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Immunological Tolerance Induced by Intestinal Protozoa in Patients with Type 1 Diabetes Mellitus

A Thesis

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بسم الله الرحمن الرحيم

﴿وَيَسْئلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي
وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً﴾

صدق الله العلي العظيم

(سورة الاسراء - الآية ٨٥)

Certification

We certify that this thesis entitled **(Immunological Tolerance Induced by Intestinal Protozoa in Patients with Type 1 Diabetes Mellitus)** was prepared by **Hiba Ali Hadi Jasim** under our supervision at the Department of Microbiology, College of Medicine, University of Babylon, as a partial requirement for the degree of Doctorate of Philosophy in **Medical Microbiology**

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Dedication

To the satisfaction of Allah and his Messenger, Mohammed, may God's prayers and peace be upon him.
Imam Mahdi, may God hasten his reappearance

To the spirit of my father
To my lovely mother
To my lovely darling husband
And To my brothers

Hiba
2023

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Summary

Type 1 Diabetes Mellitus (T1DM) occurs due to disturbance intolerance of the immune system and invasion of β-cells in the pancreatic islets by auto-reactive immune T-cells and inducing deterioration of β-cells activity and viability and prolonged therapy with external insulin.

The alterations in the host immune state might influence and be influenced by other concomitant diseases. Parasitic infections might inhibit T1DM by disrupting the pathways leading to the Th1-mediated destruction of β-cells mediated by mechanisms related to the capacity of the host to mount a Th2 response to parasites. this study aims to determine some immunological parameters in patients with T1DM and auto tolerance induced by parasitic Infections.

The study was a case-control carried out on Paediatric patients referred to Imam Al-Hassan for Diabetic and Endocrinology and Karbala Teaching Hospital for Children in Karbala province the age of patients ranged from (1-15 years) during the period from February to December 2022. The study included 180 participants [98(54%) male and 82 (46%) female] grouped into four groups (70 T1DM, 50 Intestinal protozoal infections,25 mixed T1DM infected by intestinal protozoa, and 35 apparently healthy Controls.

Two parts of parameters were included: the first part of the parameters is carried out after diagnosis of T1DM clinically according to their clinical manifestations by Fasting Blood Sugar and Glycated Haemoglobin level HbA1c including measurement of interleukins (interleukin-2, interleukin-10, and prostaglandin-E2), and the detection of islet autoantibody against GAD Glutamic acid decarboxylase, Zinc transporter family member 8 (ZnT8) Antigen, and Islet Cell Auto-antibodies ICA in the case and

controls groups by ELISA in serum and detection of intestinal protozoa in T1DM in the stool sample by General Stool Examination and confirm the diagnosis by Immunochromatographic Rapid CERTEST.

There were no significant differences between male and female distributions in study groups by Chi-square test for variables at (P=0.233, P≥0.05), and there were highly significant differences between age groups (P=0.001, P≤0.05) in the Chi-square test.

In Diabetic and Mixed groups increased level of IL-2, GAD, ZnT8, and ICA if compared with control, also increased level of IL-10 in Mixed groups due to infection by parasites, there were a strong correlation between IL-10 and IL-2 (R =0.745, p<0.01) in a mixed group, also there was a highly significant difference in IL-10 level, with decreased IL-2 level in mixed groups if compared with T1DM that determined the role of intestinal parasitic infection to modulate immune function toward Th-2, and positive correlation (R=0.470, P<0.05) between the increased serum concentrations of IL-10 and PG-E2 indicates a link between them as anti-inflammatory cytokines, while There was a negative correlation between IL-10 with GAD, ZnT8, and ICA at R=(-0.358,-0.428, and -0.035, P<0.05) respectively. In Parasitic groups increased levels of IL-10 and PG-E2 and decreased or normal levels of IL-2, GAD, ZnT8, and ICA, There were highly significant differences (P=0.000, P≥0.05) in mean anti-GAD levels between males and females and more in the females, there were no significant differences in IL-2, IL-10, PG-E2, GAD, ZnT8, and ICA concentration between *Entamoeba histolytica* and *Giardia lamblia* infections in parasitic and mixed groups.

To conclude the role of elevated levels of IL-10 in the mixed group is to decrease the severity of the disease.

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List of Abbreviations

Abbreviation	Description
ADCC	Antibody-Dependent Cell- Cytotoxicity
ADI	Arginine De-aminase
AIRE	Auto-Immune Regulatory Elements
ALA	Amoebic Liver Abscess
AMI	Antibody-mediated immunity
APCs	Antigen-presenting cells
APRIL	A proliferation-Inducing Ligand
ATPase/flippase	Adenosine Tri Phosphatase /flippase
B1 Cells	Beta 1 lymphocyte Cells
BAFF	B-cell Activating Factor of the TNF family
B-reg	Beta lymphocyte regulatory
CBC	Complete Blood Count
CCL 2,20	Chemokine (C- C motif) Ligand 2,20
CD4,8	Cluster of differentiation 4,8
ChgA	Chromogranin A
CMI	Cell-mediated immunity
CMC	Cell-Mediated Cytotoxicity
CMV	Human Cytomegalo-Virus
CP	Cysteine Protease
CTLA-4	Cytotoxic T lymphocyte antigen-4
CXCL1,2,3,8	Chemokine 1,2,3,8
CXCL3	Chemokine (C-X-C motif) Ligand 3

CXCL8	Chemokine (C-X-C motif) Ligand 8
DC	Dendritic cell
DKA	Diabetic Keto Acidosis
ECM	Extra Cellular Matrix
EDTA	Ethylene diamine tetra acetic acid
EhCP-A5	*Entamoeba histolytica* Cystine Protease-A5
ELISA	Enzyme Linked Immuno- Sorbent Assay
EP 1-4	Prostaglandin E receptor 1- 4
ES	Excretory-secretory molecules
ESPs	Execratory -Secretory Products
FBS	Fasting Blood Sugar
Foxp3	Forked box Protein 3 gene
GAD	Glutamic Acid Decarboxylase
GADA	Glutamic Acid Decarboxylase Autoantibody
Gal/Gal NAC	Galactose/Galactose N- Acetyl glucose amine
GPI	Glycosyl Phosphatidyl Inositol
HbA1C	Glycated Haemoglobin A1C
HG	Hyper-Glycemia
HLA	Human Leukocyte Antigen
HRP	Horse Reddish Peroxidase
IA-2	Insulinoma Associated 2 Auto-antibody
IAA	Insulin Auto-Antibody
IAPP	Islets Amyloid Polypeptide
IBS	Irritable Bowel Syndrome
IC	Immunochromatographic
ICA	Islet Cell Auto-antibodies

IDDM	Insulin-Dependent Diabetes Mellitus
IDF	International Diabetes Federation
IECs	Intestinal Epithelial Cells
IFN	Interferon
IFN γ,α	Interferon gamma, alpha
IgA	Immunoglobulin A
IGRP	Islets specific Glucose-6-phosphatase Related Protein
IHA	Indirect Hemagglutination Assay
IL-2,4,10	Interleukin 2,4,10
IL2R	Interleukin 2 Receptor
ILCS	Innate Lymphoid cells
iNKT	Invariant Natural Killer T-cells
iNOS	Inducible Nitric Oxide Species
IPIs	Intestinal Parasites Infections
IPs	Intestinal Parasites
LPPG	Lipo Peptido Phospho Glycan
LFI	Lateral Flow Immunoassay
MAC	Membrane Attack Complex
MALT	Mucosal Associated Lymphoid Tissues
MAPK	Mitogen Activated Pyruvate Kinase
MCP-1	(monocyte chemoattractant protein -1)
MHC	major histocompatibility complex
Mϕs,	Macrophages
MUC2	Mucin 2
MVs	Micro Vesicles
NFκB	Nuclear Factor Kappa

NKs	Natural Killer cells
NO	Nitric Oxide
OD	Optical Density
OGTT	Oral Glucose Tolerance Test
PAMPs	Pathogen-associated molecular patterns
PARP-1	Poly (ADP-Ribose) Polymerase
PCR	Polymerase Chain Reaction
PCs	Plasma Cells
pDC	Plasmacytoid Dendritic Cell
Pg/ml	Picogram/millilitre
PG-E2	Prostaglandin- E2
PRRs	Pattern recognition receptors
Prx	Peroxiredoxin
PTPN22	Protein Tyrosine Phosphatase Non – Receptor Type 22
RBS	Random Blood Sugar
ROM 1	Rhomboid protease 1
ROS	Reactive Oxygen Species
SC	Secretory Components
sIgA	Secretory Immunoglobulin Alpha
T1DM	Type 1 Diabetes Mellitus
TAC	Total Antioxidant Capacity
TCR	T-cell receptor
TD	T-cell Dependent
TEDDY	The Environmental Determinant of Diabetes in Youth
TFF3	Trefoil Factor 3

Tfh	T-cell follicular helper
TGF	Transforming Growth Factor
Th1	T Helper 1
Th17	T-Helper 17
Th2	T Helper 2
Th3	T- Helper 3
TI	T-cell Independent
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
T-reg	T- regulatory
Trx	Thioredoxin
US	United States
VNTRs	Variable Number Tandem Repeats
VSPs	Variant Surface Proteins
WHO	World Health Organization
ZnT8	Zinc Transporter 8
α-HD6	Alpha- Human Defensin 6
β-HD1	Beta- Human Defensin 1
µm	Micro meter
(IU/ml)	International Unit/millilitre

Chapter one

Introduction and Literatures Review

1. Introduction

Type 1 Diabetes Mellitus (T1DM) is a metabolic disease, organ-specific autoimmune endocrine disease, and one of the most common chronic conditions in children characterized by chronic hyperglycemia resulting from insufficient insulin secretion, which affects the metabolism of carbohydrates, lipids, and proteins. The result of a persistent inflammatory process in the pancreatic islets of Langerhans is beta-cell destruction (insulitis) (Los and Wilt, 2021).

Loss of self-tolerance to β-cell autoantigens, which results in the Th1-mediated death of insulin-producing β-cells and the generation of Th1 cytokines, has been related to the development of diabetes which results in improper glucose metabolism that can cause ketoacidosis and many additional problems, including retinopathy, nephropathy, and even cardiovascular diseases and early death (Juhas *et al*., 2019).

Several autoantigens found in the pancreatic β-cells may be crucial in the beginning or development of autoimmune islet damage as autoimmunity in T1DM evolves from an early activation to a chronic condition. Strong evidence suggests that the development of overt illness is related to islet autoantibody reactions against a variety of islet autoantigens. Islet autoantigens in β-cells proteins (Glutamic Acid Decarboxylase, Pro-insulin, Islets specific glucose-6-phosphatase Related Protein (IGRP), Islets amyloid polypeptide (IAPP) and Chromogranin A(ChgA) frequently interact with CD4+T-cells and autoantibodies by Epitope spreading mechanism (Lu *et al*., 2020).

Additionally, the development of a regulatory network might help limit inflammation that could otherwise result in pathology while assisting in the

regulation of overt immune responses to prolong the parasite's ability to survive (Elliott and Weinstock, 2017).

Diabetic patients weakened immune systems (immunocompromised) make infections more common than ever, because infectious organisms are much more active in hyperglycemia than they were in normoglycemia (Zhou *et al*., 2020).

The gastrointestinal tract is one of the systems impacted by hyperglycemia in diabetes mellitus (DM) (Yao *et al*., 2018, Ambachew *et al*., 2020).

preventing the Th1-mediated death of insulin-producing beta cells through processes related to the host's ability to develop a Th2 response to parasites, parasites may prevent type 1 diabetes (De Ruiter *et al*., 2017).

There were immunological interactions between parasites and their hosts, in which parasites can polarize the immune system toward a potent type-2 immune response that is linked to immune defense and tissue healing and induces tolerance and regulatory function in T1DM patients (Ali *et al*., 2018).

The prevalence of endoparasites in people with type 1 diabetes and a robust Th2 response to parasites may be connected to *Giardia lamblia* observed in type 1 diabetes (Hassanein and Fanaky, 2021). Numerous epidemiological studies in experimental animal models and people tend to the anti-autoimmune diabetic benefits of parasites (Berbudi *et al*., 2016).

The presence of the parasites appeared to drive the development of regulatory cells that recognize autoantigens and inhibit autoimmune disease processes, the parasites act as T-regulatory lymphocyte subpopulation (T-regs) adjuvants (Maizels and McSorley, 2016).

Immunomodulation caused by parasites may enhance insulin sensitivity, trigger immunological tolerance, and assist in avoiding complications of

diabetes. It is thought that parasite infections affect and control human immunity, suppressing autoimmune disorders as a result (Pliszka and Szablewski, 2020), by generating immune-evasion chemicals that might block an abnormal immunological response in the host, parasitic diseases have decreased in frequency during the past few decades, probably as a result of improved sanitation. In the meanwhile, autoimmune illnesses are becoming more common, which cannot just be attributed to variations in susceptibility genes (Murdaca *et al.*, 2021).

The Hygiene Hypothesis explains that increased atopy, asthma, and autoimmune illnesses like T1DM1 might result from decreased exposure to pathogens throughout childhood (Ali *et al*., 2018). Parasites are great candidates to be taken into account in the hygiene hypotheses (Rajasekaran *et al*., 2017).

Immunomodulation caused by the parasitic infection can lead to the identification of compounds with anti-inflammatory effects. Because of their ability to depress the immune system, (Caraballo, 2018).

Excretory-secretory (ES) molecules, such as ES-62, are one type of molecule that parasites use to communicate with the host immune system. These molecules can activate the immune system and potentially inhibit the growth of CD4+ T cells and conventional B2 cells in vivo, increase the production of IL-10 by peritoneal B1 cells, and prime the antigen-presenting cells to promote Th2 differentiation and suppress Th1 responses (Mukherjee *et al*., 2017, Mukherjee *et al*., 2020). The host's stromal cells, dendritic cells, macrophages, eosinophils, mast cells, basophils, epithelial cells, and innate helper cells are all involved in the parasite-induced Th2 responses (Anuradha *et al.*, 2014).

Professional APCs, dendritic cells process and deliver antigens to T cells by identifying the PAMPs (pattern-associated molecular patterns) linked to these parasites through TLRs (Zakeri *et al*., 2018).

Dendritic Cells DCs play a special function in parasite infection by displaying the Th2 phenotype and producing less IL-12 and IL-10. It's interesting to note that lower levels of IL-12 and IL-10 ultimately stop DCs from getting autologous T cells to release IFN-γ. On the other hand, ES-62 modifies immunological responses by interacting with the DCs necessary for CD4+ T cell priming and activation. Preculturing DCs with ES-62 causes DC maturation and concurrent activation of Th-2 responses dominated by IL-4 (Sutaveesup *et al*., 2020).

1.1. Aim of Study

Measuring certain immunological markers in people with T1DM1 and auto-tolerance brought on by parasite infections

These objectives helped to accomplish this aim:

1- Detection of intestinal protozoa in type 1 Diabetes Mellitus in the stool sample to document protozoal infections.

2- Measurement of interleukins (IL-2, IL-10, and prostaglandin E2) and estimation of Glutamic Acid Decarboxylase (GAD) Autoantibodies, Islet Autoantibodies of Pancreatic Beta-Cells, and Zinc Transporter Family Member 8 (ZnT8) Islet autoantibodies was detected using an ELISA method in both the patient and control groups.

3- Determining the role of elevated levels of IL-10 in the mixed group (diabetes infected with intestinal parasitic infection) to decrease the level of auto-antibodies.

4- Research any association between T1DM and intestinal parasitic infections (auto tolerance).

1.2. Literatures Review

1.2.1. Diabetes mellitus

Type 1 Diabetes Mellitus is the most prevalent endocrine disorder and one of the most prevalent chronic illnesses in children (Wherrett *et al*., 2018).

T1DM is characterized by immune system disruption intolerance, invasion of beta (β)-cells by auto-reactive immunological T cells, degradation of β-cell activity and survival, and prolonged external insulin treatment (Mirmira *et al*., 2016).

Diabetes, which is expected to impact 693 million individuals globally by 2045, is one of the illnesses with the greatest rate of growth (Cole and Florez, 2020). T1DM is an autoimmune condition that affects just one organ, the pancreas, and is brought on by the body's immune system attacking insulin-producing pancreatic beta cells. T1DM is caused by a variety of genetic and environmental variables that might appear in different combinations in people (Los and Wilt, 2021).

The goal of treatment is to reduce hyperglycemia while also lowering the chance of hypoglycemia. Numerous additional elements, like meals, insulin dosages, physical stress, and exercise, have an impact on the intricate balance of glucose. Education of the patient and their families is essential, as is an understanding of typical developmental phases and the difficulties they present in the context of day-to-day life with a chronic illness. Children and adolescents with type 1 diabetes can expect to live long and meaningful lives with the right treatment and support (Benslama *et al*., 2021).

1.2.2. Type 1 Diabetes mellitus Etiology:

In T1DM, the immune system gradually destroys the beta cells of the pancreatic islets over months or years, resulting in a complete absence of insulin. Although the precise origin of type 1 diabetes (T1DM) is still unclear, it is thought to be caused by a mix of genetic, environmental, and immunological factors. The fundamental process is an autoimmune attack on the pancreatic beta cells that make insulin. According to recent research, chronic enteroviral infections may be the cause of this autoimmune islet loss (Vehik *et al*., 2019).

A substantial correlation between certain HLA (DR and DQ) alleles, particularly DRB103-DQB10201 and DRB 10401-DQB10302H, and a hereditary susceptibility exists. Heritability is influenced by several more genes as well (Roep *et al*., 2021). Within the initial ten years following the first twin's diagnosis, there is a 30% chance of acquiring T1DM, with a lifelong danger of about 65% (Lucier and Weinstock, 2018).

Autoantibodies to pancreatic antigens in the blood may indicate that a person has T1DM or is at risk for developing it. These antibodies include antibodies to protein tyrosine phosphatase, glutamic acid decarboxylase, insulin, islet cell cytoplasmic antibodies (ICA), insulinoma-associated 2 (IA-2), and zinc transporter 8 (ZnT8). It is generally accepted that an Enterovirus or other environmental stimuli initiate autoimmune β-cell death and that the bigger the number of detectable antibodies and the higher their titers, the greater the chance of developing T1DM (Vojislav *et al*., 2020).

Environmental factors include microbial, dietary, and toxic substances the hygiene hypothesis states that increased sanitization is linked to an increase in autoimmune-mediated illnesses. It is hypothesized that insufficient immune

system development results from the loss of childhood exposure to infectious pathogens (Murdaca *et al*., 2021).

Additionally, investigating the dietary variables, and intake of cow's milk proteins increases the likelihood of developing islet autoimmunity in those with low to moderate-risk HLA-DR genotypes but not significantly in people with high-risk genotypes (Roep *et al*., 2021).

1.2.3. Type 1 diabetes mellitus pathophysiology

A chronic metabolic condition called type 1 diabetes (T1D) is brought on by the invasion and death of pancreatic islets by the autoimmune system. Genetic predisposition and autoantibodies are the two most frequent causes of the T1DM subtype (Type 1a) (Sanhueza *et al*., 2019, Kurianowicz *et al*., 2021).

A small percentage of instances of T1DM are brought on by the idiopathic death or failure of beta-pancreatic cells (Type 1b), this condition is commonly connected to substantial health consequences and is defined by the slow formation of pancreas-specific autoantibodies and severe hyperglycemia. Regardless of gender, it can be diagnosed at any age, however, it is most frequently seen in children and teenagers, when asymptomatic patients, euglycemic, and positive for the necessary auto-antibodies (Association, 2019, Haris *et al*., 2021)

It takes a long time before symptoms of hyperglycemia and frank diabetes appear, reflecting the high proportion of cells that must be killed before overt diabetes is apparent (Paschou *et al*., 2018).

Additionally, these mechanisms change amongst people; as a result, many endotypes have been hypothesized. ... other possible non-genetic contributors to the genesis of T1D, include viral infections, nutrition, and gut flora (Singh *et al*.,

2015). the potential involvement of beta cells directly in the onset of pathogenic diseases. Epigenetic impacts, which function as a bridge between environmental variables and genetic vulnerability and may contribute to part of the disease variability, are another element in T1DM risk Therefore, a move toward individualized medicines may enhance the efficacy of the latter and, consequently, produce better long-term outcomes for patients. To reverse or even prevent the onset of the illness, there is a clear need for a deeper comprehension of the preclinical stages of T1D and the discovery of novel predictive biomarkers for early diagnosis and treatment (Zajec *et al*., 2022).

The bulk of the global differences in incidence have been linked to exposure to risk factors associated with the environment. Environmental variables interact with genetically sensitive people to cause an autoimmune response (Blohmé et al., 1992, Los and Wilt, 2021).

Preclinical phases of type 1 diabetes have recently been identified. Beta-cell autoimmunity and normal glucose handling are the characteristics of stage 1, improper glucose handling and no overt symptoms are the characteristics of stage 2, and clinically obvious signs of insulinopenia are the characteristics of stage 3. It could take a long time to get through these stages. Research on therapies in the pre-clinical groups may show to postpone or prevent the onset of type 1 diabetes, even though pre-clinical staging is typically not clinically meaningful (Ilonen *et al*., 2019).

The etiopathogenesis of type 1 diabetes mellitus, which causes hyperglycemia and ongoing insulin dependency, is not entirely understood (Los and Wilt, 2021).

1.2.4 Immunologic factors for developing Diabetes Mellitus

1.2.4.1 Failure of immune tolerance

Immunological tolerance is the absence of immunological response and is caused by a complicated set of processes that make it difficult for the immune system to generate defenses against self-antigens. Most of us are born with an immune system that builds tolerance to both the items we eat and to every other system in our body. When immature lymphocytes come into contact with self-antigens in the primary lymphoid organs, they either die or stop reacting, which is known as central tolerance (Shojaeian and Mehri-Ghahfarrokhi, 2018).

When mature lymphocytes that evaded negative selection during ontogeny come into contact with self-antigens in secondary lymphoid organs, they experience anergy, deletion, or suppression, which results in peripheral tolerance. It has recently been shown that a diverse family of T regulatory cells is crucial for dampening immunological reactions toward oneself (Lucier and Weinstock, 2018).

Autoimmunity and autoimmune disorders are the outcomes of a breakdown or loss of immunological tolerance, Such occurrences are associated with both genetic and environmental causes, with infections serving as the primary example of the latter. Infectious agents may indeed trigger autoimmune reactions. They can do this by either causing tissue inflammation, which results in an unintentional bystander activation of autoreactive T cells, or by stimulating T cell responses to microbial epitopes that cross-react with self-peptides (Romagnani, 2006, Zajec *et al*., 2022).

The idea of immunological tolerance is crucial to the comprehension of type 1 diabetes and the creation of methods for its diagnosis, treatment, and prognosis. Type 1 diabetes is the outcome of an autoimmune reaction that directly or

indirectly affects the β-cell. Preventing the illness from developing stops the loss of tolerance. So tolerance induction, Islet transplantation, in particular, offers hope for a disease cure (Zajec *et al*., 2022).

T1DM can be brought on by mutations in non-MHC components like the AIRE gene and polymorphism in PTPN22, CTLA4, FOXP3, and VNTRs of insulin which can affect TCR signaling during negative selection and T-cell response as well as non-genetic events that affect central tolerance in the thymus. The possibility of viral infections, which might have an impact on the human thymus, is an intriguing theory. It has been demonstrated that coxsackievirus B4 virus and other enteroviruses may infect thymic epithelial cells and thymocytes. The maturation and differentiation of T cells were among the anomalies that were discovered after that. The T-regulatory lymphocyte subpopulation (T-regs, formerly known as suppressive lymphocytes) is crucial to the immune response network, particularly for peripheral tolerance. It has been shown that T1DM patients have quantitative and qualitative T-reg deficiencies, which might account for the heightened (unrestrained) immune response that finally results in the autoimmune response (Vicinanza *et al*., 2019).

1.2.4.2 Cellular immunity

Most likely, apoptosis, a process also known as programmed cell death and involving a cascade of cysteine-asparaginase activations known as caspases, is how the cells of the endocrine pancreas are destroyed in T1DM. necrosis and necroptosis may also be significant in people; nonetheless, necrosis is a significant cell death type for cells (Martinez *et al*., 2021).

One theory states that autoreactive T cells inside the islet microenvironment cause an inflammatory response with elevated levels of the proinflammatory cytokines IL-1, TNF (tumor necrosis factor), and IFN (interferon). The caspase

cascade is triggered by these cytokines. Other ideas contend that interaction between autoreactive T lymphocytes and cells through the perforating system or Fas/Fas ligand induces apoptosis directly (Martinez *et al*., 2021).

Even in this case, cytokines secretion disorder is required. The subpopulation of T-regs is of great interest and their quantitative and qualitative deficits in patients with the disease are very important (Szablewski, 2014).

Histologically, persistent atrophic inflammation with the involvement of T lymphocytes, macrophages, B-lymphocytes, and dendritic cells is seen inside the islets of Langerhans before the beginning of T1DM. When patients are asymptomatic and euglycemic, this syndrome often develops over several months or years. Long latency periods before symptoms of hyperglycemia appear to indicate that many functional cells must be eliminated before the disease's clinical presentation (Marca *et al*., 2018).

1.2.4.3 Humoral immunity

The disturbance of immune response includes the humoral immunity arm too, the onset of clinical manifestation of T1DM may precede for years producing autoantibodies. The main autoantibodies detected in patients with T1DM are those against Glutamic Acid Decarboxylase (GAD) Autoantibodies against GAD are a predictor of progression to overt diabetes and Zinc transporter family member 8 (ZnT8) autoantibodies In the β-cell, it plays an important physiological role since Zn, which is highly concentrated in β-cells, is needed for normal insulin storage. tyrosyl phosphatase Insulinoma Associated (IA-2), insulin (IAA), and Islets cytoplasmic autoantibodies (ICA) (Marca *et al*., 2018).

The main possible target is proinsulin the significant impact of insulin gene deletion on disease development and the ability of insulin therapy to prevent or

postpone the onset of T1DM in pre-diabetics confirms the role of insulin as an autoantigen (Gonzalez-Duque *et al*., 2018).

The GAD enzyme, which is found in β-cells, the central nervous system, and the testes, is another significant autoantigen. Approximately 70% of T1DM patients had anti-GAD autoantibodies at the time of diagnosis. IA-2 is a significant autoantigen as well, with autoantibodies it being present in about 60% of T1DM patients at the time of diagnosis (Martinez *et al*., 2021).

Autoantibodies to IA-2 often manifest later than those to insulin and GAD and are strongly correlated with disease development. Also recently discovered as an autoantigen for T1DM is the zinc transporter (ZnT8). ZnT8 autoantibodies are present in 60–80% of patients with recently diagnosed diseases. ZnT8 autoantibodies appear later than autoantibodies to insulin in children who are followed from birth to the development of T1DM, and they typically go away very soon after the disease's clinical manifestation (Szablewski, 2014;Sanhueza *et al*., 2019).

There is strong evidence that the amounts of ZnT8 autoantibodies are correlated with age, HLA genotype, and metabolic condition at diagnosis, but they are ineffective in detecting cell autoimmunity. The concurrent production of two or three autoantibodies from the aforementioned list is one of the greatest indicators of development into clinical T1DM (Martinez *et al*., 2021).

1.2.5 Type 1 Diabetes mellitus Diagnosis:

1.2.5.1 Conventional Blood Tests : the following blood tests can detect T1DM in children (Bhansali *et al*., 2016, Dong *et al*., 2021):

• **Random blood sugar test:** The main screening test for type 1 diabetes is this one. At some random moment, a blood sample is obtained. The presence of diabetes is suggested by a blood sugar level of 200 mg/dL (11.1 mmol/L) or greater.

• **The HbA1C test for glycated hemoglobin:** This test provides the child's three-month average blood sugar level. Diabetes is diagnosed when the A1C result is 6.5 percent or greater on two different tests.

• **A fasting blood sugar test:** a blood sample is taken after the child fasts overnight. Type 1 diabetes is suggested by a fasting blood sugar level of 126 mg/dL (6.0 mmol/L) or greater.

- **Rarely is an oral glucose tolerance test (OGTT)**: administered to diagnose diabetes with the OGTT, the blood glucose level 2 hours after consuming 75 g of anhydrous glucose must be more than or equal to 200 mg/dl.

1.2.5.2 Additional tests

A doctor would likely advise further tests to distinguish between type 1 diabetes and type 2 diabetes if blood sugar testing reveals diabetes since the two types of the disease have different treatment options. Once diabetes has been diagnosed, a blood test is used to differentiate type 1 diabetes from other forms (Association, 2019).

These additional procedures include blood testing to look for autoantibodies that target specific beta cell components as well as antibodies that are frequently present in type 1 diabetes (Association, 2019).

The most widely used methods for finding antibodies to glutamic acid decarboxylase are crucial. (GAD) is an enzyme with several significant functions that are found in the brain and pancreas. It continues to play a part in the conversion of the excitatory amino acid glutamate into the inhibitory neurotransmitter, gamma amino butyric acid (GABA). Total antioxidant capacity (TAC), protein tyrosine phosphatase type A2 (IA-2A), and glutamic acid decarboxylase autoantibody (GADA) indicators on -cell stress and/or death in T1D persons (Mirmira *et al*., 2016).

Islet cell cytoplasmic antibodies are included among these antibodies (ICA) Pancreatic autoantibodies in the blood may indicate that a person has T1DM or is at risk for developing it. and the transmembrane protein known as zinc transporter 8 protein (ZnT8A) is responsible for transferring zinc to insulin vesicles. In type 1 diabetes, antibodies produced against ZnT8A are thought to independently demonstrate autoimmunity. The risk of developing T1DM increases with the number of detected antibodies and their titers (Vojislav *et al.*, 2020).

The early stage of T1DM is identified by the presence of two or more islet autoantibodies. By enabling prompt glycemic control and facilitating patient enrollment in clinical trials during a crucial window when patients still possess islet cell function and are more likely to respond favorably to immunomodulatory intervention, early identification of these Stage 1 individuals lowers the risk of Diabetic Keto Acidosis DKA (Herold *et al*., 2019, Cortez *et al*., 2020).

Checking the urine or blood to look for ketones, which also point to T1DM rather than type 2 (Los and Wilt, 2021) C-peptide, a byproduct of insulin production, is also tested for by some. Type 1 diabetes may be indicated by extremely low C-peptide levels (Association, 2019).

1.2.6 Cytokines involved in the pathogenesis of type 1 Diabetes:

Manipulation of anti-inflammatory immune response aided by cytokine and chemokine afford redirection immune response toward functional tolerance and inhibit organ-specific autoimmunity in T1DM, type 1 cytokines like IL-2, IL-12, and IFN-γ have pro-inflammatory actions, whereas type 2 cytokines like IL-10, IL-4, and TGF-β exert inhibitory or anti-inflammatory activities, pro-inflammatory cytokines which contribute to the disease's development (Lu *et al*., 2020).

Cytokines play crucial roles in orchestrating complex multicellular interactions between pancreatic b cells and immune cells in the development of type 1 diabetes (T1D) and are thus potential immunotherapeutic targets for this disorder. Cytokines that can induce regulatory functions—for example, IL-10, TGF-β, and IL-33—are thought to restore immune tolerance and prevent β -cell damage. By contrast, cytokines such as IL-6, IL-17, IL-21, and TNF, which promote the differentiation and function of diabetogenic immune cells, are thought to lead to T1D onset and progression. However, Targeting these dysregulated cytokine networks, may not always provide consistent results due to the pro- or anti-inflammatory cytokine roles, which are in charge of β-cell death (DiMeglio *et al*., 2018).

1.2.6.1 The Role of IL-2 in Diabetes mellitus and Intestinal parasitic infections (IPIs):

Understanding the signaling mechanisms that enable the pleiotropic cytokine IL-2 to regulate the development and homeostasis of both pro- and anti-inflammatory T-cell cytokines is essential for understanding the molecular specifics of immune regulation. The STAT5 transcription factors are activated

by the IL-2 receptor when it connects to JAK tyrosine kinases (Churlaud *et al*., 2018).

It is a crucial T-cells growth factor (Rosenzwajg *et al*., 2020, Dong *et al*., 2021).

It is becoming more and more obvious that, within each T cell subset, IL-2 will signal within a context of other signal transduction networks, which combined will influence the transcriptional and metabolic processes that dictate T cell populations (Ross and Cantrell, 2018).

IL-2 was first identified as a cytokine that stimulated T cells, promoting their growth and differentiation into effector T cells (Bettini and Bettini, 2021).

Additional research has demonstrated that low-dose IL-2 increases the growth of T-reg populations, which in turn encourages the production and suppressive activity of T-regs. When autoreactive Th1 cells are inhibited, Th2- and Th3-type cytokine-producing cells take over and have a protective impact. Consequently, T1D disease development and onset are under control (Dong *et al*., 2021).

IL-2 has also been noted to encourage B-cell proliferation. Therefore, as shown in both preclinical and clinical investigations, IL-2 is a promising therapeutic target in T1D. Indeed, a variety of immune cell types, such as natural killer (NK) cells and effector T cells, which play pathogenic roles at the beginning of T1D, are affected by IL-2 via its receptor (IL-2R) (Habib *et al*., 2019).

IL-2 is important in Cell-Mediated Immunity CMI for host defense against intestinal protozoa by preventing dissemination and recurrence of the parasite through pMHC II with TCR During the initial stage of infection, intestinal epithelial cells (IECs) bind to and recognize the carbohydrate recognition domain via toll-like receptor (TLR)-2/4, which activates NF-κB and leads to the

production of inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α (Li *et al*., 2020).

1.2.6.2 The Role of IL-10 in Diabetes mellitus and Intestinal parasitic infections IPIs:

Interleukin -10, a cytokine with anti-inflammatory properties, plays a critical role in the prevention of inflammatory and autoimmune pathologies. This role is mediated through IL-10R and is associated with many immune cells, generating feedback regulation of diverse immune responses. However, despite the immunosuppressive effects attributed to IL-10, its ability to modulate disease progression in T1D is still debatable (Lu *et al*., 2020).

Individuals with IL-10 deficiency experience spontaneous intestinal inflammation due to weakened and extremely permeable mucosal barriers. Additionally, studies have demonstrated that IL-10 increases MUC2 synthesis, inhibits antigen-presenting cell activation, triggers B cell class switching to IgA, inhibits apoptosis in IECs, decreases pro-inflammatory NF-κB signaling in IECs, and stimulates the induction of CD4+ T-reg. cells. It's interesting to note that there was no increase in IL-10 level in asymptomatic carriers. On the other hand, individuals with dysenteric and amoebic liver abscesses (ALA) had higher levels of IL-10 (Machado *et al*., 2018)

Entamoeba histolytica invasion of the colon and liver triggers an anti-inflammatory immune response, which may be able to successfully inhibit immunological responses against the amoebae. To start an infection, the amoeba must balance the amounts of IL-10 and inflammatory cytokines. It was demonstrated that TLR2 mediated TNF-α, IL-6, IL-8, IL-12, and IL-10 secretion by peritoneal monocytes and macrophages exposed to Lipopeptidophosphoglycan. High dosages of LPPG have also been proven to

suppress TLR2 gene expression. LPPG-driven signaling may thereby initiate a negative feedback loop that dampens inflammatory responses (Khalaf AL-Majid and Hafez, 2021).

The immunosuppressive properties of IL-10 and its capacity to control the development of T1D illness. A substantial connection between elevated IL-10 expression and illness attenuation suggests that the preventive benefits of IL-10 have also been observed in people. Increases in T-reg frequencies, Th2-type cytokine (IL-4 and IL-10) levels, as well as the inhibition of Th1-type cytokines (IL-2 and IFN-γ), are all suggested as methods by which IL-10 regulates T1D. In T1D, IL-10 is linked to immature DC tolerance and IL-10-producing regulatory B lymphocytes (B-regs) (Al-Kahfaji and Al-Masoudi, 2019, Lu et al., 2020). Improvements in β-cell function, inhibition of insulitis progression, and prevention of diabetes have all been linked to some treatment approaches that stimulate IL-10 in animal models (Lu *et al*., 2020).

1.2.6.3 The role of Prostaglandin PGE2 in Diabetes mellitus and Intestinal parasitic infections IPIs:

Prostaglandin E2 (PGE2) plays a regulatory or anti-inflammatory role in Type 2 inflammation. The outcome affected the dynamics of Type 1 and Type 2 cytokine production from T cells, which subsequently enabled Th2 responses. Through the inhibition of a Th1 response in DM1, PGE2 may also be crucial in shifting the balance toward a Th2 response (Takahashi *et al*., 2022).

Prostaglandin E2 (PGE2), a lipid mediator produced by the cyclooxygenase enzyme system, is the main prostaglandin in the kidney.PGE2 functions by binding to four G-protein-coupled EP receptors (EP1 to EP4), the role of PGE2 in various contexts, such as during autoimmunity, cancer, and chronic

inflammation, it may play both pro-and/or anti-inflammatory roles depending on receptor usage, disease context, and timing of inflammation (Polese *et al.*, 2021).

Prostaglandin 2 (PGE2), which is produced and secreted by *E. histolytica* trophozoites, affects tight junction integrity and ion absorption in a contact-independent manner. Amebic PGE2 that has been secreted interacts with prostaglandin E receptor 4 (EP4) on IECs to break down tight junctions and promote luminal Cl^- secretion. PGE2 released by amebae causes IECs to produce more IL-8, which triggers an inflammatory response. PGE2 is a strong mucin secretagogue that can deplete the protective mucus barrier and, as a result, disrupt luminal barrier function. On the other hand, it has also been shown that local PGE2 during invasive amebiasis has an anti-inflammatory impact. Class II MHC II immune-associated antigen expression is decreased by an unidentified soluble protein component of *E.histolytica* trophozoites in a PGE2-dependent manner, MHC II expression and TNF-α expression can be partially recovered by inhibiting macrophage PGE2 production. the deactivated macrophage does not regain iNOS expression or amebicidal activity, however, when PGE2 production is inhibited. iNOS expression and complete recovery of MHC II and TNF-α are likely prevented by a constant supply of parasite-derived PGE2, presumably through a concentration-dependent action of PGE2. In essence, ameba-secreted PGE2 inhibits inflammation in ALA, which is necessary for life, but likely promotes Colonial cell death by contact-dependent killing (Ben Nasr *et al*., 2018).

1.2.7 Auto-Antibodies in Type 1 Diabetes mellitus

1.2.7.1 Glutamic Acid Decarboxylase (GAD)

Glutamic Acid Decarboxylase (GAD) is a better comprehension of Type 1 diabetes, it is also found in the brain and pancreas and contains the GAD enzyme, which decarboxylates glutamine acid. It is important for the development and function of islet cells in numerous ways. It still contributes to the process by which the excitatory amino acid glutamate is transformed into the inhibiting neurotransmitter GABA. The enzyme GAD produces the messenger gamma-aminobutyric acid (GABA), which is found in the central nervous system. (Yoshizaki *et al*., 2012, Mirmira *et al*., 2016).

1.2.7.2 Zinc transporter 8 (ZnT8)

Is an important transmembrane protein that carries zinc to the insulin compartments. Through the production of antibodies against ZnT8A (ZnT8A), type 1 diabetes is believed to display autoimmunity (Elmaoğulları *et al*., 2018).

It has more expression in the alpha and ductal cells, and zinc transporter 8 is mostly confined to the pancreatic islet beta cell. Using the secretory granule membrane, ZnT8 catalyzes the import of Zn^{++} ions from the cytosol into the lumen in exchange for protons delivered by the vesicular proton pump (Wenzlau, 2016).

1.2.7.3 Islet Cell autoantibodies (ICA)

Islet autoantibodies remain a reliable indicator for identifying individuals with the highest risk of type 1 diabetes (Long *et al*., 2021).

Islet autoantibodies are the main signs of pancreas autoimmunity in type 1 diabetes. (T1D). Islet autoantibodies identify antigens on secretory granules within pancreatic beta cells, such as insulin (IAA), glutamic acid decarboxylase (GADA), and ZnT8 (ZnT8A). Islet antibodies are the primary markers of the autoimmune response that are monitored for diagnosis or prognosis in T1D patients or disease forecast in at-risk individuals before the onset of T1D. Islet

autoantibodies have been the main scientific method used to examine the natural history of T1DM (Lampasona and Liberati, 2016).

1.2.8 Mucosal Immunity to Intestinal Protozoa

The mucosal immune system, also known as the mucosa-associated lymphoid tissues (MALT), which is made up of mucus layers, epithelial cells, lymphoid tissues, and immune molecules in the mucosal lamina propria, ensures the protection of these surfaces. The mucosal immune system's most prevalent antibody isotype is immunoglobulin A (IgA). IgA in serum is monomeric, but IgA on the mucosal surface has a dimeric structure. The initial line of defense for mucosal immunity is secretory IgA (sIgA) it is a polymeric IgA made up of a secretory component (SC), a J chain, and a dimeric IgA. The gut produced the majority of the sIgAs. The hydrophily of sIgA is caused by the abundance of hydrophilic amino acids in IgA Fc and the glycosylation of IgA and SC, which traps microorganisms. Peristalsis then aids in the removal of the pathogen clumps (Lycke and Bemark, 2017).

Trophozoites are eliminated from the gastrointestinal surface as a result of the cytotoxic secretory IgA and defensins that are produced. The production of nitric oxide (NO), which is also produced by immune and epithelium cells, prevents trophozoites from growing. In addition, mast cell degranulation and NO production in neurons stimulate intestinal peristalsis, which helps in the ejection of trophozoites by immune exclusion (Fink and Singer, 2017, Li *et al*., 2020).

As a first line of defense, the parasites may cause epithelial tight junctions to be broken, stimulate CD8+ lymphocytes, and cause apoptosis in cells in contact with the trophozoites. due to the shortening of brush-border microvilli, disaccharidases, and other enzymes are deficient. In the following stage, CD4+

T-cells that produce IL-17 and TNF-α and dendritic and mast cells that produce IL-6 are used to trigger adaptive immune responses. Tuft cells are another possible source of IL-17 these cells may be triggered by trophozoite metabolites, which would then heighten pro-inflammatory responses in the intestinal epithelium (Kaetzel *et al*., 2017, Schneider *et al.*, 2018)

Cell-mediated immune responses are also important for host defense against intestinal protozoa. During the initial stage of infection, intestinal epithelial cells (IECs) bind to and recognize the carbohydrate recognition domain of the parasitic lectin via toll-like receptor (TLR)-2/4, which activates NF-κB and leads to the production of inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α (Wang *et al*., 2022).

Intestinal Epithelial Cells IECs are the second line of barriers against pathogens after the mucosal layer and the first line of host cells to encounter microbial/parasite antigens, they express an array of pathogen recognition receptors (PRRs), including TLRs. IFN-γ is involved in the clearance of infection, whereas IL-4 and TNF-α are associated with the disease (Li *et al*., 2020)

1.2.9 Prevalence of intestinal parasites among type 1 in pediatric diabetic Mellitus patients:

One of the most frequent causes of human illnesses globally is intestinal parasites (IPs). However, due to variances in environmental, socioeconomic, and geographical variables, the distribution of species varies from one place to another. They are more prevalent in tropical nations and mostly spread in unsanitary environments. Thus, local information on the prevalence and dominant species of parasites aids in the development of efficient management techniques to enhance the health of chronically sick individuals like T1DM

patients, T1DM is a metabolic disorder that impairs metabolism and suppresses the immune system. It has been shown that T1DM patients have reduced innate and adaptive immune responses. Because of this, individuals are more susceptible to infectious diseases, which makes parasites more active and able to produce serious pathology (de Lourdes Ochoa-González *et al*., 2021).

Children and individuals with impaired immune systems who are taking IPIs run the risk of developing iron deficiency anemia, development impairment, and gastrointestinal issues such as diarrhea, nausea, vomiting, and abdominal discomfort (Al-Megrin *et al*., 2021).

The association between diabetes mellitus and infections is very common. These infections, even when mild, interfere with blood glucose control. During infections, an increase in blood glucose occurs and usually, an increase in insulin dose is required (Calliari *et al*., 2020).

In addition to the typical community-acquired illnesses, it has been documented that some infections preferentially affect persons with diabetes, and other common infections may be more severe in these individuals. There is evidence that T1DM patients with good glycemic management have improved immune function and reduced morbidity and death from severe infections (Dunachie and Chamnan, 2019).

Intestinal parasites are significant opportunistic pathogens that cause infections in immunosuppressed individuals, and diabetic patients are known to have impaired immune systems. There have been several reports of connections between T1DM and parasite diseases. However, a protective association existed between certain parasites and T1DM, which allowed for the immunological screening of these individuals to avoid serious and catastrophic DM outcomes (Wiria *et al*., 2015).

The prevalence of entero-parasites in people with T1DM and a robust Th2 response to parasites may be linked to the *Giardia lamblia* seen in type 1 diabetes (Allain *et al*., 2017, Alemu *et al*., 2018).

The established fecal-oral transmission of infection by contaminated food and water is the primary cause of the incidence of amoebiasis in diabetes patients in the developing world (Siddiqua *et al*., 2017, Ambachew *et al.*, 2020).

The presence of an infectious condition,(Drawany *et al*., 2019) in turn, raises blood glucose and increases the risk of decompensation, and pediatricians should be cautioned to intensify monitoring and insulin therapy and to avoid the risk of DKA (Al–Mousawi and NEAMAH, 2021).

A subject of significant interest is the link between infections and diabetes mellitus (DM). Diabetes is likely to change an individual's vulnerability to infections through a variety of immune system-related impacts (Dunachie and Chamnan, 2019).

Th2-type response is finally induced as a result of the suppression of pro-inflammatory cytokines and subsequent downregulation of costimulatory molecules (Joardar *et al*., 2021).

The intricate immune evasion systems made possible by the parasite-derived chemicals enable the parasite's survival inside the host body. Immunomodulation caused by IPIs may improve insulin sensitivity and reduce the risk of complications (Lambrecht and Hammad, 2017, Varyani *et al*., 2017).

1.2.10 *Entamoeba histolytica*

1.2.10.1 Classification of *Entamoeba histolytica* :

Domain: Eukaryota

Class: Sarcodina

Order: Diplomonadida

Phylum: Amoebozoa

Family: Entamoebidae

Genus: *Entamoeba*

Species: *E. histolytica*

Classification by Linnaeus (Avila *et al*., 2016).

1.2.10.2. Morphology of *Entamoeba histolytica*:

Entamoeba histolytica is the cause of human amebiasis (Amoebozoa, Amorphaea). *Entamoeba histolytica* is the classification for all pathogenic Entamoeba strains, whereas *Entamoeba dispar* is the classification for non-pathogenic *Entamoeba* strains. Two phases stand out: trophozoites and cysts. The colon (and subsequently other organs) is colonized by the motile, mononucleated trophozoite (10 to 20 µm, occasionally bigger), which can then develop into the cyst stage, which is similar in size but has one to four nuclei, occasionally larger (and eventually other organs), where it may develop into a cyst stage of comparable size, but one that causes human illness by ingesting excreted cysts, or fecal-oral contamination (Begum *et al*., 2020).

Upon transfer from person to person, foodborne, or waterborne sources. As a result, crowded living conditions and inadequate sanitation are socioeconomic variables that support amebiasis, nowadays, tourists from endemic regions are the main cause of amebiasis incidence in the EU and the US. Amebiasis, which was given the term "histolytica" for its capacity to disintegrate host tissues, The cause of tissue damage is probably caused by the amoebae directly destroying host cells, Amoebae bite off and absorb unique host cell pieces after adhering to host cells, It aids in cell death, the finding of amoebic trogocytosis in *E. histolytica* may also provide insight into a pathway for intercellular exchange that has been preserved throughout evolution (Bennett *et al*., 2019).

1.2.10.3. The life cycle of *Entamoeba histolytica*:

Human amebiasis is caused by the parasite *E. histolytica*. It has two cellular forms that make up its life cycle: the trophozoite (or vegetative form), which lives in the intestinal lumen, and the cyst, which is the contaminating form that can survive outside of the body and is in charge of spreading the parasite. Cysts are expelled in the feces of the host and spread from one person to another by a fecal-oral pathway, such as contaminated food, drink, or direct touch. Trophozoites are created when a tetra-nucleate cyst excysts in the small intestine, producing eight amoebae that go to the colon. Trophozoites feed on bacteria there as commensals and then multiply or encyst. Therefore, the *E. histolytica* life cycle is straightforward, consisting of an infected cyst and a trophozoite that divides. The trophozoites of 10% of people with *E. histolytica* go from being commensal to virulent, and because of their high motility, they infiltrate the intestinal barrier, including the mucus layer and eventually the epithelium and connective tissue (Aguilar-Rojas *et al*., 2016, Aguilar-Rojas *et al*., 2020).

E. histolytica does not require a vector for transmission as many other parasite species do meaning that surveillance and diagnostics for this disease should be enhanced, according to the Centers for Disease Control, because of its relatively simple transmission and impact in terms of death and morbidity (Babuta *et al*., 2020).

1.2.10.4 Pathogenesis of *Entamoeba histolytica*:

An invasive enteric protozoan is called *E.histolytica*, when mature quadrinucleated cysts are ingested in fecally contaminated food or drink, an infection usually starts. While trophozoites are quickly killed once they are outside the body or by stomach secretions if consumed, cysts can live for days to weeks in the environment (Cheepsattayakorn and Cheepsattayakorn, 2014).

Trophozoites can cling to the intestinal epithelium, lyse it, and then disseminate hematologically through the portal venous system to far-off locations such as the peritoneum, liver, lung, or brain. The Gal/GalNAc lectin, which binds galactose and N-acetyl-D-galactosamine residues on O-linked sugar side chains of mucins, promotes colonization and adhesion to the colonic mucus layer. Animals lacking N-terminal galactose or N-acetyl-D-galactosamine are less susceptible to trophozoite adhesion, giving them some protection from invasive illness (Cornick and Chadee, 2017). investigations on the virulence of *E. histolytica* species are ongoing, and invasive disease risk has been associated with the presence of certain enzymes. For instance, to get rid of the branched polysaccharides from mucin cells, glycosidases such as sialidase, N-acetylgalactosaminidase, and N-acetylglucosaminidase are required, due to the trophozoites' ability to penetrate the intestinal epithelium and weaken the

protective mucous barrier, the risk of metastasis to distant areas is increased (Kantor *et al*., 2018).

The enzyme glycoside hydrolase B-amylase was also used to measure the virulence of *E. histolytica*. These enzyme-deficient species were unable to penetrate the mucus barrier and spread invasive illness. In addition to apoptosis, contact-dependent cell killing, proteinase (cysteine proteinase), and the development of amebapores that cause cytolysis of infected cells are further processes used to kill epithelial cells and inflammatory cells (Cornick and Chadee, 2017).

Several theories have been proposed and are being tried to explain why some individuals have an asymptomatic disease while others get an invasive disease. It has been discovered that the host's genetic, immunological state, age, and gender, as well as the strain's virulence, all predict the severity of the disease (Bernin *et al*., 2014).

1.2.10.5 Diagnosis of *Entamoeba histolytica*:

The "gold standard" for diagnosing amoebiasis remains the microscopic analysis of a stool specimen. *E. histolytica* must be found to include swallowed red blood cells; nevertheless, the absence of *E. histolytica* in the feces does not rule out the diagnosis of amoebiasis (Gardiner *et al*., 2015).

For the diagnosis of *E. histolytica*, several diagnostic methods are available, including microscopy, serology, antigen detection, molecular approaches, and colonoscopy with histological analysis. Since *E. histolytica* is morphologically similar to the nonpathogenic species *E. dispar* and *E. moshkovskii*, the presence of cysts or trophozoites in stool cannot be utilized to diagnose the illness it causes (Kantor *et al*., 2018).

Assays for detecting antigens CERTEST in stool are sensitive, specific, simple to use, and may be able to detect early infection, even though these tests are more precise and sensitive than microscopy, they are costly, and serology is highly helpful for the diagnosis of invasive amoebiasis. all individuals in whom a liver abscess is suspected should also get an ultrasound of the liver both intestinal and extra-intestinal disorders may be quickly, precisely, and successfully diagnosed using PCR to identify *E. histolytica*-specific nucleic acids. Although it has great sensitivity and specificity, it is currently not commonly used for diagnostic testing because of its expensive cost and lack of standardization. Monoclonal antibodies are used in these experiments to bind to epitopes on *E.histolytica* that are absent from other nonpathogenic strains. Utilizing ELISA, radioimmunoassay, or immunofluorescence, antigens can be found (Kantor *et al*., 2018).

Last but not least, a colonoscopy can be used to directly view the colon to detect amoebiasis, especially when nonspecific gastrointestinal symptoms make diagnosis challenging. additionally, the rectum, ascending colon, sigmoid colon, and, seldom, the transverse and descending colon, are the areas where "flask-like" ulcerations or erosions are most frequently found (Horiki *et al*., 2015).

1.2.10.6 Virulence Factors Roles of *Entamoeba histolytica* in Immune Evasion Strategies:

Glycosidases disrupt the host's physical defenses and soluble immune mediators the host gut and liver epithelia are damaged, immune cells are destroyed, and soluble immunological mediators are activated or degraded by hydrolases released by *E. histolytica* trophozoites. the mucosal layer is a physical

barrier between the epithelium and lumen for the amebae to begin colonizing, the barrier's carbohydrate breakdown is essential (Corfield, 2015).

Cysteine proteases are also known to modify cell-mediated immunity and degrade immunoglobulins, complements, and cytokines. It is unknown, though, whether these modifications are beneficial or harmful for preventing amebic infection. Chronic amebiasis-induced Th2- and Th17-mediated pathogens immunological responses (Argüello-García et al., 2020, Wang *et al*., 2022).

Immunoglobulins released by cells are primarily in charge of the intestine's immune response to amebic infection. Anti-Gal/GalNAc lectin IgA has been shown to decrease trophozoite colonization in the colon. It's interesting to note that human IgA is cleaved by *E. histolytica* surface-associated CP, most likely EhCP-A5. Amebic CPs can cleave both IgA1 and IgA2 isotypes (Babuta *et al*., 2020, Cinicola *et al*., 2022).

Amebic CPs are also capable of inactivating circulating IgG, and as a result, it is thought that they play a role in survival during tissue invasion and extraintestinal proliferation. Anaphylatoxins C5a and C3a are cleaved and rendered inactive by CPs in *E. histolytica* trophozoites to prevent a complement assault (Castellanos-Castro *et al*., 2020).

Proteins Glycosylphosphatidylinositol-Anchored *E. histolytica* may also avoid complement attachment by covering its surface with proteins that are attached to glycosylphosphatidylinositol (GPI). In most eukaryotes, GPI serves as a glycolipid that anchors a variety of proteins and glycoconjugates to the cell surface, serving as cell surface decoration to subvert host immunity (Saha *et al*., 2016)

The contact-dependent killing of cells an ameba's method of avoiding immune monitoring involves immobilizing and destroying immune cells. Numerous cells, including neutrophils, T lymphocytes, macrophages, and different tissue culture lines, can be obliterated by amebic trophozoites. Immune cell killing and phago/trogocytosis. The term "amebic trogocytosis" refers to a novel method of ectoparasite death of living cells in which trophozoites consume bits (nibbling) of the host cells after ectoparasite attachment (Ralston, 2015).

This was a remarkably quick procedure since attachment-induced amebic trogocytosis started within just one minute biting off and ingesting individual host cells leading to an increase in intracellular calcium levels, which ultimately caused cell death as seen by the breakdown of cell membrane integrity. Investigations on the virulence of *E. histolytica* species are ongoing, and invasive disease risk has been associated with the presence of certain enzymes. For instance, to get rid of the branched polysaccharides from mucin cells, glycosidases such as sialidase, N-acetylgalactosaminidase, and N-acetylglucosaminidase are required this increases the risk of metastasis to distant areas by allowing the trophozoites to enter the intestinal epithelium and weaken the protective mucous barrier (Horiki *et al*., 2015).

The presence of the enzyme glycoside hydrolase B-amylase also affected the virulence of *Entamoeba histolytica*. These enzyme-deficient species were unable to penetrate the mucus barrier and spread invasive illness. In addition to apoptosis, contact-dependent target cell lysis, proteinase (cysteine proteinase), and the development of Amebapores a class of important pore-forming peptides that cause cytolysis of infected cells are further processes used to kill epithelial cells and inflammatory cells (Burgess *et al*., 2017, Cornick and Chadee, 2017).

E.histolytica trophozoites release substances that, when exposed to microbial antigens, trigger a defensive response in human IECs, the first line of host cells. The factors that activate TLR2, such as LPPG and Gal/GalNAc lectin, are candidates for this pathway as PRRs cause activation of NF-κB upon binding to their ligand. Commensal microorganisms can also disrupt NF-κB signaling to reduce pro-inflammatory IEC responses, suppressing NF-κB in IECs, which is necessary for maintaining gut homeostasis and needs constant activation of NF-κB via TLR signaling in response to intestinal bacteria. *E.histolytica* Microaerophilic and oxygen-consuming trophozoites. Low oxygen tension values are tolerated by them. A typical respiratory electron transport chain that results in the conversion of O2 to H2O is absent in *Entamoeba histolytica*. It does, however, breathe and can withstand 5% oxygen in the gas phase (Desure *et al*., 2021).

To circumvent host immunity, *E. histolytica* lacks respiration and manages oxidative stress. Prostaglandin 2 (PGE2), which is produced and secreted by *Entamoeba histolytica* trophozoites, affects tight junction integrity and ion absorption in a contact-independent manner. Amebic PGE2 that has been secreted interacts with prostaglandin E receptor 4 (EP4) on IECs to break down tight junctions and promote luminal Cl^- secretion. PGE2 released by amebae causes IECs to produce more IL-8, which triggers an inflammatory response. PGE2 is a powerful mucin secretagogue that can weaken the protective mucus barrier by producing hypersecretion and overcoming luminal barrier function (Ben Nasr *et al.*, 2018).

E. histolytica trophozoites contain high levels of cysteine, instead of glutathione, as the major thiol in the cell. They have several enzymes to protect them from oxidative damage. In *E. histolytica*, anti-oxidative stress response

proteins like peroxiredoxin (Prx), superoxide dismutase, flavoprotein A, ferredoxin, thioredoxin (Trx), and Trx reductase aid in immune escape. To protect delicate proteins from oxidative stress, the Trx/Trx reductase system is essential. Trx is known to be disrupted by the amebicidal medications metronidazole and auranofin unexpectedly, *E. histolytica* pathogenicity is increased by oxidative stress (Jeelani and Nozaki, 2019).

1.2.11 *Giardia lamblia*

1.2.11.1 Classification of *Giardia lamblia*:

Phylum: Metamonada

Class: Fornicata

Order: Diplomonadida

Family: Hexamitidae

Genus: *Giardia*

Species: *G. lamblia*

Classification by Linnaeus (Rojas-López *et al*., 2022)

1.2.11.2 Morphology of *Giardia lamblia:*

Although anaerobic, *Giardia lamblia* can also be somewhat aerotolerant. The multi-flagellated trophozoite (four pairs of flagella) and the cyst are the two morphological types of giardia. The trophozoite has a sticky disk on the ventral surface and is di-nucleated, pear-shaped, multi-flagellated, 9 to 15 µm long, 5 to 15 µm broad, and 2-4 µm thick (Jex *et al*., 2020).

Trophozoites survive on nutrients from the intestinal fluid and are connected to the duodenal and jejunal epithelial cells. They prefer the amino acid arginine as their preferred fuel (Vermathen *et al*., 2018, Hemphill *et al*., 2019).

Quadri-nucleated, thick-walled cysts are produced when trophozoites become detached (8–10 µm in diameter). The infectious stage is represented by the cysts, which are expelled in the feces. Giardiasis is thus brought on by fecal contamination of food, water, or direct contact with feces; the latter is considered to be the main source (Waldram *et al*., 2017).

1.2.11.3 The Life Cycle of *Giardia lamblia:*

G. lamblia infection can be transmitted by direct contact with sick humans or animals, contaminated water or food, or through the fecal-oral route (Kraft, 2019).

Cysts become quickly contagious when they are excreted in feces, after being ingested, cysts are digested in the stomach, where they grow into trophozoites that colonize the duodenal and proximal jejunal epithelium, motile trophozoites that have consumed the cysts emerge (excystation) and adhere to intestinal epithelial cells in the upper small intestine, where they multiply extracellularly. Giardia's life cycle is completed by creating cysts in the lower intestine area (encystation). infected people may experience frequent bouts of fatty diarrhea due to the parasite's ability to reproduce, although asymptomatic infections are also rather common (Rojas-López *et al*., 2022).

1.2.11.4 Pathogenesis of *Giardia lamblia:*

Pathogenesis and virulence are influenced by the genotype of the Giardia strain as well as the nutritional and immunological status of the recipient. So far,

eight genetic groups (A till H) have been discovered. It is widely accepted that assemblages of A and B strains cause human infections (Allain *et al*., 2019).

However, more recent information indicates that human pathogenic strains from assemblage E may also exist because these strains can be found in both humans and animals, giardiasis can be categorized as a zoonosis. giardiasis is a disease that is particularly dangerous for young children who live in unhygienic surroundings since it can be lethal when combined with malnutrition or immunosuppression. Additionally, children who have ongoing infections may have stunting (Rogawski *et al*., 2017).

G.lamblia sometimes referred to as *Giardia duodenalis* and *Giardia intestinalis*, is the most frequent cause of intestinal parasite infection in the first two years of life (Rogawski *et al*., 2017). *G.lamblia* may affect epithelial cells through cell-cycle arrest, proliferation impairment, tight-junction disruption, and apoptosis induction through direct or indirect mechanisms (Bartelt and Sartor, 2015).

Diarrheal symptoms mostly occur during the acute phase of the infection, and at least some of the effects of the infection appear to be isolate-dependent (Allain *et al*., 2017).

Giardiasis' acute phase pathophysiology is characterized by the absence of trophozoite invasion of the intestinal tissues and overt inflammatory cell infiltration, except for a slight increase in intraepithelial lymphocytes and mast cells (Allain and Buret, 2020).

These cells may be activated via Giardia arginine deaminase or through its metabolic product citrulline (Muñoz-Cruz *et al*., 2018).

Clinical signs of giardiasis often occur 1-2 weeks after infection. Giardiasis illness outcomes are modulated by a range of host and environmental variables,

like other infectious diseases. These include gut flora, age, immunological variables, nutrition, and concomitant illnesses (Barash *et al*., 2017).

G. lamblia infections can cause a variety of symptoms, such as frequent, fatty diarrhea, cramping in the abdomen, nausea, and wasting, but they can also be asymptomatic. Persistent infection may lead to alterations in the microbiota, villus blunting, leaky gut, nutritional malabsorption, and growth retardation. Food allergies, irritable bowel syndrome, and chronic fatigue syndrome are examples of postinfectious issues. In contrast to most other intestinal infections, giardia infection triggers substantial innate and adaptive immune responses but little to no inflammation (Jex *et al*., 2020).

Catalytic proteins, metabolic enzymes, surface molecules, and soluble mediators that bind to host cell receptors and extracellular matrix components are among the secreted molecules that play a virulent role in the cytotoxic effect on epithelial cells by *G.lamblia* excretory/secretory products (ESP), these parts are divided into five major categories: enzymes involved in energy metabolism, tenascins, toxins-like compounds, cysteine-rich surface proteins, and cysteine proteases (Ortega-Pierres and Argüello-García, 2019, Jex *et al*., 2020)

The wide variety of early symptoms, which can include diarrhea and weight loss as well as longer-term consequences including growth stunting in kids who did not exhibit any symptoms and a high incidence of postinfectious irritable bowel syndrome (IBS) (Adam, 2021).

1.2.11.5 Diagnosis of *Giardia lamblia* :

To properly diagnose this protozoan illness, a variety of diagnostic techniques should be used. The best laboratory test for giardiasis diagnosis is the flotation of zinc sulfate (Ortega-Pierres and Argüello-García, 2019), this approach works well when testing serial fecal samples and is a low-cost diagnostic test that can identify numerous parasite illnesses, the diagnostic

performance of an immunochromatographic (IC) test may be assessed using immunological techniques, particularly immunochromatography, although they are pricy, sensitive, and specific assays, It is quick, accurate, and convenient to diagnose amoebiasis, giardiasis, and cryptosporidiosis using the Rapid Immuno Card CERTEST *Entamoeba/ Cryptosporidium /Giardia*, when other techniques are not accessible, notably during a waterborne outbreak, it might be employed as a fast service test or in conjunction with other diagnostic modalities to confirm the diagnosis. when light microscopy produces unfavorable results, immunological tests should be applied. *G.lamblia* isolates may be molecularly characterized using PCR, which is crucial for epidemiological investigations. (Hanevik *et al*., 2017).

However, in the feces of asymptomatic carriers with low parasite burdens, PCR failed to identify mild infections (Rojas-López *et al*., 2022).

1.2.11.6 Virulence Factors Roles of *Giardia lamblia* in Immune - evasion strategies

The pathogenicity and trophozoite adhesion to host epithelial cells of *G.lamblia* is thought to be aided by the secretion of tiny extracellular, membrane-bound vesicles by the parasite, these immune-evasive effects will aid in the spread of infection and are hence crucial to pathogenesis. The impacts of this parasite's proteolytic activity may thus be used to simulate a large portion of the pathophysiological reactions that it causes (Gavinho *et al*., 2020). *G.lamblia* evades host inflammatory responses by producing antioxidants, cleaving interleukin-8 (IL-8), depleting arginine via arginine deaminase (ADI), and shifting variant surface protein (VSP) expression (Bartelt and Sartor, 2015).

G.lamblia has developed an escape strategy based on the diversity of these surface proteins since IgA detects them as major antigens. One (main) variation

surface protein (VSP) is expressed on a single trophozoite, it is generally accepted. Changes in the chromatin state and/or RNA interference are two epigenetic processes that cause the production of various VSPs, and hence antigenic variation. To circumvent the immunological reaction, trophozoites that survive exposure to IgA react by expressing several variations of these so-called "variant surface proteins, an antigenic switch is the name given to this tactic. The antigenic flip is most likely epigenetically controlled and can result from exposure to medicines as well as antibodies (Müller *et al*., 2019).

Variant Surface Proteins VSPs may actively take part in harming epithelial cells through proteolytic activities in addition to playing a passive role in evading host immune responses. There is another great summary of parasite tactics for avoiding host immune responses (Cabrera-Licona *et al*., 2017).

The role of Giardia excretory-secretory products (ESPs) in disease pathogenesis remains incomplete, exposure to host enterocytes activates *G.lamblia* trophozoites to modify the expression of a broad range of their genes and to release a variety of secretory compounds (Ma'ayeh *et al*., 2017).

Surface lectins and parasite proteins including giardin, variant-specific surface proteins (VSPs), tenascins, and secreted cysteine proteases are involved in the adhesion of trophozoites to the host's enterocyte (Dubourg *et al*., 2018).

Additionally, Giardia exploits antigenic variation (VSPs) and arginine deiminase, an enzyme that utilizes luminal arginine, a metabolite needed for intestinal epithelial cells to produce anti-Giardia nitric oxide (NO), to elude host immune responses. (Allain *et al*., 2019).

Indeed, *G.lamblia* cysteine proteases are one of the ESPs that significantly contribute to the pathophysiology of giardiasis that are membrane-bound as well as secreted have been linked to encystation and intestinal barrier dysfunction through the disruption of villin and apical junctional complexes in intestinal

epithelial cells, activation of apoptosis (i.e., caspase-3 activation and PARP-1 cleavage), cleavage of mucin and mucus depletion, breakdown of microbiota biofilm Giardia CPs also show significant immunomodulatory qualities that enable the parasite to elude the host's immune system and, as a result, influence local inflammatory responses, in addition to the functions played by VPSs and metabolic enzymes in Giardia's host immune evasion. recent research has shown that Giardia cysteine proteases, which are released as secreted proteins and include thiol proteinases and cysteine proteases (such as cathepsin B and cathepsin L), can degrade host immune factors like immunoglobins (IgG, IgA1, and IgA2), inflammatory mediators (CXCL1, CXCL2, CXCL3, CXCL8, CCL2, and CCL20), and host defensins (α-HD6, β-HD1, and TFF3) (Allain *et al*., 2019).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Equipment and Instruments

The table (2-1) below lists the instruments and equipment utilised in the current investigation, their manufacturer, and their place of origin.

Table 2-1: Laboratory Equipment and Instruments.

NO.	Equipment and Instruments	Company	Origin
1.	Automatic Micropipette	Slammed	(Germany)
2.	Centrifuge EBA 20	Hettich	(Germany)
3.	Cuvette in optical glass	Citoclase	China
4.	Cool Box	Cool Box	India
5.	Cover Glass	Microscopy	China
6.	Disposable specimen's dropper	Microscopy	China
7.	Disposable Syringe5 ml	Dolphin	UAE
8.	EDTA vacuum tube	Xinle	China
9.	ELISA Mixer	Denley	U. K
10.	ELISA Reader	Beckman	Germany
11.	ELISA Shaker	Jean Robin	France
12.	ELISA system	Biotek	USA
13.	ELISA Washer	Beckman	Germany
14.	Eppendorf tube (2.5) ml	Sterile	S. Korea
15.	Filter paper	Afco	(Jordan)
16.	Flat -head Vortex mixer	Elabascience	USA
17.	Gel tube	Afco	(Jordan)
18.	Graph paper	CALBIOTECH	Italia

19.	Gloves	Sempermed	Thailand
20.	Haemoglobin Analyzer H8	Lifotronic	China
21.	Incubator	Memmert	Germany
22.	K3EDTA tube	Dia Tech	(USA)
23.	Medical cotton	MAY	Turkey
24.	Micro ELISA Plate	Elabscience	USA
25.	Microscope	Olympus	Germany
26.	Microscope Slides	Abron export	India
27.	Microwave	Argose	Germany
28.	Multichannel pipettes	Human	Germany
29.	Rack	Afco	Jordan
30.	Screw cup tubes	Abron export	India
31.	Spectrophotometer	Humalyzer	(Germany)
32.	Sterile Plain tube	Dolphin	Syria
33.	Tips (different sizes)	Dolphin	China
34.	Timer	Citoclase	China
35.	Tourniquet for blood drawing	Yyfmed	China
36.	Vacuum gel tube	Xinle	China
37.	Water Bath	Memmert	Germany
38.	Wooden stick	Citoclase	China

2.1.2. Diagnostic Kits:

Table (2-2) lists the diagnostic kits utilized for this investigation alo, their manufacturers, and countries of origin.

Table 2-2: Diagnostic Kits used during this study

No	Kitts	Company	Origin
1-	Glucose	Human	Germany
2-	IL-2 ELISA Kit	Biont	China
3-	IL-10 ELISA Kit	Biont	China
4-	Prostaglandin E2 ELISA Kit	Biont	China
5-	Anti-GAD (Glutamic Acid Decarboxylase) ELISA	Euroimmun	Germany
6-	Anti ZnT8 (Zinc Transporter System) ELISA Kit	Bioassay	China
7-	Anti-ICA (Islets Cell Autoantibodies) ELISA Kit	Bioassay	China
8.	CERTEST Crypto+ Giardia+ Entamoeba, Immuno Chromatography (IC)	COMBO CARD TEST	Zaragoza (Spain)

2.2.Method

2.2.1. Ethical Approval and study design (6553):

The ethical approval in this study was achieved by:

1. The College of Medicine Ethics Committee at University of Babylon.
2. Karbala Health Directorate's ethical committees.
3. Control as well as written permission from each patient
4. This study was a case-control study conducted in College of Medicine at the University of Babylon in Iraq.

2.2.2. Questionnaire:

Questionnaires were designed to search from the children at Kerbala Teaching Hospital for Children and the Research Committee, taking the international and local standards into account, for collecting data from children with diabetic children and /or their parents. (Appendix I).

2.2.3. Clinical Specimens and Study Groups

The current study is a case-control study that involved 180 children 95 with diabetes mellitus,50 with intestinal parasitic infections and 35 apparently healthy controls aged (1-15) years participants came from Karbala Teaching Hospital for Children and Imam Al-Hassan Center for Diabetic and Endocrine Disease in Karbala province. Patients were selected with type 1 diabetes treated with insulin between February 2022 and December 2022. The practical portion was carried out in the laboratories of Karbala Teaching Hospital for Children.

2.2.3.1. Collection of blood specimens

Each healthy control subject and the patient had up to 5mL of venous blood drawn using plastic disposable syringes to puncture veins. RBS and HbA1c detection required the addition of 2 ml to the EDTA tube. The gel tube is filled with the leftover blood (3 mL), which is then kept at room temperature for 30 minutes to start the clotting process. The serum was then separated from the specimens by centrifuging it at 3,000 (round/minute) for (15) minutes. The sera were kept at (-20 ºC) until the IL-2, IL-10, prostaglandin levels, anti-GAD, anti-ZnT8 (Zinc Transporter System), and anti-ICA tests were performed (Hu *et al.*, 2014).

2.2.3.2. Collection of stool specimens

Patients with diabetes who had abdominal pain and diarrhea with intestinal parasitic infections, as well as from non-diabetic kids in the same age range (1 -

15) years, a stool specimens was taken in a sterile screw cap for microscopic examination of stools to identify the etiologic agents and confirm the diagnosis using Rapid CERTEST strips for *Cryptosporidium parvium + Giardia lamblia + Entamoeba histolytica*, Immuno Chromatography (IC) (Bahadır and Sezgintürk, 2016).

2.2.3.2.1. General Stool examination GSE:-

Smears with normal saline and/or Lugol's iodine were used to test each stool specimens for *Entameba histolytica* and *Giardia lamblia.* Under low-power and high-power microscopes, the smear was extensively studied, the current work has employed a modified Ziehli-Neelsen stain to identify cryptosporidium oocysts. Slides were inspected with a high-power lens and a drop of oil under microscopy (Hu *et al.*, 2014).

2.2.4 Patient Group

One Hundred Forty-Five patients (70 Diabetic Patients,50 with Intestinal Parasitic infection, and 25 Mixed) in the current study (77 male and 68 female, 1 to 15 years old) 70 patients and 25 Mixed were diagnosed with type 1 diabetes by a consultant doctor based on clinical signs and laboratory findings and 50 patients diagnosed with intestinal parasitic infections depending on clinical manifestation and laboratory diagnosis by General Stool Examination.

2.2.5 Control Group

Thirty-five participants who had normal FBS and HBA1C results as well as negative results from a general stool examination made up this group (20 men and 15 women, ages (1 - 15) years.

2.2.6 Inclusion and Exclusion Criteria:

All patients had to meet the inclusion requirements:

1- Having T1DM and intestinal parasitic infections.

2- Relative individuals were allowed to participate.

The excluded Criteria:

1- Any patient presented with any kind of tumor or malignancy.

2- Acute inflammation rather than Amoebiasis and Giardiasis.

3- Anti-inflammatory drugs were used to treat the patient.

4- Other different autoimmune conditions.

2.2.7 The Steps of the Present Study

The study steps of the present work are illustrated in Figure (2-1).

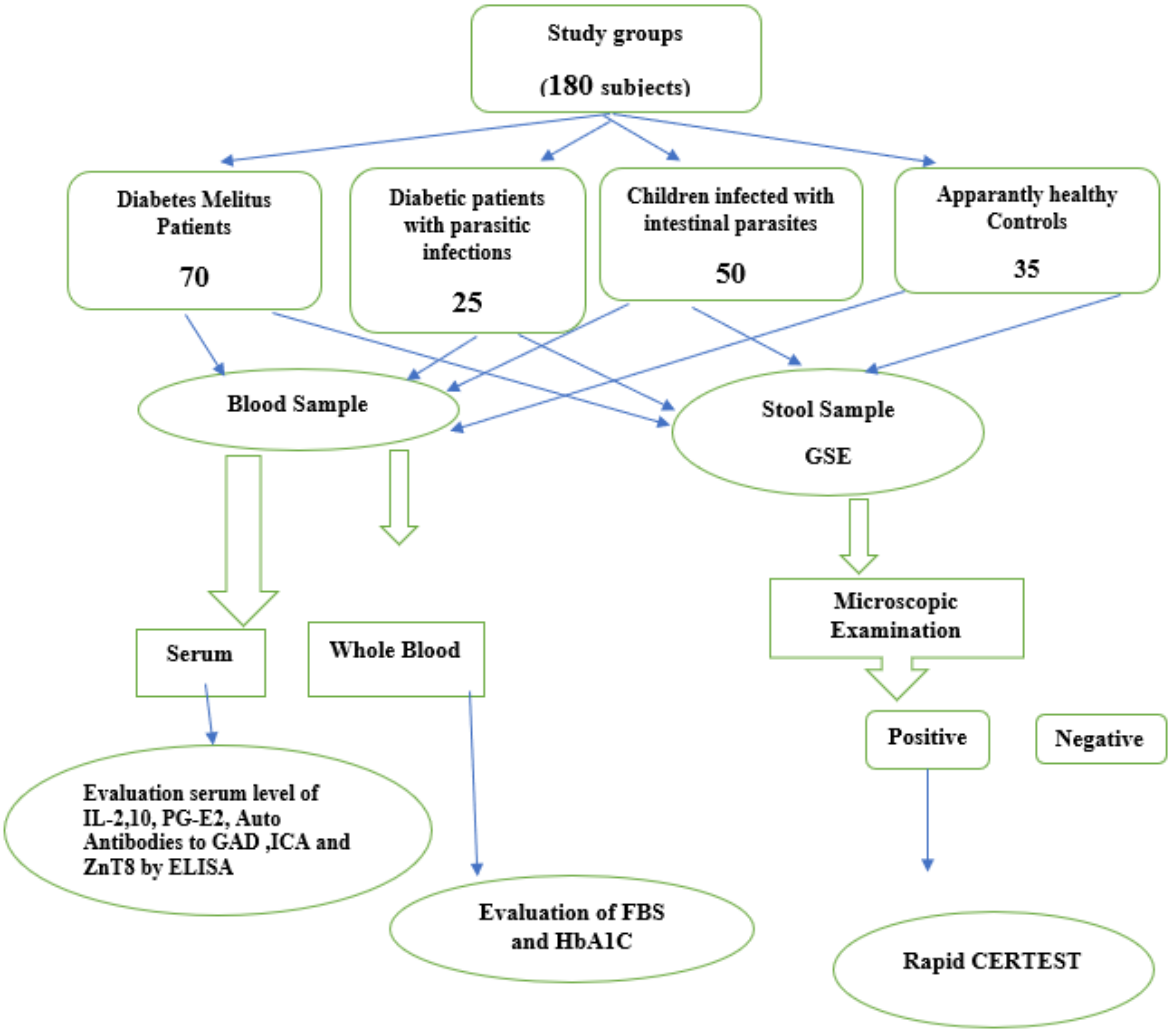

Figure 2-1: The Steps of the Study

2.2.8 Diagnostic Study Tests

2.2.8.1 CERTEST *Crypto+Giardia +Entamoeba* COMBO CARD TEST, Zaragoza(Spain) Immuno Chromatography (IC) Assay

The Triage parasite panel is a new qualitative enzyme immunoassay (EIA) panel for the detection of *Giardia lamblia* (α1-giardin), *Entamoeba* (*histolytica/E. dispar*), and *Cryptosporidium parvum* in fresh or frozen, unfixed human fecal specimens. to examine the parasite, a triage immunochromatography assay was used to identify the three types of parasite.

A. Assay Principle:

This technique is called a lateral flow assay (LFA) and is composed of a chromatographic system (separation of components of a mixture based on differences in their movement through reaction membrane) and immunochemical reaction (between antibody-antigen It is based on the movement of the specimens across the membrane via capillary force.is composed of four parts: a sample pad, which is the area on which the specimens is dropped; a conjugate pad, on which labeled tags are combined with biorecognition elements; a reaction membrane (usually nitrocellulose membrane pre-coated with monoclonal antibodies on the test line T) containing test line and control line for target antigen-antibody interaction; and absorbent pad, which reserves waste (Singh *et al*., 2015, Hu *et al*., 2014). For the construction of LFAs gold nanoparticles, colored latex beads, carbon nanoparticles, quantum dots, and enzymes are used as a label for increasing the sensitivity.(Bahadır and Sezgintürk, 2016, Mao et al., 2013).

B. Specimen preparation :

- The stick was used to pick up a sufficient specimens quantity.
- The specimens was added to the stool collection tube (for liquid specimenss, add 125 µl in the stool collection tube using a micropipette).

- The tube was closed with the diluent and stool specimens. Shaking the tube to ensure good sample dispersion

C. Test Procedure:

1- The stool collection tube was shaken to ensure good sample dispersion.

2- The sealed bag was removed before using it.

3- The stool collection tube was taken, the end of the cap was cut, and pour three drops into the circular window with the letter A, three drops into the window with the letter B, and three drops into the window with the letter C.

4- The result was read for 10 minutes.

D. Interpretation of the results:

Strip A consists of a nitrocellulose membrane pre-coated with monoclonal antibodies against *Cryptosporidium parvium*.

Strip B consists of a nitrocellulose membrane pre-coated with monoclonal antibodies against *Giardia lamblia*.

Strip C consists of a nitrocellulose membrane pre-coated with monoclonal antibodies against *Entamoeba histolytica* as shown in Figure (2-2).

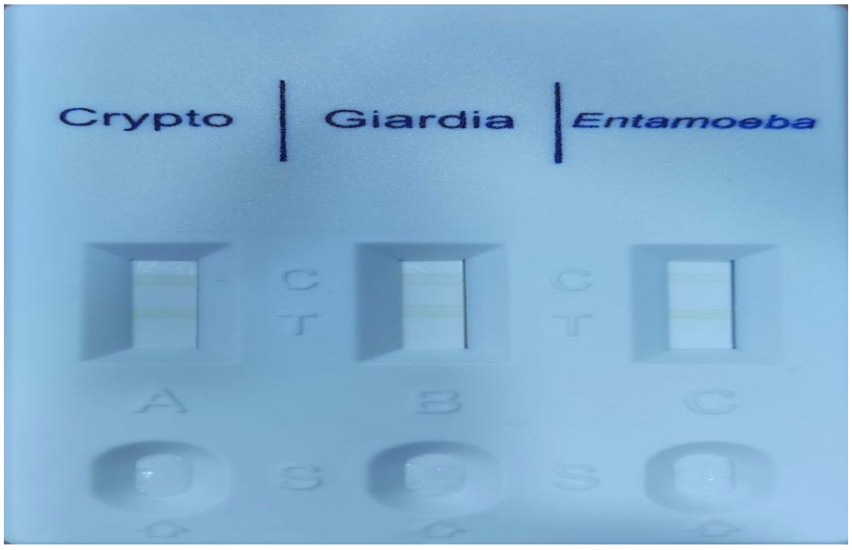

Figure 2-2: The Triage Parasite Panel

2.2.8.2. Biochemical Test for Measurement of Fasting and Random Blood Glucose Level.

After enzymatic oxidation in the presence of glucose and oxidase, glucose levels were measured. Under the catalysis of peroxidase, the generated hydrogen peroxide combines with phenol and 4-amino antipyrine to produce a red-violet quinone imine dye that serves as an indicator.

The reference range for normal blood (70–100 mg/dl) (Martinov and Fife, 2020).

2.2.8.3 Detection of Glycated Hemoglobin HbA1c

It's a reliable marker to indicate the control of blood glucose levels in a whole blood specimens (EDTA Tube) in the past three months. H8 Analyzer adopts the High-Performance Liquid Chromatography (HPLC) method to measure the level of HbA1ab, HbA1c, and HbA0. The Analyzer calculates the rest items. Chromatography is used to separate proteins, nucleic acids, or small molecules in complex mixtures. Liquid chromatography can be used for analytical or preparative applications. (Horváth, 2013, Abaza, 2020)

A. Assay Principle of High-Performance Liquid Chromatography(HPLC):

The term chromatography is employed to describe a wide variety of separation techniques but all of these techniques share the same principle, this principle can be described as the difference in interaction properties of the analyte to two phases.

One of these phases is relatively static and called the stationary phase and the other is relatively mobile and called the mobile phase. All the chromatography types that have a liquid mobile phase are called liquid chromatography or LC (Horváth, 2013).

2.2.8.4 Immunological Tests

Several tests were included in this study especially immunological tests involving IL-2, IL-10, Prostaglandin -E2 (PG-E2), GAD, ZnT8, and ICA. Collected blood specimenss of patients infected with *Entamoeba histolytica* and *Giardia lamblia* and healthy controls (70 DM1,50 Parasitic,25 DM1, and Parasitic infections, and 35 healthy controls).

2.2.8.4.1 Measurement of interleukin-2, interleukin-10 and Prostaglandin-E2.

Using the ELISA method, the levels of interleukin-2, interleukin-10, and prostaglandin in the serum of patients and controls were determined. The IL-2, IL-10, and PG-E2 kits all employ the same test techniques and share the same components, and all reagents are ready to use. Thus, to prevent repetition, state them in one paragraph.

A. Assay Principle of interleukin - 2, interleukin-10 and Prostaglandin PG-E2 assay.

Direct Enzyme-Linked Immunosorbent Assay (ELISA) is the name of immunoassay in which looking for IL-2, IL-10, and PG-E2 antigens. The plate has been pre-coated with antibodies against human IL-2, IL-10, and PG-E2. The relevant Micro-ELISA strip plate wells are filled with standards or samples and then the particular antibody is added. The samples are introduced and bind to antibodies that have been coated on the wells. Then human IL-2, IL-10, and PG-E2 antibody that has been biotinylated is added, and it binds to the samples. The biotinylated IL-2, IL-10, and PG-E2 antibodies are then bound by the addition of streptavidin-HRP. After incubation, the washing procedure removes unbound Streptavidin-HRP. A substrate solution (Chromogen A+ Chromogen B) is then added and color develops in proportion to the amount of human IL-2, IL-10, and

PG-E2. The reaction is terminated by the addition of an acidic stop solution and absorbance is measured at 450 nm. The optical density OD value is proportional to the concentration of IL-2, IL-10, and Prostaglandin-E2. The calculation of the concentration of IL-2, IL-10, and Prostaglandin-E2 in the samples by comparing the OD of the samples to the standard curve.

B. Reagent Provided: The components of the reagent used in the present study are shown in Table (2-3)

Table 2-3: Kit Components, of interleukin, - 2 IL-10, and Prostaglandin have the same constituents

No.	Item	Specifications (96 T)
1-	Coated ELISA Micro-plate	8×12 (1)
2-	Standard：2400 ng/L for IL-2 Standard：640 ng/ml for IL-10 Standard：2400 pg/ml for Prostaglandin	0.5ml×1 bottle 0.5ml×1 bottle 0.5ml×1 bottle
3-	Standard Dilution	3ml×1 bottle
4-	Anti-IL-2 antibodies labeled with biotin Anti-IL-10 antibodies labeled with biotin Anti-PG-E2 antibodies labeled with biotin	1ml×1 bottle 1ml×1 bottle 1ml×1 bottle
5-	Chromogen Solution A	6ml×1 bottle
6-	Chromogen Solution B	6ml×1 bottle
7-	Streptavidin-HRP	6ml× 1bottle
8-	wash solution	20ml (30X)×1bottle
9-	Stop Solution	6ml×1 bottle
10-	Sealed bags	1
11-	Seal plate membrane	2
12-	Instruction	1

C. Assay Procedure

1. All reagents, standard solutions, and serum were Prepared as instructed at room temperature before use.

2. The number of wells required was determined for the assay. The wells were inserted in the frames for use. The unused wells should be stored at 2-8°C.

3. 50µl of the standard was added to the standard well.

4. 40µl sample was added to sample wells and then add 10µl anti- IL-2, IL-10 and PG-E2 antibody to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells. Mix well. the plate was covered with a sealer. Incubate for 60 minutes at 37°C.

5. The dish was washed five times with wash buffer after the sealer was removed. For every cleaning, soak wells in a minimum of 0.35 ml of wash buffer for 30 seconds to 1 minute. For automatic washing, aspirate every well before washing it five times with wash buffer while overfilling each well. Place paper towels or another absorbent substance nearby to blot the plate.

6. 50µl of substrate solution A was added to each well and then 50µl substrate solution B was added to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. 50µl of Stop Solution was added, and the blue colour will change into yellow immediately.

8. Determine the optical density (OD value) of each well immediately using a microplate reader at 450 nm within 10 minutes after adding the stop solution.

1. Dilution of Standards

For IL-2

	Concentration	Dilution
Standard No.5	1200 ng/L	120 µl Original Standard + 120µl Standard diluent
Standard No.4	600 ng/L	120 µl Standard No.5+ 120 µl Standard diluent
Standard No.3	300 ng/L	120 µl Standard No.4+ 120 µl Standard diluent
Standard No.2	150 ng/L	120 µl Standard No.3 120 µl Standard diluent
Standard No.1	75 ng/L	120 µl Standard No.2+ 120 µl Standard diluent

For IL-10

	Concentration	Dilution
Standard No.5	320 ng/ml	120 µl Original Standard + 120µl Standard diluent
Standard No.4	160 ng/ml	120 µl Standard No.5+120 µl Standard diluent
Standard No.3	80 ng/ml	120 µl Standard No.4+ 120 µl Standard diluent
Standard No.2	40 ng/ml	120 µl Standard No.3+ 120 µl Standard diluent
Standard No.1	20 ng/ml	120 µl Standard No.2+ 120 µl Standard diluent

For Prostaglandin PG-E2

	Concentration	Dilution
Standard No.5	1200 pg/ml	120µl Original Standard + 120µl Standard diluent
Standard No.4	600 pg/ml	120µl Standard No.5+ 120µl Standard diluent
Standard No.3	300 pg/ml	120µl Standard No.4+ 120µl Standard diluent
Standard No.2	150 pg/ml	120µl Standard No.3+ 120µl Standard diluent
Standard No.1	75 pg/ml	120µl Standard No.2+ 120µl Standard diluent

2. Sample preparation

1. 40µl samples were added in sample wells without touching with gentle shaking.

2. Then 10 µl of Biotinylated human Antibody was added.

3. 50 µl of Streptavidin-HRP was added.

4. The plate was sealed by a Closure plate membrane and then incubated for 60 minutes at 37 °C.

5. The Closure plate membrane was carefully peeled off, and the plate was washed 5 times for 30 seconds by Wash Buffer

3. Coloring:

Fifty µl Chromogen Solution A and Fifty µl Chromogen Solution B were added to each well, by gently shaking and then the plate was covered and incubated at 37°C in the dark for 10 minutes.

4. Termination:

Fifty µl Stop Solution was added to each well and mixed thoroughly. The color was changed from blue to yellow.

5. The optical density (O.D) was read using microplate reader at a wavelength 450 nanometers (nm)

D. Interpretation of results:

The standard curve of IL-2, IL-10, and PG-E2 was constructed by blotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing a through the points on the graph, these calculations can be best performed with computer-based curve-fitting software and the best-fit line can be determined by regression analysis. The curves are shown in Figures (2 – 3), (2 – 4), and (2 – 5) respectively.

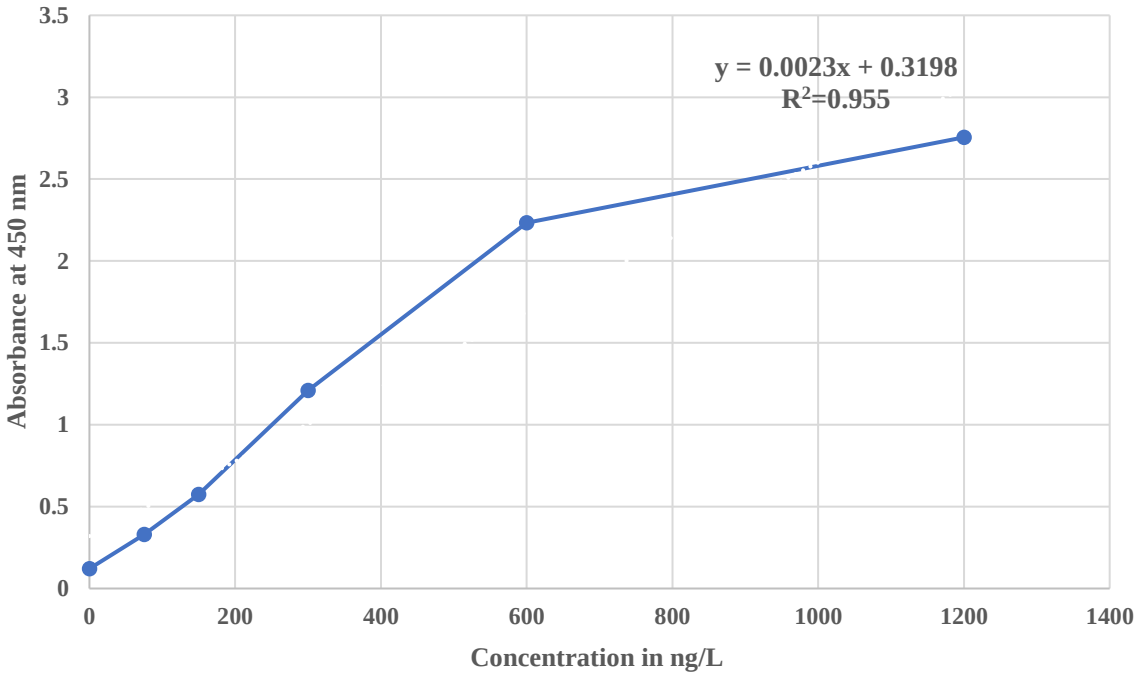

Figure 2-3: Standard Curve for IL-2

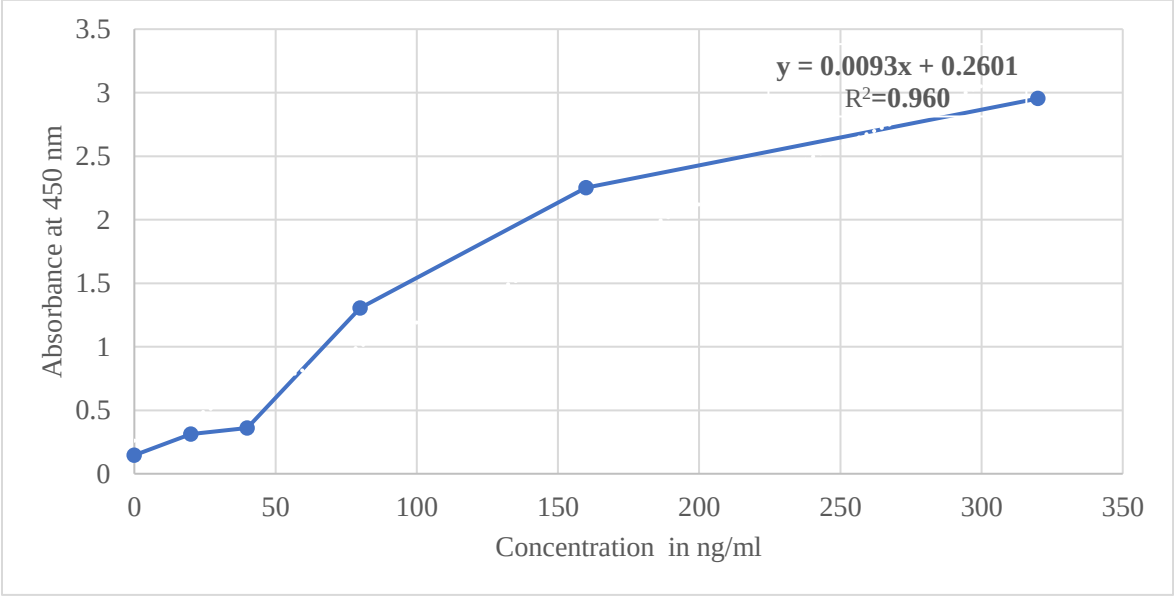

Figure 2-4: Standard Curve for IL-10

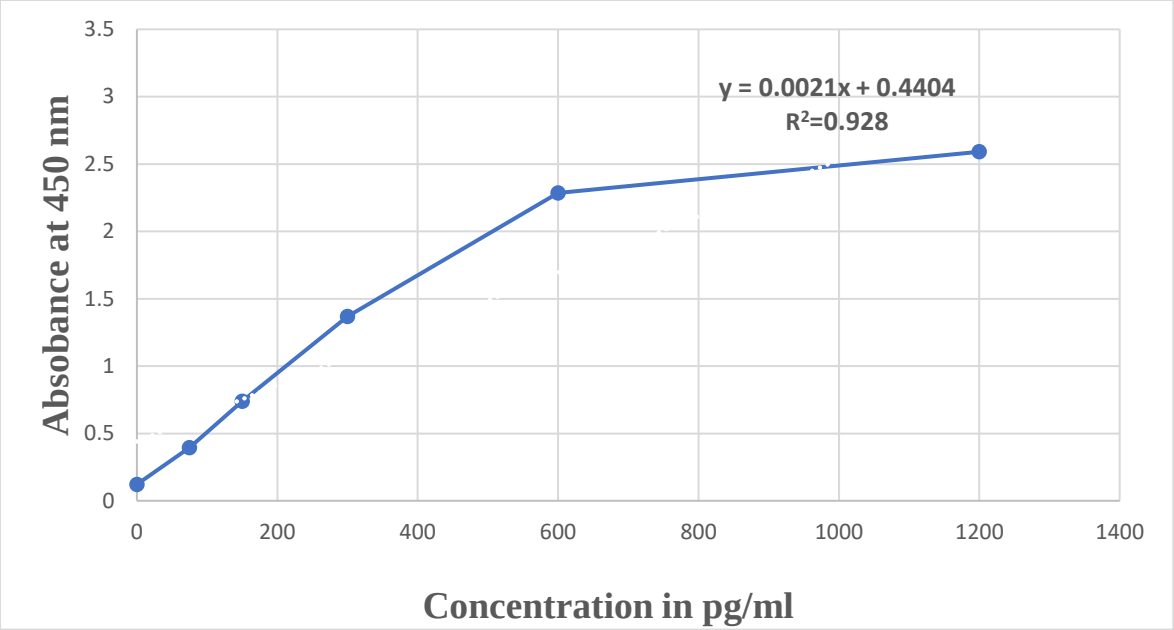

Figure 2-5: Standard Curve for PG-E2

2.2.8.4.2 Determination of the serum levels of Anti-GAD (Glutamic Acid Decarboxylase) IgG:

Following the manufacturer's instructions, EUROIMMUN Human GAD ELISA kits from Medizinische Labor diagnostika AG (Lubeck, Germany) were used to quantitatively assess the levels of GAD IgG in the serum of the patients and control participants. the specifics of the kit's parts as well as the reagents and solutions utilized are listed in Table (2-4)

A. Assay principle of the GAD test kit:

This kit offers an indirect ELISA technique-based semi-quantitative or quantitative in vitro test for human IgG class antibodies against GAD. GAD antigen-coated microplate wells. Patient samples are incubated in the sample's positive wells in the first reaction phase, where particular antibodies bind to the GAD. A bridge between the GAD on the reagent wells and the biotin-labeled GAD that is introduced during the second incubation stage can be created by bound antibodies acting divalently. A third incubation is performed utilizing

enzyme-labeled avidin (enzyme conjugate), which catalyzes a colour reaction, to detect the bound biotin. The quantity of antibodies against GAD is proportionally correlated with the intensity of the colour that results.

B. Reagent Provided

The components of the reagent used in the present study are shown in Table (2-4)

Table (2-4) Anti-GAD (Glutamic Acid Decarboxylase) IgG: EA1022-9601 G Content of the test kit:

NO.	Component	Format\Description
1.	Microplate wells coated with antigen	12×8 strips
2.	Calibrator 1to 6 2000 \250\120\35\15\5 IU\ml (IgG, human).	6×0.7 ml
3.	Negative control 0 IU\ml	1×0.7 ml
4.	Positive control (IgG, human).	1×0.7 ml
5.	GAD (biotin-labeled GAD)	3×5.5 ml
6.	GAD buffer	2×15 ml
7.	Enzyme conjugate (peroxidase-labeled avidin , 20X concentrated)	1×0.7 ml
8.	Conjugate buffer	1×15 ml
9.	Wash buffer 10 ×concentrate	1×125 ml
10.	Chromogen \substrate solution TMB\H2O2	1×15 ml
11.	Stop solution 0.25M sulphuric acid	1×12 ml
12.	Plastic foil	3 pieces
13.	Quality control certificate	1 protocol
14.	Test instruction	1 booklet

C. Assay procedure for measuring serum Anti-GAD IgG antibodies:

1. 25 µl of calibrator, positive control, negative control, and patient samples serum were dispensed into appropriately labeled wells of a microwell plate.
2. The frame was covered and incubated for I hour at room temperature.
3. The plate was then cleaned in an automatic plate washer using the originally prepared Wash Buffer 1x. The plate's contents were fully disposed of by tapping it on absorbent paper with the openings facing downward after three rounds of washing.
4. 100 µl of the GAD (biotin-labeled GAD) was added to each well. The plate was then incubated at room temperature for 1 hour.
5. After incubation, 3 cycles of washing were executed as described above.
6. 100 µl of the Enzyme Conjugate (peroxidase-labeled avidin) was added to each well. The plate was then incubated at room temperature for 20 minutes.
7. After incubation, 3 cycles of washing were executed.
8. Then 100 µl of chromogen/ Substrate Solution was added to each well and the plate was incubated at room temperature in the dark for 20 minutes.
9. After incubation, 100 µl of Stop Solution was quickly added to each well, the plate was lightly shacked to ensure a homogenous distribution of the solution.
10. Using a microplate reader and a reference wavelength of 450 nm, the wells' photometric values were measured.

D. Calculation of results:

By "point-to-point" plotting the extinction values (OD) measured for the 6 calibrators against the equivalent units (linear/linear) for calculation of the standard curve, the quantity of anti-GAD IgG antibodies in the test and control samples can be assessed. The upper limit of the normal range for the Euro-Immun assay, as in figure (2-6) 10 relative units (IU/ml), was used for

quantitative interpretation of the findings. The interpretation range as recommended by Euroimmun is; —Positive for ≥ 10 IU/ml and —negative —for < 10 IU/ml.

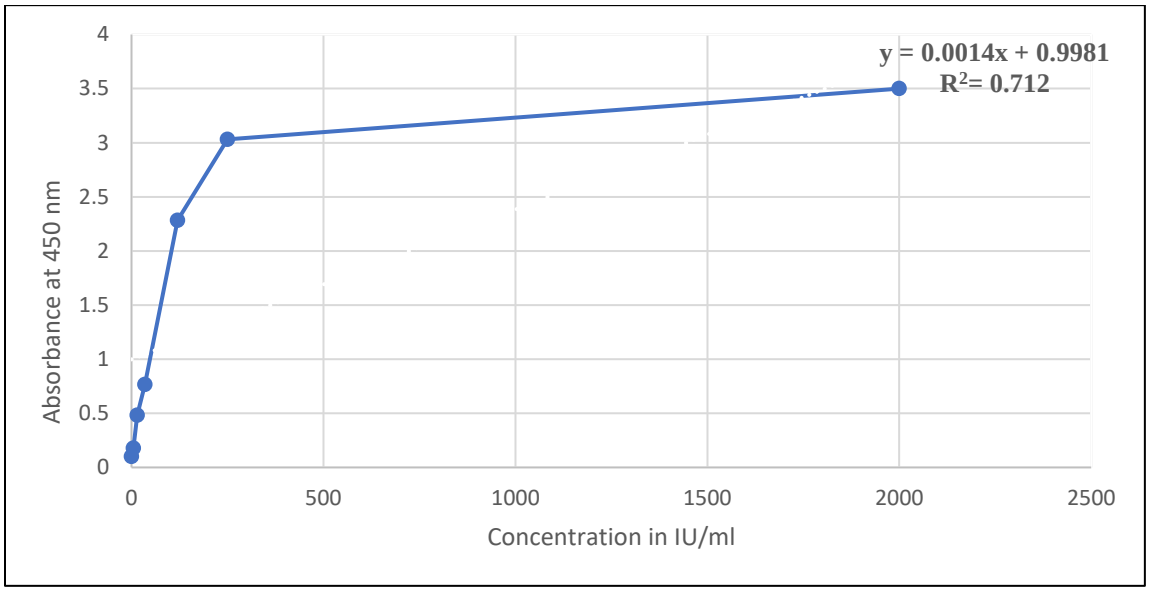

Figure 2-6: Standard Curve for Glutamic Acid Decarboxylase

2.2.8.4.3 Determination of the serum levels of Human Zinc Transporter 8 ELISA Kit:

Following the manufacturer's instructions to the letter, the Bioassay Technology Laboratory ZnT8 ELISA kits (China) This sandwich kit is for the accurate quantitative detection of Human zinc transporter 8(also known as ZNT8) in the serum of the test and control participants. the kit's parts as well as the reagents and solutions employed in Table (2-5).

A. Human Zinc Transporter 8 ZnT8 principle:

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA), The plate has been pre-coated with Human ZnT8antibody. ZnT8 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human ZnT8 Antibody is added and binds to ZnT8 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated ZnT8 antibody. After incubation, unbound

Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of HumanZNT8. The reaction is terminated by the addition of an acidic stop solution and absorbance is measured at 450 nm.

B. Reagent Provided:

The components of the reagent used in the present study are shown in Table (2-5)

Table (2-5) Human zinc transporter 8 ELISA Kit ZnT8 Cat.No E3428Hu Content of the test kit:

NO.	Component	Quantity(96T)
1.	Standard Solution (320 U/ml)	0.5ml x1
2.	Pre-coated ELISA Plate	12 * 8 well stripsx1
3.	Standard Diluent	3mlx1
4.	Streptavidin-HRP	6mlx1
5.	Stop Solution	6mlx1
6.	Substrate Solution A	6mlx1
7.	Substrate Solution B	6mlx1
8.	Wash Buffer Concentrate (25x)	20mlx1
9.	Biotinylated Human ZNT8 Antibody	1mlx1
10.	User Instruction	1
11.	Plate Sealer	2pics
12.	Zipper bag	1pic

C. Assay Procedure:

1. Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. The number of strips required for the assay was determined. the strips were inserted in the frames for use. The unused strips should be stored at 2-8°C.

3. 50µl of the standard was added to the standard well.

4. 40 µl of the sample was added to the sample wells, followed by the addition of 10 µl of anti-ZnT8 antibody and 50 µl of streptavidin-HRP to the sample wells and standard wells. (Not blank control well). Mix thoroughly. Apply a sealant to the plate. At 37 °C, incubate for 60 minutes.

5. Remove the sealer, then use a wash buffer to wash the plate five times, soak wells in 300ul of wash buffer for 30 to 60 seconds. Aspirate each well for automatic washing, then use the wash buffer five times. paper towels or another absorbent substance nearby were placed to blot the plate.

6. Each well received 50 µl of substrate solution A before receiving 50 µl of substrate solution B plate should be incubated for 10 minutes at 37 °C in the dark.

7. Each well received 50 µl of Stop Solution; the blue colour instantly turned yellow.

8. Within 10 minutes of adding the stop solution, measure the optical density (OD value) of each well using a microplate reader at 450 nm.

Reagent Preparation

- The reagents should be brought to room temperature before use.
- Standard Reconstitute the 120µl of the standard (320U/ml) with 120µl of standard diluent to generate a 160 U/ml standard stock solution. Allow the standard to sit for 15 minutes with gentle agitation before making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (160 U/ml) 1:2 with standard diluent to produce 80 U/ml, 40 U/ml, 20 U/ml, and 10 U/ml solutions. Standard diluent serves as the zero standard (0 U/ml). Any remaining solution should be frozen at -20°C and used within one month. The dilution of standard solutions suggested is as follows:

Standard No.	Concentration	Dilution
Standard No.5	**160 U/ml**	120 µl Original Standard + 120µl Standard diluent
Standard No.4	**80 U/ml**	120 µl Standard No.5+120 µl Standard diluent
Standard No.3	**40 U/ml**	120 µl Standard No.4+ 120 µl Standard diluent
Standard No.2	**20 U/ml**	120 µl Standard No.3+ 120 µl Standard diluent
Standard No.1	**10 U/ml**	120 µl Standard No.2+ 120 µl Standard diluent

Wash Buffer Dilute 20 ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

D. Interpretation of results:

A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawing a best-fit curve through the points on the graph. These

calculations can be best performed with computer-based curve-fitting software and the best-fit line can be determined by regression analysis as in Figure (2-7).

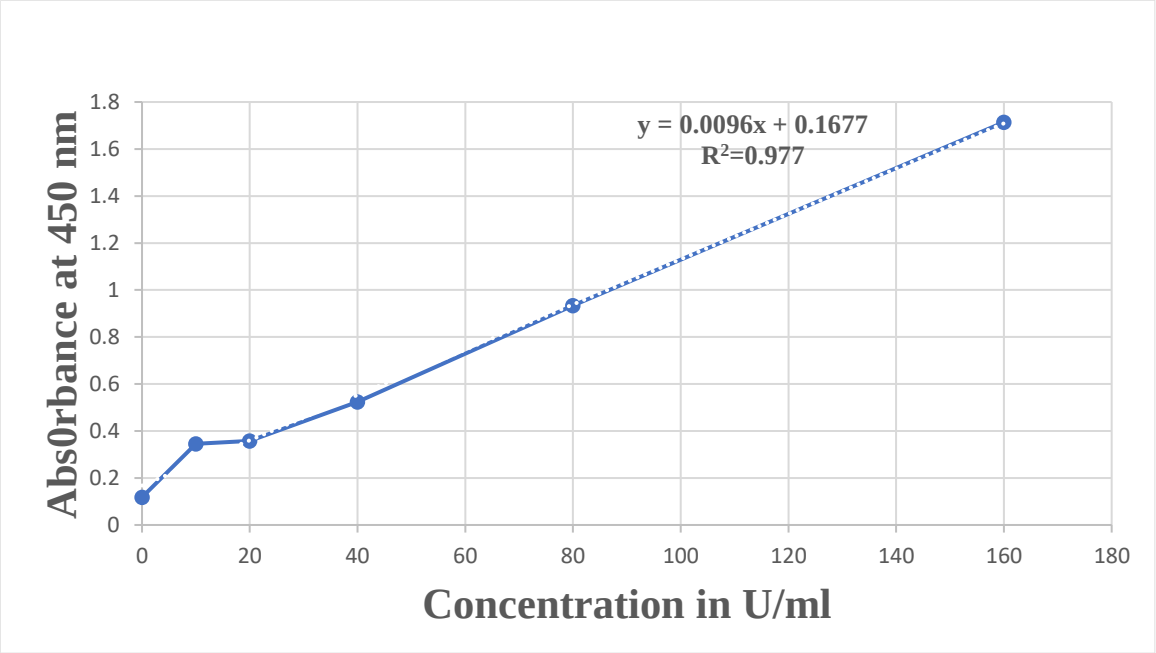

Figure 2-7: Standard Curve for Zinc Transporter 8

2.2.8.4.4 Determination of Human Islet Cell Antibody, ICA ELISA Kit

The Laboratory for Bioassay Technology ICA With careful respect to the manufacturer's instructions, ELISA kits (China) was used to semi quanitatively assess the levels of ICA in the serum of the test and control participants. the specifics of the kit's parts as well as the reagents and solutions employed in Tables (2-6).

A. Assay Principle of ICA :

This kit is based on a semi-quantitative enzyme immunoassay technique by indirect ELISA. the microtiter plate has been pre-coated with a target antigen. Positive/Negative Controls or samples are added to the wells and incubated. Antibodies in the samples bind to the antigen on the plate. Unbound antibody is washed away during a washing step. a Horseradish Peroxidase (HRP)

conjugated detection antibody is then added and incubated. Unbound HRP is washed away during a washing step. TMB substrate is then added and colour develops. The reaction is stopped by the addition of an acidic stop solution and colour changes into yellow which can be measured at 450 nm. The OD of an unknown sample can then be compared to the OD of the positive and negative controls to determine the presence of islet cell antibody ICA.

C. Reagent Provided:

The components of the reagent used in the present study are shown in Table (2-6)

Reagents Preparation

- All reagents should be brought to room temperature before use.
- **Wash Buffer 25x** Dilute 25x wash buffer with distilled water to yield 500 ml of 1x wash buffer. If crystals have formed in the concentrate, gently mix until all crystals have been fully dissolved. Dilute the Wash Buffer Concentrate 1:25 with reagent-grade water to obtain 500 ml of 25x wash buffer.

Table (2-6) Human Islets Cell Antibody ELISA Kit ICA Cat.NO ED0360Hu Content of the test kit:

NO.	Component	Quantity(96T)
1.	Pre-coated ELISA Plate	12 * 8 well strips x1
2.	Negative Control	0.5ml × 1 vial
3.	Positive Control	0.5ml × 1 vial
4.	HRP Conjugated	6ml × 1 vial
5.	Stop Solution	6mlx1
6.	Substrate Solution A	6mlx1
7.	Substrate Solution B	6mlx1
8.	Wash Buffer Concentrate (25x)	20mlx1
9.	Sample Diluent	6ml × 1 vial
10.	User Instruction	1
11.	Plate Sealer	2pics
12.	Zipper bag	1pic

C. Assay Procedure

1. All reagents, standard solutions, and samples were prepared as instructed at room temperature before use. The assay is performed at room temperature.

2. the number of strips required for the assay was determined and inserted the strips in the frames for use. The unused strips should be stored at 4°C for up to one month.

3. A blank well was set without any solution.

4. 50µl of negative control was added to each of the negative control wells and 50 µl positive control to each of the positive control wells. Then 40µl sample

diluent was added and then 10µl of the sample was added to the sample well, and mixed.

5. A plate was covered with sealer, and incubated for 30 minutes at 37°C.

6. The sealer was removed and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 60 seconds for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

7. 50µl of HRP was added to each well (except the blank well). Cover with a plate sealer, and incubate for 30 minutes at 37°C.

8. The sealer was removed and washed as described above.

9. 50µl of substrate solution A was added to each well and then 50µl substrate solution B was added to each well. Mix well. Incubate plate covered with a new sealer for 10 minutes at 37°Cin the dark.

10. 50µl of stop solution was added to each well, and the blue colour changed into yellow immediately.

11. Determine the optical density (OD value) of each well immediately using a microplate reader at 450 nm within 15 minutes after adding the stop solution.

D. Calculation of Results:

Average the reading samples. for calculation of the valence of the Human islet cell antibody, ICA, compare the sample well with the control.

Quality Control

- The average OD positive≥1.00
- The average OD negative ≤0.10 (OD: Optical Density)

Results

- **Cut-off Value = average Negative Control value + 0.15.**
- **While OD sample < Cut off Value: Negative.**

  While OD sample ≥ Cut off Value: Positive

Semi-quantitively Result= OD sample/ Cut-off Value

2.2.9 Statistical analysis:

Data was introduced into IBM SPSS (version 24.0) (San Diego, California, USA), and the figure was constructed by Microsoft EXCEL program 2019, a statistical analysis of this case-control study was conducted. All numerical or quantitative data were presented as an (m ± SD) graph. The lower degree of statistically relevant variance is generally acknowledged to be 0.05 or less. An independent student's t-test and one-way ANOVA Test were employed to find a statistically significant connection between the interleukins and serum autoantibodies of the test and control samples. The Chi-square test was used to examine qualitative variables, while quantitative data were presented as numbers (No.) and percentages. The link between two or more related quantitative variables was discovered using Pearson correlation analysis.

*Statistics were considered significant with a p-value of 0.05 or below.

Chapter Three
Results and discussion

3. Results and Discussion

3.1 Sex and Age Distribution of Study Groups

A total of one hundred eighty cases were divided into four groups (70 T1DM, 50 parasitic infections, 25 mixed (diabetes with parasites), and 35 normal as control groups cases at age1-15 years), there were 98 (54%) cases were males and the other 82 (46%) of cases were females. There were no significant differences between male and female distributions by Chi-square test for variables at (P=0.233, P≥0.05) as shown in Table (3-1)

Table (3-1): Age and Sex distribution among study's groups

Characteristics	Study's groups				Total	P-value
Sexes	**Diabetes**	**Parasitic**	**Mixed**	**Controls**	98(54%)	**0.233 NS ¥**
Males	33(48%)	29 (58%)	15(60%)	21 (60%)		
Females	37(52%)	21 (42%)	10(40%)	14 (40%)	82(46%)	
Total No.	70	50	25	35	180(10%)	
Age groups (Years)						
≥ 5	8 (11%)	25 (50%)	5 (20%)	17 (48%)	55 (30%)	**0.000 HS ANOVA**
6-10	39 (56%)	19 (38%)	18(72%)	16 (45%)	92 (52%)	
11-15	23 (33%)	6 (12%)	2 (8%)	2 (7%)	33 (18%)	
Total No.	70	50	25	35	180 (100%)	

(No: Number of cases; ¥: Chi-square test ; ANOVA: One way Analysis Of Variance; NS: Non-Significant at p ≥ 0.05; HS: Highly Significant at p ≤ 0.05

There was no significant correlation between sex and other parameters in the study's groups as in Table (3-2).

Table (3-2): The correlation of Sex with other parameters in the Study's groups.

Parameters	R^2Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Age	0.173	0.064	- 0.124	0.137
IL-2(ng/l)	- 0.122	- 0.054	- 0.023	- 0.339
IL-10(ng/ml)	0.128	0.117	0. 084	0.009
PG-E2(pg/ml)	- 0.035	- 0.122	0 .235	- 0.085
GAD(IU/ml)	0.336	- 0.073	0.200	0.207
ZNT8(U/ml)	- 0.067	- 0.147	- 0.080	- 0.321
ICA	- 0.222	- 0.056	0.012	- 0.213
(-): Negative Correlation, R^2: Pearson Correlation Test				

In T1DM males 33(48%),37(52%) females and Mixed patients15(60%) males,10(40%) females, there are no significant differences in males and females but the number of females is slightly more than males that consistence with Turtinen *et al*. (2018) who certify the immunologic aggressiveness of the disease is more variable and varies between sexes with a higher frequency in girls.

Szablewski, (2014) Sanhueza *et al*. (2019) mentioned that T1DM was higher for the female gender, when a few high-risk HLA-DR and HLA-DQ subtypes were present, while it was inversely related to the age of the first appearance of positive autoantibodies. Girls seemed to have poorer glycemic control and signs of a more severe metabolic derangement at the diagnosis of the

disease. Boys, however, more often tested positive for three of four biochemical autoantibodies (IAA, IA-2A, and ZnT8A) while only GADA positivity was more common in girls (Turtinen, 2021).

Ages of the study's groups were classified into three groups: less than 5 years 55 (30%),6-10 years 92 (51%), and 10-15 years 33 (19%) There was a highly significant difference (p=0.000, P value ≤ 0.05) in ages distribution between study groups by ANOVA test as shown in Table (3-1), and positive correlation between age and ICA in mixed group (R^2= 0.336) and negative correlation with PG-E2 level at (R^2=-0.450) meaning the serum levels of (ICA) increased with age while decrease PG-E2 with age progression as in Table (3-3).

Table (3-3): The correlation of Age with other parameters in the Study's groups.

Parameters	R^2Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	0.173	0.064	- 0.124	0.137
IL-2(ng/l)	- 0.023	- 0.117	- 0.095	- 0.282
IL-10(ng/ml)	- 0.019	0.133	- 0.332	0.459
PG-E2(pg/ml)	0.221	0.050	- 0.450*	- 0.166
GAD(IU/ml)	0.011	- 0.033	- 0.030	0.078
ZNT8(U/ml)	0.176	- 0.158	0.274	0.113
ICA	0.061	- 0.023	0.336*	0.109
(-): Negative Correlation , R^2 :Pearson Correlation Test , *. Correlation is significant at the 0.05 level (P-value).				

The percentage of age result of T1DM children ≥ 5 years was 8(11%), 6-10 years was 39(56%), while it was 23(33%) in ages more than 11-15 years and

mixed the percentage of children ≥ 5 years was 5(20%), 6-10 years was 18(72%), while it was 2(8%) in ages more than 11-15 years as in table (3-1).

The age results were certified by Baechle *et al.* (2022) who mentioned the incidence of type 1 diabetes has increased rapidly over recent decades, particularly in young children, and the prevalence of type 1 and type 2 diabetes has increased significantly in young children but at a lower rate in recent years. Continued surveillance of the prevalence is essential for the planning of healthcare resources and prevention measures.

Hoffmann *et al*. (2019), and Bonifacio *et al*. (2021) proved the same age groups The peak incidence of the development of islet autoantibodies occurs in the first years of life (4–6 years),(Hou *et al.*, 2021) the increased of T1DM in children aged between 0 and 14 years, and Turtinen. (2021) The youngest children (aged 0.5–4 years).

This early peak also indicates that the risk of developing islet autoantibodies attenuates with age and that age influences the child's risk for developing islet autoantibodies, as demonstrated in relatives of patients with type 1 diabetes. there was an increase in the number of females than males in T1DM groups.

Type 1 diabetes can appear at any age, but it appears at two noticeable peaks. The first peak occurs in children between 4 and 7 years old. The second is in children between 10 and 14 years old (Staff, 2023).

In Parasitic patients, 58% of males were infected with intestinal parasitic infection, and 42% of females, that consistence with Tigabu *et al*. (2019) found that male was significantly associated with intestinal parasitic infection at 56.9% and age less than 10 years.

Also, the current results the percentage of children ≥ 5 years was 25(50%), and in 6-10 years was 19(38%), while it was 6(12%) in ages 11-15 years the present results are consistent with Pakmehr *et al*. (2022) who mentioned the

parasitic infections are global health problems, especially in developing countries. Parasitic infections affect 3.5 billion people worldwide, and 450 million of them, mostly children, suffer from intestinal parasites with the highest prevalence in the age group 1-9 years, Sitotaw *et al*. (2019) mentioned the higher prevalence rate was recorded among 6 to 18 years old children and Gebretsadik *et al*. (2018) who mentioned all age groups were affected by intestinal parasites but children who were at the age of below 2 years and the age between 2 and 3 years were 4.7 times and 2.6 times at risk of acquiring infection with intestinal parasites in comparison at the age of 3–5 years children.

Intestinal parasitic infections are common infectious diseases causing many health problems and impaired growth and physical development. Children under five years old are the most vulnerable to infections, due to their immature immunity and feeding and exploratory behaviors (Fauziah N *et al*., 2022).

The study involved a specimens of 50 patients infected by intestinal parasites. the most predominant parasite determined in this study was *Entamoeba histolytica/dispar* 36/50 (72%) followed by *Giardia lamblia* 14/50 (28%) as in Figure (3-1). with the same percent in mixed groups 18/25 (72%) was *Entamoeba histolytica/dispar*, followed by *Giardia lamblia* 7/25 (28%). The prevalence rate of parasitic infection in males was 29 (58%), whereas females were 21(42 %). The association between intestinal parasitic infections (IPIs) and sexes was found statistically insignificant.

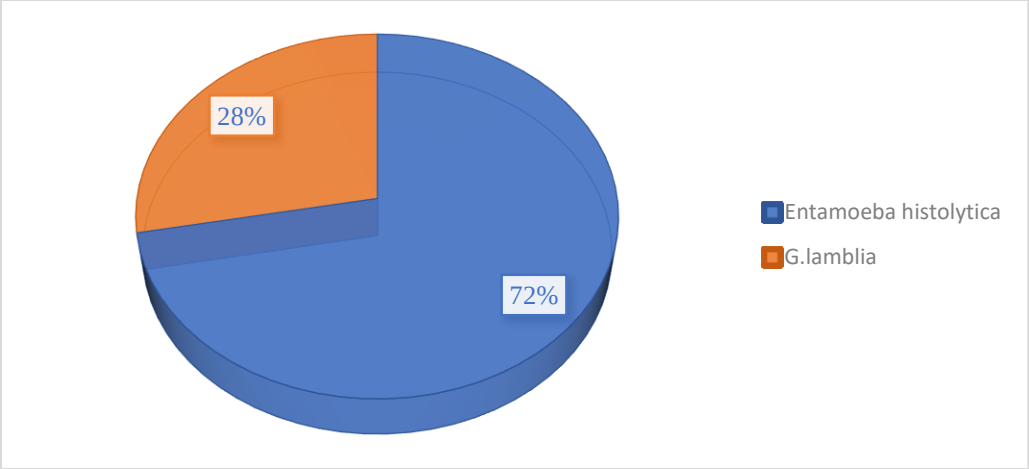

Figure (3-1): Type and Percentage of parasitic infections in parasitic and mixed groups

These results matched with Khan *et al*. (2019) who found nearly the same results prevalence of *Entamoeba histolytica* was (73%). Amoebiasis was significantly associated with eating unwashed raw vegetables and average toilet facilities. Among clinical complications, are hemodynamic changes, jaundice, vomiting, hemoglobin level, loose motion, intolerance to oral feeding, and a history of antibiotics.

The obtained results about *Giardia lamblia* incidence were also approved by (Al-Kahfaji and Al-Masoudi, 2019), another study also approved the same results by Fauziah. (2022) Giardiasis is the second most reported infection, with a prevalence ranging from 4.43% in Ethiopia to 66.33% in the Central African Republic.

3.2. Interleukin IL-2 Concentration

Interleukin IL-2 was high concentration and highly significant differences (P = 0.000, P≤0.05) in diabetic patients and mixed (diabetic patients with intestinal parasitic infections) (89.5±37.8 ng/l and 48.5±29 ng/l) respectively if compared with control (25.4±7.6 ng/l) While the concentration of IL-2 was low in parasitic infections at (19.2±4.2 ng/l) if compared with controls (25.4±7.6 ng/l) as shown in table (3-4).

Table (3-4): Interleukin-2 concentration in the study's groups

Study's Groups	IL-2 (ng/l) M* ± SD**	ANOVA F	P-Value
A- Diabetes Mellitus	89.5±37.8	85.048	0.000 HS
B- Intestinal Parasitic Infections	19.2±4.2		
C- Mixed (T1DM infected by parasites)	48.5±29		
D- Controls	25.4±7.6		

HS: Highly Significant at p ≤ 0.05; * Mean; ** Standard deviation, One-way ANOVA Test, (ng/l): nanogram per litre

Interleukin IL-2 was high in concentration in diabetic patients and diabetic patients with intestinal parasitic infections these results were consistences with Pérez *et al*. (2004), Lu *et al*. (2020), Los and Wilt. (2021)and Kurianowicz *et al.* (2021) they were found that the concentration of IL-2 in children with long-duration type 1 diabetes was higher than in healthy subjects. Duo to People with type 1 diabetes experienced an autoimmune reaction that was characterized by the release of Th1 cytokines. and the obtained result inconsistent with Khalil *et al.* (2021) who found the IL-2 levels revealed a significant (P < 0.001) decrease in all diabetic children.

While the concentration of IL-2 was low in parasitic infections ,the obtained results were consistent with Benson *et al*.(2012), and Lee *et al.* (2020) who mentioned that A temporary decrease in the frequency of IL-2 and a decrease in the absolute amount of T-reg cells were brought on by the acute reactions to the parasitic, bacterial, and viral pathogens. The start of powerful Th1 responses and host defense against the pathogens depended on the infection-induced partial loss of T-reg cells(Ali *et al*., 2018). Due to the proliferation of pathogen-specific CD4+ T cells with a constrained ability to produce IL-2 was the source of the observed decrease of T-reg cells.

Duo to immunological interactions between parasites and their hosts, in which parasites can polarize the immune system toward a potent type-2 immune response that is linked to immune defense and tissue healing and induce tolerance and regulatory function in DM patients. while, these obtained results inconsistence with other study which found significant increases in the level of interleukins IL-2, IL- 4 and IL-10 in patients with parasitic infections than those of healthy control (Khalaf *et al*., 2021).

Hulme *et al.* (2012) found that augmenting IL-2R signaling can prevent and reverse T1DM disease, with protection conferred primarily by the restoration of regulatory T-cell (T-reg) function.

Also, Dwyer *et al*. (2016) and Tahvildari and Dana. (2019) found that low doses of IL-2 therapies dysregulation of the immune system contributes to the breakdown of immune regulation, leading to autoimmune diseases, such as (T1DM).

There were no significant differences in IL-2 concentration between *Entamoeba histolytica* (28.7± 22 ng/l) and *Giardia lamblia* infections

(29.6±22.2 ng/l) (P= 0.829, P≥0.05) in parasitic and mixed groups by independent sample T-test as shown in table (3-5).

Table (3-5): IL-2 Concentration in Patients infected by *Entamoeba histolytica* and *Giardia lamblia*

	Type of Parasites	No.	M* ± SD** (ng\l)	P- value
IL2	*Entamoeba histolytica*	53	28.77± 22.040	0.829 Ŧ NS
	Giardia lamblia	22	29.65± 22.200	

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number of infected patients ,Ŧ : Independent sample T-Test ,(ng\l):nanogram per litre

There were no significant differences at (P=0.463, P≥0.05) in mean IL-2 levels between males and females by independent sample T-Test as in Table (3-6) that inconsistence with Khalaf. (2021) he mentioned that the rate of parasitic infection was higher in males than in females.

Table (3-6): IL-2 Concentration in Males and Females

	Sexes	No.	M* ± SD** (ng\l)	P-value
IL2	MALES	98	51.98 ± 42.024	0.463 Ŧ NS
	FEMALES	82	51.71 ± 39.700	

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number of infected patients,Ŧ :Independent sample T-Test,(ng\l):nanogram per litre

The current results show there were highly significant differences among age groups of patients 0-5 years,6-10 years, and 11-15 years (P=0.000) by one-way ANOVA as in figure (3-2).

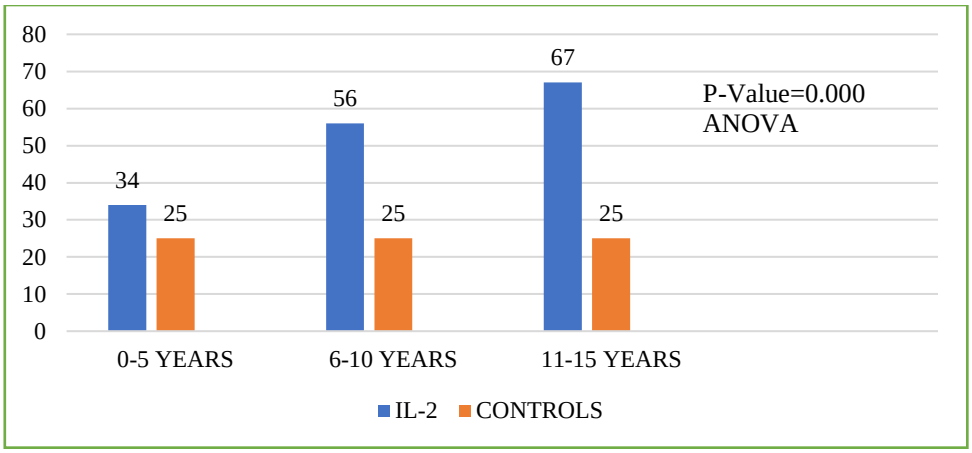

Figure (3-2) Interleukin -2 Concentration in Age Groups

The present study's correlation with other parameters in the study's groups results found a highly positive correlation (0.745) between serum levels of IL-2 and IL-10 in the Mixed group and no significant correlation with other parameters in study groups as illustrated in Table (3-7).

Table (3-7): The correlation of IL-2 with other parameters in the study's groups.

Parameters	R^2 Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	- 0.122	- 0.054	- 0.023	- 0.339
Age	- 0.023	- 0.117	- 0.095	- 0.282
IL-10 (ng/ml)	0.098	- 0.120	**0.745 ****	- 0.032
PG-E2 (pg/ml)	- 0.030	- 0.108	0.320	0.144
GAD(IU/ml)	- 0.068	0.168	0.349	0.186
ZnT8(U/ml)	- 0.236	- 0.213	- 0.145	- 0.108
ICA	- 0.046	- 0.069	0.086	0.409
(-): Negative Correlation ,R^2 :Pearson Correlation Test ,means high significance correlation at p=0.01**				

The present study's results correlation found that serum levels of IL-2 with other study's parameters (GAD, ZnT8, and ICA) were positively correlated

when the serum levels of IL-2 increased, levels of (GAD, ZnT8, and ICA) tended to increase that matched with Marek-Trzonkowska *et al*. (2016) findings [Zinc Transporter 8 Autoantibody (anti-ZnT8) levels were correlated with IL-2 levels], and negative correlation with (IL-10 and PG-E2) the results mean when the serum levels of IL-2 increased, the level of (IL-10, PG-E2) tended to decrease as illustrated in the table (3-8)

Table (3-8): Correlation of IL-2 with other study's parameters

	Parameters	IL-10	PG-E2	GAD	ZnT8	ICA
IL2	R^2	**-0.247***	**-0.312***	**0.541****	**0.417***	**0.356***
	P-value	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
R^2**:Pearson Correlation **. Correlation is significant at the 0.01 level (P-value).**						
***. Correlation is significant at the 0.05 level (P-value).**						

3.3. Interleukin IL-10 Concentration:

A significant increase in the level of interleukin IL-10 at (P=0.000, P≥0.05) in patients with parasitic infections (93.4±64.6 ng/ml), was higher than those of healthy control (25.4 ± 6 ng/ml) also the significantly elevated at (P=0.000, P≥0.05) level of IL-10 in mixed groups (82±59 ng/ml) while the levels of the anti-inflammatory cytokine interleukin-10 (IL-10) are decreased in T1D (21.5±7.1 ng/ml) as shown in table (3-9).

Table (3-9) Interleukin-10 concentration in the study's groups

Study's Groups	IL-10 (ng/ml) M* ± SD**	ANOVA	P-Value
A-Diabetes Mellitus	21.5±7.1	39.681	0.000 HS
B-Intestinal Parasitic Infections	93.4±64.6		
C-Mixed(T1DM infected by parasites)	82±59		
D-Controls	25.4±6		
HS: Highly Significant at p ≤ 0.05; * Mean; ** Standard deviation,(ng/ml):nanogram per millilitre			

A significant increase in the level of interleukin IL-10 in patients with parasitic infections is consistence with Khalaf *et al*. (2021) found that a significant increase in the level of interleukins and the amount of IL-10 in patients, was higher than those of healthy control. Also, Corrêa *et al*. (2020) mentioned the parasite-infected patients had the highest concentration of every cytokine analyzed.

The obtained elevated level of IL-10 in mixed groups proved the results of Mauri and Bosma. (2012) and Yoshizaki *et al*. (2012) approved that Parasites or their products can increase IL-10 levels in vivo in various ways.

Increased IL-10 can promote the transformation of anti-inflammatory cells, such as T-regs, and inhibit the development of autoimmune diseases (Matsumoto *et al*., 2014, Liu *et al*., 2021).

Regulatory B cells' (B-regs) primary biological function is to suppress inflammatory Th1 and Th17 responses that are mediated by cells through homologous interactions with T cells and the production of IL-10, Regulatory B cells (B-regs) is a subset of B cells that generate and secrete the inhibitory factor interleukin-10 (IL-10), hence having an anti-inflammatory impact.

While Levels of the anti-inflammatory cytokine interleukin-10 (IL-10) are decreased in T1D which is consistence with Rios-Arce *et al.* (2020) and Liu *et al.* (2021) approved that Levels of the anti-inflammatory cytokine interleukin-10 (IL-10) are decreased in T1D**.**

Also Wynalda. (2022) had the same results the primary concept is that certain parasites and their byproducts can be used as immunomodulatory agents to create innovative, hopeful therapeutic medicines for the treatment of autoimmune diseases.

There is some evidence that suggests parasites control interleukin-10, an anti-inflammatory cytokine. this alteration of IL-10, which is controlled by T helper cells, lowers the risk of autoimmune illness and aids in maintaining low levels of inflammation in the gut. also, parasites can reduce the host's immunological response to themselves (parasite-specific immunoregulation).

These seem capable of inhibiting immunological responses through the stimulation of regulatory T cells or Th2-type cells, parasites cause immunoregulation (or both). However, parasites may also directly activate signaling pathways to increase IL-10 in host cells to modify their hosts' immune responses. This is done through secreted or expelled parasite metabolites, proteins, or extracellular vesicles (or a mix of these) (Marek-Trzonkowska et al., 2016, Elliott and Weinstock, 2017, Gazzinelli-Guimaraes and Nutman, 2018).

Reduced cellular immune responses and a change in T-cell responses to two kinds of T helper cells are frequently associated with parasite infection. Additionally, an inverse relationship between the prevalence of parasitic infections and autoimmune disorders was discovered, raising the possibility that

"parasitic treatment" may be helpful for those with autoimmune diseases (Abdoli *et al*., 2022).

The hygiene hypothesis probably explains the autoimmune disorders' unequal regional distribution in the world is likely explained by the hygiene theory. If migration takes place at a young age and within a threshold that varies depending on the condition, people who move from places with low prevalence of autoimmune diseases to places with high incidence get the disease with the frequency of the host nation (Bach, 2018)

There are no significant differences in IL-10 concentration between *Entamoeba histolytica* (89.8 ±65.5 ng/ml) and *Giardia lamblia* infections (89.4±56.6 ng/ml) (P= 0.558, P≥0.05) in parasitic and mixed groups by Independent sample T-test as shown in table (3-10) This inconsistence with (Babaei et al., 2016, M'bondoukwé et al., 2022) they improved that *G. lamblia* had significantly elevated levels of serum IL-10, (mean 100 ng/ml) compared to healthy controls and The prevalence of endoparasites in people with type 1 diabetes a robust Th2 response to parasites may be connected to *Giardia lamblia* observed in type 1 diabetes (Hassanein and Fanaky, 2021).

Table (3-10) IL-10 Concentration in Patients infected by *Entamoeba histolytica* and *Giardia lamblia*

	Type of Parasites	No.	M* ± SD** (ng/ml)	P-value
IL10	***Entamoeba histolytica***	**53**	**89.88±65.531**	**0.558 Ŧ NS**
	Giardia lamblia	**22**	**89.43±56.63**	

NS: non-significant at p ≥0.05; * Mean; ** Standard deviation; No.:Number of infected patients,Ŧ: Independent sample T-Test,(ng/ml): nanogram per millilitre

There were no significant differences (P=0.445 P≥0.05) in mean IL-10 levels between males and females by Independent sample T-Test as illustrated in table (3-11) that consistence with M'bondoukwé, (2022) who found no significant differences between sex at (p=0.4).

Table (3-11) IL-10 Concentration between Males and Females

	Sexes	No.	M* ± SD** (ng/ml)	P value
IL10	MALES	98	50.24±40.379	0.445 Ŧ
	FEMALES	82	51.24±47.955	NS

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number, Ŧ: Independent sample T-Test,(ng/ml): nanogram per millilitre

Also, the results confirm there were no significant differences among age groups of patients 0-5 years,6-10 years, and 11-15 years at (P=0.121) by one-way ANOVA as in figure (3-3) that inconsistence with M'bondoukwé, (2022) who mentioned that Among children under five years old, the IL-10/TNF-α and IL-10/IL-6 ratios were higher in those with intestinal protozoan infections than in uninfected children. The IL-10/TNF-α ratio was also higher in children aged 5–15 years.

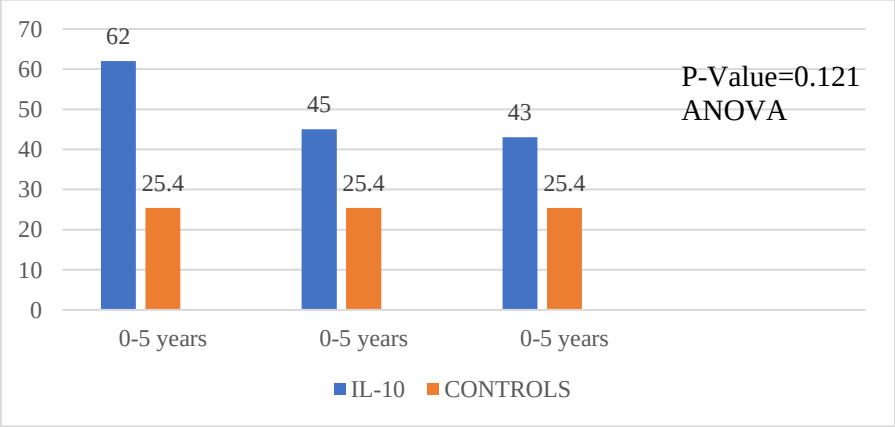

Figure (3-3) Interleukin -10 Concentration in Age Groups

The present study's correlation results with other parameters in the study's groups found a positive correlation between serum levels of IL-10 and IL-2 concentration in mixed groups and a non-significant correlation between IL-10 and other parameters in the study's groups as in Table (3-12)

Table (3-12): The correlation of IL-10 with other parameters in the Study's groups.

Parameters	R^2 Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	0.128	0.117	0.084	0.009
Age	- 0.019	0.133	- 0.332	0.459
IL-2(ng/l)	0.098	- 0.120	**0.745 ** **	- 0.032
PG-E2(pg/ml)	0.037	0.379	0.470	- 0.160
GAD(IU/ml)	0.022	0.117	- 0.358	0.067
ZnT8(U/ml)	- 0.242	0.324	- 0.428	0.237
ICA	0.139	0.161	- 0.035	0.045
(-):Negative Correlation ,R^2 :Pearson Correlation Test ,**means high significance correlation at p=0.01				

The present study's correlation with other study's parameters results found a positive correlation between serum levels of IL-10 and PG-E2 when the serum levels of IL-10 increased, PG-E2 levels tended to increase, and a negative correlation with (IL-2, GAD, ZnT8, and ICA) when the serum levels of IL-10 increased, the level of (IL-2, PG-E2, GAD, ZnT8, and ICA) tended to decrease as in table (3-13).

Table (3-13): Correlation of IL-10 with other study's parameters

	Parameters	IL-2	PG-E2	GAD	ZnT8	ICA
IL10	R^2	-0.247*	0.640**	-0.298*	-0.256*	-0.122
	P-value	0.001	0.001	0.001	0.001	0.104
R^2: Pearson correlation **. Correlation is significant at the 0.01 level (P-value).						
*. Correlation is significant at the 0.05 level (P-value).						

3.4. Prostaglandin PG-E2 Concentration

Prostaglandin concentration was low (14.5±6.3 pg/ml) in T1DM patients as compared with controls, the higher concentration of PGE2 in parasitic infected patients and mixed groups (94.5± 77,89±37 pg/ml) respectively if compared with controls (15.9±6.8 pg/ml) at (P=0.000, P ≤0.05) if compared with controls (15.9 ± 6.8 pg/ ml) and diabetic (14.5±6.3 pg/ml) as shown in table (3-14).

Table (3-14) Prostaglandin PG-E2 concentration in the study's groups

Study's Groups	PG-E2 (pg/ml) M* ± SD**	ANOVA	P-Value
A-Diabetes Mellitus	14.5±6.3	46.953	0.000 HS
B-Intestinal Parasitic Infections	94.5±77		
C-Mixed(T1DM infected by parasites)	89±37		
D-Controls	15.9 ± 6.8		
HS: Highly Significant at p ≤ 0.05; * Mean; ** Standard deviation, (pg/ml):picogram per millilitre			

Prostaglandin PG-E2 Concentration was highly significant among study groups a(p-value=0.000), PG-E2 Concentration was low in T1DM patients which is consistence with Ben Nasr *et al*. (2018) who mentioned the anti-inflammatory effect of PG-E2.

That inconsistence with Salvi *et al*. (1997) who found that diabetics had significantly higher levels of both PGE2 and IL-1 beta as compared to non-diabetic controls the condition known as sterile inflammation, which is characterized by a mild inflammatory response, is considered to be brought on by hyperglycemia. While tissue healing and pathogen clearance are both dependent on inflammation, persistent and long-lasting inflammatory responses harm tissue, which results in immunopathology and poorer disease outcomes (Brandt *et al*., 2018, Gurumurthy and Lloyd, 2019).

Diabetes mellitus with poor management causes many comorbidities, including an increased risk of infections. Although hyperglycemia improves phagocyte reactivity, the antibacterial capacity of immune cells from diabetic individuals is suboptimal through the inhibition of a Th1 response, PGE2 may also be crucial in tipping the balance toward a Th2 response.

Prostaglandins are a group of vital lipid mediators that play a key role in influencing the onset, activation, maintenance, effector functions, and resolution of type 2 inflammation. They are secreted during type 2 inflammation (Oyesola and Tait Wojno, 2021).

Higher concentration of PGE2 in parasitic-infected patients and mixed groups as compared with controls that consistence with Bonyek-Silva *et al*.(2020) who mentioned that prostaglandins concentration was statistically significantly increased in patients infected with the parasite if compared with controls. also consistence with Yesuf and Kenubih. (2019) mentioned that Lipid droplets are also exploited by the pathogen to aid in adhesion, promote

pathogenesis, and alter host metabolism as a means of immune evasion. and this result clarifies the modulatory effect of parasitic infections and the evasion of the immune response to chronic infections that improved by (Rodrigues *et al.*, 2021).

The apicomplexan parasites used lipid particles for a variety of functions, such as altering the permeability and fragility of host cells, facilitating the parasite's insertion into the host cell membrane, and fostering growth, invasion, and the organism's most effective reproduction. The lipid has a significant function in anaerobic groups of parasites as a growth stimulant, boosting virulence, promoting encystation and vesicle formation, as well as aiding immune system induction and dendritic cell maturation. prostaglandins may control the inverse link between parasite infection and allergies, as well as the possible involvement of prostaglandins generated by parasites in the regulation of host-pathogen interactions. novel treatment options for those suffering from Type 2 inflammatory illnesses, such as allergic rhinitis and asthma, that include a large prostaglandin-driven component (Gill et al., 2016, Bonyek-Silva et al., 2020).

However, PGE2 causes a wide spectrum of biochemical reactions linked to inflammation. PGE2 production is increased, and it has the potential to signal via four separate main receptors (EP1, EP2, EP3, or EP4, which are expressed by a wide variety of cell types) to trigger either pro-inflammatory or regulatory immune responses, PGE2 concentrations, and particular receptor signaling are essential. PGE2 weakens the ability of innate phagocytes, making the host more vulnerable to bacterial, fungal, and viral infections as well as protozoan parasites.

Regarding parasite infections, PGE_2 has been implicated in the downregulation of ROS production from phagocytes, compromising a protective host response (Dwyer *et al*., 2016).

The current results show there were no significant differences in PG-E2 concentration between *Entamoeba histolytica* (88.9± 43.7 pg/ml) and *Giardia lamblia* infections(101.91±53.49 pg/ml) at (P= 0.066, P≥0.05) by Independent sample T-Test in parasitic and mixed groups as shown in table (3-15).

Table (3-15) Prostaglandin Concentration in Patients infected by *Entamoeba histolytica* and *Giardia lamblia*

	Type of Parasites	No.	M* ± SD** (pg/ml)	P- value
PG-E2	*Entamoeba histolytica*	53	88.91±43.721	0.066 Ŧ NS
	Giardia lamblia	22	101.91±53.49	

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number of infected patients,Ŧ : Independent sample T-Test ,(pg/ml):picogram per millilitre

Also, the results confirm there were no significant differences (P=0.375, P≥0.05) in mean PG-E2 levels between males and females by Independent sample T-Test as illustrated in Table (3-16).

Table (3-16) Prostaglandin Concentration in Males and Females

	Sexes	No.	M* ± SD** (pg/ml)	P value
PG-E2	MALES	98	51.42±45.604	0.375 Ŧ NS
	FEMALES	82	42.56±40.446	

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number Ŧ :Independent sample T-Test ,(pg/ml):picogram per millilitre

Also, the results confirm there were no significant differences among age groups of patients 0-5 years,6-10 years, and 11-15 years (P=0.093) by one-way ANOVA test, as in Figure (3-4).

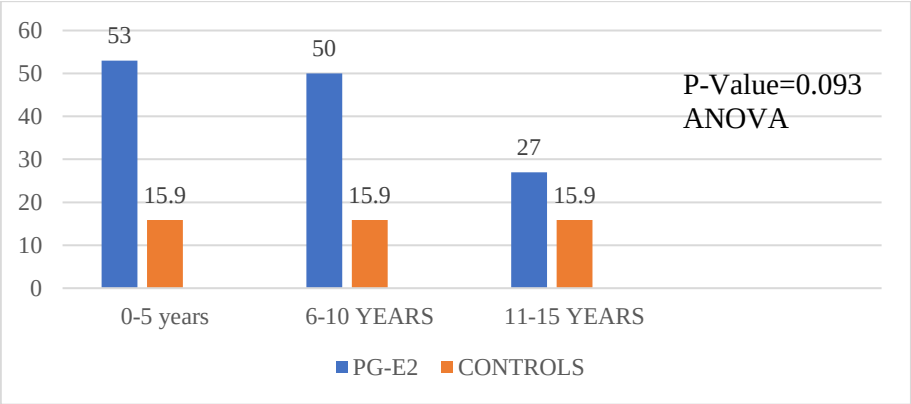

Figure (3-4) Prostaglandin E2 Concentration in Age Groups

The present study's correlation results with other parameters in the Study's groups were a significant correlation between PG-E2 and IL-10 and a negative correlation with Age, ZnT8, and ICA in mixed groups as in Table (3-17)

Table (3-17): The correlation of PG-E2 with other parameters in the Study's groups.

Parameters	R^2 Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	- 0.035	- 0.122	0.235	- 0.085
Age	0.221	0.050	- 0.450*	- 0.166
IL-2(ng/l)	- 0.030	- 0.108	0.320	0.144
IL-10(ng/ml)	0.037	0.379	0.470*	- 0.160
GAD(IU/ml)	0.031	- 0.106	- 0.060	0.050
ZNT8(U/ml)	0.183	0.038	- 0.311	- 0.205
ICA	0.093	0.090	- 0.265	- 0.079
(-):Negative Correlation ,Pearson Correlation Test .* Correlation is significant at the 0.05 level (P-value).				

The present study's correlation with other study's parameters found a positive correlation between serum levels of PG-E2 and IL-10 when the serum levels of PG-E2 increased, IL-10 levels tended to increase, and a negative correlation with(IL-2, GAD, ZnT8, and ICA) when the serum levels of PG-E2 increased, the level of (IL-2, PG-E2, GAD, ZnT8, and ICA) tended to decrease as in table (3-18).

Table (3-18): Correlation of PG-E2 with other study's parameters

	Parameters	IL-10	IL-2	GAD	ZnT8	ICA
PG-E2	R^2	**0.640****	**-0.312***	**-0.301***	**-0.222***	**-0.148***
	P-value	**0.001**	**0.001**	**0.001**	**0.003**	**0.048**
R^2**: Pearson Correlation **. Correlation is significant at the 0.01 level (P-value).**						
***. Correlation is significant at the 0.05 level (P-value).**						

3.5. Glutamic Acid Decarboxylase GAD IgG Autoantibodies Concentration:

The prevalence of anti-GAD in T1DM was (66/70) 94% and the level of anti-GAD was higher in type 1 diabetes and mixed patients (139.9±95.4 IU/ml, 45.5±6 IU/ml respectively) than in control (1.4±0.9 IU/ml) and The mean of anti-GAD IgG in parasitic groups (0.6±0.4 IU/ml) was low if compared with control (1.4±0.9 IU/ml) at (P=0.000, P≤ 0.05) among groups as shown in table (3-19).

Table (3-19) Glutamic Acid Decarboxylase GAD IgG concentration in the study's groups

Study's Groups	GAD (IU/ml) M* ± SD**	ANOVA F	P-Value
A- Diabetes Mellitus	139.9±95.4	69.792	0.000 HS
B- Intestinal Parasitic Infections	0.6±0.4		
C- Mixed (T1DM infected by parasites)	45.5±6		
D- Controls	1.4±0.9		

HS: Highly Significant at p ≤ 0.05; * Mean; ** Standard deviation,(IU/ml):International Unit per milliliter

The obtained results consistence with Basu *et al*. (2020) and Peng *et al*. (2022) and the duration of diabetes positively correlated with the number of T1DM-specific antibodies and also with GAD antibody positivity.

Generally, glutamate decarboxylase autoantibodies (GADAs) are the most prevalent autoantibodies after diagnosis, However, when studies with islet autoantibody data at diagnosis are considered, it becomes clear that overall islet antigen-2 autoantibodies (IA-2A) tend to persist for longer than GADA or ZnT8A (Long *et al*., 2021).

The research by which they have confirmed that GAD or IA2 antibodies are highly sensitive markers for Type 1 diabetes mellitus, in pediatric age and have identified a group of patients who lack ICA (Zanone *et al*., 2003, Peng *et al*., 2022).

Glutamate decarboxylase autoantibodies (GADAs) assays help improve the value of risk stratification in autoimmune diabetes mellitus and protect islet function. Identification and early intervention are important for Latent Autoimmune Diabetes in Adults (LADA).

The mean of anti-GAD IgG in parasitic groups in current results matched with Cortez *et al*.(2020), Long *et al.* (2021) who mentioned that negative islets antigen in normal and at low risk of developing autoimmune diabetes.

The current results of the prevalence of anti-GAD in T1DM that nearly consistence with Basu *et al*.(2020) the prevalence of GAD autoantibodies and ZnT8 autoantibodies are 70%–80%, 60%–70% respectively in children with new-onset T1DM.

Also, Zanone *et al*.(2003) go with the results of an Iraqi study that demonstrated 88.6% of T1DM patients were positive for anti-GAD, Also The Tunisian study results by the Sheet and Khudhair. (2019) found that 84.6 percent of children with newly diagnosed diabetes had seropositive anti-GAD antibodies (within six months of diagnosis), and Elkadhi *et al*. (2002) accords with abroad study found (an 89%) seropositivity rate. also among the patients with new-onset T1DM using the anti-GAD IgG ELISA test (Palomer *et al*., 2004).

In contrast, other Iraqi studies exhibited a lower percentage of anti-GAD seropositivity (Kareem *et al.*, Mohammed, 2019).

Those conflicting results might be explained by the varying cut-off values used in diagnostic kits to determine test sensitivity and/or varying the autoimmunity progression level from person to person among different populations (Thabit *et al*., 2012).

Also, the results confirm There were highly significant differences (P=0.000, P≥0.05) by Independent sample T-Test in mean anti-GAD levels between males and females and more in the females which inconsistence with other studies that found no significant differences in mean anti-GAD levels between males and females of Type 1 diabetic patients, although there was a slight trend towards higher values in females (De Block *et al*., 2001, Mohammed, 2019), While the results consistence with Turtinen *et al*., (2018) mentioned the immunologic

aggressiveness of the disease is more variable as the predominance of different autoantibodies varies between sexes with a higher frequency of GADA in girls, while the three other biochemical autoantibodies were more common in boys. as shown in table (3-20).

Table (3-20): GAD Concentration in Males and Females

	Sexes	No.	M* ± SD** (IU/ml)	P value
GAD	MALES	98	46.49±33.733	0.000 Ŧ
	FEMALES	82	78.82±69.642	HS

HS: Highly Significant at p ≤ 0.05* Mean; ** Standard deviation; No. : Number
Ŧ : Independent sample T-Test,(IU/ml):International Unit per milliliter

The current results show there were highly significant differences among age groups of patients 0-5 years,6-10 years, and 11-15 years at (P=0.000) by one-way ANOVA test as in figure (3-5), that inconsistence with several studies have shown that the incidence of anti-GAD IgG autoantibodies reduced as the period of T1DM increased, with the prevalence of anti-GAD in patients with disease duration less than 5 years being 78.3 percent, and beginning to decline in those with disease duration more than 12 years, attributable to the depletion of islet cell autoantibodies with time (Mahdi *et al.*, 2015).

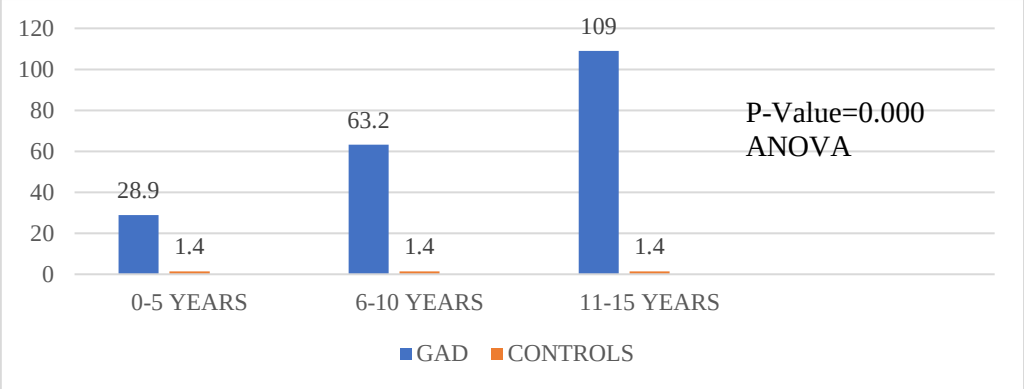

Figure (3-5) Glutamic Acid Decarboxylase GAD IgG concentration in Age Groups

According to the results of the correlation between GAD concentration and other parameters in the Study's groups, there was no significant correlation between GAD concentration and other parameters in the study groups as shown in Table (3-21).

Table (3-21): The correlation of GAD with other parameters in the Study's groups.

Parameters	R^2 Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	0.336	- 0.073	0.200	0.207
Age	0.011	- 0.033	- 0.030	0.078
IL-2(ng/l)	- 0.068	0.168	0.349	0.186
IL-10(ng/ml)	0.022	0.117	- 0.358	0.067
PG-E2(pg/ml)	0.031	- 0.106	- 0.060	0.050
ZNT8(U/ml)	- 0.043	0.046	0.205	- 0.229
ICA	0.054	- 0.127	0.234	0.026
(-):Negative Correlation ,R^2 :Pearson Correlation Test				

The present study correlation with other study's parameters found that serum levels of GAD, IL-2, ZnT8, and ICA were positively correlated when the serum levels of GAD increased, levels of IL-2, ZnT8, and ICA tend to increase, and negative correlation with(IL-10 and PG-E2) when the serum levels of GAD increased, the level of (IL-10 and PG-E2) tended to decrease as illustrated in the Table (3-22).

Table (3-22): Correlation of GAD with other study's parameter

	Parameters	IL-10	IL-2	PG-E2	ZnT8	ICA
GAD	R^2	-0.298*	0.541**	-0.301*	0.471*	0.392*
	P-value	0.000	0.000	0.000	0.000	0.000
R^2: Pearson Correlation **. Correlation is significant at the 0.01 level (P-value).						
*. Correlation is significant at the 0.05 level (P-value).						

3.6 Zinc transporter 8 protein (ZnT8 Ag) Concentration:

Zinc transporter 8 protein positivity prevalence was detected in 41/70 (58%) of patients with T1DM and 15/25 (60%) of the mixed group. ZnT8A concentration was high in T1DM and mixed groups (81±41 U/ml.,67±21 U/ml) respectively as compared with controls (24±7 U/ml) while the low or normal concentration in parasitic infections (24.3±7.6U/ml) there are highly significant differences (P=0.000, P≥0.05) among study's groups, As shown in table (3-23).

Table (3-23): Zinc transporter 8 protein (ZnT8) Concentration in the Study's groups

Study's Groups	ZnT8 (U/ml) M* ± SD**	ANOVA	P-Value
A-Diabetes Mellitus	81±41.4	56.295	0.000 HS
B-Intestinal Parasitic Infections	24.3±7.6		
C-Mixed (T1DM infected by parasites)	67.5±21.4		
D-Controls	24.9±7.6		
HS: Highly Significant at p ≤ 0.05; * Mean; ** Standard deviation,(U/ml): Unit per milliliter			

These obtained results consistence with Elmaoğulları *et al*. (2018) who found that ZnT8A positivity was detected in 58% of the patients with new onset T1DM and 8% of the control group. also, Gu *et al*. (2021) ZnT8 Antibody was the earliest antibody to appear in children.

The identification of ZnT8 as a cell surface target of humoral autoimmunity in the earliest phase of antibody responses opens a new avenue of investigation into the role of antibodies in the development of β-cell autoimmunity.

Also, Abd Elhameed *et al*. (2020) mentioned (ZnT8A) measurement can be helpful in the detection of suspected new cases of type 1 diabetes when other islet autoantibodies are negative.

And Lampasona and Liberati. (2016) The primary indicators of pancreatic autoimmunity in type 1 diabetes are islet autoantibodies. Insulin (IAA), glutamic acid decarboxylase (GADA), protein phosphatase-like IA-2 (IA-2A), and ZnT8 (ZnT8A) are all antigens located on secretory granules within pancreatic beta cells that are recognized by islet autoantibodies.

The essential markers of the autoimmune response monitored for diagnosis or prognostics in T1D patients or disease prediction in at-risk persons before T1DM onset are islet antibodies, Islet autoantibodies have been the primary tool used to investigate the natural history of T1DM.

while low or normal concentrations in parasitic infections consistence with Cortez *et al*. (2020) and Long *et al*. (2021) mentioned that negative ZnT8 antigen is normal and at low risk of developing autoimmune diabetes.

There were no significant differences in Sex groups (male, female) by Independent sample T-Test at ($P=0.708$, $P\geq0.05$) these results were consistence with Elmaoğulları *et al*. (2018) who found that no association was found between ZnT8 positivity and age, gender, presence or degree of ketoacidosis as illustrated in the table (3-24).

Table (3-24): Zinc transporter 8 protein Concentration in Males and Females.

	Sexes	No.	M* ± SD** (U/ml)	P value
ZNT8	MALES	98	51.67±35.280	0.708 Ŧ NS
	FEMALES	82	53.53±41.964	

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number of specimens, Ŧ :Independent sample T-Test,(U/ml): Unit per milliliter

There were highly significant differences in 0-5 years old,6-10 years, and 11-15 years (P=0.000) by one-way ANOVA test as in figure (3-6), so the positivity of ZnT8 concentration increased with age progression that not matched with Pöllänen *et al*. (2020) ZnT8A and IAA appeared commonly as the first autoantibody, but in the preschool years, IA-2A- and especially GADA-initiated autoimmunity increased. GADA-positive seroconversions continued to appear steadily until ages 10 to 15 years (Long *et al.*, 2021). Whilst zinc transporter 8 autoantibodies (ZnT8A) prevalence declines more rapidly with age progression Fakhfakh *et al*. (2022) and Huang *et al*. (2019) suggested a strong inverse correlation between the age of T1DM onset and the prevalence of ZnT8-Ab. and inconsistence with a recent study on T1DM patients by Bravis *et al*. (2018) from sub-Saharan Africa (Amhara, the second-largest ethnic group in Ethiopia) showed that the prevalence of ZnT8-Ab is low in all age groups, including those with the childhood-onset disease as in figure (3-6).

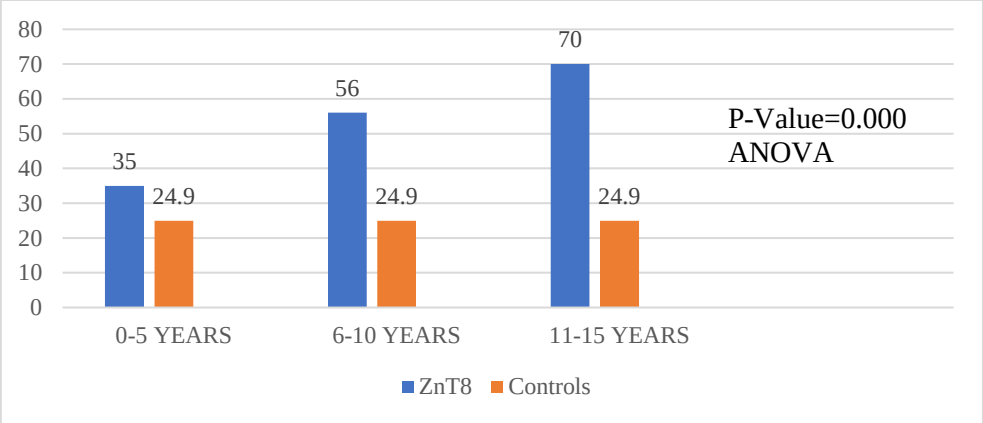

Figure (3-6) Zinc Transporter 8 Autoantibodies Concentration in Age Groups

According to the results of the correlation between ZnT8 concentration and other parameters in the study's groups, there was no significant correlation between ZnT8 and other parameters in the study groups as in Table (3-25).

Table (3-25): The correlation of ZNT8 with other parameters in the Study's groups.

Parameters	R^2 Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	- 0.067	- 0.147	- 0.080	- 0.321
Age	0.176	- 0.158	0.274	0.113
IL-2(ng/l)	- 0.236	- 0.213	- 0.145	- 0.108
IL-10(ng/ml)	- 0.242	0.324	- 0.428	0.237
PG-E2(pg/ml)	0.183	0.038	- 0.311	- 0.205
GAD(IU/ml)	- 0.043	0.046	0.205	- 0.229
ICA	- 0.031	- 0.096	0.220	0.066
(-): Negative Correlation ,R^2: Pearson Correlation Test				

The present study's correlations found a positive correlation with other study's parameters between serum levels of ZnT8 and (IL-2, GAD, and ICA)

when the serum levels of ZnT8 increased, the level of (IL-2, GAD, and ICA) tend to increase and negative correlation with (IL-10 and PG-E2) when the serum levels of ZnT8 increased, the level of (IL-10 and PG-E2) tended to decrease as in table (3-26).

Table (3-26): Correlation of ZnT8 with other study's parameters

	Parameters	IL-10	IL-2	PG-E2	GAD	ICA
ZnT8	R^2	**-0.256***	**0.417****	**-0.222***	**0.471****	**0.356***
	P-value	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
R^2**: Pearson Correlation **. Correlation is significant at the 0.01 level (P-value).**						
***. Correlation is significant at the 0.05 level (P-value).**						

3.7 Islet Cell Autoantibodies ICA Concentration:

Islet autoantibody developed in 30/70 (42%) T1DM children with high concentration (1.04±0.55) and mixed groups 15/25 (60%) was (1.03±0.58) if compared with controls (0.42±0.25) with high significant differences at ($P \leq 0.05$, P=0.000), while the low or normal concentration in parasitic infections (0.43±0.25), the study results illustrated in the Table (3-27)

Table (3-27): Islet Cell Autoantibodies ICA Concentration in the study's groups

Study's Groups	ICA M* ± SD**	ANOVA	P-Value
A-Diabetes Mellitus	1.04±0.55	28.186	0.000 HS
B-Intestinal Parasitic Infections	0.43±0.25		
C-Mixed (T1DM infected by parasites)	1.03±0.58		
D-Controls	0.42±0.25		
HS: Highly Significant at p ≤ 0.05; * Mean; ** Standard deviation			

The obtained results for T1DM were also proved by Winkler *et al*. (2019) and Long *et al*. (2021) approved that Islet autoantibodies may provide biomarkers for long-term β-cell function and insights into how to prevent ongoing islet autoimmunity, While the low or normal concentration in parasitic infections that consistence with Cortez *et al*. (2020) and Long *et al*. (2021) mentioned that negative ICA antigen is normal and at low risk of developing autoimmune diabetes.

The biochemically characterized autoantibodies peaked at a young age, although the ICA seroconversion rate climbed near adolescence. ZnT8A and IAA often occurred as the first autoantibodies before the age of two, but during the preschool years, IA-2A and notably GADA-initiated autoimmunity surged. After that, GADA-positive seroconversions persisted until between the ages of 10 and 15.

In contrast to chronic Insulin Auto-Antibodies, inverse IAA seroconversions were more common (49.3% resulted in a negative seroconversion), and this resulted in a longer time between seroconversion and diagnosis Al Alwan *et al.* (2012) showed that 67% were positive for ICA.

There were no significant differences between male and female patient groups at (P=0.773, P≥0.05) by Independent sample T-Test as shown in Table (3-28) this obtained results inconsistence with Zamanfar *et al*. (2020) who showed that More than 80% of pediatric patients with type 1 diabetes were autoantibody-positive. ICA and GADA were the most frequently detected autoantibodies. the presence of antibodies was significantly higher in females**.** Also, the obtained results inconsistence with Turtinen. (2021) frequency of ICA has been observed to be higher in females, and Dearden *et al*. (2018) females seem particularly susceptible to developing increased adiposity and disrupted glucose homeostasis as a result of exposure to in-utero undernutrition or high sugar environments.

Table (3-28): Islet Cell Autoantibodies ICA Concentration in Males and Females

	Sexes	No.	M* ± SD** (U\L)	P value
ICA	**MALES**	**98**	**0.75±0.53**	**0.773 Ŧ NS**
	FEMALES	**82**	**0.75±0.55**	

NS: non-significant at p ≥0.05 ; * Mean; ** Standard deviation; No.:Number
Ŧ: Independent sample T-Test

There were highly significant differences (p=0.001) between ages 0-5 years,6-10 years, and 11-15 years by one-way ANOVA test as in figure (3-7), these results consistence with Pöllänen *et al*. (2020) which mentioned that at a median age of 4.4 years (range, 0.3-5.1 years) the ICA seroconversion rate increased toward puberty, but the biochemically defined autoantibodies peaked at a young age. Before age 2 years.) and the obtained results did not match with

Al Alwan *et al*. (2012) who mentioned the presence of ICA was predominant in children aged under six years.

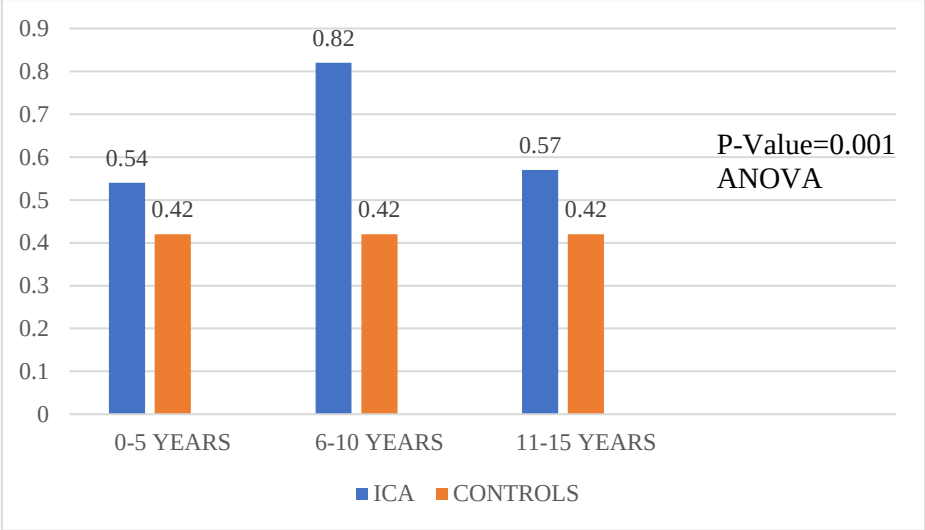

Figure (3-7) Islet Cell Autoantibodies ICA Concentration in Age Groups

According to the results of the correlation between ICA concentration and other parameters in the study's groups, there was a significant correlation between ICA and (Age, GAD, and ZnT8) and a negative correlation with PG-E2 in the Mixed group as in Table (3-29).

Table (3-29): The correlation of ICA with other parameters in the study's groups.

Parameters	R^2 Correlation among Study's groups			
	T1DM	Parasitic	Mixed	Control
Sex	- 0.222	- 0.056	0.012	0.137
Age	0.061	- 0.023	0.336	0.109
IL-2(ng/l)	- 0.046	- 0.069	0.086	0.409
IL-10(ng/ml)	0.139	0.161	- 0.265	0.045
PG-E2(pg/ml)	0.093	0.090	- 0.035	- 0.079
GAD(IU/ml)	0.054	- 0.127	0.234	0.026
ZNT8(U/ml)	- 0.031	- 0.096	0.220	0.066
(-):Negative Correlation ,R^2 :Pearson Correlation Test				

The present study's correlation with other study's parameters found that serum levels of ICA and (IL-2, GAD, and ZnT8) were positively correlated when the serum levels of ICA increased, the level of (IL-2, GAD, and ZnT8) tend to increase, and negative correlation with (IL-10, PG-E2) when the serum levels of ICA increased, the level of (IL-10, PG-E2) tended to decrease as in table (3-30).

Table (3-30): Correlation of ICA with other study's parameters

	Parameters	IL-10	IL-2	PG-E2	GAD	ZnT8
ICA	R^2	-0.122*	0.356**	-0.148*	0.392**	0.356**
	P-value	0.104	0.001	0. 048	0.001	0.001
R^2: Pearson Correlation **. Correlation is significant at the 0.01 level (P-value).						
*. Correlation is significant at the 0.05 level (P-value).						

3.8. Correlation of IL-10 with IL-2 and PG-E2 in Mixed Group

There was a strong correlation between IL-10 and IL-2 (R^2 =0.745, p<0.01) as shown in Figure (3-8), Also there was a high significant difference in IL-10 level in mixed groups if compared with diabetic groups at P value=0.000, decreased IL-2 level in mixed groups if compared by diabetic groups at P value=0.000 that determined the role of intestinal parasitic infection to modulate immune function toward Th-2.

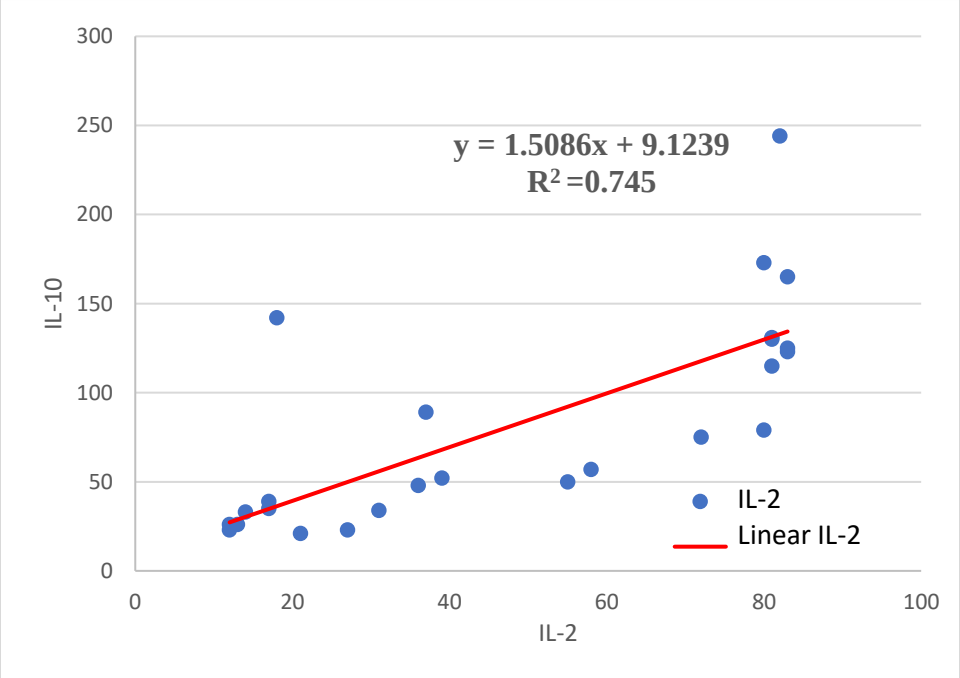

Figure (3-8): Correlation of IL-2, IL-10 in Mixed Groups

The importance of interleukin-2 (IL-2) is a T cell growth factor necessary for the expansion of T cells and the production of effector and memory cells (Abbas *et al*., 2018).

The abnormality in their development or function causes various autoimmune diseases, including T1DM (Ren *et al*., 2019).

They are essential for maintaining immunologic self-tolerance as well as for regulating abnormal or excessive immune responses to commensal or invasive human microorganisms as well as harmless environmental chemicals (Trotta et al., 2018, Neumann et al., 2019, Skartsis et al., 2023).

Interleukin 10 (IL-10) cytokine promotes the broad inhibition of immunological responses, which is necessary for controlling immune activities. The major role of this cytokine is thought to be its autocrine/paracrine properties, which involve direct binding to leukocytes and the consequent containment of immunological responses. Reduced antigen presentation and enhanced T-cell anergy have been associated with IL-10 production from CD4+CD25+FoxP3+ regulatory cells (T-regs), macrophages, and other leukocytes and subsequent binding to IL-10 receptors on macrophages and dendritic cells (DCs). The obtained results consistence with those (Bijjiga and Martino, 2013, Neumann et al., 2019).

The cytokine also functions to minimize the development of Th1 responses by decreasing Th1-related cytokines (IL-2, IL-12, and IFN-γ) and encouraging Th2 responses by increasing levels of Th2-related cytokines (IL-4, IL-5 & IL-13) Additionally, Because IL-10 is essential for maintaining the delicate balance between tissue protection and effective immunity, its expression is highly dynamic and tightly regulated.

The observed positive correlation ($R=0.470$, $P<0.05$) between the increased serum concentrations of IL-10 and PG-E2 indicates a link between them as the anti-inflammatory cytokines as in Figure (3-9)

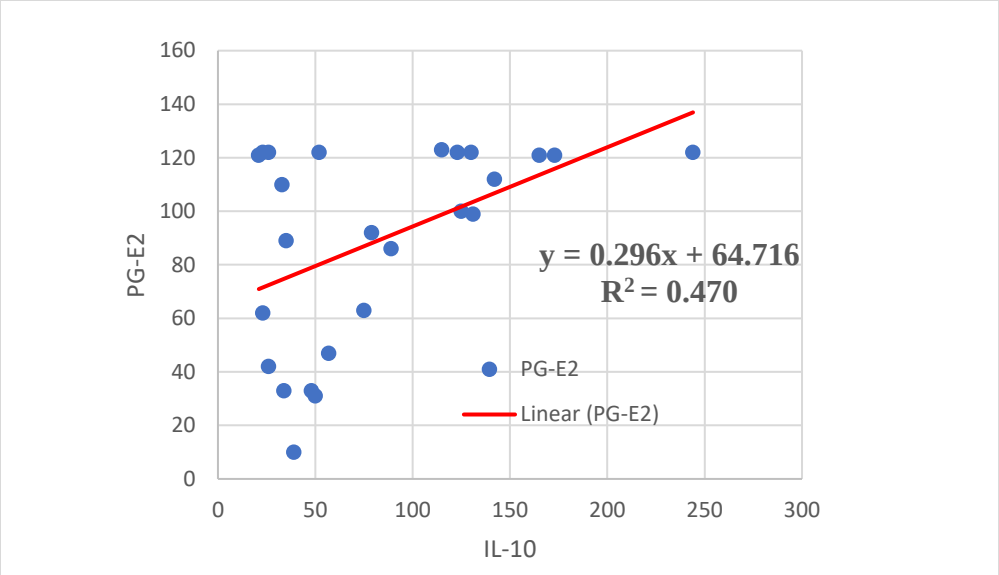

Figure (3-9): Correlation of PG-E2, IL-10 in Mixed Groups

The results consistence with Yesuf and Kenubih. (2019) determined the role of Prostaglandins as powerful lipid molecules that work through a variety of receptors to exert their effects. Prostaglandins may modify fluid secretion and chloride absorption, which may contribute to the pathophysiology of secretory diarrhea.

Also, Maric *et al*. (2018) and Zhou *et al.*(2018) mention that Prostaglandins can also upregulate or suppress the expression of inflammatory cytokines.

collectively offer emerging proof that intestinal inflammation is promoted by PGE2-mediated alteration of microbiota-Treg communication (Crittenden *et al.*, 2021).

3.9. Correlation of IL-10 with T1DM Auto-Antibodies in Mixed Groups:

There was a negative correlation between IL-10 with (GAD, ZnT8, and ICA) (R=-0.358, -0.428, and -0.035 at P<0.05) respectively when the increased serum concentrations of IL-10 accompanied by decreased (GAD, ZnT8, and ICA).

The present study was focused on determining the role of elevated levels of IL-10 in the mixed group (diabetes infected with intestinal parasitic infection) to decrease the level of auto-antibodies, which was significant between them(p-value ≤0.05) as shown in Figures (3-10),(3-11) and (3-12) respectively.

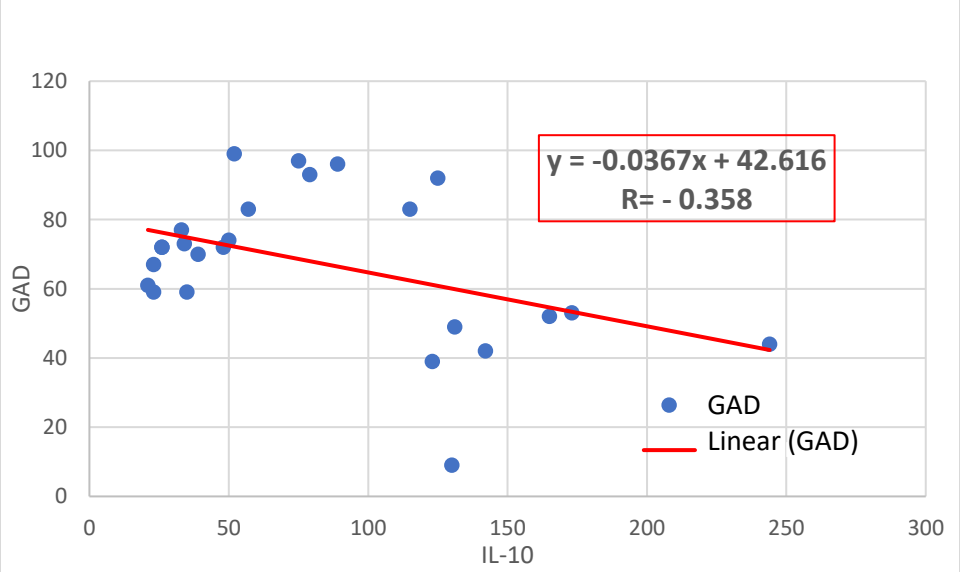

Figure (3-10): Correlation of GAD, IL-10 in Mixed Groups

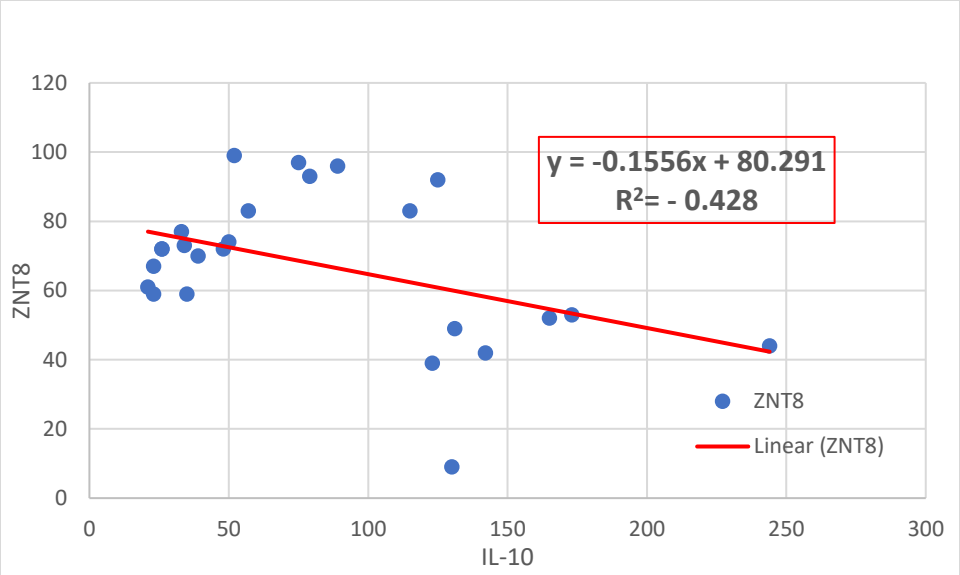

Figure (3-11): Correlation of ZnT8, IL-10 in Mixed Groups

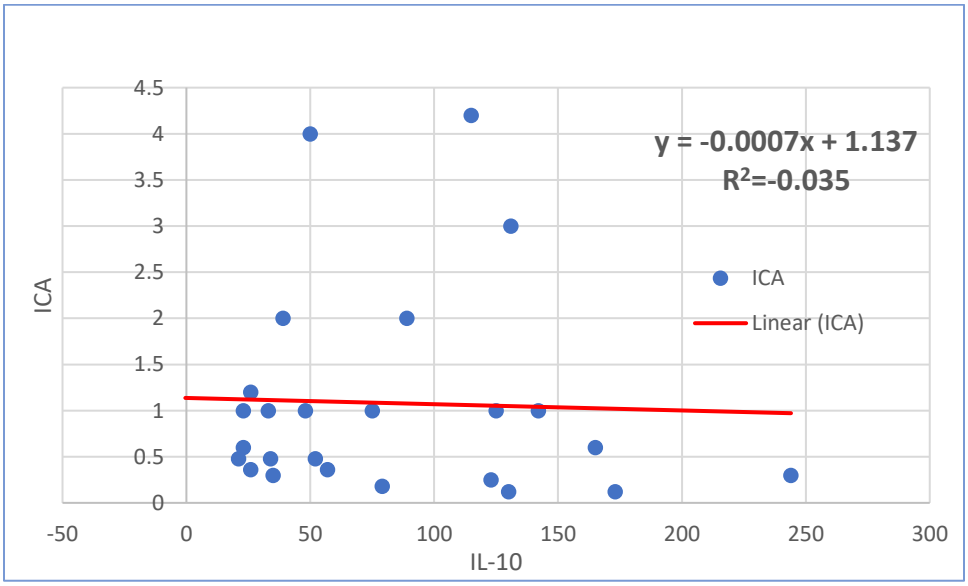

Figure (3-12): Correlation of ICA, IL-10 in Mixed Groups

These obtained results clarify the role of interleukin-10 in decreasing the levels and severity of immunological auto-antibodies effects that consistence with Casas *et al*. (2020) who mentioned when IL-10 was initially discovered its activity was accompanied by a considerable increase compared to baseline levels and drop in the percentages of GAD. Retaining residual beta cell function

reduces the severity of the condition, makes therapy easier, avoids complications, and improves survival.

The current study consistence with the recent study of Chen *et al*. (2022) who clarified that the experimental group had lower anti-GAD autoantibodies when an elevated level of IL-10 and transforming growth factor (TGF- β) were discovered.

The results were close to the finding of El-Mokhtar *et al*. (2020) which found a negative correlation between pan IL- 10 with autoantibodies GAD, ZnT8, and ICA

Also, the current results were close to the finding of Arif *et al*.(2014) which showed the ZnT8 ($P < 0.05$) is significantly less frequent in the elevated IL-10 level.

In support of the current study observation, Andrade Lima Gabbay *et al*. (2012) have shown (IA2) and zinc transporter-8 (ZnT8) present in the pancreatic β-cells correlated negatively with CXCL10 and CCL2, IA-2A titers and were correlated negatively with circulating IL-10.

And close to the finding of dos Santos Haber *et al*.(2023) performed that, antibodies involved in the pathogenesis of T1DM, such as anti-glutamic acid decarboxylase (GAD) antibody, anti-zinc transporter 8 (ZnT8A), anti-islet antibody ICA negatively correlated with anti-inflammatory cytokines (IL-10).

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions:

According to the obtained results in the present study, the following conclusions have been detected:

1- The present study determined the role of intestinal parasitic infection to modulate immune function toward Th-2 and determining the role of elevated levels of IL-10 in the mixed group.

2- In Diabetic and Mixed groups increased levels of IL-2, GAD, ZnT8, and ICA, if compared with controls also increased levels of IL-10 in Mixed groups as compared with Diabetic, Parasitic, and healthy controls.

3- There was a strong correlation between IL-10 and IL-2, Also there was a highly significant difference in IL-10 level in mixed groups if compared with diabetic groups, with decreased IL-2 level in mixed groups if compared to the diabetic group and there was a negative correlation between IL-10 with GAD, ZnT8 and ICA.

4- In the Parasitic group increased levels of IL-10 and PG-E2 if compared with Diabetic, Mixed, and healthy control.

5- There were no significant differences in mean IL-2, IL-10, PG-E2, ZnT8, and ICA levels between males and females But there were highly significant differences in the mean of anti-GAD levels between males and females and more in the females.

6- There were no significant differences in IL-2, IL-10, PG-E2, GAD, ZnT8, and ICA concentration between *Entamoeba histolytica* and *Giardia lamblia* infections in parasitic and mixed groups.

Conclusions and Recommendations

Recommendations:

1- Study the polymorphism of Foxp3 and Cytotoxic T-Lymphocyte-Associated Antigen-4 (CTLA-4) mediated inhibition of T cell response, development and activation of T-reg in T1DM.

2- Additional studies are needed to detect the IL-10 and PG-E2 family gene expression with a large sample of the study population to establish an obvious idea about the prevalence of this polymorphism among intestinal parasitic infections in diabetic patients.

3- Various immunological and hematological indicators, such as IgA, IgE, IL-4, IL-5, IL-1β, IFN-γ, and complete blood count (CBC), should be done to assess the immune response in diabetes patients with intestinal parasite infections.

4- Studying the phagocytic activity in diabetic patients and the level of myeloperoxidase, nitric oxide dismutase, and Reactant Oxidative Stress ROS.

5- Using purified parasitic antigens as immunotherapy to decrease the harmful effect of parasite and benefit from parasitic immunomodulation.

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Appendices

……..*Appendix*……..

استمارة معلومات

اسم المريض :

العمر : | الجنس | ذكر ☐ | انثى ☐

عدد افراد العائلة : | ذكور ☐ | أناث ☐

اسم ونوع المرض :

التشخيص المعتمد للمرض :

تاريخ حدوث الإصابة بالمرض :

العلاج المستخدم | حقن ☐ | حب ☐ | اخرى ☐

نوع الانسولين المستخدم | صافي ☐ | خابط ☐ | مخلوط ☐

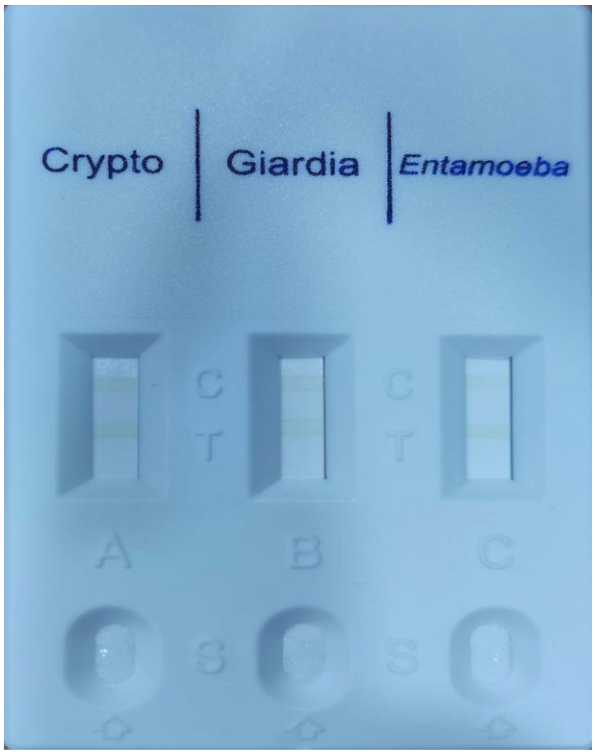
هل توجد امراض وراثية اخرى لدىالمريض | نعم ☐ | لا ☐

اسم المرض :

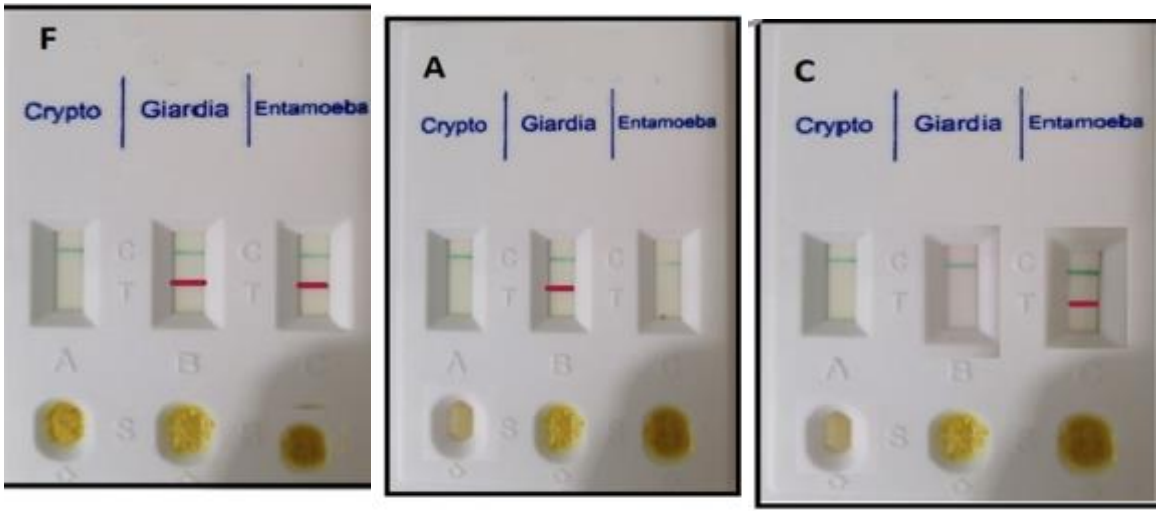
هل توجد امراض وراثية لدىافراد العائلة : | ذكور ☐ | اناث ☐

الملاحظات :

……..*Appendix*……..

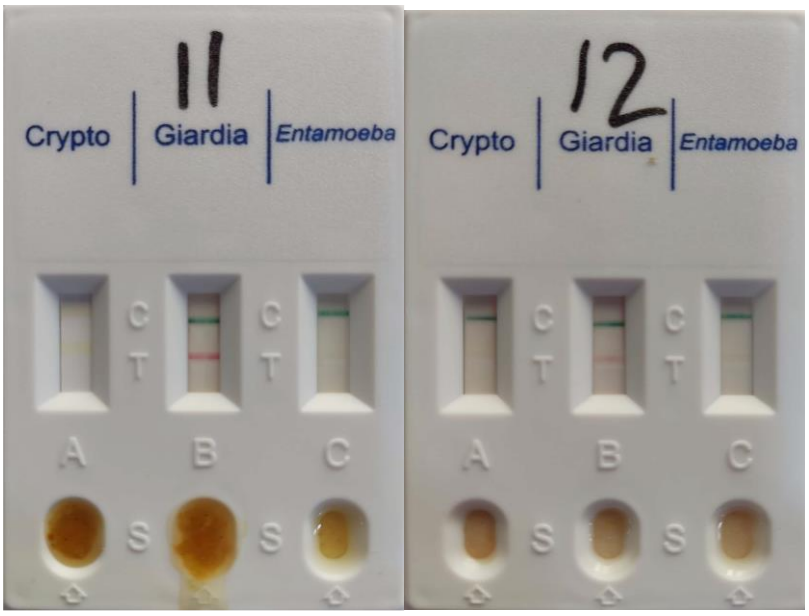


CERTEST *Cryptosporidium* + *Giardia* + *Entamoeba* COMBO CARD TEST

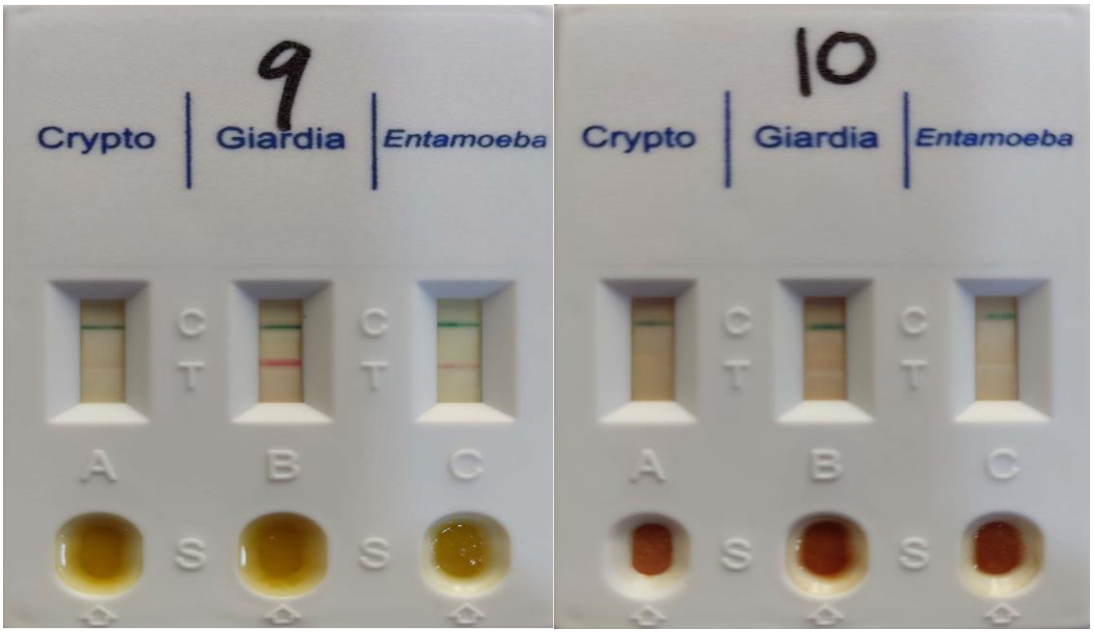


Interpretation of positive results for *Entamoeba histolytica* ,*Giardia lamblia* and both of them

……..*Appendix*……..



Positive results for *Giardia lamblia*



Positive results for *Entamoeba histolytica* and *Giardia lamblia*

الخلاصة

يحدث داء السكري من النوع الاول (T1DM) بسبب اضطراب عدم تحمل الجهاز المناعي وغزو الخلايا بيتا β في جزيرات لانكرهانز في البنكرياس بواسطة الخلايا التائية المناعية ذاتية التفاعل وإحداث تدهور في نشاط الخلايا β وصلاحيتها مما يتطلب العلاج لفترات طويلة بالأنسولين الخارجي. قد تؤثر التغيرات في الحالة المناعية للمضيف وتتأثر بالأمراض المصاحبة الأخرى. قد تمنع العدوى الطفيلية داء السكري من النوع الاول عن طريق تعطيل المسارات المؤدية إلى التدمير بوساطة Th1 لخلايا β المنتجة للأنسولين بوساطة آليات مرتبطة بقدرة المضيف على تحفيز استجابة Th2 للطفيليات.

كانت هذه دراسة حالات وشواهد أجريت على مرضى الأطفال المحالين الى مستشفى كربلاء التعليمي للأطفال في محافظة كربلاء ومركز الامام الحسن لمرضى السكري والغدد الصماء الذين تتراوح أعمارهم بين (1-15 سنة) خلال الفترة من شباط الى كانون الاول ٢٠٢٢ حسب مظاهرهم السريرية.

شملت الدراسة 180 مشاركا (98, 54% ذكور و 82 , 46% إناث) مقسمة الى أربع مجموعات (70 مريضا مصابا بالسكري النوع الاول و 50 مصابا بعدوى طفيلية معوية و 25 مريضا مصابا بالسكري النوع الاول والطفيليات المعوية وكذلك 35 اصحاء .

تضمنت الدراسة الحالية جزأين من المعلمات: كان الجزء الأول من المعلمات هو قياس الحركيات الخلوية (2 ، 10 ، وبروستاجلاندين E2) ويتم ذلك بعد تشخيص T1DM اعتمادا سريرياعلى سكر الدم الصائم ومستوى الهيموغلوبين السكري.HbA1c واكتشاف الاجسام المضاده الذاتيه لخلايا بيتا البنكرياس التي تتضمن الأجسام المضادة الذاتية لحمض الجلوتاميك ديكاربوكسيلاز ضد GAD وعضو عائلة ناقل الزنك 8 (ZnT8) والأجسام المضادة التلقائية للجزيرة ICA في الحالة ومجموعات التحكم بواسطة الممتزالمناعي المرتبط بالانزيم ELISA في مصل مجاميع الدراسة, والكشف عن الاوالي المعوية في داء السكري من النوع الأول في عينة البراز عن طريق الفحص العام للبراز وتأكيد التشخيص عن طريق فحص الكروماتوغرافي المناعي السريع.CERTEST

لا توجد فروق ذات دلالة إحصائية بين توزيعات الذكور والإناث بواسطة اختبار مربع كاي للمتغيرات عندالقيمة الاحتمالية (P = 0.45، P≥0.05)و كانت هناك اختلافات ذات دلالة إحصائية كبيرة بين الفئات العمرية عندالقيمة الاحتمالية (P = 0.000، P≤0.05) في اختبار أنوفا ذا الاتجاه الواحد.

في المجموعتين السكرية والمختلطة ارتفع مستوى IL-2 و GAD و ZnT8 و ICA إذا ما قورنت بالاصحاء ، كما ارتفع مستوى IL-10 في المجموعات المختلطة للعدوى نتيجة للاصابة بالطفيليات.

كان هناك ارتباط قوي بين IL-10، IL-2 (R = 0.745 ، p<0.01) ، كما كان هناك فرق معنوي للغاية في مستوى IL-10 في المجموعات المختلطة إذا ما قورنت مع مجموعات مرضى السكري عندالقيمة الاحتمالية (P = 0.000) ، مع انخفاض مستوى IL-2 في المجموعات المختلطة إذا ما قورنت بمجموعات مرضى السكري عند القيمه الاحتمالية (P = 0.000)التي حددت دور العدوى الطفيلية المعوية لتعديل وظيفة المناعة تجاه Th-2.

يشير الارتباط الإيجابي الملحوظ عند القيمه الاحتمالية (R = 0.470، P<0.05) بين تركيزات المصل المتزايدة من IL-10 و PG-E2 إلى وجود صلة بينهما كحركيات خلوية مضادة للالتهابات والارتباط الإيجابي (، P<0.05) .

كان هناك ارتباط سلبي ملحوظ بين IL-10 مع GADو ZnT8 و ICA (0.358-،0.428- و 0.035 -) عند القيمه الاحتمالية (P<0.05) على التوالي .

في المجموعات الطفيلية ارتفع مستوى IL-10و PG- E2 وانخفاض أوضمن المستوى الطبيعي من IL-2, GAD ، ZnT8 و ICA.

كانت هناك فروق ذات دلالة إحصائية عالية (P = 0.000، P≥0.05) في متوسط مستويات GAD بين الذكور والإناث وأكثر في الاناث.

لم تكن هناك فروق ذات دلالة دلالية إحصائية في تركيز IL-2 و IL-10 و PG-E2 و GAD و ZnT8 و ICA بين عدوى *Entamoeba histolytica* و *Giardia lamblia* في المجموعات الطفيلية والمختلطة.

في الدراسة الحالية تم تحديد دور المستوى المرتفع من IL-10 في المجموعة المختلطة (مرض السكري المصاب بعدوى طفيلية معوية) لتقليل شدة المرض.

جـــمـــهـــوريـــة الـعـراق
وزارة التعليم العالي والبحث العلمي
جـــامـــعـــة بـــابـــل
كـــلـــيـــــة الـــطـــب

التحمل المناعي المحفز بالاصابات الطفيلية المعوية لمرضى داء السكري من النوع الاول

أطروحة

مقدمة إلى مجلس كلية الطب / جامعة بابل

كجزء من متطلبات نيل شهادة الدكتوراه /الأحياء المجهرية الطبية
تقدمت بها

هبة علي هادي جاسم القاضي

بكالوريوس تقنيات الصحية والطبية/ الجامعة التقنية الوسطى بغداد (٢٠٠٤)
ماجستير/ احياء مجهرية طبية /كلية الطب /جامعة بابل (٢٠١٠)

بإشراف

الأستاذ الدكتور
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الدكتورالأستشاري
محمد صالح مهدي
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مستشفى الأمام الحسين التعليمي/ كربلاء

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