursor can be induced in myeloid cells during inflammation. Conversely, IL-1β is not constitutively expressed in healthy states and is exclusively produced by myeloid cells during inflammation.Both IL-1α and IL-1β are synthesized as intracellular precursors. However, the IL-1α precursor is biologically active, whereas the IL-1β precursor is not (Kim *et al.*, 2013).

Inside the cell, the activity of the IL-1α precursor is kept in check by the type 2 IL-1R (IL-1R2), a decoy receptor which binds the cytokine but does not transduce inflammatory effects, thus functioning as a 'sink' (Molgora *et al.*, 2018). However, IL-1α exiting from the cell can directly bind the IL-1R1 and exert its biologic effects. Conversely, the IL-1β precursor must undergo intracellular processing and activating cleavage by the NLRP3 inflammasome in order to generate biologically active IL-1β (Cavalli *et al.*, 2021).

Alarmins are proteins or molecular components normally found inside the cell, whose presence in the extracellular compartment signals loss of membrane integrity to nearby cell . Thus, alarmins function as *de facto* danger-associated molecular patterns (DAMPs), and trigger sterile inflammation. Notable examples

include HMGB1 , as well as IL-1α (Bianchi *et al.,* 2017). Since the IL-1α precursor is constitutively present in a biologically active form in epithelial cells, necrosis (i.e. following trauma, ischemia or viral infection) results in the immediate release of IL-1α in the extracellular space, and consequent engagement of IL-1R1 on adjacent live cells. This results in induction of tissue inflammation . Conversely, IL-1β is neither present in most cells nor biologically active as a precursor, and thereby does not function as an alarmin (Cavalli *et al.*, 2021).

2-9-2-3 Interferon gamma (IFN-γ)

Interferon-γ (IFN-γ) is the sole member of the type II interferon family discovered almost 60 years ago. E. Frederick Wheelock was the first to describe IFN-γ as a phytohemagglutinin-induced virus inhibitor produced by white blood cells after they have been stimulated (Wheelock, 1965). IFN-γ is a protein encoded by the IFNG gene, composed of two polypeptide chains associated in an antiparallel fashion (Zaidi & Merlino, 2011). In human blood, IFN-γ is present in three fractions with different molecular mass. One fraction represents the active free form of IFN-γ, while the other two are considered mature IFN-γ molecules. The fully synthetized protein is glycosylated at amino termini where the level of glycosylation determines the final weight of the defined fractions (Lilkova *et al.*, 2019).

Notably, it has been reported that glycosylation itself does not affect the activity of interferon, but rather prevents its degradation by proteinases. Therefore, this chemical modification increases interferons half-life in the bloodstream and prolongs IFN-γ-mediated effects (Alspach *et al.*, 2019). The production of IFN-γ is mainly regulated by natural killer (NK) and natural killer T (NKT) cells in innate immunity while CD8+ and CD4+ T-cells are major paracrine sources of IFN-γ during adaptive immune response (Burke & Young, 2019).

These cells are stimulated by interleukins produced in situ, such as IL-12 , IL-15, IL-18, and IL-21 , tumor- or pathogen- secreted antigens , and partially by IFN-γ itself through an established positive feedback loop (Hosking *et al.,* 2014).From a biological point of view, IFN-γ is a pleiotropic cytokine with antiviral, antitumor, and immunomodulatory functions. Hence, it plays an important role in coordinating both innate and adaptive immune response (Mendoza *et al.,* 2019).

In an inflammatory environment, IFN-γ triggers the activation of the immune response and stimulates the elimination of pathogens; it also prevents over-activation of the immune system and tissue damage. This balance is maintained by complex mechanisms which are not yet fully understood (Ivashkiv, 2018). In the tumor microenvironment (TME), IFN-γ consistently orchestrates both pro-tumorigenic and antitumor immunity. IFN-γ acts as a cytotoxic cytokine together with granzyme B and perforin to initiate apoptosis in tumor cells , but also enables the synthesis of immune checkpoint inhibitory molecules and indoleamine-2,3-dioxygenase (IDO), thus stimulating other immune-suppressive mechanisms (Mojic *et al.,* 2018).

Masi *et al.,* (2017) and Saghazadeh *et al.,* (2019) mentioned immune abnormalities in the postnatal period involves activation of the monocytic and Th1 arm of the immune response, *via* increased IFN-γ, and this has been found in children with ASD . Few studies have reported increased levels of cytokines that can precipitate inflammatory processes in ASD, such as interferon gamma (IFNγ) or IL-12 , or a decreased production of cytokines that negatively regulate inflammation, such as transforming growth factor β1 (TGFβ1) (Abd-Allah *et al.,* 2020).

2-9-2-4 Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor (TNF) alpha is one of the first discovered cytokines shown by Carswell in 1975 and was named for tumor regression activity induced in the serum of mice treated with Serratia marcescens polysaccharide (Aggarwal *et al.,* 2012). TNF being one of the prominent cytokine has about 19 different members of the TNF superfamily that includes tumor necrosis factor alpha (TNF-α), tumor necrosis factor beta (TNF-β), TNF-related weak inducer of apoptosis (TWEAK), TNF-related apoptosis-inducing ligand (TRAIL), lymphotoxin-β (LT- β), CD40L, CD30L, 4-1BBL, CD27L, glucocorticoid-induced TND receptor ligand (GITRL), fibroblast-associated ligand (FasL), OX40 ligand (OX40L), LIGHT, A proliferation-inducing ligand (APRIL), B-cell-activating factor (BAFF), receptor activator of NFκB ligand (RANKL), vascular endothelial cell-growth inhibitor (VEGI), and ectodysplasin A ((EDA)–A1, EDA-A2) (Muhammad, 2019).

TNF-α is a potent mediator of inflammation, as well as many normal physiological functions in homeostasis and health and antimicrobial immunity (Ursini *et al.,* 2017). TNF-α plays important roles in various biological processes, such as immunomodulation, fever, inflammatory response, inhibition of tumor formation, and inhibition of virus replication (Bradley, 2019). Inflammation is a classical host defense response of vascularized living tissue to infection and injury, and in the central nervous system (CNS), the term neuroinflammation is used to denote cellular and inflammatory responses of vascularized neuronal tissue through activation of resident cells in the brain (microglia, astrocytes, and endothelial cells), the recruitment of blood-derived leukocytes including neutrophils, lymphocytes, and macrophages, and a plethora of humoral factors (Muhammad, 2019). TNF-α promotes the growth of intestinal epithelium in fetuses by stimulating the development of intestinal stem cells (Schreurs *et al.,* 2019). TNF-α also promotes the apoptosis of cardiac valve

interstitial cells (VICs). TNF-α-knockout mice developed VIC hypertrophy at 16 days post-partum, indicating that TNF-α plays an important role in the development of cardiac valves (Wang *et al.*, 2017). TNF-α promotes the proliferation of bone marrow-derived mesenchymal stem cells (BMSCs), osteoclast progenitor cells, and chondrocytes (Fang *et al.*, 2019).

Study results mentioned higher TNF-α levels were found in children with ASD in comparison with controls, suggesting that elevated levels of serum proinflammatory agents may be implicated in the pathophysiology of ASD (Zhao *et al.*, 2021). Data results provide further support for altered innate immunity being an important autism pathogenic factor, with autistic children showing increased blood TNF-α concentrations associated with symptom severity, and decreased expression of the THRIL gene involved in regulating TNF-α (Xie *et al.*, 2017).

Materials and Methods:

3- 1 Instruments and Materials

3-1-1 Instruments:

Table (3-1) list of the devices used.

Table 3-1: A list of the devices

Instruments	Country	Company
Centrifuge	Germany	Hettich
ELISA Combiwash device	Germany	Human
ELISA Reader HS device	Germany	Human
Kroma Plus Automatic Clinical Chemistry Analyzer device	Spain	Linear
ABX Micros 60 Hematology Analyzer device	France	HORIBA Medical
Refrigerator	China	Shark
Incubator	Germany	Human

3-1-2 Laboratory Kit

Table (3-2) List of laboratory equipment used.

Table 3-2: List of laboratory equipment

Laboratory Kit	Country	Company
Serum CD4+ Measurment Kit	China	SHANGHAI
Serum IL-1α Measurment Kit	China	SHANGHAI
Serum IFN-γ Measurment Kit	China	SHANGHAI
Serum TNF-α Measurment Kit	China	SHANGHAI
Serum ALP Measurment Kit	Spain	Linear
Serum ALT Measurment Kit	Spain	Linear
Serum AST Measurment Kit	Spain	Linear
Serum TBIL Measurment Kit	Spain	Linear
Serum Creatinine Measurment K	Spain	Linear
Hematological test kit	France	HORIBA

3-1-3 Equipments

Table (3-3) tools used.

Table 3-3: Equipments

Equipments	Country	Company
Cold rack box	Canada	BioBasic
Test tubes	Germany	Labcco
Torniquet	Syrian	Medical lect
Rack tubes	Korea	Bioneer
Plastic disposable syringes 5ml	China	Meheco
Rack tube	Korea	Bioneer
EDTA Test tubes	Germany	Labcco
Micro pipette 20-200µl	Germany	Human
Pipette tips	Germany	Human

3-2 Methods

3-2-1 Work Plan

As shown in Figure (3-1), which research design.

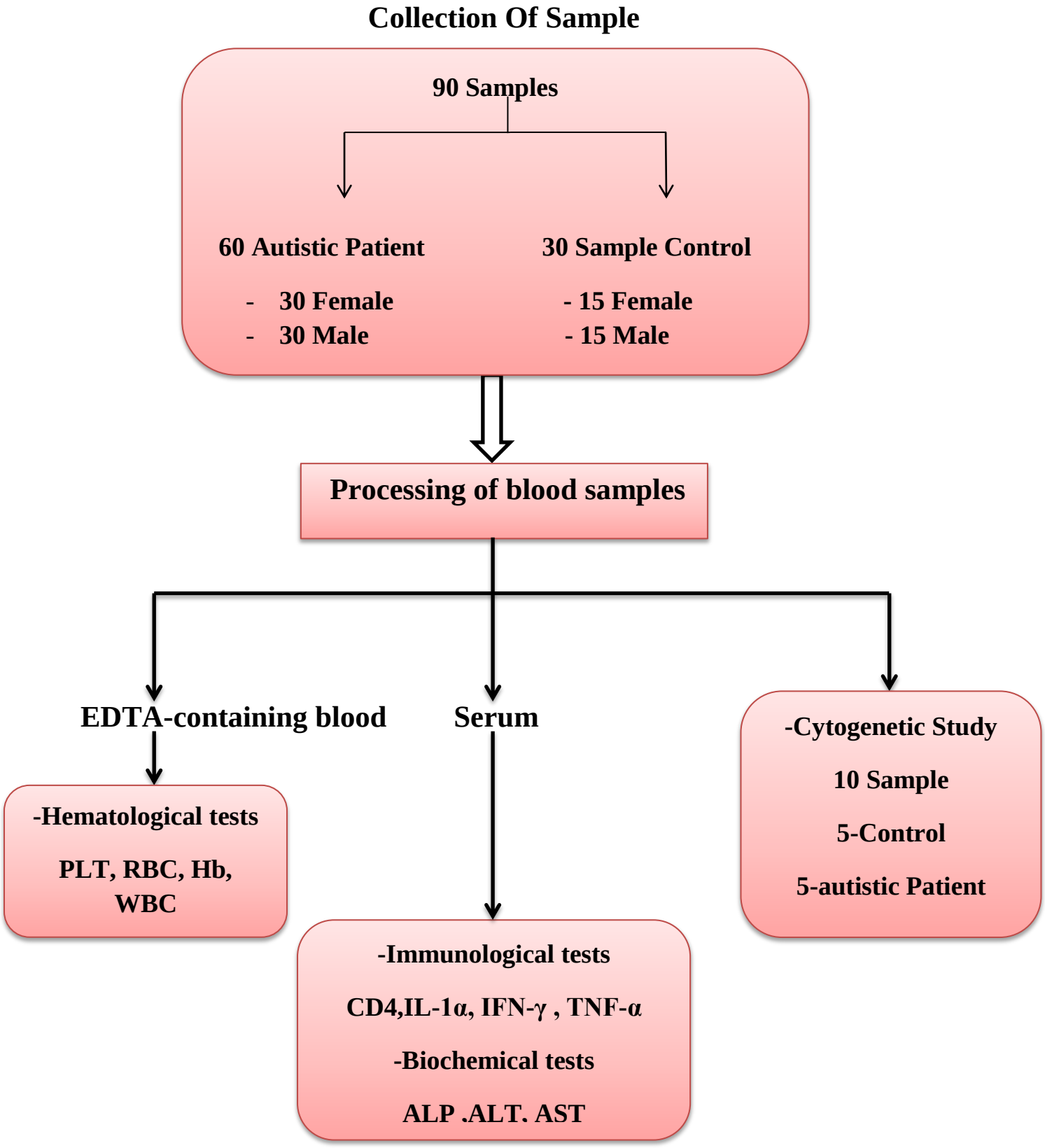


Figure 3-1: shows the research design.

3-2-2 Collection of Samples

This study was conducted for the period September 2020 to March 2021, as (60) blood samples were collected from the autistic patients after diagnosis by the specialist doctor at Ibn Rushd Hospital in Baghdad governorate, as the number of males was (30) and the number of females was (30), and (30) blood samples were collected healthy people of both sexes and used as a control group, and the number of males were (15) and females were (15), and they were within the age range of (2-14) years. All examinations were performed and they were not suffering from any chronic or acute disease at the time of collecting the form. All samples were collected by drawing venous blood after sterilizing the area with 70% alcohol. (5ml) of blood was withdrawn by using plastic medical syringes, and the drawn blood was placed in test tubes (gel tube), then the serum were separated and placed in tubes by Centrifuge for (5) minutes at a rate of (3000 rpm), while tubes containing EDTA for the purpose of conducting blood tests. And stored at a temperature of 20c^{o}.

3-3 Laboratory Methods

3-3-1 Immunological Tests

3-3-1-1Determination of the level of immunological biomarkers (CD4, IL-1α, IFN-γ, TNF-α) in the studied group:

The level of immunological indicators (CD4,IL-1α ,IFN-γ, TNF-α) was determined in the serum of patients with autism using the sandwich ELISA test for (90) people, 60 samples of autistic patients, 30 a sample of males and 30 samples of females. And 30 samples of the control group, 15 samples from males and 15 samples from females, according to the instructions received from the examination kit in the company (Shanghai), and the test principles shown in Figure (3-2).

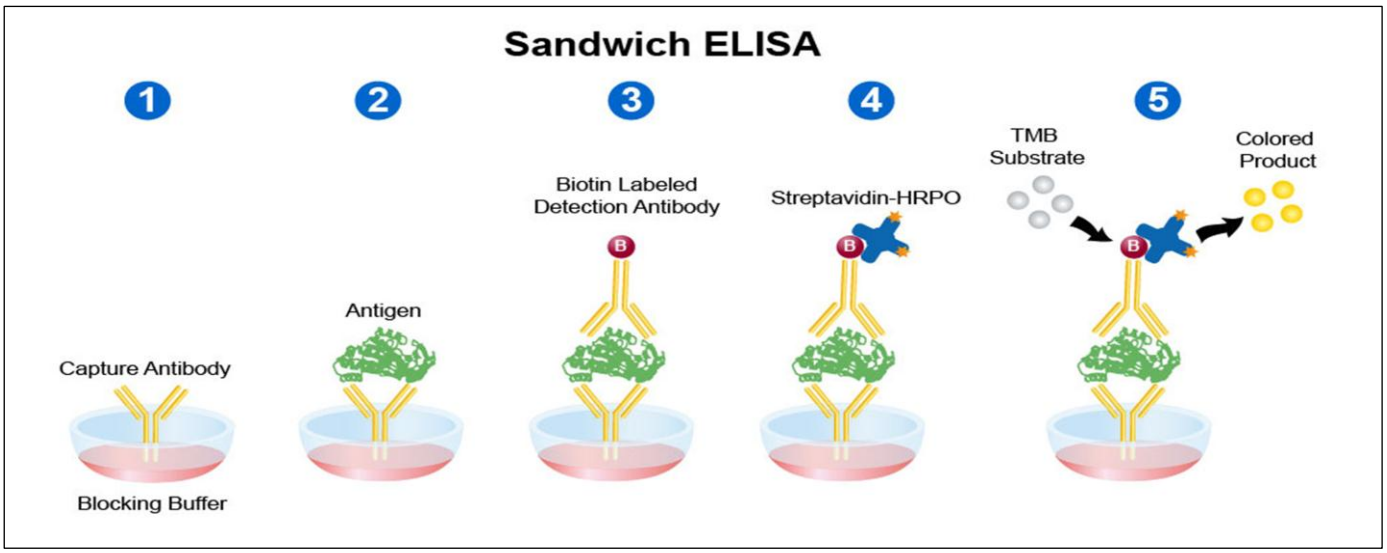


Figure 3-2 ELISA Sandwich test principle

The method of work:

A-Working Principle

1- The samples were prepared for (60) minutes before work to dissolve the serum, as well as the working materials, at room temperature (18-25)ºC.

2- Dilution of standard solution, by drawing 120µl of the standard solution and 120µl of the dilution solution into a tube, then withdraw 120µl of it into a tube containing 120µl of the dilute solution, and so on. And according to the company's instructions as shown in figure (3-3) which works as a zero standard (parts of gram/ml).

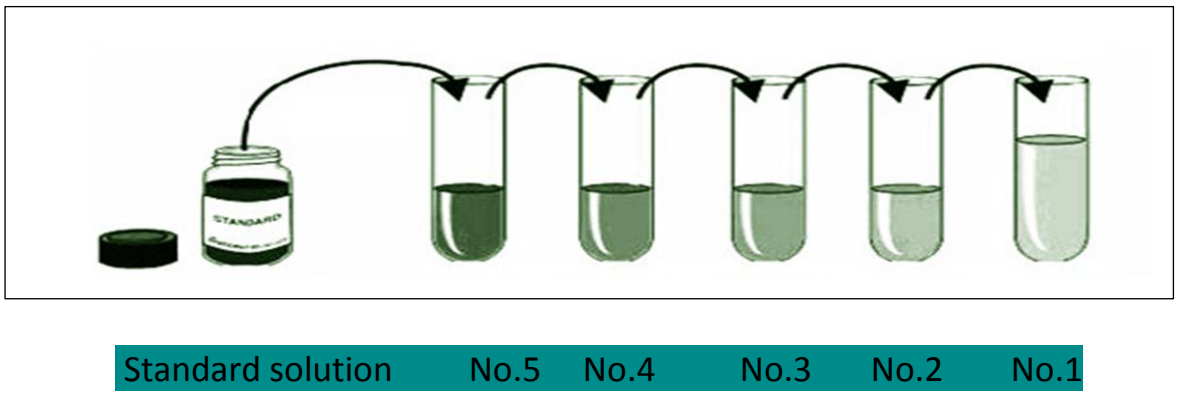


Figure 3-3 the process of preparing the standard solution (CD4, IL-1α, IFN-γ, TNF-α).

3- 40µl of samples were added into each pit. Except for the standard.

4- Put 50µl of the prepared (diluted) standard solution into the standard pits, as well as 50µl of the concentrated standard solution into one pit of the standard pits, except for the first and second.

5- 10µl of antibody labeled with biotin was added to each pit containing the samples and added 50µl streptavidin-HRP, (was added to each pit except for standard)(biotin antibodies have united advance in the standard so no biotin antibodies are added).

6- Move the plate to ensure mixing between the samples and control group with antibody labeled with biotin and streptavidin-HRP.

7- Incubation for 60minutes at 37ºC.

8- Washing solution was prepared, then washing concentration (30X) was diluted with distilled water for later use.

9- Washing (5) times using the COMBIWASH device, which is characterized by accuracy and speed in the washing process for two minutes.

10- 50µl of chromogen reagent A was added to each pit except for Blank.

11- 50µl of chromogen reagent B was added to each pit except for Blank.

12- The plate was moved several times to ensure that the solutions are mixed.

13- Incubation for 10minutes at 37ºC away from light for color development.

14- 50µl of the stop solution was added to each pit to stop the reaction (color changes from blue to yellow immediately at that moment).

15- After 10 minutes the results were read at a wavelength of 450nm, by Human Reader HS.

B- calculation: Appendix number (1,2,3,4) respectively.

3-3-2 Biochemical test

The biochemical tests were done using KROMA PLUS automatic clinical chemistry analyzer device, which analyzed the results of the following tests: (ALP, ALT, AST, Total Bilirubin, Creatinine).

Working Principle:

Is a fully automated benchtop clinical chemistry analyzer that can do 240 photometric tests per hour and up to 360 tests per hour with the integrated ISE modulate. The blood sample was drawn from the patient and placed in test tube then the sera were separated by centrifuge for (5)minutes and at a rate of 3000(rpm). KROMAplus has bar code scanner for identifying positive samples and reagents, as well as reagent refrigeration on board.

A- Determination the level of (ALP) in blood serum:

Working Principle(Klin, 1972)

Alkaline phosphatase (ALP) catalyzes the hydrolysis of 4-nitrophenylphosphate (4-NPP) to free 4-nitrophenol and inorganic phosphate, with the alkaline buffer serving as a phosphate-group acceptor. The rate of synthesis of 4-nitrophenol. Which is proportional to the activity of ALP present in the sample, is monitored kinetically at 405 nm.

$$\text{4-Nitrophenilphosphate} + H_2O^+ \xrightarrow[\text{PH} > 9]{\text{ALP, Mg+}} \text{4-Nitrophenol} + P_i$$

The method is based on the DGKC's proposed optimized formulation.

Reagents:

Table 3-4 reagents used in the measurement of ALP

Reagents	Materials	Concentration
	DEA buffer	1.25 mol/L
	pH	10.2
	Magnesium chloride	0.6 mmol/L

R1. ALP buffer	Biocides	
R2. ALP substrate	4-NPP	50mmol
	Biocides	

Calculation:

KROMA plus automatically measures the concentration of the analyzes from each sample.

Measuring range (<130- <281) U/L. If we want to get the unit of µKat/L, the result were multiplied by 0.01667.

B- Determination the level of (ALT) in blood serum

Working principle (Winn-Deen, *et al.*, 2005)

Alanine aminotransferase (ALT/GPT) catalyzes the transfer of alanine's amino group to oxoglutarate, resulting in glutamate and pyruvate. Lactate dehydrogenase (LDH) converts nicotinamide adenine dinucleotide to lactate in the presence of decreased nicotinamide adenine dinucleotide(NADH). The rate of decrease in absorbance caused by the oxidation of NADH to NAD+, which is proportional to the activity of ALT present in the sample, is monitored kinetically at 340nm.

L-Alanine + 2-Oxoglutarate $\xrightarrow{\text{ALT/GPT}}$ L- Glutamate + pyruvate

Pyruvate + NADH + H^+ $\xrightarrow{\text{LDH}}$ Lactate + NAD^+

The method is based on the IFCC's proposed optimized formulation.

Reagents:

Table 3-5 reagents used in the measurement of ALT

Reagents	Materials	Concentrations
R1. ALT substrate	TRIS buffer	150 mmol/L
	pH	7.3
	L-alanine	750 mmol/L
	Lactate dehydrogenase	>1350U/L
R2. ALT coenzyme	NADH	1.3 mmol/L
	2-Oxoglutarate	75mmol/L
	Biocides	

Calculation:

KROMA plus automatically measures the concentrations of the analyzes from each sample.

Measuring range (<39- <46) U/L , If we want to get the unit of µKat/L, we multiply the result by 0.01667.

C- Determination the level of (AST) in blood serum

Working Principle(Young, 2000)

The transfer of the amino group from aspartate to oxoglutarate is catalyzed by aspartate aminotransferase (AST/GOT), which results in glutamate and oxaloacetate.

In the presence of decreased nicotinamide adenine dinucleotide, malate dehydrogenase (MDH) converts it to malate (NADH). The rate of decrease in absorbance caused by the oxidation of NAD to NAD+, which is proportional to the sample, is monitored kinetically at 340nm.

L-Aspartate + 2 –Oxoglutarate $\xrightarrow{\text{AST/GOT}}$ L-Glutamate + Oxalacetate

Oxalacetate + NADH + H^+ $\xrightarrow{\text{MDH}}$ L- Malate + NAD^+

This test has been developed in accordance with the International Federation of Clinical Chemistry and Laboratory Medicine(IFCC's) standard method.

Reagents:

Table 3-6 reagents used in the measurement of AST

Reagents	Materials	Concentration
R1. AST substrate	TRIS buffer	121 mmol/L
	pH	7.8
	L-aspartate	362mmol/L
	Malate dehydrogenase	>460 U/L
R2. AST coenzyme	NADH	1.3mmol/L
	2-oxoglutarate	75 mmol/L
	Biocides	

Calculation:

KROMA plus automatically measures the concentration of the analyzes from each sample.

Measuring range (0-40)U/L. If we want to get the unit of µKat/L, we multiply the result by 0.01667.

D- Determination the level of (TBIL) in blood serum

Working Principle (Walters & Gerarde, 1970)

Bilirubin is photometrically measured after being transformed to coloured azobilirubin by diazotized sulfanilic acid. Only the former of the two bilirubin fractions in serum, bilirubin-glucuronide and free bilirubin linked to albumin, responds immediately, whereas the latter reacts after being dislodged from protein by an accelerator.

Reagents :

Table 3-7 reagents used in the measurements of TBIL

Reagents	Materials	Concentration
RT.	Sulfanilic acid	29mmol/L
	HCL	0.24mol/L
	Duposol®	3%(w/v)
RN.	Sodium nitrite	11.6 mmol/L

Calclation:

KROMA plus automatically measures the concentration of the analyzes from each sample.

Measuring range (0 - 21) µmol/L.

E- Determination the level of (Creatinine) in blood serum:

Working Principle (Jaafer, 1886)

This process is based on an improved enzymatic method for determining creatinine levels in the blood and urine. The assessment is carried out in two stages. During the initial minutes of the preincubation stage of the sample with creatinase, creatine is eliminated.

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatininase}} \text{Creatine}$$

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow[\text{Ph8.0}]{\text{Sar OD}} H_2O_2 + \text{Glicine} + \text{HCHO}$$

The hydrogen peroxide produced from the oxidase reaction is cuantified by a Trinder's type reaction in which the chromogenic derivative HTIB* and 4-

aminoantipyrin (4-AA) are condensed in the presence of peroxidase (POD) to form a red quinoneimine dye.

4-AA + HTIB $\longrightarrow$ Red quinoneimine + H_2O

The rate at which the color changes is proportional to the amount of creatinine in the sample. *Hydroxy-triiodobenzoic acid.

Reagents:

Table 3-8 reagents used in the measurement of Creatinine

Reagents	Materials	Concentration
R1.Chromogen reagent	TAPS buffer	35 mmol/L
	PH	8.0
	Creatinase	≥300µKat/L
	Sarcosine oxidase	≥100 µKat/L
	Ascorbate oxidase	≥20 µKat/L
	HTIB	2 mmol/L
	detergent	0.5 g/L
R2. Enzyme reagent	TAPS buffer	50mmol/L
	pH	8.0
	creatininase	≥400µkat/L
	peroxidase	≥15µkat/L
	4-amino antipyrine	2mmol/L
	Potassium ferrocyanide	0.1 mol/L
	detergent	1.5 g/L

Calculation:

KROMA plus automatically measures the concentration of the analyzes from each sample.

Measuring range (17.7 – 123.8) mmol/L .

3-3-3 Hematological tests

The blood tests were done using the ABX Micros 60 Hematology analyzer device, which analyzed the results of the following tests: (WBC, RBC, Hb, PLT).

Working principle

A fully automatic ABX Micros 60 Hematology analyzer device was used. This device uses resistive technology and photometry as measurement methods in addition to the stepper motor technology that enables the system to work without a compressor. The blood sample was drawn from the patient and placed in special tubes containing (EDTA). The solutions used in this apparatus are: Lyse solution, cleaner solution, diluent solution.

3-4 Cytogenetic

The practical part of cytogenetics was completed in the laboratories of the Iraqi Center for Cancer and Medical Genetics Research.

3-4-1 Chemicals

Table 3-9 A list of the Chemicals used

Instruments	Country	Company
RPM1 1640 Culture medium	USA	US Biological
Foetal Bovin Serum	USA	Gibco
Trypsin (1:250)	USA	Difco
colcemid	Germany	Fluka
Giemsa Stain	UK	BDH

Phytohaemaglutinin (PHA)	Sweden	Iraqi Center for Cancer & Medical Genetics Research
Absolute Methanol	Irland	LabScan
Glacial Acetic Acid	UK	GCC
Concentrated sulfuric acid H2SO4	Poland	Poch
Absolute Ethanol	UK	GCC
Heparin	France	Panpharma
Sodium chloride NaCl	UK	BDH
Potassium Chloride KCl	UK	BDH
Sodium Phosphate mono Hydrogen Na2HPO4	Germany	Reidel de Haen
Sodium bicarbonate Na2HCO3	UK	BDH
Potassium Dihydrogen Phosphate KH2PO4	Germany	Reidel de Haen
Potassium Dichromate K2Cr2O7	Germany	Reidel de Haen
Streptomycin	Germany	Troge medical GMBH
Crystalline Penicillin	Germany	Troge medical GMBH
Nystatin	Iraq	S.D.I.

3-4-2 Laboratory devices and tools

Table 3-10 A list of the devices used

Device	Country	Company
Centrifuge	Germany	Universal 16A
Cooled incubator	Korea	K & K
Sensitive Balance	Switzerland	Precisa
Light Microscope	Japan	Olymps

Oven	Germany	Memmert
Water Bath	Germany	Tafesa
PH-meter	USA	ThermoElectron co.
Magnetic stirrer with hot plat	Korea	Vision
Cooled box	AOV international	India
Vacuum tubes (10ml)	Egypt	MEDICONex

3-4-3working method

3-4-3-1 Samples of The Study

The study samples included 10 patients (5 males, 5 females) with autism, in the early diagnosis stage (before treatment) and during treatment, collected from Ibn Rushd Hospital after their diagnosis through clinical and laboratory tests, for the period from September / 2020 to March / 2021.

3-4-3-2 Method of Samples Collection

Blood samples were collected from autistic patients using a syringe washed with heparin. 2-3 milliliters of blood were withdrawn. The sample was shaken to prevent clotting, and kept in the cooler box until it was transferred to the laboratory.

3-4-3-3 Culturing

3-4-3-3-1 Blood Samples Culturing

Blood samples (for autistic patients) were cultured using the Direct culture technique without using a mitotic stimulator, and the Short-term culture

technique using cleavage stimulator. The culture was carried out according to the following steps:

1- Under sterile conditions and using the culture cabinet, 0.3 milliliters of PHA were added to each tube containing 5 milliliters of culture medium (in case of cultivation for a short period only).

2- The blood sample was shaken well and 1-12 drops of blood were added to the culture medium tube in the case of transplanting patients' blood samples (the amount of blood added depends on the number of white blood cells, which are often in abnormal proportions), The tubes are marked by writing the sample number, time and date of implantation. The tubes were incubated in the incubator at 37°C for 1 hour (according to the direct culture method), and for 72 hours, according to the culture method, for a short period. The tubes were shaken.

3-4-3-4 Harvesting and Fixation

Colcemide was added in the last 25 minutes of the incubation time to the blood transplant tubes in the case of the culture method for a short period, and colcemide was added at the beginning of the incubation time in the case of the direct culture method of blood samples, as 0.1 ml of the working solution was added solution of colcemide to each tube containing 5 mL of culture medium and the tubes were returned to the incubator to complete the incubation time. The harvesting process was carried out according to the following steps:

1- The culture tubes were taken out after the incubation period and centrifuged at 1500 rpm for 10 minutes.

2- The filtrate was withdrawn using a Pasteur pipette, and the precipitate was mixed with the residues of the filtrate, and then potassium chloride solution was

added and heated to 37°C gradually with continuous shaking until the total volume reached 10 ml, then the tubes were placed in the water bath (at a temperature of 37°C). for 30 minutes.

3- The tubes were taken out and centrifuged at a speed of 1500 rpm for 10 minutes.

4- The filtrate was disposed of and the precipitate was mixed with the residues of the filtrate. The fixative solution was gradually added with continuous shaking of 5 ml to each tube, and the tubes were placed in the refrigerator for 30 minutes.

5- The tubes were centrifuged at a speed of 1500 rpm for 10 minutes.

6- The filtrate was disposed of and the stabilizing solution was added. The washing was repeated with the stabilizing solution until a clear filtrate was obtained. Cells were suspended in 5 mL of fixative solution.

7- The final suspension of cells can be used directly to prepare glass slides, or preserved by freezing.

3-4-3-5 Slides Preparation

The glass slides were prepared according to the following steps:

1- The sample was centrifuged at a speed of 1500 rpm to replace the fixative solution with another instantaneous preparation.

2- The filtrate was disposed of while keeping an amount of it (0.5 - 1 milliliter) depending on the amount of cell precipitate so that the suspended cell became foggy.

3- The glass slides were taken out of the refrigerator . I held the glass slide using forceps in a horizontal manner, and using a Pasteur pipette, 5-8 drops of the suspended cells were dribbled equally on the glass slide.

The glass slides were left to dry at room temperature, after which they were marked.

3-4-3-6 Staining

The glass slides were stained using giemsa stain, which was prepared upon use by mixing 1 ml of a buffer solution of the dye with 4 ml of Sorensen buffer heated at 37 C. After that, the glass slides were washed using Sorensen buffer solution heated at 37 C and left to dry. Slides were examined using a light microscope.

3-4- 4 Preparation of solutions

The solutions were prepared according to the method used by the Iraqi Center for Cancer Research and Medical Genetics (Yaseen 1990, 1999).

3-4-4-1 Fixative Solution

The fixative solution was prepared upon use by mixing three volumes of absolute methanol alcohol with one volume of glacial acetic acid.

3-4-4-2 Hypotonic potassium chloride solution KCL (0.075)

5,5875g of potassium chloride is dissolved in one liter of distilled water, this solution is prepared before use, and it is used warm at 37℃.

3-4-4-3 Colcemide

The stock solution 1 for colisimide was prepared by dissolving 1 g of colisimide in 20 ml of distilled water, and kept by freezing. The second stock solution 2 is prepared by mixing 1 ml of the first stock solution with 19 ml of distilled water, and kept by freezing. The working solution of colisimide was prepared by mixing 1 ml of the second stock solution with 19 ml of distilled water, distributed in equal volumes in containers, and kept by freezing.

3-4-4-4 Giemsa stain

2 grams of dye powder were dissolved in 100 milliliters of absolute methanol alcohol in an opaque vial, and mixed using a magnetic stirrer for three days at room temperature, then filtered using filter papers and kept in an opaque vial. The dye was used immediately, diluted in a ratio of 1 ml dye to 4 ml of warm Sorensen buffer solution at 37˚C.

3-4-4-5 Sorensen's Buffer (PH 6.8)

Sorensen buffer solution was prepared by dissolving the following substances in one liter of distilled water. The solution was kept in the refrigerator until useas.

Table 3-11 A list of chemicals used

Chemical	Weight (g)
KH2PO4	6.74
Na2HPO4	7.08

3-4-4-6 Phosphate-Buffered Saline (PBS) (PH 7)

A phosphate buffer solution was prepared by dissolving the following substances in one liter of distilled water. The solution was kept in the refrigerator until use.

Table 3-12 A list of chemicals used

Chemical	Weight(g)
NaCL	8
KCL	0.2
Na2HPO4	0.92
KH2PO4	0.2

3-4-4-7 Phytohaemaglutinin (PHA)

Packages of 3ml PHA solution prepared and prepared by the Iraqi Center for Cancer and Medical Genetics Research were obtained and kept by freezing.

3-4-4-8Chromic acid

Chromic acid was prepared by dissolving 100 g of potassium dichromate in 1 liter of distilled water, and slowly 500 ml of concentrated sulfuric acid was added to the solution and mixed well.

3-4-4-9 Trypsin solution

The trypsin solution was prepared before use by dissolving 0.25 g of trypsin powder in 100 ml of phosphate buffer solution PBS, mixed using a magnetic stirrer until dissolved and distributed in containers.

3-4-4-10 Sodium Bicarbonate Solution

The solution was prepared by dissolving 4.4 grams of sodium bicarbonate powder in 100 milliliters of distilled water, then sterilized with an autoclave at a temperature of 121 ºC and keeping the solution in the refrigerator until use.

3-4-4-11 Antibiotics

1- Streptomycin

Using, one packet (1 g) of streptomycin was dissolved in 5 ml of sterile distilled water to obtain a solution with a concentration of 200 mg/ml.

2- Penicillin

Prepare when using by dissolving one package (1000,000 IU) of crystalline penicillin in 5 ml of sterile distilled water to obtain a solution of concentration (200,000 IU / ml.).

3- Nystatin

A nystatin solution was prepared upon use by dissolving one tablet of nystatin (500,000 IU) in 100 milliliters of distilled water to obtain a solution with a concentration of (5000 IU/ml), mixed using a magnetic stirrer until dissolved.

3-4-4-12 Rosswell Park Memorial Insttute (RPMI-1640)

Using RPMI-1640 medium with hepes, with L-glutamine, without sodium bicarbonate produced by US Biological Company, one liter of culture medium was prepared by mixing the following materials:

Table 3-13 A list of materials used.

Materials	Volume
Fetal bovine serum	200 ml
Penicillin (100,000 IU/ml.)	0.5 ml.
Streptomycin (20 mg./ml.)	0.5 ml .
Nystatin (5000 IU/ml.)	0.5 ml
Sodium bicarbonate (4.4%)	(Used to adjust pH to 6.8 - 7.2)

The materials were mixed well in a one-liter glass bottle and the volume was filled up to one liter using distilled water. Under sterile conditions and using the culture cabinet, the culture medium was sterilized by filtering through a 0.22 µm filter, and distributed in sterile 10 ml tubes, with a volume of 5 ml per tube, kept frozen until use.

3-4-4-13 Slide's Preparation

The glass slides were cleaned by immersing them in a chromic-sulfuric acid detergent solution (Dichromate-sulphuric acid cleaning solution "Chromic acid")

for a period of no less than three days, then washed with hot water and immediately after that with cold water, then kept in a beaker containing distilled water and placed in the freezer until it is about to freeze and then transferred to the refrigerator at 40 ° C until use. A cleaned chromic acid solution was prepared by dissolving 63 g of sodium (or potassium) dichromate by heating in 35 ml of water, then cooling, and completing the volume to a liter by adding concentrated sulfuric acid (Conc. H2So4).

3-5 Photography

A light microscope of Olympus supplemented by a camera of nixon has selected to determine the results of cytogenetic study.

3-6 Statistical analysis

The data of the current study were analyzed using T-test to compare two variables and F to compare more than two numerical variables, the variables were described by (detection of the best diagnostic test). Pearson's correlation (R) explains the type and strength of the relationship between the variables. Significant differences a = 0.05 were applied to the test. (SPSS v.22 and Graph pad prism v.6) Software used to analyze current data (Levesque, R. (2007).

Results and Discussion:

4-1 Distribution of study Samples according to age groups and gender:

The results of the current study showed that patients with age period ≥10 (10-15 years) years scored high percentage (53.3%) than <10 (2-8 years) (46.7%). In contrast, our results show the controls with age period ≥10 (10-15 years) years scored lowest percentage (40.0%) than <10 (2-8 years) (60.0%) with no significant difference (p>0.05) between study groups dependent on age. In our results there is no differences between study groups depending on gender table (4-1).

Table 4-1 comparative age periods and gender between study Samples by using chi-square test.

			Groups		Total	P value
			Patients	Controls		
Age periods (years)	<10 (2-8 years)	n	28	18	46	0.233
		%	46.7%	60.0%	51.1%	
	≥10 (10-15 years)	n	32	12	44	
		%	53.3%	40.0%	48.9%	
Gender	Males	n	30	15	45	1.00
		%	50.0%	50.0%	50.0%	
	Females	n	30	15	45	
		%	50.0%	50.0%	50.0%	

There were no significant difference ($p>0.05$) between study groups according to age and gender. Early detection of ASD can lead to early treatment (Rogers *et al.,* 2014).

The subgroup analysis for studies that only included children (⩽10 years) found a mean age at diagnosis of 43.18 months (95% CI: 39.79–46.57) with a range of 30.90–74.70 months (van't Hof *et al.,* 2021) and that not compatible to our results that showed the autism disorder occur in age children ⩽10 years and >10 years . Studies that reported a lower or partly lower mean or median age children aged ⩽10 years (Christensen *et al.,* 2019) or had a majority of the children aged ⩽10 years (Jo *et al.,* 2015). Thus, the age at ASD diagnosis among these younger populations is logically lower. Conversely, studies that reported a higher mean or median age included 26% to 53% adults in their populations (Kentrou *et al.,* 2019), which explains the higher age at ASD diagnosis.

Study result suggest that there are subtle, yet potentially meaningful, quantitative, and qualitative phenotypic differences between females and males that common screening tests are not always sensitive enough to recognize (de Giambattista *et al.,* 2021). Further studies to improve practice and course for the assessment of females, reducing sex/gender-based inequities in ASD care, are required. The gender ratio for the ASD, as a whole is consistently reported at around 4:1 M:F (Idring *et al.,* 2015), ranging according to intelligence quotient (approximately 2:1 in ASD with intellectual impairment; up to 11:1 ASD without intellectual impairment) and age (nearly 5:1 in child and adolescents; around 2:1 in adults) (Rutherford *et al.,* 2016).

4-2 The immunological study included (CD4, IL-1α, IFN-γ, and TNF-α):

Result of conducted study showed the high mean value for CD4 (8.68±3.06), IL_1a (5.69±2.50), IFN_y (606.51±286.62) and TNF-α(84.07±41.86) parameters in patients than controls for CD4 (4.56±1.99), IL-1α (2.45±1.11), IFN-γ (378.40±126.35) and TNF-α (29.80±13.76) with high significant different (P<0.05) table (4-2) and figure (4-1).

Table 4-2 comparative immunological parameters between study Samples by using student t test.

Groups		N	Mean	SD	P value
CD4 (ng/ml)	Patients	60	8.68	3.06	0.001***
	Controls	30	4.56	1.99	
IL_1a (ng/l)	Patients	60	5.69	2.50	0.001***
	Controls	30	2.45	1.11	
IFN_y (pg/ml)	Patients	60	606.51	286.62	0.001***
	Controls	30	378.40	126.35	
TNF_a (ng/l)	Patients	60	84.07	41.86	0.001***
	Controls	30	29.80	13.76	

*There is a significant difference and below the probability level ($p < 0.05$).

** There is high significant difference and below the probability level ($p<0.01$).

*** There is a very high significant difference and below the probability level ($p<0.001$).

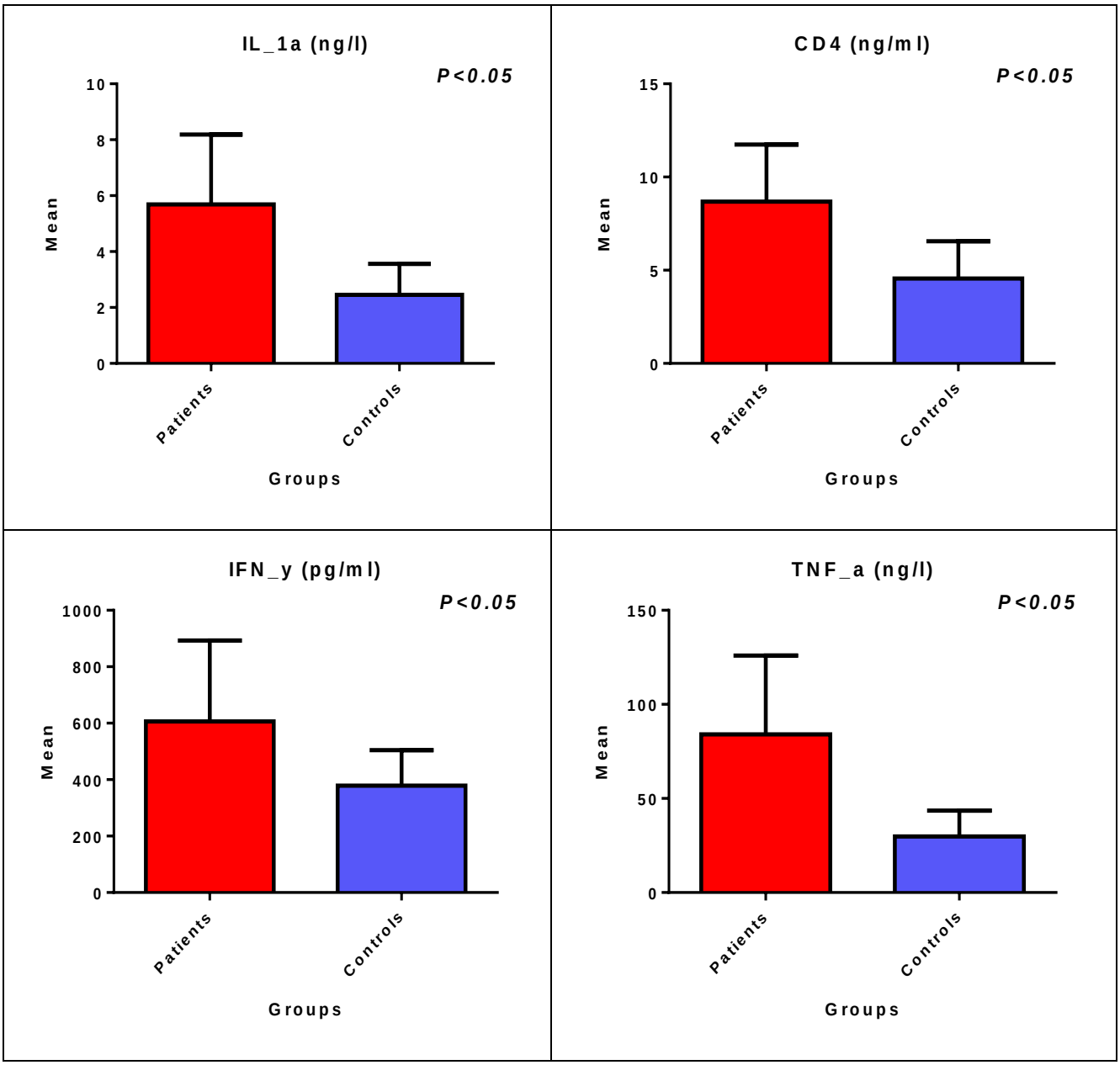


Figure 4-1 comparative immunological parameters between study groups

Immune dysfunction has been reported in approximately 60% of children with ASD (Careaga *et al.*, 2017). Although immune abnormalities in ASD have been reported since the late 1970's (Stubbs & Crawford, 1977) a consensus for differences in cellular activation has not yet been reached (Rose & Ashwood, 2014). This was true for both children with ASD who displayed increased TH1 cytokine profiles and those

with increased TH2 cytokine profiles. Researchers hypothesize that immune activation involving several arms of the immune system is observed in ASD, and that the unifying denominator among these studies is in fact a decrease in immune regulation and inability to control immune responses (Rose *et al.,* 2020).

Corroborating this hypothesis, decreased levels of the regulatory cytokines transforming growth factor (TGF)β1 , interleukin (IL)-10 , and IL-35 (Rose & Ashwood, 2018) have been reported from blood components or in stimulated immune cell cultures from individuals with ASD. In addition, decreased frequency of regulatory T cells (Tregs) have also been found in individuals with ASD (Ahmad *et al.,* 2017). On a background of decreased regulation, the varying pro-inflammatory immune abnormalities reported in ASD may reflect the heterogeneity of ASD, genetic background or environmental exposures, and illustrate the need to find common subgroups within ASD, that may help define more targeted treatments to benefit more individuals across the spectrum (Ousley & Cermak, 2014).

In the ASD with gastrointestinal disorders , reporters found elevated frequencies of TH17 and TH17 1 populations, and coupled to this were decreased populations of IL-10 producing CD4 T cells, reductions in regulatory T cells, and decreased ratios of regulatory T cells compared to inflammatory TH17 subsets (Rose *et al.,* 2020). Considering our previous findings of increased mucosa-related cytokines and decreased regulatory cytokines, mainly active TGFβ1, in children with ASD who experience GI symptoms (Rose *et al.,* 2018), these data are suggestive of impairments in immune regulation. Alterations in the ASD with no gastrointestinal disorders included elevated populations of CD4 T cells that could represent a shift to a TH2 response (Rose *et al.,* 2020) and these results compatible to our results that showed high levels of CD+4 in patients with ASD than controls and that suggest that ASD patients have decreased regulation and increased inflammatory responses but that

inflammatory signals may be different and lead to different co-morbidities. Study findings support the hypotheses that 5-Aminoisoquinolinone (5-AIQ) has promising novel therapeutic effects on neuroimmune dysfunction in autism and is associated with modulation of Treg and Th17 cells, where the 5-Aminoisoquinolinone decreased the abundance of IL-9 (significantly increased in brain tissue) -producing CD4+ T cells (5-AIQ) (Alhosaini *et al.,* 2021).

Results data show that there is increased levels of IL-6 receptors and CD4+ T cells , and these increased associated with upregulated Th17A development in ASD subjects. Furthermore, treatment of CD4 + T cells in vitro with IL-6 leads to much greater upregulation of *p*-STAT3, and IL-17A in ASD subjects than controls, and in finally, this treatment blockade of IL-6 mediated effects on CD4+ T cells (Nadeem *et al.,* 2021).

Data results strongly support the lead induced CD4 + CD3 + T-cell dependent immune system activation and chronic inammation as the key pathogenetic events in autism spectrum disorders. Moreover, data supports the CD4 + CD3 + T-cell dependence of mitochondrial dysfunction development in ASD patient reported in previous study (Harutyunyan *et al.,* 2021). Abd El-Aziz and El-Din, (2012) showed the children with autism have significantly reduced levels of serum IgG, IgA and IgM compared to the control children, suggesting an underlying defect in the immune function, also the cell-mediated immunity is impaired as evidenced by low numbers of CD4+ cells and increased CD8+ T cell subpopulations and decreased CD4+/CD8+ ratio and that not compatible to our study results.

The immunological role in the etiopathogenesis of ASD is a matter of ongoing debate among researchers. There is evidence of an alteration in the immune system of children with ASD, which includes increased cytokine levels in both the

brain and plasma (Guloksuz *et al.*, 2017). This theory of immunological alteration is based on the knowledge that the brain is able to recognize cytokines, such as the proinflammatory cytokines IL-1a, IL-1β, TNF-α, and IL-6, as molecular signals of sickness (Masi *et al.*, 2017).

Altered cytokine profiles have been consistently linked to ASD in children during this period . In high-functioning male children with ASD, the plasma levels of IL-1β, IL-1 receptor antagonist (IL-1RA), IL-5, IL-8, IL-12(p70), IL-13, and IL-17 are elevated relative to matched controls (Masi *et al.*, 2017). In addition to elevated expression of IL-1β, as identified by Suzuki and colleagues, IL-6, IL-12, TNF-α, and IL-23 are also elevated in ASD compared to healthy controls, suggesting a dysregulated immune response (Ricci *et al.*, 2013).

TNF-α is a central regulator of inflammation and is elevated in the children with ASD (Zhao *et al.*, 2021) and that compatible to our results. Excessive TNF-α has been proven to be involved in autistic behaviors, such as impaired social interaction and repetitive behaviors (Wang *et al.*, 2010), which may also participate in the imbalance between the inhibitory and excitatory behaviors of the brain. IL-6, IL-1β, IL-12p70, MIF, eotaxin-1, MCP-1, IL-8, IL-7, IL-2, IL-12, TNF-α, IL-17, and IL-4 may be identified as a series of potential biomarkers for ASD in peripheral blood. This may provide some important clues for ASD therapeutic discovery (Zhao *et al.*, 2021).

The increase in cytokines may indicate an impaired immune response, with a predominant response of Th2 cells. Some authors have suggested the existence of an endophenotype of ASD linked to an autoimmune dysregulation (Angelidou *et al.*, 2012). Others have described altered levels of immune mediators linked to greater deterioration in behavior. It has thus been suggested that a dysregulation of the

immune response may be related to the behavior and cognitive impairment of children with ASD (Gomez-Fernandez *et al.*, 2018).

Data results provide further support for altered innate immunity being an important autism pathogenic factor, with autistic children showing increased blood TNF-α concentrations associated with symptom severity (Xie *et al.,* 2017, Othman et al., 2021). Synaptic dysfunction is consistently thought to be the main underlying mechanism of ASD. Pro-inflammatory mediators, including cytokines, might be involved in different synaptic processes, such as long-term potentiation (LTP), that is involved in synaptic plasticity in learning and memory processes ; cytokines, such as IL-18, IL-1β, and TNF-a, act on many molecular components of LTP in variable fashions (Abd-Allah *et al.*, 2020).

IL-1β has also been shown to play a key role in mediating severe placental damage and neurodevelopmental anomalies in offspring (Arrode-Brusés & Brusés, 2012). IL-1β showed the highest concentration levels in fetal brains and was the only cytokine that was significantly upregulated 24 h after maternal poly (I:C) injection, suggesting that IL-1β may have a deleterious impact on central nervous system development (Xu *et al.,* 2015). Study results provides evidence for the lower concentration of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist in autistic patients compared with control subjects (Saghazadeh *et al.,* 2019).

Leveles of IFN-γ were continously increased and this result was disagree with Halbot *et al.*, (2015) who mentioned that detection of IL-12 and IFN-γ in autistic patients serum showed significantly decrease level ($P < 0.05$) compared with healthy control.

Masi *et al.*, (2017) and Saghazadeh *et al.*, (2019) mentioned immune abnormalities in the postnatal period involves activation of the monocytic and Th1 arm

of the immune response, *via* increased IFN-γ, and this has been found in children with ASD and that compatible to our results. Few studies have reported increased levels of cytokines that can precipitate inflammatory processes in ASD, such as interferon gamma (IFNγ) or IL-12 , or a decreased production of cytokines that negatively regulate inflammation, such as transforming growth factor β1 (TGFβ1) (Abd-Allah *et al.,* 2020).

Choi et al (2016) also showed that their maternal immune activation (MIA) model induced the production of several other cytokines, finding increased levels of TNF-*α*, IFN-*β*, and IL-1*β* 3 h following MIA induction, but not the anti-inflammatory IL-10. More recent human studies found increased levels of IFN-*γ*in sera from mid-gestational mothers with a child that would later be diagnosed , and elevated MCP-1 , IL-4, TNF-*α*, and TNF-*β* levels in amniotic fluid (Meltzer & Van de Water, 2017). Interferon-γ is a soluble cytokine that functions in the immune response. It is a predominant inducer of indoleamine dioxygenase (IDO) that converts tryptophan to kynurenine in kynurenine pathway (Wilson *et al.,* 2018). *IFNG-AS1* plays an important role in regulation of *IFNG* expression in human $CD4^+$ T cells (Peng *et al.,* 2015). It has been shown that there was significant *IFNG* upregulation and *IFNG-AS1* downregulation in Iranian children with ASD compared with controls. The male autism children had also higher levels of *IFNG* expression compared with healthy subjects. Moreover, there was a significant direct association between *IFNG-AS1* expression level and age in ASD group (Fallah *et al.,* 2020).

Warre-Cornish *et al.,* (2020) found evidence that IFN-γ disproportionately altered the expression of genes associated with schizophrenia and autism, suggesting convergence between genetic and environmental risk factors. Mid-pregnancy maternal serum IFN-γ is increased during gestation of offspring with ASD , and circulatory IFN-γ levels are elevated in neonates subsequently diagnosed with ASD relative to

developmental delay controls (Heuer *et al.,* 2019). Intriguingly, within the brain, many antiviral IFN-γ signaling targets also play important roles in neuronal development and synaptic activity, independent of microbial infection (Bilousova *et al.,* 2012).

4-3 Measurement of biochemical indicators, including (ALP, ALT, AST, TBIL, and Creatinine):

Result of our study shows the high mean value for ALP (259.98±95.11), ALT (38.60±16.75), AST (46.43±22.99) and TBIL (35.77±15.18) parameters in patients than controls with high significant different (P<0.05). in contrast, our study shows the low mean value for Creatinine (44.29±10.91) parameters in patients than controls with high significant different (P<0.05) table (4-3) and figure (4-2).

Table 4-3 comparative biochemical parameters between study Samples by using student t test.

Groups		N	Mean	SD	P value
ALP (U/L)	Patients	60	259.98	95.11	0.001***
	Controls	30	107.90	38.51	
ALT (U/L)	Patients	60	38.60	16.75	0.04*
	Controls	30	29.73	5.54	
AST (U/L)	Patients	60	46.43	22.99	0.001***
	Controls	30	23.15	6.05	
TBIL (umol/L)	Patients	60	35.77	15.18	0.001***
	Controls	30	11.24	3.96	
Creatinine (mmol/L)	Patients	60	44.29	10.91	0.61
	Controls	30	57.99	22.60	

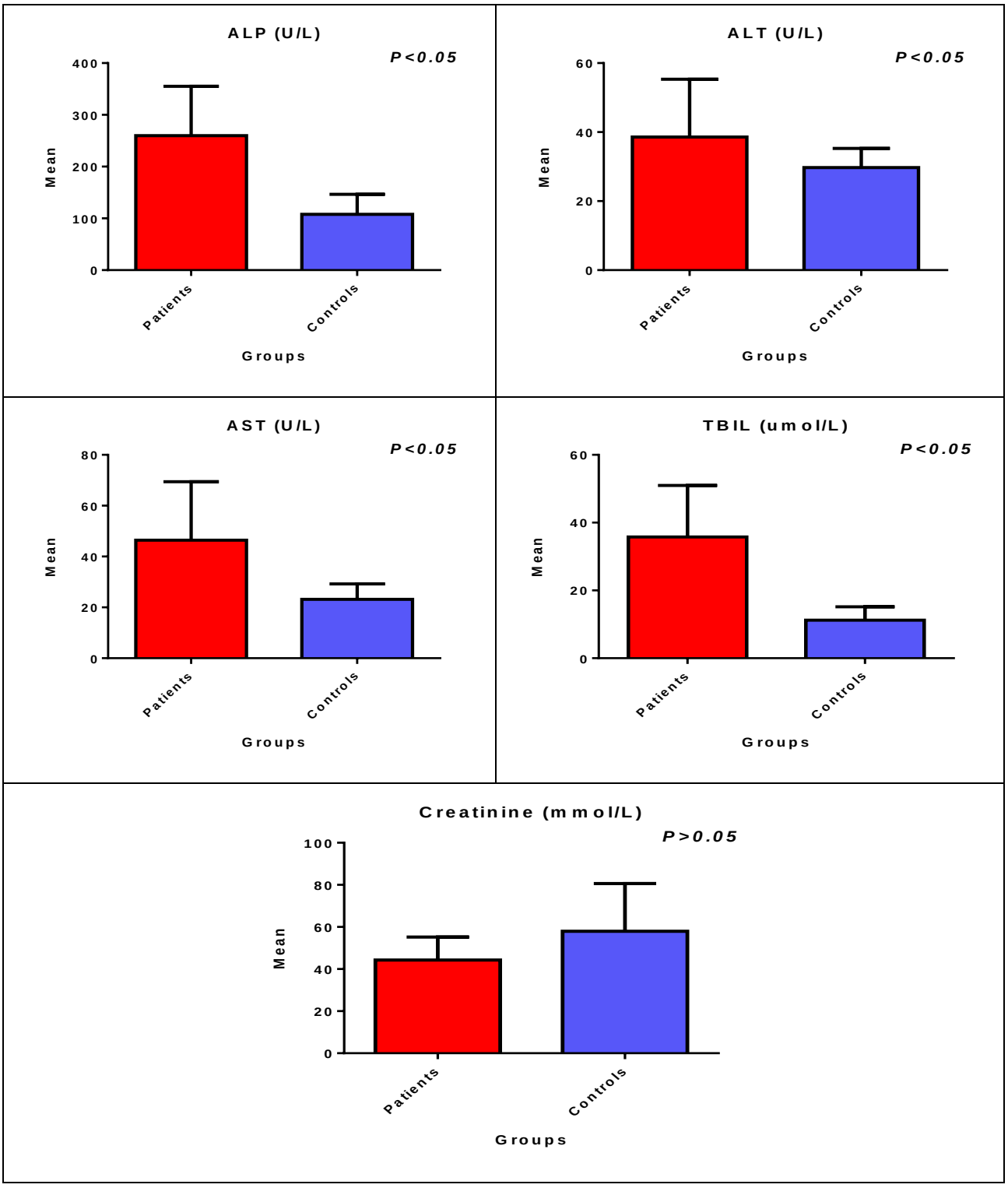


Figure 4-2 comparative biochemical parameters between study Samples

Moses *et al.,* (2014) revealed the people with ASD have unaltered lipid profiles and lower glucose and liver enzyme levels compared to a community-based population and that not compatible to our study that mentioned high levels of liver enzyme levels in ASD than compared . In contast, Dobre *et al.,* (2009) &Baeza *et al.,* (2018) showed high levels of AST, ALT and GGT in patients than controls. Less than 3% of children and adolescents on antipsychotics (AP) during 1-year follow-up showed an increase in ALT or AST levels in one or more of the assessments, and none of these increases was of clinical significance. Patients with metabolic syndrome (MetS) and at risk of meeting MetS criteria (AR-MetS) increased during this period, and the possible role of ALT levels to monitor these patients deserves further study (Baeza *et al.,* 2018).

The metabolic disorders like obesity is predictor of liver testing and abnormal test results, irrespective of ALT threshold. Children with ASD have an increased risk of obesity and obesity-related metabolic disorders , and these disorders are associated with increased levels of liver parameters; ALT, AST, ALP in ASD than controls (Frye *et al.,* 2015; Li *et al.,* 2020).

Lv *et al.,* (2016) show high bilirubin in ASD than controls and that compatible to our results. After adjustment for the effects of sociodemographic factors and birth weight, neither hyperbilirubinemia nor phototherapy was an independent risk factor for ASD (Wu *et al.,* 2016). Neonatal hyperbilirubinemia, or jaundice, has been implicated as a potential environmental risk factor for ASD. Hyperbilirubinemia is associated with a 43% increased risk of ASD, based on a meta-analysis of 13 studies (odds ratio: 1.43; 95% confidence interval [CI]: 1.22–1.67) (Amin *et al.,* 2011). Yet previous studies of hyperbilirubinemia and ASD have important limitations. Past studies relied on administrative diagnoses or parental report of jaundice instead of analyzing total serum bilirubin (TSB) levels; evaluated a dichotomous hyperbilirubinemia variable based on traditional cutoffs or infant weight; did not control for confounding; were not

population-based; or included a relatively small sample ($N < 120$) of children with ASD (Vandborg *et al.*, 2015). Thus, the heterogeneity ($P = .002$) and limitations of previous studies limit the conclusions that can be drawn (Wu *et al.*, 2016).

The study revealed that children with ASD exhibit alterations in the serum levels of certain amino acids, and the divergence can be sex-related or associated with different cognitive function, which might provide clues for further etiological research of ASD (Yu et *al.*, 2021). In our results we noticed low levels of creatinine in ASD than controls and these results compatible tor results (Aydin, 2018). Creatine transporter deficiency (CTD) is a treatable, X-linked, inborn error of metabolism. Two brothers with autism spectrum disorder were diagnosed with CTD at the ages of 17 and 12 years. Both were found to have a previously reported hemizygous p.408delF (c.1216_1218delTTC) deletion mutation. Both patients were given creatine monohydrate, L-arginine, L-glycine and S-adenosylmethionine, which partially improved the behavioral problems. Serum creatinine levels, creatine peak at brain MR spectroscopy or creatine/creatinine ratio in urine should be evaluated to identify CTD in children with autistic behavior and language disorders (Aydin, 2018).

Preliminary evidence suggests that the mitochondria may be a fruitful target for treatment and prevention of ASD (Rose *et al.*, 2018).Study result confirms that several mitochondrial biomarkers are abnormal in children with ASD and suggest that certain mitochondrial biomarkers can differentiate between ASD and typically developing children, making them possibly useful as a tool to diagnosis ASD and identify ASD subgroups (Khemakhem *et al.*, 2017). The routine screening for abnormal urinary creatine (CR) and guanidinoacetate (GAA) could be considered in ASD diagnose protocols; however, individuals positive for Creatine deficiency syndrome (CDS) are likely to be rare in an ASD cohort (Wang *et al.*, 2010).

4-4 Measurement of hematological indicators, including (WBC, RBC, PLTs, and HGB):

Result of our study shows the high mean value for PLTs (371.91±144.19), parameter and low mean value of RBCs (2.24±0.40) and HGB (9.42±1.21) in patients than controls with high significant different (P<0.05). finally, the mean value of WBCs parameter was no significant (p>0.05) table (4-4) and figure (4-3).

Table 4-4 comparative hematological parameters between study groups by using *student t* test.

Groups		N	Mean	SD	P value
PLTs x 10^3/uL	Patients	60	371.91	144.19	0.001***
	Controls	30	263.33	51.02	
RBCs x 10^6/uL	Patients	60	2.24	0.40	0.001***
	Controls	30	4.64	0.53	
HGB (g/dL)	Patients	60	9.42	1.21	0.001***
	Controls	30	12.61	1.65	
WBCs x 10^3/uL	Patients	60	6.69	2.51	0.06
	Controls	30	7.86	2.26	

*There is a significant difference and below the probability level ($p < 0.05$).

** There is high significant difference and below the probability level ($p<0.01$).

*** There is a very high significant difference and below the probability level ($p<0.001$).

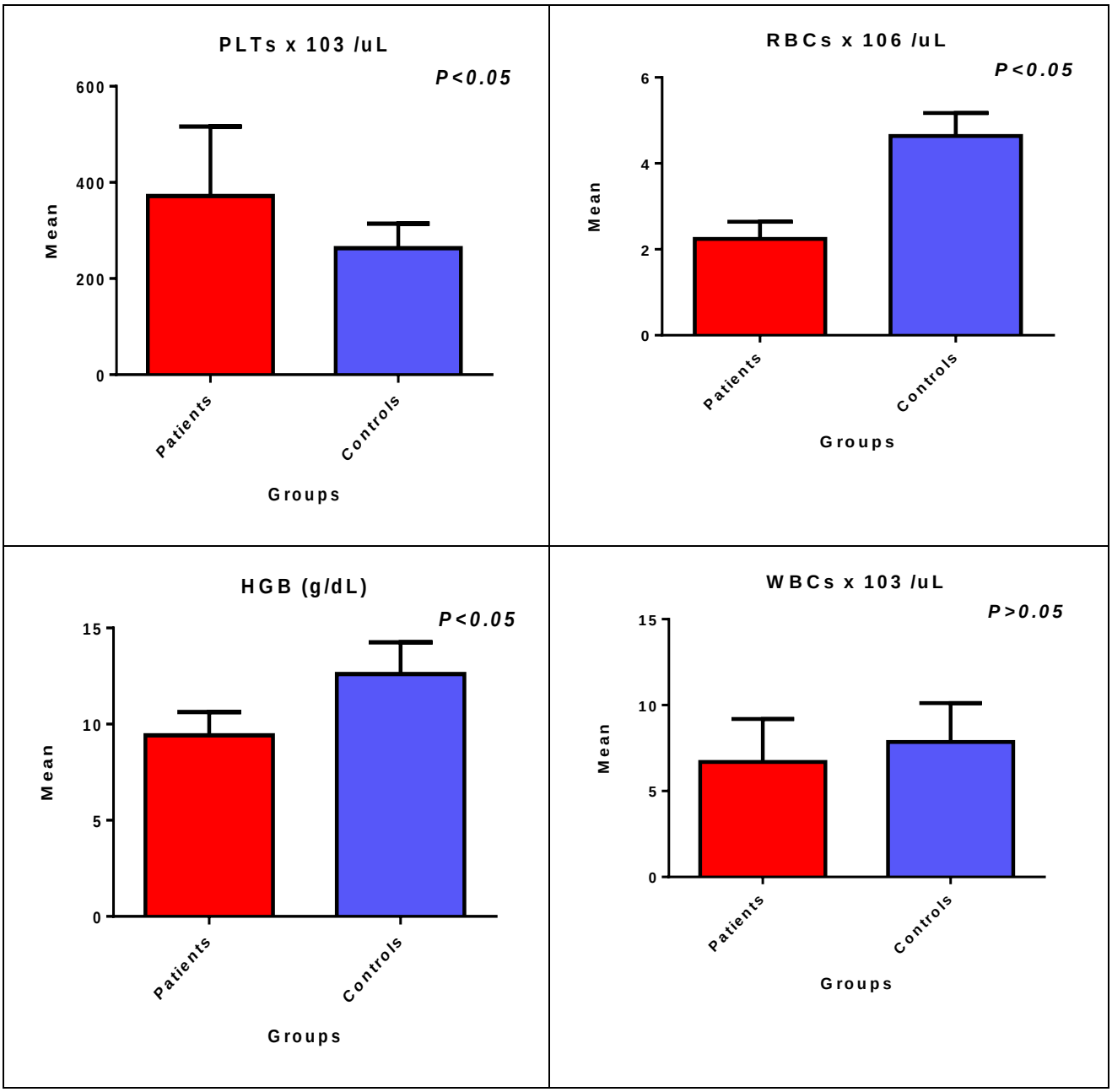


Figure 4-3 comparative hematological parameters between study Samples

The analysis was explained significant different in some RBC variables (HGB, HCT, MCV and MCH) in autistic children compare with healthy control, the study suggested may qualified to anemia. The food selectivity may be caused for iron deficiency in children with autism that increase the severity of psychology and behavioral problems (Munshed *et al.*, 2018).

Gunes *et al.,* (2017) revealed the hemoglobin levels of children with ASD were lower than healthy children, but this was not sufficient to result in anemia. Iron deficiency anemia in children with ASD might be associated with intellectual disability instead of ASD symptom severity. Our results compatible to results (Gunes *et al.,* 2017). Multivariate logistic regression analysis revealed that serum iron deficiency, serum Calcium levels, serum Vitamin D levels; ferritin, reduced physical activity; child order, body mass index percentiles, and parental consanguinity can all be considered strong predictors and major factors associated with autism spectrum disorders (Bener *et al.,* 2017).

Our study showed high levels of PLTs in patients with ASD than controls and these results compatible to results (Coban *et al.,* 2019). These results may suggest an impairment in platelet functions rather than in platelet morphology for children with ASD. Considering these results, further investigation of thrombocyte functions in the ASD may lead to a better understanding of the pathogenesis of ASD and to the development of our limited knowledge of this disorder (Coban *et al.,* 2019).

Report increased platelet counts, decreased platelet ATP dense granule secretion, and increased serotonin plasma levels not only in ASD patients but also in their first-degree relatives. This suggests that potential genetic factors associated with platelet counts and granule secretion can be associated with, but are not fully penetrant for ASD (Bijl *et al.,* 2015). It was shown for the first time that clotting (hypercoagulability) of the blood plasma in patients with autism and childhood schizophrenia was increased. This can cause thrombosis in small vessels of the brain. Early spontaneous clots appear in many patients that indicating the presence of systemic inflammation, possibly associated with an exacerbation of neuroinflammation. The thrombodynamics test allows detection of predisposition to hypercoagulability in the early stages when other methods are not sensitive enough (Brusov *et al.,* 2019).

4-5 Study of sensitivity and specificity of immunology, chemical, and hematology variables:

Result of current study shows the highest Sensitivity was for ALP (95%), TBIL (95%), AST (80%), TNF_a (78%), creatinine (76%), IFN_y (75%), IL_1a (72%) and CD4 (71%) than others parameters . Based on specificity, the highest specificity scored was for RBCs (93%), HGB (91%) , ALT (73%) and WBCs (66%) than others parameters with significant different ($p<0.05$ table (4-5) and figure (4-4).

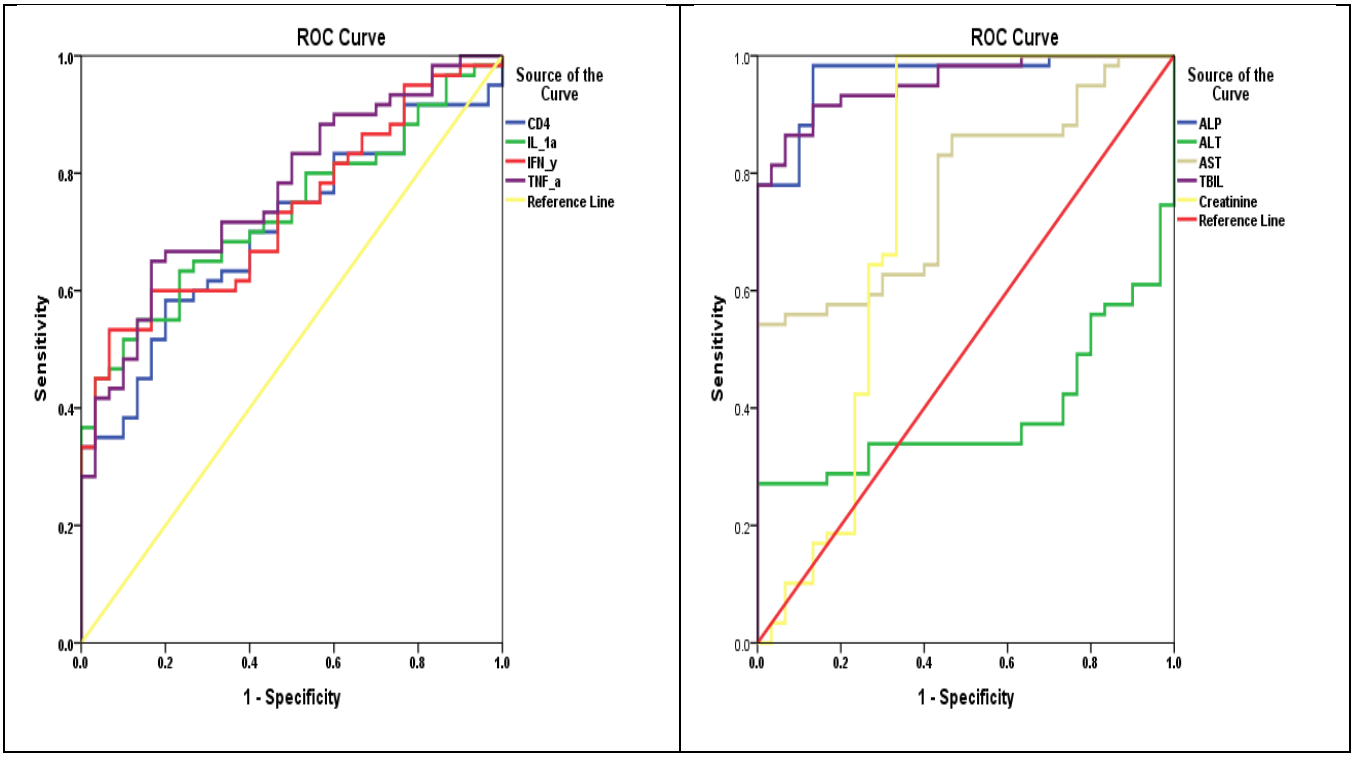
Table 4-5 ROC curve, sensitivity and specificity of variables

Variables	AUC	St. Error	Sig.	Sensitivity	Specificity
CD4	0.702	.054	.002**	71%	47%
IL_1a	0.732	.051	.001***	72%	46%
IFN_y	0.733	.052	.001***	75%	50%
TNF_a	0.769	.050	.001***	78%	46%
ALP	0.964	.018	.001***	95%	14%
ALT	0.389	.059	.087	40%	73%
AST	0.771	.049	.001***	80%	43%
TBIL	0.954	.019	.001***	95%	43%
Creatinine	0.750	.070	.001***	76%	33%
WBCs	0.342	.059	.015*	36%	66%
RBCs	0.000	0.000	.001***	0%	93%
PLTs	0.621	.058	.063	65%	63%
HGB	0.005	.006	.001***	0.001%	91%

*There is a significant difference and below the probability level ($p < 0.05$).

** There is high significant difference and below the probability level ($p<0.01$).

*** There is a very high significant difference and below the probability level ($p<0.001$).



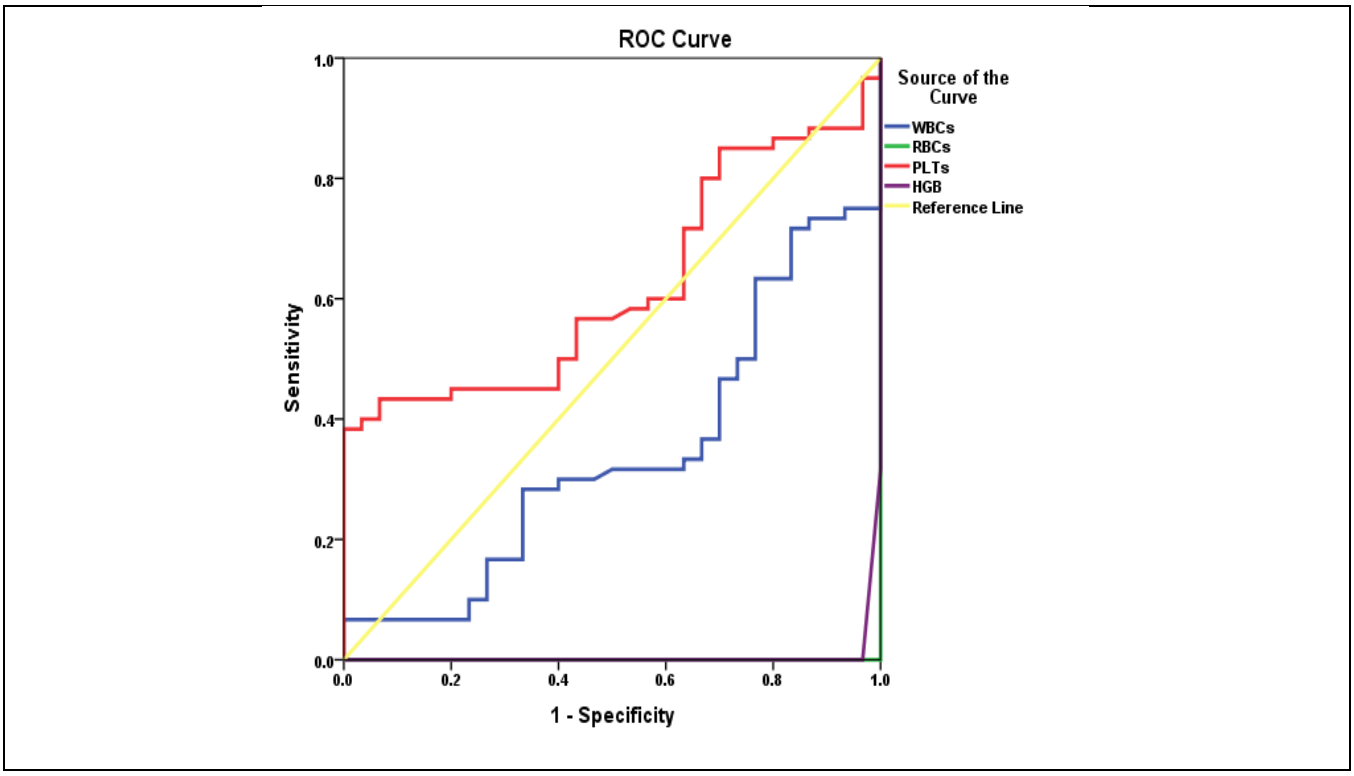


Figure 4-4 ROC curve, sensitivity and specificity of variables

In our opinion, ROC curve should become the special standard for the identification of parameters that are sensitive and specific enough to support ASD diagnosis, while its utility in prognosis, risk assessment, and evaluation of therapeutic interventions still awaits further studies. ROC curves emphasize the most significant statistical differences between cases and controls. The Area Under the Curve (AUC) provides a useful metric to compare different biomarkers. While the AUC value close to 1 indicates an excellent predictive marker, a curve that lies close to the diagonal () has no diagnostic utility. AUC value close to 1.00 is always accompanied by satisfactory values of specificity and sensitivity of the biomarker (Metz, 1978).

For a discussion of the use of ROC curves in translating biomarkers to clinical practice, When studying the perspective ASD biomarkers, high sensitivity means that autism will be identified in most cases, while high specificity means that

few, if any, healthy individuals will be positive to the test. Very interestingly, the combined ROC analysis of two distinct parameters increased their specificity, which suggests that one might resort to the combination of a panel of (related) parameters rather than to a single parameter alone (Bara *et al.*, 2018).

The observed signatures are consistent with the hypothesis of immune alterations in autism and an increased risk of developing autism in subjects exposed to prenatal infections or stress (Filosi *et al.*, 2020). Study findings suggest that increases or decreases in cytokines owing to genetic mutations or downstream of maternal immune activation (MIA) could have deleterious effects on brain development and function independent of any role in inflammation (Estes & McAllister, 2015).

Zhao *et al.*, (2021) show abnormal levels of IFN-Y, TNF-a and IL-1B in patients ASD than controls, and these abnormal cytokines may be potential biomarkers for the diagnosis and treatment of ASD in the future. Data results show alterations in the IL-1β system, with abnormally increased serum levels of IL-1β and IL-1RA in the children with ASD. Further, polymorphisms in the IL-1β-511 and IL-1RA genotype variants correlated positively with autism severity and behavioral abnormalities. IL-1β-511 and IL-1RA gene polymorphisms could impact ASD risk and may be used as potential biomarkers of ASD. Variations in the IL-1β and IL-1RA systems may have a role in the pathophysiology of ASD (Saad *et al.*, 2020).

TNF-*α* was specifically selected to activate the classical NF-*κ*B signaling cascade of the innate immune system and to stimulate the nuclear translocation of NF-*κ*B in primary cortical neurons (Young *et al.*, 2012). NF-*κ*B DNA binding activity was significantly increased in the peripheral blood samples of children with autism . NF-*κ*B has also been shown to be aberrantly expressed in the orbitofrontal cortex in patients with autism, which indicates that NF-*κ*B could be part of a putative molecular cascade

leading to inflammation in brain regions associated with the behavioral and clinical symptoms of autism (Young *et al.*, (2011). However, our laboratory results show that the expression of NF-*κ*B (p65) and the phosphorylation/activation of NF-*κ*B (p65) at Ser536 are not significantly changed in the cerebellum and cortex of both autistic subjects and BTBR mice in an autism model (Malik *et al.*, 2011).

These findings imply that NF-*κ*B may be involved in the abnormal inflammatory response processes suggested in autistic brain but do not play an important part (Xu *et al.*, 2015). Xie *et al.*, (2017) showed the 11 cytokines measured only concentrations of TNF-α ($p=0.002$), IL-1β ($p=0.02$) and IL-17a ($p=0.049$) were significantly increased in ASD children compared to typically developing controls, but only TNF-α concentrations were positively correlated with severity of ASD symptoms and were predictive of an ASD phenotype (area under the curve = 0.74) and that compatible to our results that showed AUC of TNF-α is 0.769. TNF-α has a critical role in regulating synaptic strength and plasticity , and his levels have been positively correlated with ASD severity (Inga Jácome *et al.*, 2016).

Elevated serum alkaline phosphatase levels are essential markers for diagnosis of vitamin D deficiency that is crucial in autistic children, especially adolescents, to take protective measures and treat this condition early (Şengenç *et al.*, 2020).

Result study showed that neonatal jaundice may be associated with ASD and may increase the risk of ASD among children (Jenabi *et al.*, 2020). It is found a high risk of publication bias, selection bias, and potential confounding in all studies. Based on the *low risk of bias studies* there was no convincing evidence to support an association between neonatal jaundice and autism (Kujabi *et al.*, 2021).

Significantly, it is found increased levels of creatinine, and a decrease in creatine were observed in the female ASD subjects. Therefore, it is shown the creatinine:creatine ratio has a potential to be a good predictor of ASD in the female subjects (AUC=0.942) (Xiong *et al.,* 2019). Urinary creatine/creatinine ratio should be measured in patients with hypotonia, developmental delay, seizure and autism whose family history indicates an X-linked inheritance (Nozaki *et al.,* 2015).

Study results suggest that the gene expression profiles identified from the peripheral blood samples of young adults with ASD can be used to identify a biological signature for ASD. Further study using a larger cohort and more homogeneous datasets is required to improve the diagnostic accuracy (Oh *et al.,* 2017). Padmakumar *et al.,* (2019) mentioned the platelets could serve as a peripheral biomarker or cellular model for autism as they share common biological and molecular characteristics with neurons and it has high AUC= 0.712 and these study compatible to our results that showed high AUC=0.65 for PLTs.

4-6 Study of relationship among immunological parameters:

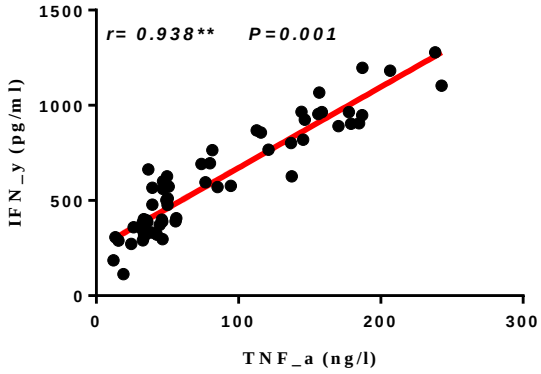
Results of our study revealed significant correlation among immunological parameters, where the CD4 is positive significant correlate with IL_1a (r=0.826**, p=0.001), IFN_y (r=0.876**, p=0.001) and TNF_a (r=0.928**, p=0.001). TNF_a is positive significant correlate with IFN_y (r=0.826**, p=0.001), and TNF_a (r=0.878**, p=0.001). Finally, IFN_y is positive significant correlate with TNF_a (r=0.938** , p=0.001) table (4-6) and figure (4-5).

Table 4-6 correlation among immunological parameters by using Pearson correlation

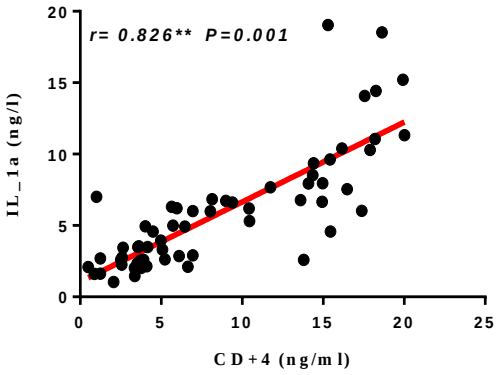
		CD4	IL_1a	IFN_y
IL_1a	r	.826**		
	p	.000		
IFN_y	r	.876**	.925**	
	p	.000	.000	
TNF_a	r	.858**	.878**	.938**
	p	.000	.000	.000

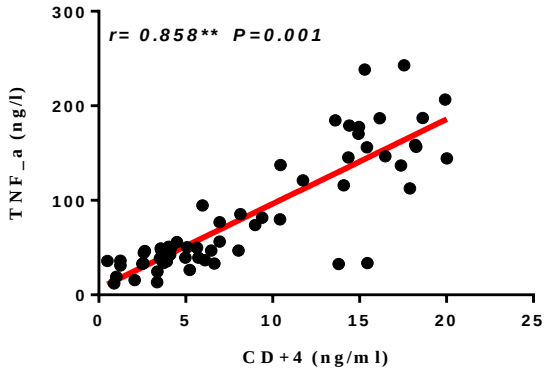
Zhao *et al*., (2021) show increase levels of pro-inflammatory cytokines (IL-1, TNF-a and IFN-Y) and CD4 T cell subsets in patients with autism than controls and these findings strengthen the evidence for an abnormal cytokine profile in ASD and may be use these cytokines as potential biomarkers for the diagnosis and treatment of ASD in the future.

a) Correlation relationship among IFN-γ and TNF-α

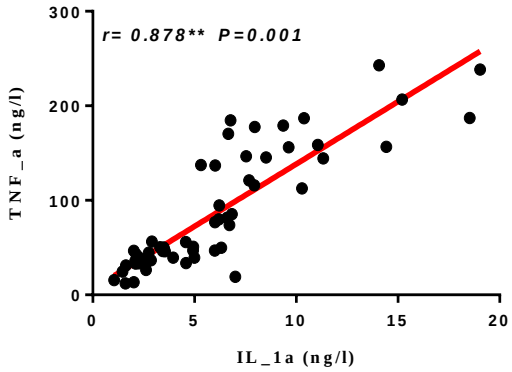


b) Correlation relationship among IL-1α and CD4

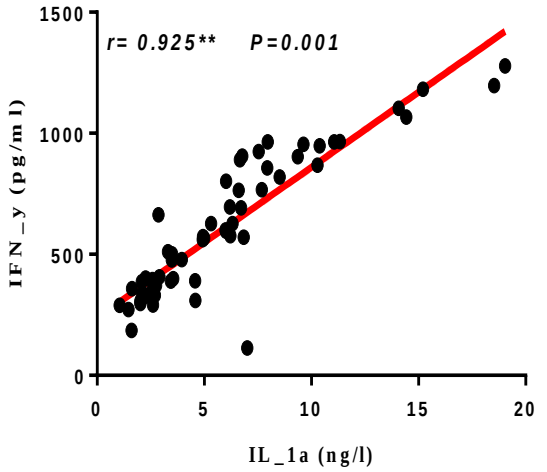


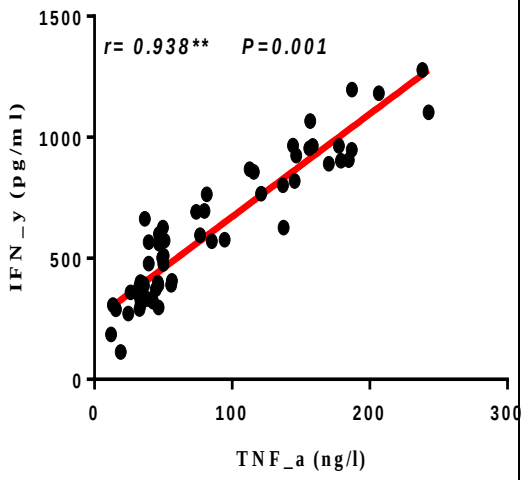


c) Correlation relationship among TNF-α and CD4



d) Correlation relationship among TNF-α and IL-1α





e) Correlation relationship among IFN-γ and IL-1α	**f) Correlation relationship among IFN-γ and TNF-α**

Figure 4-5 correlation relationship among immunological parameters

4-7 Comparative immunological parameters with patients gender:

Based on immunological parameters and sex difference, in our results there is no significant between parameters and sex difference ($p>0.05$) table (4-7) .

Table 4-7 comparative immunological parameters with patients gender by using student t test.

Gender		N	Mean	Std. Deviation	P value
CD4	Males	30	7.98	3.89	0.37
	Females	30	9.38	2.25	
IL_1a	Males	30	6.14	2.17	0.43
	Females	30	5.23	2.83	
IFN_y	Males	30	607.85	281.19	0.97
	Females	30	605.17	296.76	
TNF_a	Males	30	84.21	40.80	0.98
	Females	30	83.94	42.91	

Clinical observations have provided compelling evidence that humoral and cell-mediated immune responses markedly differ across sexes, with women showing more pronounced inflammatory and innate immune responses during bacterial and viral infections than men (Klein & Flanagan, 2016). Females have an increased immune response to viral infection and vaccination compared to males, but show an increased risk of developing certain autoimmune diseases (Flanagan *et*

al., 2017). The augmented inflammatory response is thought to be beneficial by limiting pathogen spread and by accelerating pathogen clearance, resulting in lower infection-related mortality rates in women compared with men (Angele *et al.,* 2014). This is supported by findings from population-based studies indicating that female sex is associated with a better prognosis after sepsis or severe trauma. However, there seems to be a downside of this heightened inflammatory responsiveness as chronic inflammatory and autoimmune diseases are more prevalent in women than in men (Keselman & Heller, 2015).

Intravenous administration of LPS provoked in both men and women an acute systemic inflammatory response that was characterized by a transient rise in plasma concentrations of pro-inflammatory cytokines. Importantly, female participants mounted a substantially stronger *in vivo* cytokine response, with significantly greater increases in TNF-α and IL-6. This not only confirms and extends the findings from earlier endotoxin studies in healthy humans, but is also consistent with clinical observations in septic patients (Wegner *et al.,* 2017).

Study results demonstrate complex interplays between oestradiol, testosterone and X chromosome number and discovered that female sex and pubertal development associate with an increased production of and response to type 1 IFN in a manner that may underpin the increased prevalence of autoimmune diseases in females after puberty (Webb *et al.,* 2019).

Data results indicate that the regulation of IL-1 secretion is fundamentally different in women compared with men and alludes to the possibility that IL-1 may serve different biologic functions in women than men (Lynch *et al.,* 1994) and that not compatible to our results showed no significant difference between gender and IL-1. Study result suggest that an altered cytokine (IL-1B) response or profile is associated

with the severity of ASD-related symptoms, with sex a potential modifier of this relationship (Masi *et al.*, 2017).

The association of reduced levels of IL-1β, IL-8, MIP-1β, and VEGF, with higher severity levels of ASD in females suggests sex differences in cytokine expression in children with ASD. This was also supported by the exploratory optimal network visualization graphs of cytokine correlations for males and females. Results from recent DNA analysis suggests that females could be more resilient to genetic insults due to carrying more extreme neurodevelopmental-related genetic mutations than males with the same symptoms (Jacquemont *et al.*, 2014).

4-8 Comparative immunological parameters with patients age :

Based on immunological parameters and age periods , in our results there is no significant between immunological parameters and age periods ($p>0.05$) table (4-8) .

Table 4-8 comparative immunological parameters with patients age periods by using student t test.

Age periods (years)		N	Mean	Std. Deviation	P value
CD4	<10	28	8.73	3.22	0.94
	≥10	32	8.63	3.01	
IL_1a	<10	28	5.34	2.58	0.57
	≥10	32	5.99	2.47	
IFN_y	<10	28	597.49	288.96	0.82
	≥10	32	614.40	288.94	
TNF_a	<10	28	89.34	42.69	0.56
	≥10	32	79.47	40.97	

Four studies show an age-associated increase of IFN-γ and TNF-α release (Djuardi *et al.*, 2016), which is also consistent with the findings from flow-cytometry studies. Interestingly, this age association is not seen in some of the more recent studies, particularly when TLR-agonists instead of mitogens are used as stimulatory antigens and/or when children in the first year of life only were included (Lisciandro *et al.*, 2012). This suggests that age association can only be detected in studies in which the populations have a large-enough age difference. The importance of the age-interval of investigated populations is further supported by a recent publication in Indonesian children showing that the greatest age-associated increase for IFN-γ and TNF-α was seen after the first year of life (Djuardi *et al.*, 2016).

In addition, all the more recent studies showing no age influence or an age-associated decrease of IFN-γ and TNF-α releases were performed in resource-limited settings. Evidence suggests that cytokine release is influence by continent of origin and in general, cytokine concentrations were lower in African children compared to children from other continents, thereby potentially limiting the detection of any age association in these studies (Ge *et al.*, 2014). Both IFN-γ and TNF-α are key cytokines produced by cells from the innate and adaptive immune system and involved in the immune response to many pathogens in early childhood. The lower expression of these two cytokines in infants and young children has been postulated to be associated with the susceptibility to numerous infections in early childhood and the failure of diagnosis including those cytokines to perform well (Muthukuru, 2019).

Importantly, as both cytokines are included in diagnostic assays and being drug targets, developmental changes during childhood need to be accounted for when choosing test cut-offs for dosing of drugs interfering with those cytokines. IFN-γ and TNF-α-producing T cells are detected in varying frequency; however, an age-associated increase

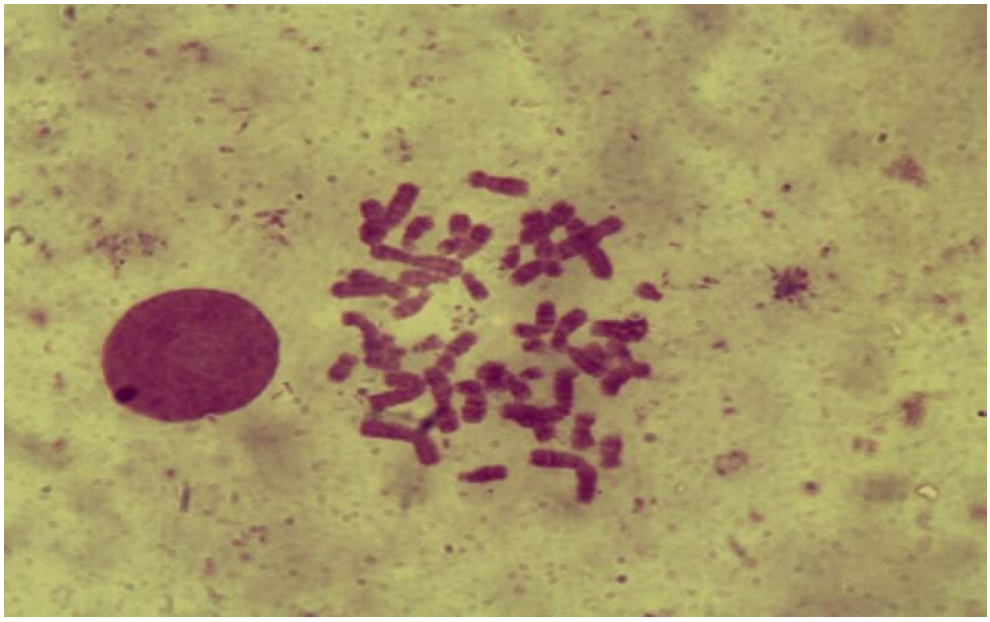
was seen in all studies (Decker *et al.*, 2017). Further to age, other factors have been described to influence cytokine concentrations such as sex , household sanitation standard , sport , dietary supplements maternal cytokine profile , and method of blood sampling (Nguyen *et al.*, 2010).

It is found that CD4+ and CD8+ T cell in the circulation have relatively stable frequencies, and the absolute number of CD8+ T cell decreased with age; however, the ratio of CD4+ or CD8+ naïve population increased with age. Unlike the obvious changes in other T cell subsets with age and gender, the stable level of T cell in peripheral blood may support their capacity for sustaining long-term immunological memory, while their importance may increase together with ageing (Li *et al.*, 2019).

As we age, the innate immune system becomes dysregulated and is characterized by persistent inflammatory responses that involve multiple immune and non-immune cell types and that vary depending on the cell activation state and tissue context. This ageing-associated basal inflammation, particularly in humans, is thought to be induced by several factors, including the reactivation of latent viral infections and the release of endogenous damage-associated ligands of pattern recognition receptors (PRRs) (Shaw *et al.*, 2013).

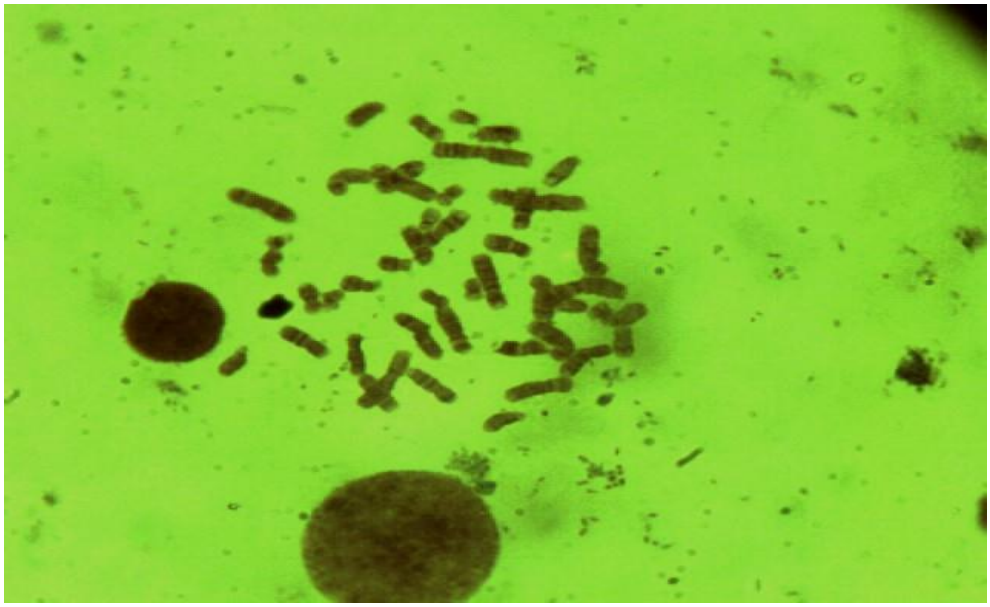
4-9 Cytogenetic Study

Results of current study revealed no significant different in shape of chromosomes between patients and controls as shown in figure (4-6).



(a)

Chromosomes in a healthy person(100X)



(b)

Chromosomes in a patient with autism (100X)

Figure 4-6 Chromosomes in a healthy person and Chromosomes in a patient with autism

(ASD) is highly genetically heterogeneous and may be caused by both inheritable and de novo gene variations. In the past decade, hundreds of genes have been identified that contribute to the serious deficits in communication, social cognition, and behavior that patients often experience. However, these only account for 10–20% of ASD cases, and patients with similar pathogenic variants may be diagnosed on very different levels of the spectrum (Rylaarsdam & Guemez-Gamboa, 2019).

Copy number variations (CNVs) are submicroscopic structural variants in chromosomes that include duplications, deletions, translocations, and inversions, sometimes stretching several kilobases (Marshall *et al.*, 2008).

Recently, CNVs have been shown to contribute to autism, and CNVs appear to be a more important risk factor in sporadic cases than in family forms of ASD. Epidemiological studies have shown gender bias in autism as well as in identity, where the number of autistic affected males is 3-4 times greater than females, and for identity, the ratio is close to 1.3: 1, although this is believed to decrease with low IQ and some Studies indicate that severe ID may be more prevalent among females,(Mak, 2015). Also, a form of identity has been reported in up to 70% of children with autism. This overlap between autism and identity justifies the search for common causative factors and sex ratios in these disorders make the X chromosome an excellent candidate. (Do *et al.*, 2017).

Many genes may be affected with these changes, but not all are necessarily drivers of disease. Studies have found a higher load of rare, genic CNVs in autistic individuals, implicating these variants in ASD pathology (Pizzo *et al.*, 2019). CNV is now understood as an extremely important contributing factor in ASD susceptibility,

and current estimates postulate that these variations directly cause roughly 10% of ASD cases (Geschwind, 2011).

Neurocytogenomic variations are nowadays thought to play a critical role in human brain development and aging. Chromosome instability is likely to be an element of pathogenetic cascades in a variety of brain diseases (Yurov *et al.,* 2018). Chromosome 15q11-q13 has been identified as a candidate region that increases the risk of autism (Devlin & Scherer, 2012). This region contains several critical genes, such as $GABA_A$ receptor genes cluster, *UBE3A* and *CYFIP1*, which might be correlated with the development and function of the brain (Hsiao *et al.,* 2016). The postmortem of autistic individuals revealed a reduced expression of *GABRB3*, *GABRA5*, and/or *GABRG3*, which was detected in several specific brain regions, such as the superior frontal cortex, parietal cortex, and cerebellum (Fatemi & Folsom, 2015).

Many CNV carriers do not meet full diagnostic criteria for autism but nevertheless meet clinical cutoffs for autistic traits. Although profile differences between variants were observed, there is considerable variability in clinical symptoms in the same variant (Chawner *et al.,* 2021).

Sherman *et al.,* (2021) indicate that mosaic (early-developmental) copy number variants (mCNVs) contribute a previously unexplained component of ASD risk. In epidemiological sample, Mahjani *et al.,* (2021) discovered the rare potentially damaging single nucleotide variation (pdSNV) were more common than potentially damaging copy number variation (pdCNV) and the combined yield of potentially damaging variation was substantial at 27%. The results provide compelling rationale for the use of high-throughout sequencing as part of routine clinical workup for ASD and support the development of precision medicine in ASD (Mahjani *et al.,* 2021).

ASD shows familial and genetic association not only with other neurodevelopmental disorders, but also with other psychiatric disorders, such as anxiety, depression, and intentional self-harm. Family history of ASD comorbid with intellectual disability, epilepsy, congenital malformations, or chromosomal abnormalities is less related to other psychiatric disorders, potentially suggesting a different etiology for this subgroup of patients (Ghirardi *et al.*, 2021).

Conclusions:

We conclude from our current study the following:

1. There were no statistically significant differences with regard to gender and age groups on autistic patients.
2. High levels of CD4 and pro-inflammatory cytokines (IL-1α, IFN-γ, TNF-α) in autistic patients compared to healthy controls.
3. High levels of ALP, ALT, AST, and TBIL in autistic patients compared to healthy people with a high significant difference, while creatinine, it was observed that it was preserved in autistic patients compared to healthy people with no significant difference.
4. High level of blood PLTs and low levels of HGB and RBC in autistic patients compared to healthy people with a significant difference with a statistical significance.
5. The immunological indicators as well as the levels of ALP, AST, TBIL, and creatinine as prognostic factors in the diagnosis of autism.
6. There are significant associations between immunological indicators in autistic patients.
7. Gender and age groups had no significant effect on immunological indicators in autistic patients.
8. There is no difference between the shape of the chromosome cell in autistic patients and healthy persons.

Recommendations:

The current study recommends following:

1. Measuring the level of the immunological biomarkers (Interleukin-1β).
2. Genetic study of Autism by specific approach .
3. Conducting wide awareness campaigns for this disease by the Ministry of Health as well as organizations and its danger, taking into account the psychological aspect of patients.
4. Ministries and government agencies for Autism disorder by the Ministry of Health.
5. Extensive awareness campaigns for this disease by the Ministry of Health as well as an organization for patients' families.

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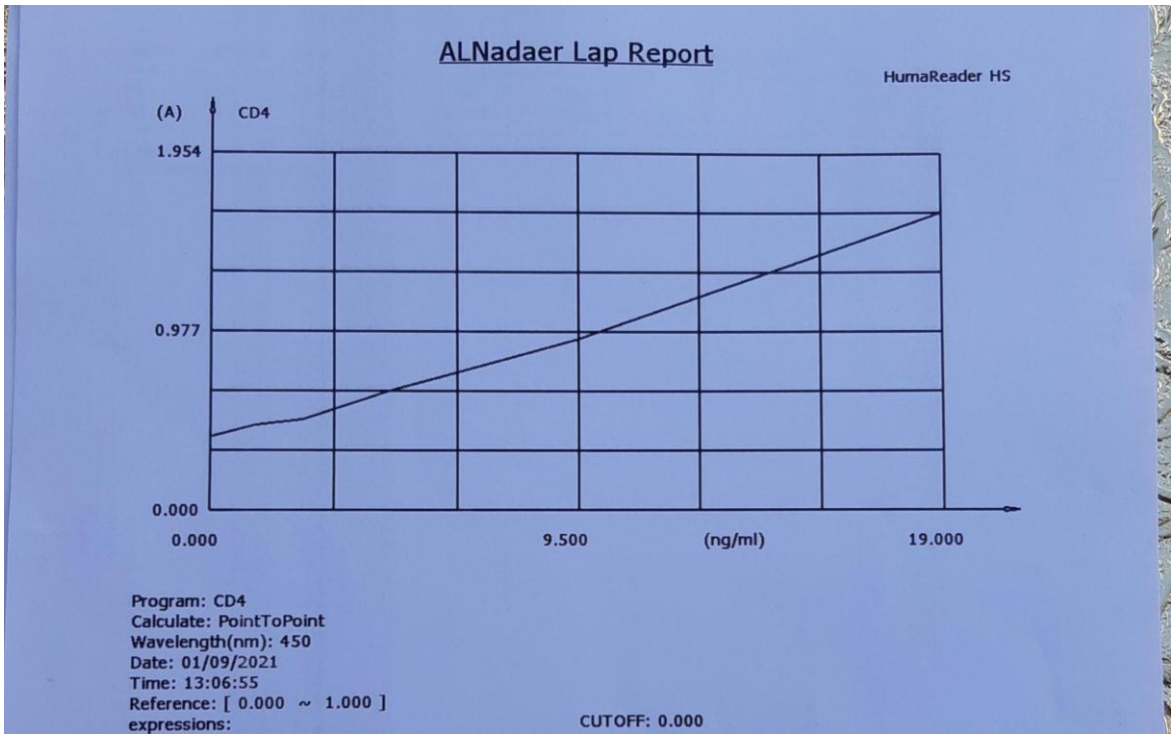
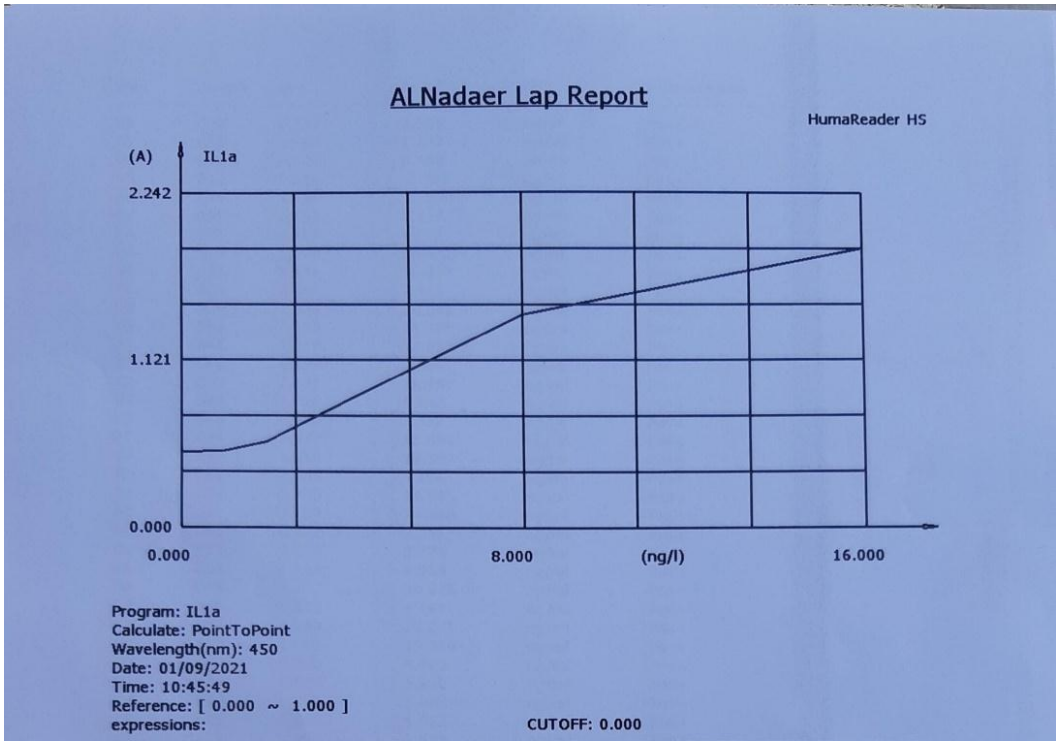
Appendices

Appendix(1) CD4 Standard curve

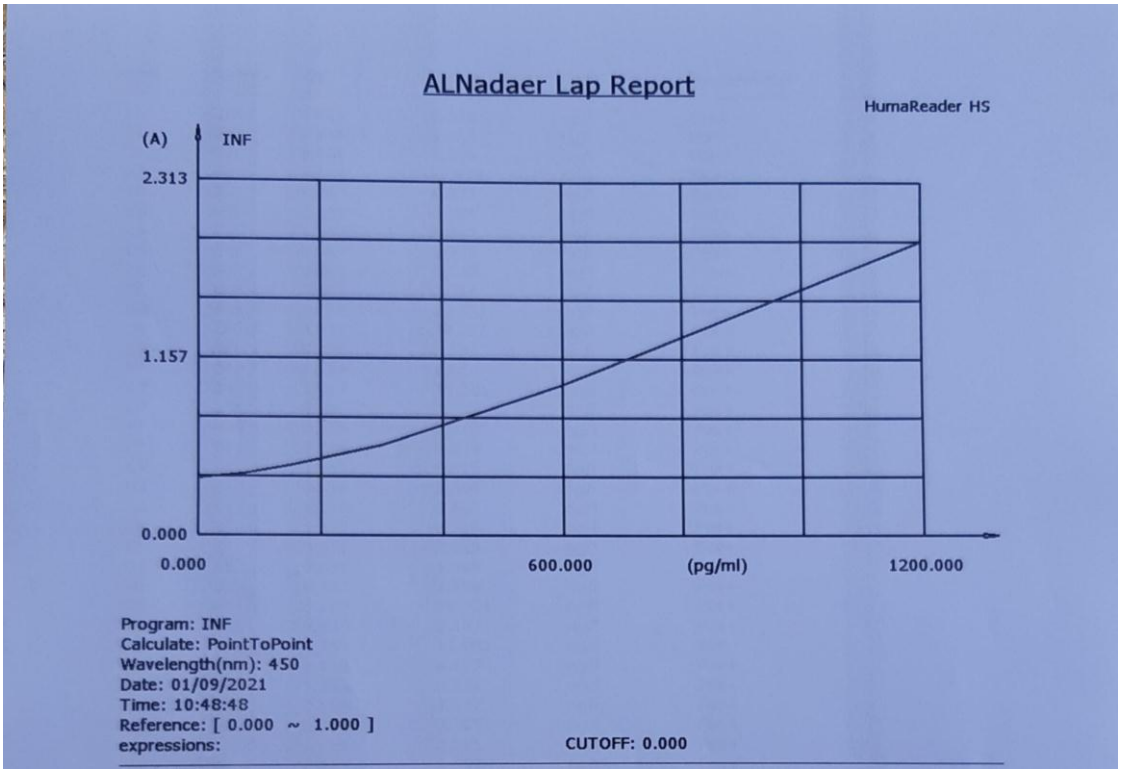


Appendix (2) IL-1α Standard curve

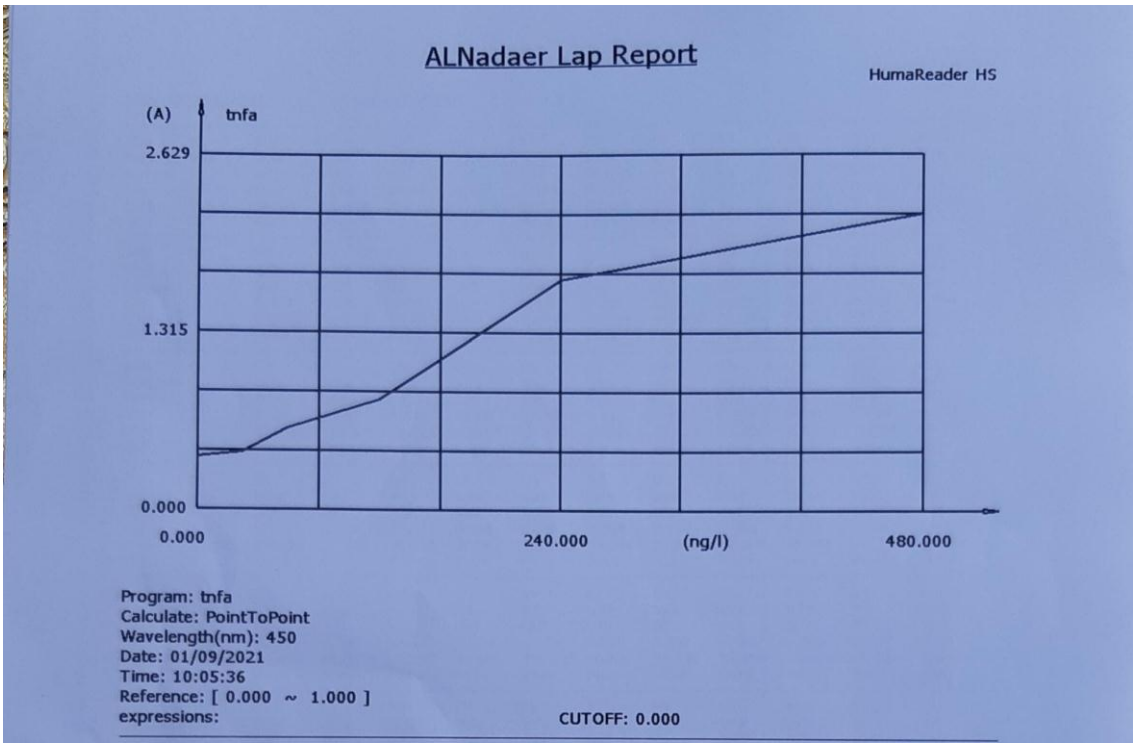


Appendices

Appendix (3)IFN-γ Standard curve



Appendix (4) TNF-α Standard curve



الخلاصة

هدفت الدراسة الحالية إلى تقييم المؤشرات المناعية وبعض المؤشرات الحيوية لمرضى التوحد. تم جمع الدم من المرضى في مستشفى ابن رشد في بغداد ، وأجريت الدراسة من أيلول 2020 إلى آذار 2021 ، حيث تم تقييم الدور المناعي لدى مرضى التوحد لقياس المؤشرات المناعية. CD4 ، المستوى (IL-1α و IFN-gamma بالاضافة الى TNF alpha) ويتم قياسه بواسطة تقنية المقايسة الامتصاصية المناعية المرتبطة بالإنزيم (ELISA).

تضمنت المؤشرات الكيميائية الحيوية ALP و ALT و AST و TBIL و Creatinine) ومؤشرات الدم (PLT، RBC، HGB، WBC).

اشتملت الدراسة على 90 عينة ، منها 60 عينة لمرضى التوحد (30 ذكر و 30 أنثى) تتراوح أعمارهم بين (2-14 سنة) ، تم جمع 30 عينة من 15 ذكرًا و 15 أنثى ضمن الفئة العمرية (2 إلى 14 عامًا) ، تم استخدامهم كمجموعة سيطرة حيث تم جمع عينات من مرضى التوحد قبل إعطائهم العلاج وبعد موافقة المرضى.

أظهرت نتائج الدراسة الحالية عدم وجود تأثير للعمر والجنس على التوحد، لوحظ أن المؤشرات المناعية (CD4 و IL_1α و IFN_γ و TNF_α) ارتفعت في مرضى التوحد (8.68 ± 3.06 و 5.69 ± 2.50 و 606.51 ± 286.62 و 84.07 ± 41.86) مقارنة بالأشخاص الأصحاء (4.56 ± 1.99 ، 2.45 ± 1.11 ، 378.40 ± 126.35 و 29.80).

أظهرت نتائج الدراسة الحالية زيادة في المؤشرات الكيميائية (ALP و ALT و AST و TBIL) لدى مرضى التوحد مقارنة بالضوابط الصحية ، وأظهرت أيضًا انخفاض مستوى الكرياتينين لدى مرضى التوحد مقارنةً بالضوابط الصحية.

أظهرت نتائج الدراسة الحالية أيضًا ارتفاع مستوى الصفائح الدموية (PLTs) في مرضى التوحد مقارنةً بالضوابط الصحية ، ومستوى أقل من (RBC، HGB) في مرضى التوحد مقارنةً بالضوابط الصحية.

ظهر نتائج الدراسة الحالية أيضًا أن (95) ALP٪ (، 95) TBIL٪ (، 80) AST٪ (، TNF-α 78)٪) ، الكرياتينين (76٪) ، 75) IFN-γ٪ (، 72) IL-1α٪) و 72) CD4٪)) أكثر حساسية في تشخيص التوحد. كما وجد أن هناك ارتباطات ذات دلالة إحصائية بين المؤشرات المناعية.

أظهرت نتائج الدراسة الحالية عدم وجود اختلاف كبير في الكروموسومات بين المرضى و الاصحاء.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ديالى

كلية العلوم

قسم علوم الحياة

ملامح مناعية وكيميائية حياتية ووراثية لدى مجموعة من مرضى التوحد

رسالة مقدمة إلى مجلس كلية العلوم– جامعة ديالى

وهي جزء من متطلبات نيل شهادة الماجستير في علوم الحياة

من قبل الطالب

ميلادالنبي مظفر طه سعيد

بكالوريوس علوم الحياة / كلية العلوم/ جامعة ديالى ٢٠١٨

بأشراف

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١٤٤٣/ شعبـا ن **٢٠٢٢/اذار**