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Evaluation of Autoimmune markers and frequency of HLA-DQ2\DQ8 in patients with Type 1 Diabetes \or CeliacDisease.

A thesis

Submitted to the Council of the College of Medicine, University of Kerbala, as Partial Fulfillment of the Requirements for the Master Degree of Science in Medical Microbiology

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بسم الله الرحمن الرحيم

(وَمَا تَوْفِيقِي إِلا بِاللَّهِ عَلَيْهِ تَوَكَّلْتُ وَإِلَيْهِ أُنِيبُ)

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Committee certification

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DEDICATION

To the prophet Mohammed and his progeny (peace be upon them).

To the leader of humanity imam Mohammed AL-Mahdi (peace be upon him).

To whom I draw strength and safety from him ……My father.

To my life my kindly mother to my lovely brother and sister who always support and help me.

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SUMMARY

The prevalence of celiac disease (CD) is significantly higher in patients with Type 1 diabetes mellitus (T1DM) compared to the general population and as a result, the interrelationship of these two autoimmune disorders has attracted the interest of many researchers over the years. The most pressing questions have been whether the concomitant existence of CD pose challenges to the management of T1DM or whetherthe existence of celiac disease results to the activation of genetic triggersfor T1DM in already susceptible individuals. Also, there are concerns onthe possible effects T1DM on the pathophysiology and response totreatment of CD in patients having comorbidity of both diseases. This study aimed to address these questions by evaluating the bio demographical characteristics, genetic predisposition and distribution of diagnostic parameters for CD and T1DM in patients having CD, T1DM as well as comorbidity of both conditions. Fourty-five patients with T1DMonly, 45 with CD only and 45 with both conditions were recruited for thisstudy. The demographical, anthropometric and biochemical characteristic, as well as distribution of HLA-DQ2 and DQ8 genes amongst the patients were measured. Also, duodenal biopsy as well as serum IgA and IgG classes of anti-tissue transglutaminase (anti-tTG) and anti- gliadin autoantibodies were evaluated for patients having CD only and those having comorbidity with type 1 diabetes (CD+T1DM). Serum levels of anti-glutamic acid decarboxylase (GAD) autoantibody and c- peptide were also evaluated in patients with T1DM and those having CD+T1DM. The results obtained showed significant differences in glucose (94.82±3.89 mg/dl CD vs. 173.74±1.97 T1DM), HbAlc levels (5.43±0.66% CD vs. 7.90±0.24), vitamin D3 (7.15±0.99 ng/mL T1DM vs. 11.04±2.82 CD+T1DM), and urea (35.23±4.36 mg/dL T1DM vs.

23.24±4.75 mg/dL CD+T1DM) and creatinine (1.06±0.06 mg/dL T1DM vs. 0.61±0.01 mg/dL CD+T1DM). HLA-DQ2 was skewed towards patients with CD while HLA-DQ8 was skewed towards patients with T1DM and 80% of CD+T1DM patients were HLA-DQ2/DQ8 heterozygous. Majority of the patients with Marsh III grading i.e. 71%, sero positivity to anti-tTG IgA (88.9%), IgG (60%) and anti-gliadin IgA (51.1%), IgG (100%) were those with CD+T1DM) although titer values showed significantly lower level compared to CD patients ($p < 0.05$). Also, anti-GAD levels were significantly higher in patients with T1DM only compared with those with CD+T1DM ($p < 0.05$). From the findings, it was inferred that the concomitant existence of CD and T1DM may result to difficulty in glycemic control but slow down progression of CD symptoms. Also while T1DM may confer susceptibility to CD, the pre-existence of CD may trigger the pathogenesis/ initiation of T1DM. Therefore, this study recommends that the early diagnosis of CD or T1DM should be sufficient to warrant suspicion of subclinical T1DM or CD respectively

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ABBREVIATIONS

Abbreviations	Terms
T1DM	Type 1 diabetes mellitus
CD	Celiac disease
HLA	Human leukocyte antigen
Anti- GAD	Anti –glutamic acid decarboxylase
Anti _tTG	Anti –tissue transglutaminase
GLUT	Glucose transporter
Ca	Calcium
CD4	Cluster Differention 4
ADHD	Attention deficit hyperactivity disorder
mRNA	Messenger RNA
NCGS	Non celiac gluten sensitivity
GFD	Gluten free diet
HbA1c	Hemoglobin A 1c
INF	InterFerron
DAG	Dominated anti-gliadin
TMB	Tetra methyl benzidine
DGP	Deamidated gliadin peptide
ELISA	EnzymeLinked Immunosorbents assay
EMA	Endomysial Antibodies
PCR	Polymerase chain reaction
BMI	Body mass index
PK	Proteinase K
WBC	White blood cells
PCV	Packed Cell Volume
Ig	Immunoglobulin
NCWS	Non celery wheat sensitivity
GSIS	Gliadin stimulated insulin secretion
TCR	T Cell Receptor
Treg	T-Regulatory cell
NKG2D	Natural Killer group 2D
SD	Standard deviation
CTLA4	Cytotoxic Lymphocyte associated protein 4
PTPN22	Protein tyrosine phosphate non receptor type 22
IAA	Insulin autoantibodies
IA2	Islet antigen 2
EDTA	Ethylene Diamine tetra acetic acid
DRC	Dose Receptor curve

ECL	Electrochemiluminescence
ELA	Enzyme immunoassay
VDR	Vitamin D Receptor
GABA	Gamma Amino Butyric Acid
ZNT8A	Zinc Transport 8 Antibodies
K	potassium
ATP	Adenosine triphosphate
TH17	T Helper 17
FOXP3	Fork Head box 3
APC	Antigen presenting cells
CD8	Cluster of Differention 8
GLP	Glucagon _like peptide
GLUT 1	GLUCOSE Transporter 1
GLUT2	GLUCOSE Transporter 2
GLUT4	GLUCOSE Transporter 4
C-PEPTTIDE	Carbohydrate peptide
DC	Dendetric Cell
ADP	Adenosine diphosphate
RPM	Revolutions per minute
TH	T-helper
IL22	Interleukin 22
CD1	Cluster Differention 1
SUR1	Sulfonylurea receptor -1
VDCC	Voltage _gated calcium cannels
SNAP25	Synaptosomal,25

Chapter one
Introduction
and
Literature Review

1.1 Introduction

Celiac disease, an autoimmune condition involving intestinal inflammation related to gluten ingestion, has been routinely considered to be an autoimmune disorder that is associated with other autoimmune disorders especially in children. Epidemiological and genetic data shows that type 1 diabetes mellitus (T1DM) is the most common autoimmune disease associated with celiac disease (Haladová *et al.*, 2014; Grode *et al*., 2018). The association between celiac disease and T1DM has long been established as the prevalence rates of celiac disease is 4 to 6 times higher in adults with T1DM than in the general population (DeMelo *et al*., 2015). Also, due to a common genetic background and interplay between environmental and immunological factors, patients with T1DM have an increased risk of developing other autoimmune disorders, out of which celiac disease is most common (Barker, 2006).

Concerns on the spread of cases of comorbidity of these two autoimmune conditions is increasing and the Canadian Diabetes Association guidelines recommend targeted celiac disease screening in patients with T1DM who have classic symptoms, such as abdominal pain, bloating, diarrhea, unexplained weight loss or labile metabolic control (Bakker *et al*., 2013). However, despite this increased prevalence of celiac disease in patients with T1DM, the absence of symptoms remains the major challenge in establishing the diagnosis of celiac disease in patients with T1DM and as a result, the co-existence of the tow diseases goes undiagnosed until clinical manifestations become obvious. Studies have shown that a higher proportion of patients with diabetes report subtle or no complaints at celiac disease diagnosis, with the prevalence of asymptomatic celiac disease patients with T1DM ranging from 35.7% to 62.5% (DeMelo *et al*., 2015; Prieto *et al*., 2021).

There are concerns on the implication of the comorbidity of celiac disease and T1DM on the pathophysiological of the individual conditions as well as disease severity and response to treatment. Recent data have emerged and showed that beyond the short-term metabolic andlifestyle implications, a concomitant diagnosis of celiac disease and T1DM may increase the risk for diabetes-related complications (Szaflarska-Popławska, 2014).

Additionally, evidence suggests that adult patients with both conditions are at higher risk for diabetes microvascular comorbidities, increased mortality and impaired bone health if the celiac disease remains untreated (Prieto *et al*., 2021). Interestingly, the pressing questions however, remains whether the screening of celiac disease in T1DM patients is necessary and which of the two conditions is more detrimental or beneficial to the progress and response to treatment of the other. Also, whether the interrelationship of celiac disease and T1DM occurs at the onset of the disorders or results from progression of the disease remains unresolved.

To address this questions the present study identified patients with celiac disease alone, T1DM alone as well as those with both conditions and assessed their bio demographical characteristics along with distribution of the genetic genes - HLA-DQ 2 and DQ8 haplotypes that confer susceptibility to both celiac disease and T1DM. The study also explored the possible effects of T1DM on celiac disease by analysing degrees of duodenal lesions, distribution and expression levels of celiac disease associated autoantibodies in celiac disease patients and comparing same With those having T1DM comorbidity. Similarly, the possible effect of celiac disease on T1DM was investigated by analysing the distribution of

Seropositivity and titers of serum anti-GAD and c-peptide in T1DM in relation to those with T1DM and celiac disease.

1.1.2 General Objective

To evaluate the bio demographical characteristics, genetic predisposition and distribution of diagnostic parameters for celiac disease and T1DM in patients having celiac disease, T1DM as well as comorbidity of both conditions.

1.1.3 Specific Objective

i. To measure body mass index (BMI) and some biochemical Parameters in patients having celiac disease, T1DM as well as Comorbidity of both conditions.

ii. To evaluate the frequency of HLA-DQ2 and DQ8 haplotypes in having celiac disease, T1DM as well as comorbidity of both Conditions.

iii. To investigate the effect of T1DM on celiac disease by analysing duodenal lesion and serum levels of anti-transglutaminase (anti-tTG) , anti-gliadin autoantibodies in patients having celiac disease in Comparison with those having celiac disease and T1DM.

iv. To investigate the effect of celiac disease on T1DM by analysing Serum levels of anti-glutamic acid decarboxylase (anti-GAD) autoantibodies and c-peptide in patients having T1DM in comparison with those having celiac disease and T1DM.

1. 2 Literature review

1.2.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is a chronic disease caused by auto immune mediated destruction of pancreatic β-cells, which usually leads to absolute insulin deficiency (Mejía-León *et al*., 2015). Type 1 diabetes usually occurs in children and adolescents, although it can appear at any age. In the past type 1 diabetes was called juvenile diabetes or insulin-dependent diabetes mellitus (Massoud and Massoud, 2012).

The hallmark of T1DM is the deficiency in the secretion of insulin, which is a peptide hormone that is synthesized and secreted by β-cells in the islets of Langerhans of the pancreas. It regulates the metabolism of carbohydrates, fats and proteins by delivering glucose in the blood to fatty tissue,liver and skeletal muscle cells. Due to insufficient insulin activity in the body or decreased insulin secretion in β cells, a metabolic disease called diabetes occurs (Qaid and Abdelrahman, 2016). The human insulin molecule is composed of two polypeptide chains, i.e. A and B chain are connected by two disulfide bonds. The A chain contains 21 amino acids and the B chain contains 30 amino acid (Daghlas and Mohiuddin, 2020). The A chain has an N-terminal helix connected to an anti-parallel C-terminal helix; the B chain has a central helix segment. The two chains are connected by two disulfide bonds, which connect the N and C terminal helices of the A chain to the central helix of the B chain (Wilcox, 2005). Figure 1.1 shows the structure of insulin, highlighting its amino acid sequence and disulfide linkages.

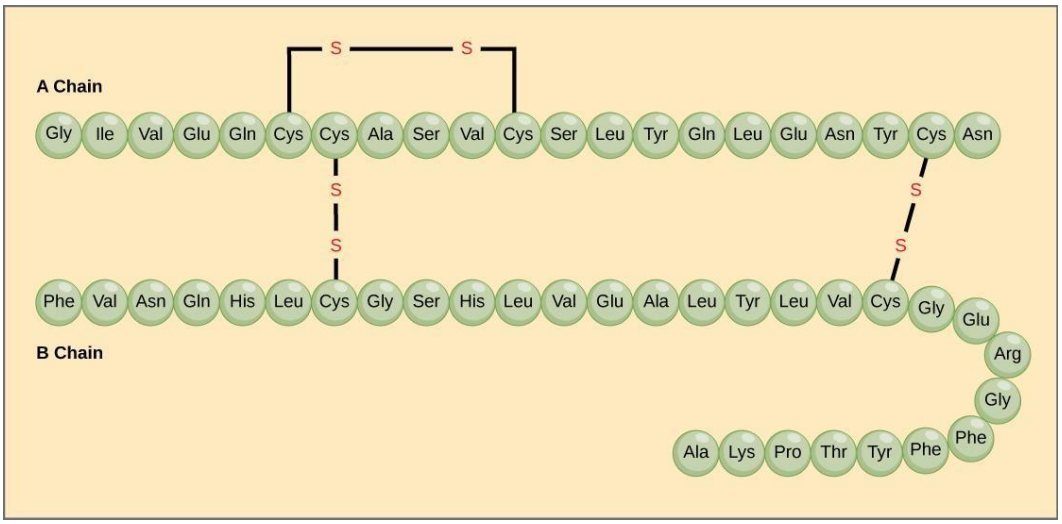


Figure 1.1: Structure of insulin
The amino acid sequences of the A and B chain of insulin. The A chain has one disulfide linkage while the two disulfide linkages connects the A and B Chains *(*Srivastava, 2021).

Insulin is synthesized in the β-cells of the islets of Langerhans. The illustration of insulin biosynthesis is presented in Figure1.2 below. Firstly, the insulin mRNA is translated as a single chain precursor called preproinsulin. Then the removal of its signal peptide at the N-Terminus during insertion at the endoplasmic reticulum occurs, thereby generating proinsulin. In the endoplasmic reticulum, endopeptidase cuts off the connecting peptide (c-peptide) between the A chain and the B chain. This breaks down the single chain into two chains (A and B) joined by disulfide bonds-the mature form of insulin is produced. Equimolar amounts of insulin and free c-peptide are packaged in the Golgi apparatusand stored in vesicles in the cytoplasm (Sirhan and Piran, 2020).

The first manifestations of T1DM develop when lack of insulin prevents cells from taking up glucose adequately, which is necessary and vital to cell function. Therefore, the classic symptoms include polyuria, polydipsia, weight loss, fatigue, and hyperglycemia, which, when left untreated, could result in a coma and ultimately death (Foster, 1878).

Diagnosis of diabetes includes fasting blood glucose higher than 126 Mg/dL, any blood glucose of 200 mg/dL or an abnormal oral glucose-Tolerance test (Marathe et al., 2017). Since 2009, the American Diabetes Association has modified the guidelines for diabetes diagnosis to include the measurement of glycated hemoglobin levels (HbA1C). This reflects the amount of blood glucose attached to hemoglobin and it is considered positive if higher than 6.5% on two occasions (Paschou et al., 2018).

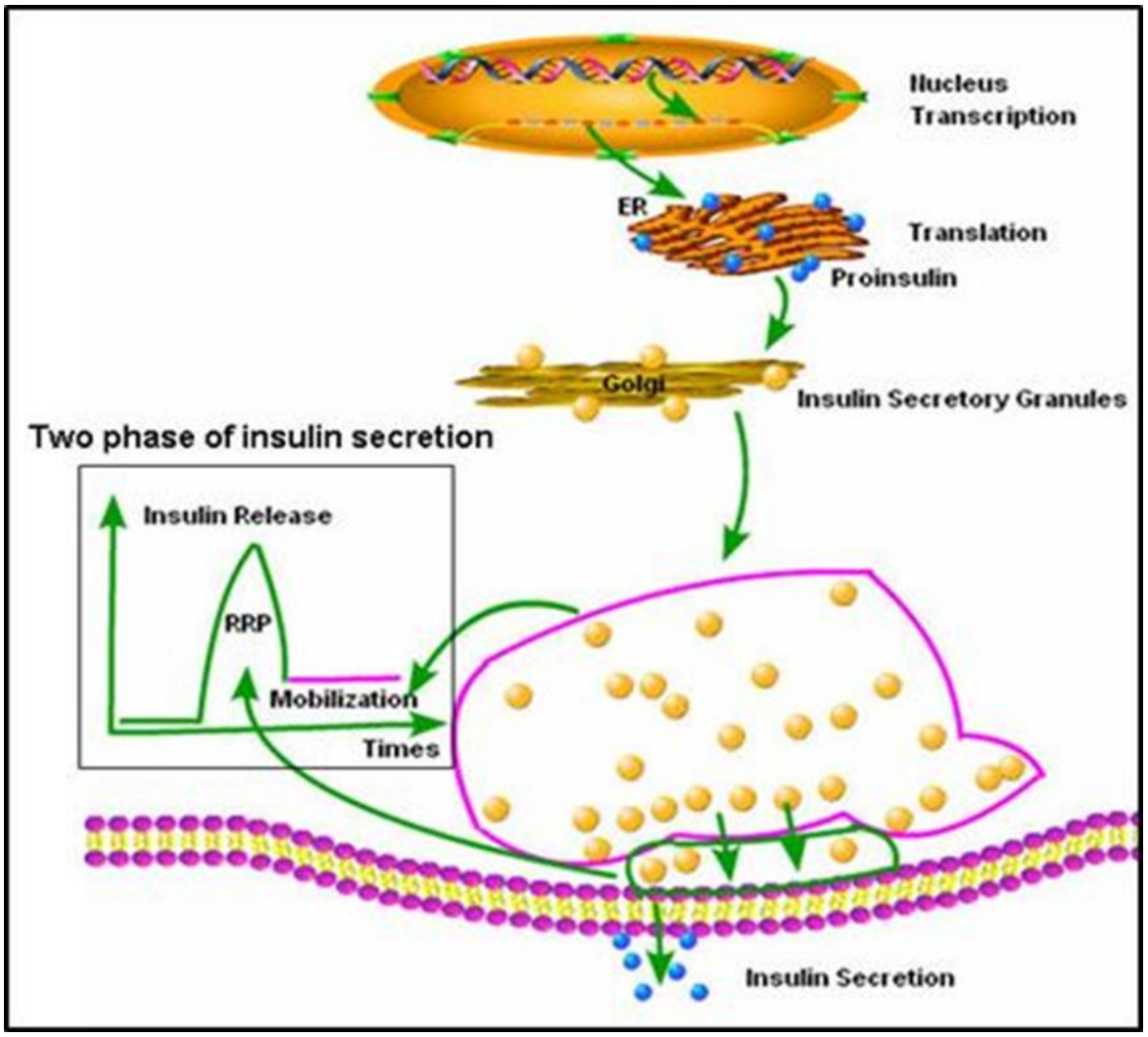


Figure 1.2: Biosynthesis and secretion of insulin
Synthesis of insulin starts with the transcription of the insulin gene followed by the translation of the mRNA into proinsulin the endoplasmic reticulum. Insulin secretorygranules facilitate the secretion of insulin from pancreatic β-cells.(Koo, 2010)

1.2.1.1 Genetics of T1DM

The most important gene that causes susceptibility to T1DM is located at the HLA class II locus on the short arm of chromosome 6 (Skog *et al*., 2013). These genetic associations between HLA and insulin alleles predict that HLA-DQ2- and HLA-DQ8-restricted proinsulin-specific CD4 T cells will infiltrate human pancreatic islets, which play a pathogenic role in T1D. The HLA-DQ antigen is a surface receptor of antigen-presenting cells (APC) for recognition by the T cell receptor (TCR), it is composed of two polypeptide subunits: the chain (of 32–34 kD) and the β chain (of 29–32 kD) (Gualandris *et al*., 2021). Each presents a peptide binding domain, an Ig-like domain and a transmembrane region with a cytoplasmic tail. These structures are subject to non-covalent association leads. Unlike major histocompatibility complex (MHC) class I molecules, both polypeptide chains are encoded by genes strictly located in the HLA-DQ region on chromosome 6. The pocket that binds to the peptide bond consists of one half of the chain and one half of the other chain.

Each DQ heterodimer is composed of two chains, namely α and β, and the corresponding amino-terminal external domains (α1 and β1) create the peptide-binding cleft, once assembled together (Abbas *et al*., 2014; Hardy and Tye-Din, 2016). The DQ molecules can be formed, with α- and β-chains encoded on the same chromosome (encoded in cis) or on opposite chromosomes (encoded in Trans). Some that not every α- and β-chain pairing will form a stable heterodimer. It is generally considered that alleles of DQα- and DQβ-chains pair up predominantly in cis rather than in trans (Tollefsen *et al*., 2012). The DQ2 and DQ8 heterodimers consist of α and β chains encoded by two HLA-DQA1 and two HLA-

DQB1 alleles that each individual possesses (Di Sabatino and Corazza, 2009).

Although predictable, CD4 T cells have never been isolated from human pancreatic islets, leaving a critical piece in the T1D autoimmune causality chain. This gap in knowledge has led some People to question the autoimmune basis of human T1DM (Koeleman *et al*., 2004; van Lummel *et al*., 2012). In T1DM, compared with homozygous HLA-DQ2 or HLA-DQ8 individuals, the risk associated with the HLA-DQ2/8 heterozygous haplotype was found to increase, indicating epistasis or synergy (Ilonen *et al*., 2002).

1.2.2 Celiac disease

Celiac disease (CD) is considered one of the most common autoimmune diseases related to small bowel triggers caused by ingestion of gluten-containing foods (Castillo *et al*., 2015). The disease was first described by the Greek physician Aretaeus in ancient Rome (Gasbarrini *et al*., 2012). CD is also known as coeliac disease, celiac sprue, non-tropical sprue and gluten sensitive enteropathy (Taylor *et al*. 2012).

It is characterized by an immune response to ingested wheat gluten and related proteins of rye and barley, leading to inflammation, villus atrophy and crypt hyperplasia. The proximal symptoms and signs of CD include diarrhoea, bloating, abdominal pain, weight loss, and fatigue and malnutrition (Alaedini and Green, 2005).

The classification of CD is based on the presence of gastrointestinal symptoms: ``classic" or ``symptomatic" CD refers to the appearance of

Diarrhea and malabsorption syndrome; while the typical asymptomatic form has no Gastrointestinal manifestations (Van Buiten, 2017). When classic bowel disease is found in patients with apparently healthy underlying CD, CD is defined as silent, which is the risk of developing classic CD.

1.2.2.1 Gluten

Gluten is composed of two main parts: gliadin protein and glutenins protein. When it comes to allergies and food sensitivities, most people react to the gliadin part (but some people also react to the gluten part (Wilser, 2016). Gluten can be found in many types of foods, even unpredictable. Wheat is commonly found in (bread, baked goods, soups, pasta, cereals, seasonings, salad dressings and broths). Rye is commonly found in rye and bread, such as pumpernickel. Barley is commonly found in (food colouring , soups , beer , Brewer's Yeast , and malted barley flour, malted milk and milkshakes, malt extract, malt syrup, malt flavouring, malt vinegar) (Passananti *et al*., 2012). Gluten can be subdivided into low and high molecular weight proteins, while the gliadin protein family contains, alpha, beta, gamma types. Both glutenins and gliadins are characterized by a high amount of prolines (20%) and glutamines (40%) that protect them from complete degradation in the gastrointestinal tract and make them difficult to digest. (Camarca *et al*., 2012).

When people with CD eat gluten, their body emits an immune response that attacks the small intestine. These attacks can cause damage to the Villi, which line up along the small intestine, forming small finger-like protrusions to promote nutrient absorption (Knowles et al., 2019).

1.2.2.2 Symptoms of celiac disease

In adults, there are rarely digestive symptoms, and only one-third of them have diarrhea. Adults are more likely to suffer from: unexplained iron deficiency anemia, fatigue, bone pain or joint pain, arthritis, osteoporosis, osteopenia (bone loss), hepato biliary diseases (transaminase, fatty liver, primary sclerosis Cholangitis), depression or anxiety, peripheral neuropathy (tingling), numbness or pain in the hands and feet), seizures or migraines, missed menstruation, infertility (Smith and Good fellow 2011). According to the World Gastroenterology Organization, celiac disease can be divided into three types: silent, classic and non-classical.

Silent celiac disease, which is also known as asymptomatic celiac disease show no symptoms, although the patient still suffers from malnutrition of the small intestine (Di Stefano *et al*., 2013). Studies have shown that even if patients think they are asymptomatic, after adhering to a strict gluten-free diet, they still show better health and reduce acid reflux, bloating, flatulence and flatulence. First-degree relatives (parents, siblings, children), regardless of symptoms, should always be screened, since there is a 1 in 10 risk of developing Celiac disease (Gutierrez-Achury *et al*., 2015).

In classic celiac disease, patients experience signs and symptoms of malabsorption,including diarrhea, steatorrhea (paleness, foul smell, fatty stools), and weight loss or growth failure in children (Cheng *et al*., 2010).

In non-classical Celiac disease, patients may have mild gastrointestinal symptoms without obvious signs of malabsorption, or they may have seemingly unrelated symptoms. They may suffer from bloating and pain, and/or other symptoms such as iron deficiency anemia, chronic fatigue, chronic migraine, peripheral neuropathy (numbness, numbness or pain in the hands or feet), unexplained chronic hyper ammonia (increased liver

enzymes), reduce bone mass and fractures, vitamin deficiency (folic acid and B12), late menarche/early menopause and unexplained infertility, dental enamel defects, depression and anxiety, dermatitis herpetiformis (Smith *et al*., 2017).

The symptoms of wheat sensitive people are similar to those of CD.
The symptoms will be relieved after gluten is removed from the diet, but the CD is positive. Some people experience symptoms of CD, such as "confused mind", depression, inattention/hyperactivity disorder (ADHD)-behavior, abdominal pain, bloating, diarrhoea, constipation, headache, bone or joint pain, and gluten The chronic fatigue of their diet, but did not test positive for CD. When removing gluten from the diet can relieve symptoms, the terms non-celiac gluten sensitivity (NCGS) and non-celery wheat sensitivity (NCWS) are often used to refer to this condition (Mollazadegan *et al*.,2013).

1.2.2.3 Genetics of celiac disease

Celiac disease is characterized by an interaction of a certain genes. Gluten and environmental factors (Green *et al*., 2015). Human leukocyte antigen (HLA)-DQ2.5 protein(encoded by HLA-DQA1*05and DQB1*02alleles) and HLA-DQ8 protein (encoded by HLA-DQA1*03 and DQB1*03:02 alleles) are recognized predisposing factors for CD (Kupfer and Jabri, 2012). The genes encoding for HLA-DQ molecules are found in a region known as class II on chromosome 6, known as HLA-DQA1 and HLA-DQB1 genes encode for α- and β-heterodimers (Kupfer and Jabri, 2012).

These are cell surface receptors located on antigen-presenting cells that form cracks that bind to gliadin peptides. An essential step in the pathogenesis of CD is HLA DQ-mediated presentation of gluten peptides to CD4 T lymphocytes: Delaminated gluten peptides (delaminated by transglutaminase 2) bind to HLA-DQ molecules and present them Stimulation of gluten-specific CD4 T cells leads to an immune response to CD (Sollid *et al*., 2015; Sollid, 2017). About 90% of individuals with CD carry HLA-DQ2.5. HLA-DQ2.5 can be expressed in cis configuration, where the alleles are located on the same chromosome (DQA1*05:01–DQB1*02:01) or in trans-configuration, where the alleles are located on opposite chromosomes(DQA1*05:05–DQB1*03:01/DQA1*02:01–DQB1*02:02) (Sollid *et al*., 2012).

Strong association was observed between HLA-DQ2.5 and predisposition to CD, explained by its affinity to binding gluten proteins (Romanos and Wijmenga, 2010). Figure 1.4 show the major histocompatibility complex class II DQ genes that confer susceptibility to CD.

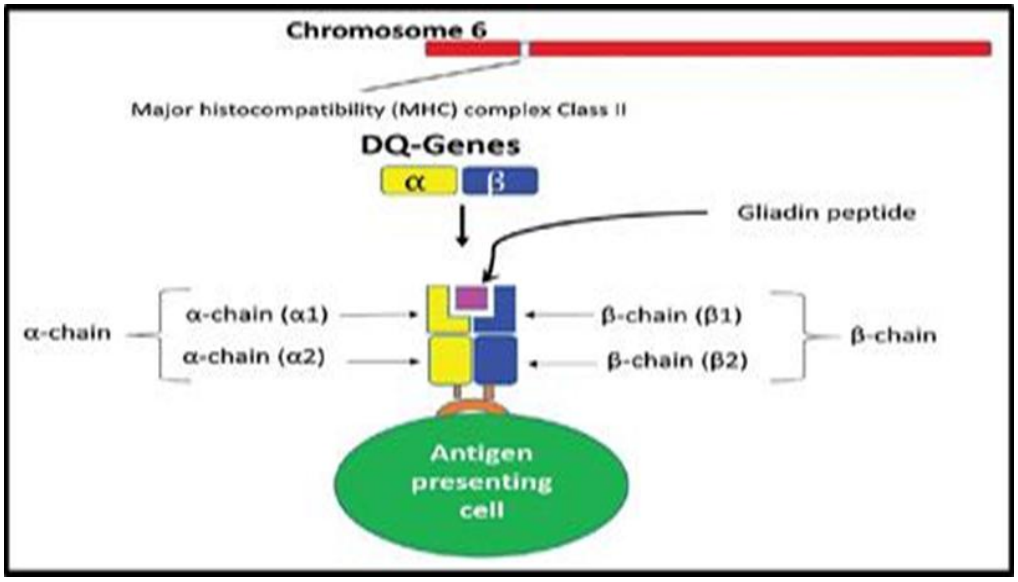


Figure1.4: The class II HLA-DQ gene

The genes encoding for HLA molecules are found in the major histocompatibility (MHC) complex on the short arm of chromosome 6 (6p21.3). HLA moleculesinvolved in celiac disease are encoded in a region known as class II by genes known as -DQ.HLA- DQA1 gene encodes the α chain (α1 and α2), while DQB1 encodes theβ chain (β1 and β2) of HLA-DQ protein. Both chains are associated as heterodimers on the surface of antigen-presenting cells and form a cleft that binds antigens and presents them to T-cell(Al-Hussaini *et al*., 2018).

1.2.3 Type 1 diabetes and celiac disease

The association between CD and T1DM was first reported in the late 1960s (Fassano and Catassi, 2012). These two disorders are immune-Mediated diseases, and have common susceptibility factors. HLA genetics and environmental factors play an important role in the Pathogenesis of the disease. The gut microbiome and infectious agents regulate innate and adaptive immunity, thereby increasing the risk of CD and T1DM (Cohn et al., 2014). Due to a common genetic background and interplay with environmental and immunological factors, patients with T1DM are high risk of developing other autoimmune diseases. CD is the most common autoimmune disease occurring with T1D (Dubé et al.,

2005; Volta et al., 2011). Both diseases have genetic patterns associated with HLA-DQ2 and HLA-DQ8, which are reported to cause the prevalence of CD in T1DM to be five to seven times higher than the general population (Fasano et al., 2003).

It is confirmed that the prevalence of HLA-DQ2 haplotypes is high in T1DM and CD patients, and it is reported that HLA-DQ2 homozygosity makes the risk in CD patients the highest. Many studies have listed DQ2 as the main susceptibility factor for CD. HLA-DQ8 is another important allele of CD and is considered to be more sensitive to T1DM (Dezsofi et al., 2008). The DQ8 heterozygosity is considered the strongest risk factor for the development of T1DM (Koeleman et al., 2004). Gliadin peptides pass through the intestinal barrier after oral gavages, and then locate in the pancreas and to a lesser extent the islets (Bruun et al., 2016). The ability of gluten peptides to cross the intestinal barrier has been independently confirmed (Freire et al., 2016). Gliadin also appears to cross the human intestinal barrier, as evidenced by the observation of gliadin in breast milk and serum of healthy mothers. Due to the high degree of vascularization in the pancreatic islets, gluten peptides may be in close contact with beta cells (Marichal, 2010).

Gliadin increases glucose-stimulated insulin secretion (GSIS) by closing the ATP-dependent K channel (Dall et al., 2013). Gluten-containing STDs and GF diets are known to increase insulitis and inflammatory cell-stress increases. The expression and enzyme activity of tTG (Ientile et al., 2007). The tTG has been shown to induce posttranslational modifications of human islet antigens and thereby increase the affinity to HLA-DQ (van Lummel et al., 2014).

Gluten-free diet reduces beta-cell stress and this may result in increased numbers of islets, besides reduced insulitis and autoimmune diabetes incidence; an effect that has also been observed when the diet was applied in utero as shown in Figure1.5

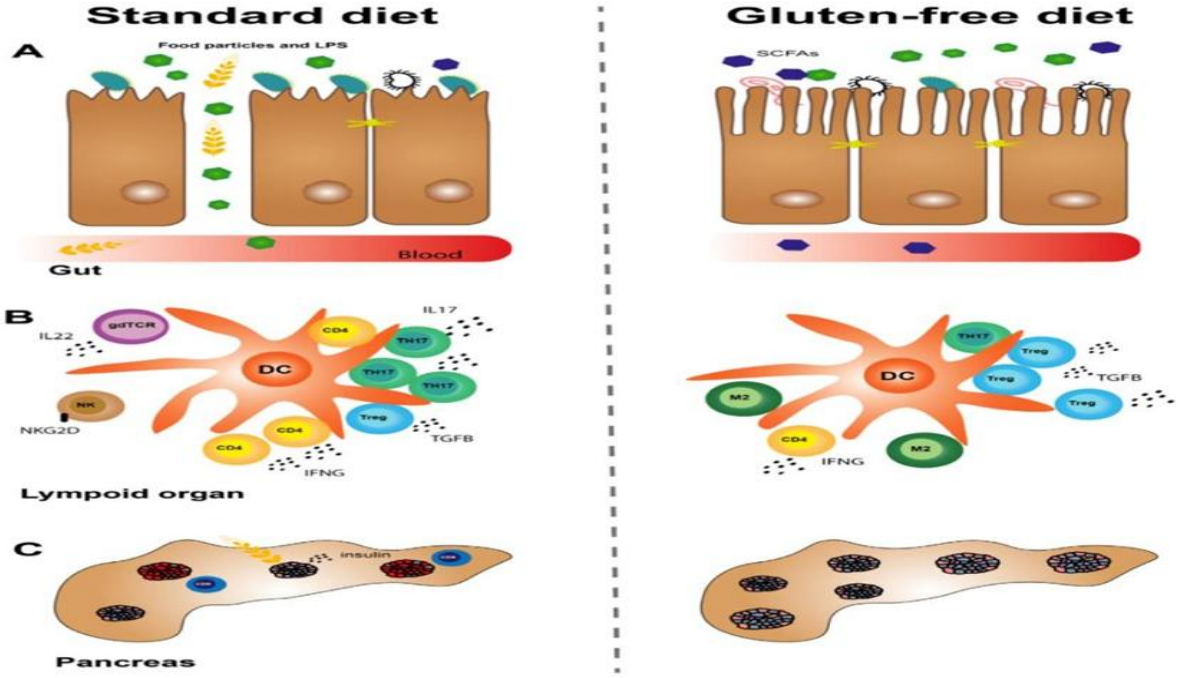


Figure1.5: The development of gluten-free (GF) diet andT1DM

(A) Gluten-free diet reduces intestinal permeability and increases the ratio of villi to crypts, thereby preventing food particles (such as gliadin peptides) from passing through the intestinal barrier and causing pancreatic reactions.
(B) GF diet regulates the innate and adaptive immune system, resulting in a decrease in the secretion of interferon gamma (IFNG) in CD4 T helper (TH) cells and a decrease in the secretion of interleukin (IL) 22 in TCR T cells, and reduce the number of activated (NKG2D) natural killer (NK) cell characteristics. The number of TH17 cells decreased, the number of immunosuppressant M2 macrophages and the number of for knead box P3 (FOXP3) regulatory Tcells (Treg) increased.
(C) GF diet can reduce β-cell stress by reducing insulin secretion, which can preserve the number of islets, reduce insulitis and improve T1DM.
(Haupt-Jorgensen et al., 2018)

1.2.3.1 Pathophysiology of celiac disease and T1DM

The common characteristics of T1DM and CD is selectively destroying of β cells of islets and enterocytes respectively. The triggering factor of the event cascade is not clear in T1DM, but the triggering factor is wheat gluten in CD. Because the pathogenic factors in CD are well known, the pathogenic mechanism of CD is more precise than that of T1DM, in addition to gluten, other infectious agents include viruses (adenovirus type 12, hepatitis C virus, rotavirus) are also implied as a risk factor for CD. This is evident from the fact that not all individuals with genetic risk factors develop CD (Knip and Simell, 2012).

Similarly, viruses such as enterovirus and herpes virus are also described as a trigger of T1DM (De Beeck and Eizirik, 2016; Rodriguez-Calvo et al., 2016). Due to some trigger, even the β-cells upregulate interferon (INF)-α and subsequently, MHC class I on cell surface exposes the β cells to attack by the autore-active CD8+ T cells with specificity for antigens in the pancreas (Peakman, 2013). Type 1 interferon also activates dendritic cells (DC) and promotes the presentation of β-cell antigens to T cells. Antibodies bind to β-cells and Fc receptors on macrophages and mediate complement killing. Activated B cells can also serve as antigen presenting cells. The B cells interact with antigen-specific CD4 T cells , and they up-regulate their cytotoxic properties. These cytotoxic CD8 T cells can release cytotoxic particles containing perforin and granzyme through the apoptotic Fas-Fas ligand cell death pathway, thereby killing the β-cells through the apoptotic Fas-Fas cell death pathway, thereby releasing these immune mechanisms (Nokoff and Rewers, 2013).

Regulatory T cells (Treg s) may become incapacitated in the presence of Certain cytokines (such as IL-21). These cytokines release a destructive

Immune response from β cells (Babaya et al., 2005). Even after the onset of the disease, since the titers of autoantibodies and autoreactive T cells lasted for a long time**,** immune response was observed in patient due to continuous exposure to the "trigger" or continuous automatic antigen exposure. Among Celiac disease patients with long-term disease or other new-onset diabetes, there are very few T1DM patients. the dysfunctionalstate of Treg s and "inflammatory blood cells" are also favored. The dysfunction state of Treg s and the persistence of the autoimmune state caused by "inflammatory myelopathy" are also favored. Due to immune interference and neuropathy caused by hyperglycemia, intestinal permeability is affected, and the site of autoimmune reaction is now transferred from the pancreas to the intestine. also been described by researchers that the gut immune system is activated in T1DM patients, asinitial priming of diabetogenic cells takes place in the gut and further activation of the immune response takes place in the regional lymph nodes (Jaakkola *et al*., 2003).

The pathogenesis of CD can be divided into three stages: intraluminal mucosal events and early mucosal events, pathogenic CD4 T cell activation and events leading to tissue damage, involving innate and adaptive immunity. The pathogenic mechanism of CD is largely attributed to the triggering of wheat gluten. Gluten refers to the protein complex of wheat, which may contain more than 100 different molecules.These proteins can be divided into gliadin protein and gluten according to solubility (Bernardo *et al*., 2009). Some incompletely digested wheat gluten; rye or barley peptides pass through the epithelium and enter the lamina propria of the small intestine due to increased intestinal permeability. When gluten is ingested in the first stage, it will be digestedto form peptides, but due to the lack of proline peptidase, residues rich inproline and glutamine remain undigested (Barker and Liu, 2008). These glutamine residues can be converted into negatively

charged glutamate by T t G . TtG is a calcium-dependent enzyme that mediates the deamination of gliadin and ultimately leads to the formation of an epitope that effectively binds to DQ2. It is then recognized by intestinal T cells (EIIi etal., 2012).

Peptides produced like 19-mers trigger an innate immune response, which is characterized by the production of IL-15. By destroying tight junctions to increase permeability, or by acting on intraepithelial lymphocytes to promote IFN-γ, thereby affecting the epithelial barrier. Especially the effective cytotoxic activity of natural killer group 2, member D, natural killer group 2, member C, these receptors recognize (MHC class I chain related antigen A and MHC class I chain related antigen B and HLA-E in Epithelial cells), through T cell receptor killing or cytokine production (such as IL-21 and IFN)(Thomas et al., 2006). Due to the increased permeability caused by the above-mentioned events, peptides like 33 mer can now reach the lamina propria. These peptides then bind to HLA molecules to form peptide complexes, which can activate the gluten-specific CD4 T cells in the host. These activated CD4 T cells lead to the production of a variety of cytokines, which can release metalloproteinase through fibrin b to promote inflammation and villous damage in the small intestine. Activated gluten-specific CD4 T cells can also stimulate B cells to produce anti-gluten and anti-tTG antibodies (Schuppan, 2000).

It has been shown that the anti-tTG antibody in CD interferes with tTG activity and has a deleterious effect on the differentiation of epithelial cells (Kagnoff, 2007; Tjon *et al*., 2010). Also more evidences show that the gut microbiome has a great influence on the formation of autoimmune responses to many diseases including T1DM and CD (McLean *et al*., 2015). There are many contraindicated reports. Whether changing themicroflora is the cause or consequence of autoimmunity is still under debate. The initiation of autoimmune effector cells occurs in the intestine in both T1DM and CD. The hypersensitivity innate receptors of bacteria and viruses (such as toll receptors) trigger an autoreactive immune response. Gram-positive bacteria have been shown to be strong inducers of Th1 response (Calder *et al*., 2006).

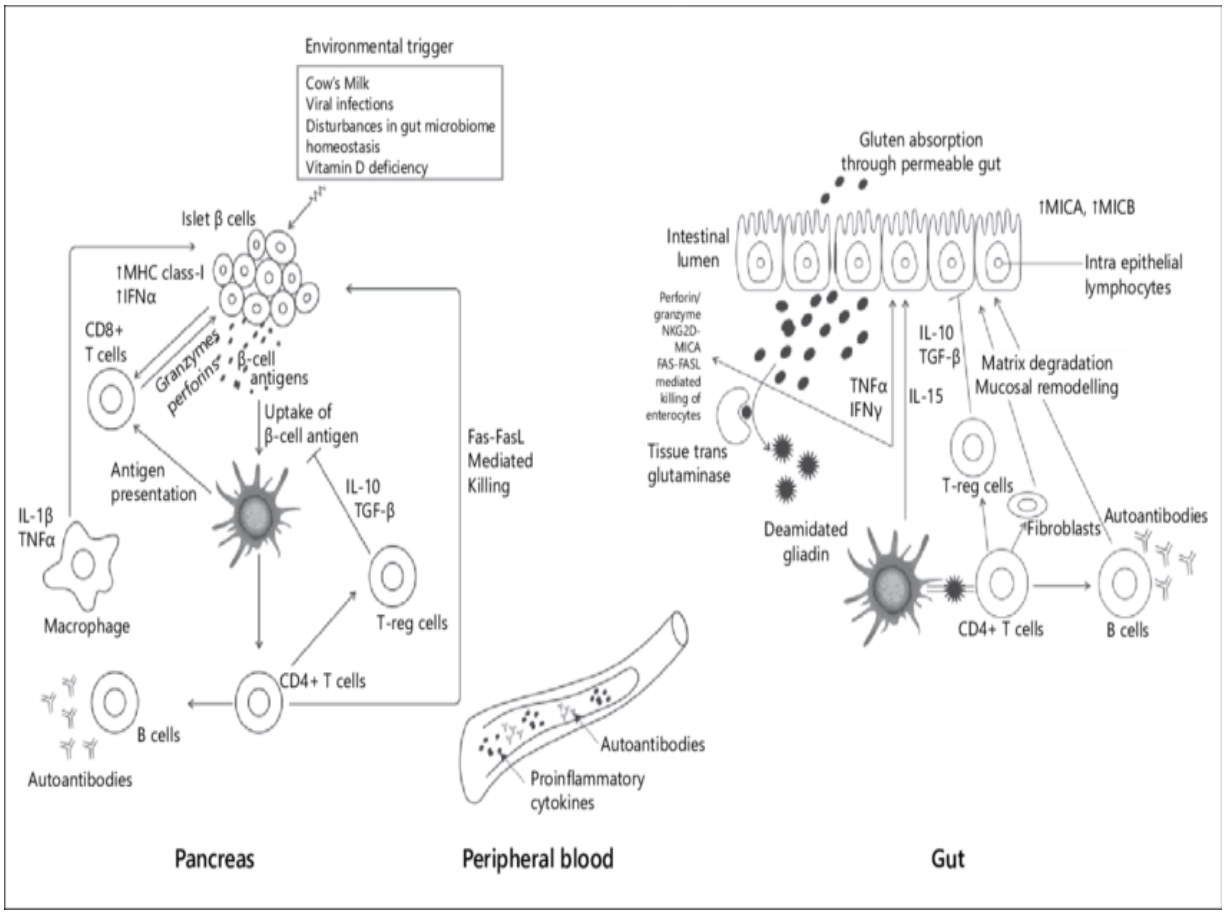


Figure1.6: The mechanism of T1DM and CD pathophysiology
Pathophysiological mechanisms in type 1 diabetes and celiac disease that lead to immune-mediated killing of β cells of islets and enterocytes. For T1DM, genetic predisposition as well as some environmental factors trigger autoimmune mediated attack on Islet B cells by cytotoxic CD8+ cells through antigenic stimulation by antigen presenting cells (APC). While in CD, the breakdown of gluten after absorption, into gliadin results to auto immune activation of CD4+ cells by APC, leading to matrix degradation and mucosal remodeling.*(Csorba 2010).*

1.2.3.2 Symptoms of CD in T1DM

The clinical features of CD in T1DM patients may be subtle, atypical or completely lacking. This means that patients with T1DM may carry a small flower CD, a silent CD or a potential CD. Regardless of the form, the detection of CD is crucial in T1DM patients, because the inclusion of a gluten-free diet (GFD) in T1DM patients can improve blood sugar control and has a positive effect on biochemical indicators.

Therefore, it is strongly recommended to regularly screen CD T1DM patients. The evaluation features that require CD diagnosis are mild Abdominal Discomfort and bloating, weight loss, fatigue, growth abnormalities that mimic physical growth delay, infertility, recurrent aphtha's stomatitis, low bone minerals hyperthyroidism and compensatory hyperthyroidism, rarely T cell lymphoma associated with bowel disease. Iron and folic acid deficiency due to anemia or iron deficiency is also one of the most serious diseases (Kakleas *et al*., 2010).

T1DM patients with CD are more likely to have hypoglycemic activity and have an increased risk of diabetic retinopathy and kidney disease. Table 1.1 summarizes the classic and non-classical symptoms of T1DM and CD diagnosis is done by following a widely recommended and accepted protocol. It includes two stages, which are serological testing and histopathological research. In the first stage, patients are screened for anti- tTG immunoglobulin A (IgA) and anti-Endomysial antibodies (inner membrane) IgA**a**utoantibodies. *(Saeed, Anjum, et al (2017)*.

Table 1:1 symptom of T1DM and celiac disease *(Saeed, Anjum, et al(2017)*.

S/N	Type 1 diabetes mellitus	Celiac disease
1.	Hyperglycemia	Abdominal discomfort/bloating
2.	Osmotic symptoms-polyuria	Weight loss, fatigue, growth abnormalities
3.	Polydipsia, polyphagia	Infertility, hypogonadism
4.	Vomiting/abdominal discomfort	Recurrent aphtous stomatitis
5.	Constipation/headache	Low bone mineralization
6.	Nocturia, pyogenic skin infections	Compensatory hyperthyroidism
7.	Recurrent candida rash	Dermatitis herpetiformis
8.	Urine ketones	Dental hypoplasia

1.2.3.3 Diagnosis of CD and T1DM

For serological diagnosis, if any patient is negative, but CD is still suspected, IgA levels should be checked for selective IgA deficiency. If selective IgA deficiency occurs, tTG IgG and EMA IgG are checked. Other than these 2 antibodies, anti-gliadin IgA and IgG as well as anti Deamidated gliadin peptide IgA antibodies are also detected in these patients and may be useful in the diagnosis of atypical CD. If the patient is detected positive for antibodies, then biopsy of small intestine is required to confirm the diagnosis.

The characteristic histological changes include an increased no of intraepithelial lymphocytes (>25 per 100 enterocytes), elongation of the crypts, and partial to total villous atrophy. Although there are no strict guide-lines formulated by any of the organizations like Ameri-can Diabetes Association, International Society for Pediatric and Adolescent Diabetes, Canadian Diabetes Association, North American Society of Pediatric Gastroenterology, Hepatology and Nutrition regarding

Screening of CD in T1DM patients in lieu of controversial reports, periodic screening is recommended irrespective of symptomatic presentation (Kakleas *et al*., 2010). Generally, the major distinguishing features of Cd and T1DM are presented in Table 1.2.

Table 1.2 General features of CD and T1DM

Feature	Celiac disease	T1DM
Worldwide incidence	0.6% –1%	<1%
Contribution of HLA genes	HLA DQ2 (DQA1*05-DQB1*02) HLA DQ8 (DQA1*03-DQB1*03:02)	HLADQ2 and/or DQ8 (DRB1*0401-DQB1*03:02 and DRB1*0301-DQB1*0201)
Non-HLA candidate genes	CTLA4,PTPN22,CD28, ICOS, MYO9B	CTLA4, PTPN22, MIC-A
Symptoms	Diarrhea, steatorrhea, weight loss, failure to thrive, iron deficiency, abdominal pain, reduced bone density, chronic fatigue, growth failure.	Polyuria, polydipsia, extensive hunger, weight loss, chronic fatigue, reduced bone density, growth failure, Hyperglycemia.
Diagnosis	Small intestinal biopsy, generally with supporting serological testing. IgA anti-tTG, IgG anti-tTG, IgA Anti-EMA, IgG DGP.	Blood test: Fasting blood glucose Level, oral glucose tolerance test, A1C. Serologic tests: ICA, IAA, GADA, IA2 antibodies
Pathogenesis	Enteropathy is due to dysregulation of The innate and adaptive immune system. Alteration of intestinal permeability.	Autoimmune destruction of pancreatic insulin-producing _-cells by an Adaptive and innate immune response.

Chapter Two

Materials and Methods

2.0 Materials and methods

2.1 Subject and study design

2.1.1 Subject:

This study was a cross-sectional study on patients with diabetes mellitusand Celiac disease recruited from three locations i.e. the Diabetes Canter+in Thi-Qar Province, the Al-Hussein Hospital in Karbala and the digestive center in Baghdad. A total of 135 patients were recruited and categorized into group A (Type 1 Diabetes only), group B (Type 1 Diabetes + Celiac disease) and group C (Celiac disease only) withthe groups having 45 patients each. Diabetes and celiac disease were diagnosed based on the criteria set by American College of Diabetes andCeliac Disease (Kabbani *et al*., 2014; Cefalu *et al*., 2019).

Ethical approval was obtained from the appropriate regulatory bodies in Thi-Qar Diabetes Centre and the Kerbala Health Directorate. Informed consent was sort from the patients or their parents/ guardians (for minors), prior to recruitment into the study. The exclusioncriteria included aged individuals (above 65 years), patients with other autoimmune disease as well as those without endoscopic result.

2.2 Sample collection

The study sample was collected between December 2020 and February 2021 with strict adherence to standard health and safety measures. Demographic and clinical data (such as name, age, gender, medical history, endoscopic result and treatment type) were collected through an interview which was done to patients and /or their parents through a questionnaire (Appendix A).

Blood samples were obtained by venipuncture following disinfection of the antecubital fossa with 70% ethanol. Five millilitres of blood were drawn and dispensed into EDTA containing tubes and 3 mL blood was drawn into a gel tube for whole blood analysis and serum preparation respectively. Serum were prepared by centrifugation at 3000 rpm for 15 minutes and the supernatants were dispensed into Eppendorf tubes and stored at -20ºC until use.

The study design, as well as techniques and procedures used in this study are schematically summarized in Figure 2.1

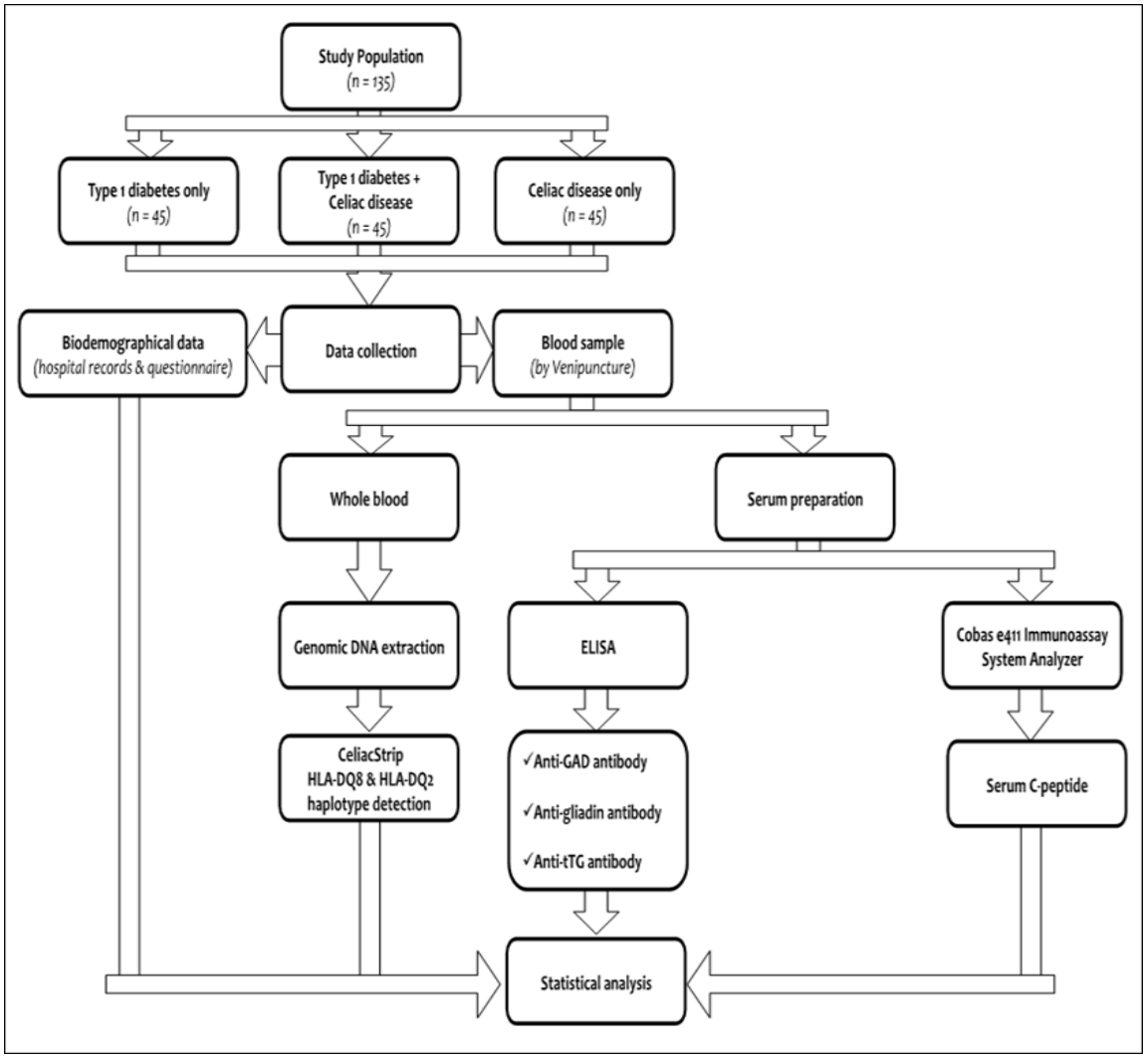


Figure 2.1: Schematic presentation of the study design as well as techniques and procedure.

2.3 Materials

2.3.1 Equipment and instruments:

In the present study, the following Equipment and Instruments were used (Table 2.1).

Table 2:1 Equipment and instruments with their and contrary of origin.

Equipment and Instrument	Country
Disposable syringe (5ml)	China
EDTA tube	China
Gel tube	China
Micropipettes (different size)	Germany
Plastic rack	China
Centrifuge	Japan
Deep freezer	Japan
ELISA reader and printer	Germany
Micro centrifuge	India
Thermo shaker	Germany
ELISA automated washer	USA
Vortex	Germany
70% alcohol for sterilization	China
Gloves	China
Eppendorf tubes(1.5 ml)	China
Filter Tips for PCR (100MlAnd 200Ml)	China
Tips (Yellow and blue)	China
U.V PCR cabinet	U.K
Cold medical box	China
Water bath	China
Incubator	China

2.4: DNA extraction kit:

Table 2.2: DNA extraction kit

DNA extraction kit contents	Volume	Manufacturing company	Country
Reliaprep binding columns (50\packs)	5 packs	Promega	U.S.A
Collection tubes (200\packs)	5 pack s		
Cell lysis buffer	55 ml		
Proteinase K solution	5.5ml		
Binding buffer	68.75ml		
Column wash solution	412.5ml		
Nuclease free water	50 ml		

2.4.1 Polymerase Chain Reaction Kits:

Table 2.3: celiac strip kit content for the detection HLA-DQ2\DQ8

Content		Quantity
Strips	Strip s	30(4X15)
Sol DN	Denaturation solution	1ml
BUF HYB	Hybridization buffer	60 ml
BUF wash1	Washing buffer 1	100 ml
CONJ HRP	Conjugate	60 ml
BUF wash2	Washing buffer 2	130 ml
SUBS TMB	Chromogen substrate	30 ml
PCR Mix	PCR Mix	0.80 ml
TAQ	TAQ	0.30 ml
PRIMERS	Primers	0.110 ml

2.5 Elisa kit

2.5.1 ELISA Kit content of serum Anti _ Tissue trans glutamines Antibody IgA\IgG.

Table 2.4: Elisa kit for detection serum (Anti _TtG IgA\IgG)

Reagent	Quantity
SORB MT 1 divisible micro plate	8 wells X12 strips
Calibrator A-F	6x1.5
Controls positive ,negative	2x1.5
Sample buffer P	5x20
Enzyme conjugate	15ml
TMB substrate	15ml
Stop solution	15ml
Wash buffer	50x20
1 instruction for use	
1 certificate of analysis	

2.5.2 Elisa kit content of serum Deamidated gliadin peptide lgA\IgG.

Table 2.5: Elisa kit for detection serum (DGP IgA\IgG)

Symbol	Components	Volume \Qty.
SORB Mt	Gliadin antigen coated microliter strips	12
Cal A	Calibrator A(negative control)	2 ml
Cal B	Calibrator B(cut -off standard)	2 ml
Cal C	Calibrator c (weak positive control)	2 ml
Cal D	Calibrator D(positive control)	2ml
ENZ CONJ	Enzyme conjugate	15 ml
SUB TMB	Substrate solution	15 ml
Stop SOLN	Stop solution	15 ml
SAM DIL	Sample diluent	60ml
WASH SOLN 10 X	Washing Buffer (10x)	60 ml

2.5.3 Elisa kit content of serum Anti _GAD Antibody

Table 2.6: Elisa kit for detection serum (Anti_GAD Ab).

Component	Colour	Format	symbol
1.Microplate wells coated with antigens 12 Micro plate strips each containing 8 individual break _off wells in a frame read for use.	-------	12x8	STRIPS
2.Calibrator 1 to 6 IU\ml(IgG, human) ready for use**.**	colourless	6x0.7 ml	CAL1-CAL6
3.Negative Control 0 IU\ml (IgG, human) ready for use.	colourless	1x0.7 ml	NEG CONTROL
4.Postive control (IgG, human) ready for use.	colourless	1x0.7 ml	POS CONTROL
5.GAD Biotin-labeled GAD, lyophilised.	colourless	3x 5.5 ml	GAD
6.GADbuffer Ready for use.	Pink	2x15 ml	GAD BUFFER
7.Enzyme conjugate Peroxidase-labeled avidin,20x concentrated	colourless	1x0.7 ml	CONJUGATE 20x
8.Conjugate buffer , ready for use**.**	colourless	1x15ml	CONJ BUFFER
9. Wash buffer, 10 x concentrate.	colourless	1x125ml	WASH BUFFER 10x
10.chromogen\substrate solution TMB\H2O2, ready for use.	colourless	1x15ml	SUBSTRATE
11.stop solution 0, 25 M sulphuric acid, ready to use.	colourless	1x12ml	STOP SOLUTION
12.**Plastic foil**	--------	3 pieces	FOIL
13.Quality control	------	1 protocol	

2.6 Material component of C-peptide accessories of cobase E 411.

Table 2.7 Cobase kit for detection serum C-peptide

REF C-peptide Cal set ,for 6X380mL
REF clean cell ,6X380mL measuring cell cleaning solution
REF elecsys wash ,1X500 ML wash water additive
REF Adapter for sys clean
REF assay cup ,60X60 reaction cups
REF assay Tip,30X120 pipette tips
REF clean liner

2.6.1 Reagent s working solutions using for C-peptide

Table 2.8: The reagent rack pack is labeled as c-peptide.

M	Streptavidin-coated micropaticles (transparentcap),1 bottle ,6.5 mL: streptavidin coated micro particles 0.72 mg\mL:preservative .
R1	Anti c -peptide Ab -biotin (gray cap),1 bottle 9mL Biotinylated monoclonal anti C-peptide antibody (mouse)1 mg \L phosphate buffer 50 m mmol\L, PH 6.0;prespective .
R2	Anti -C peptide -Ab Ru(by)(black cup),1 bottle ,9mL monoclonal anti c- peptide antibody (mouse)labeled with ruthenium complex 0.4 mg\L phosphate buffer 50 mmol\L,PH 6.0;prespective.

2.7 Methods

2.7.1 Determination of Anti-GAD antibody using ELISA

To quantitatively detect the presence of circulating autoantibodies to glutamic acid decarboxylase (GAD) antigen in the serum samples, the indirect enzyme linked immune reaction using the Anti-GAD Ab ELISA Kit (Euroimmun manufacturer and made in Germany) was used as per manufacturer's procedures.

2.7.2Assay procedure (Anti-GAD antibody)

One hundred microliters (100 µL) of controls, pre-diluted samples and calibrator were dispensed into the appropriate micro wells with wells on roll A containing no sample (reserved for blank). The plates were then covered, sealed with paraffin and incubated for 1 hour at room temperature. After incubation, the contents of the well were discarded and the wells were washed thrice with 300 mL wash buffer solution after which 100 µL of reconstituted Enzyme Conjugate reagent were added and the plate was covered and incubated for 1 hour at room temperature. At the end of the incubation, the micro wells were washed three times and 100 µL of substrate solution was added into all the wells at a rapid and

Steady pace void of any interruption. The plates were then covered and Placed in the dark for 30 minutes after which 50 µL of the stop solution into each well at a rapid, steady pace without interruption. The absorbance of the plates where then read at 450 nm from (Huma Reader manufacturer and made in Germany of Micro well plate reader). A dose response curve (DRC) was Plotted on a linear graph paper, plotting each calibrator value (as indicated on the calibrator vial label) on the X-axis And its corresponding absorbance value on the Y-axis.

A line of best fit was drawn between the three points and the GAD value of each serum sample was determined using its absorbance value and extrapolating fromthe DRC on the X-axis.

2.7.3 Determination of Anti-tTG antibody using ELISA

To quantitatively detect the presence of circulating autoantibodies to tissue Trans glutaminase (tTG) antigen in the serum samples, the indirect enzyme linked immune reaction using the Anti-tTG Ab ELISA Kit (Euroimmun manufacturer and made in Germany) was used as per manufacturer's procedures.

2.7.4 Assay procedure (Anti-tTG antibody)

One hundred microliters (100 µL) of controls, pre-diluted samples and calibrator were dispensed into the appropriate micro wells with wells on roll A containing no sample (reserved for blank). The plates were then covered, sealed with paraffin and incubated for 1 hour at room temperature. After incubation, the contents of the well was discarded and the wells were washed thrice with 300 mL wash buffer solution after which 100 µL of reconstituted Enzyme Conjugate reagent were added and the plate was covered and incubated for 1 hour at room temperature. At the end of the incubation, the micro wells were washed three times and 100 µL of substrate solution was added into all the wells at a rapid and steady pace void of any interruption. The plates were then covered and placed in the dark for 30 minutes after which 50 µL of the stop solution into each well at a rapid, steady pace without interruption. The absorbance of the plates where then read at 450 nm from (Huma Reader Hs manufacturer and made in Germany of Micro well plate reader). A dose response curve (DRC) was plotted on a linear graph paper, plotting each calibrator value (as indicated on the calibrator vial label) on the X-axis

and its corresponding absorbance value on the Y-axis. A line of best fit was drawn between the three points and the tTG value of each serum sample was determined using its absorbance value and extrapolating from the DRC on the X-axis.

2.7.5 Determination of Deamidated gliadin antibody using ELISA

To quantitatively detect the presence of circulating IgA/IgG antibodies to Deamidated gliadin antigen in the serum samples, the indirect enzyme linked immune reaction using the DGP IgA/IgG ELISA Kit (Euroimmun manufacturer and made in Germany) was used as per manufacturer's procedures

2.7.6Assay procedure (Deamidated gliadin antibody)

Hundred microliters (100 µL) of controls, pre-diluted samples and calibrator were dispensed into the appropriate micro wells with wells on roll A containing no sample (reserved for blank). The plates were then covered, sealed with paraffin and incubated for 1 hour at room temperature. After incubation, the contents of the well were discarded and the wells were washed thrice with 300 mL wash buffer solution after which 100 µL of reconstituted Enzyme Conjugate reagent were added and the plate was covered and incubated for 1 hour at room temperature. At the end of the incubation, the micro wells were washed three times and 100 µL of substrate solution was added into all the wells at a rapid and steady pace void of any interruption. The plates were then covered and placed in the dark for 20 minutes after which 50 µL of the stop solution into each well at a rapid, steady pace without interruption. The absorbance of the plates where then read at 450 nm from (Huma Reader Hs manufacturer and made in Germanyof Micro well plate reader). A dose response curve (DRC) was plotted on a linear graph paper, plotting each calibrator value (as indicated on the calibrator vial label) on the X-axis and its corresponding absorbance value on the Y-axis. A line of best fit was drawn between the three points and the tTG value of each serum sample was determined using its absorbance value and extrapolating from the DRC on the X-axis.

2.8 Determination of C-peptide using Cobas e411 Immunoassay System Analyser

The concentration of C-peptide in the serum samples was determined using Cobas e411 (Roche diagnostic manufacturer and made in Germany). The automatic immunoassay system utilises the electrochemiluminescence (ECL) technology and was ran over total assay duration of approximately 18 minutes in a 4-step procedure.

Step 1: First incubation - The serum sample 20 μL was loaded along with a biotinylated monoclonal C-peptide-specific antibody, and monoclonal C-peptide-specific antibody labeled with a ruthenium complex read to form a sandwich complex.

Step 2: Second incubation - Streptavidin -coated micro particles were added to the complex which then became bound to the solid phase via interaction of biotin and streptavidin.

Step 3: The reaction mixture was aspirated into the measuring cell where the micro particles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with pro cell and application of a voltage to the electrode then induced chemiluminescent emission which was measured by a photomultiplier.

Step 4: The results were determined via a calibration curve which is instrument -specifically generated by 2 -point calibration and a master curve provided via the reagent barcode or e-barcode.

2.9 Determination of Celiac disease associated HLA haplotypes (HLA-DQ8 and HLA-DQ2) using Celiac Strip

The Celiac Strip kit (Operon Immuno & Molecular diagnostics, Spain) allows for the detection of the presence or absence of haplotypes that encode the HLA-DQ2 and HLA-DQ8, which are the main HLA-haplotypes associated with Celiac disease. These include DQA1*05 – DQB1*02 – DRB1*03 (HLA-DQ2 [cis] haplotype 1), DQA1*05 – DQB1*0301 – DRB1*11/DRB1*12 (HLA-DQ2 [Trans] haplotype 2), DQA1*03 – DQA1*0302 – DRB1*04(HLA-DQ8). The Celiac Strip test was carried out in three procedures namely; DNA extraction, PCR amplification, as well as Hybridization and development, based on manufacturer's guidelines.

2.9.1 DNA Extraction

The genomic DNA was extracted from the nucleated cells in the whole blood samples collected under the aseptic condition and according to the protocol of ReliaPrep™ Blood gDNA Miniprep System (Promega, U.S.A.).

The blood sample was mixed thoroughly for at least 10 minutes at room temperature and 20 μL of Protein-ase K (PK) solution was then dispensed into a 1.5 mL microcentrifuge tube. Whole blood (200 μL) was added to the tube containing the Protein-ase K Solution, and mixed briefly, after which 200 μL of Cell Lysis Buffer was added. The tube was then vortexed

for at least 10 seconds and incubated at 56 °C for 10 minutes. After the incubation period, 250 µL of Binding Buffer was dispensed into the collection tube and spun briefly at maximum speed. The ReliaPrep™ Binding Column was then removed gently using tweezers to prevent touch and placed into a tube rack. The entire content of the collection tube was dispensed into the column and centrifuged at 14000 rpm for 1 minute. The binding column was checked to ensure that the lysate has completely passed through the membrane and the liquid was discarded. The binding column was placed into a fresh collection tube, then 500 µL of Column Wash Solution was added, and centrifuged for 3 minutes at 14000 rpm; this process was repeated thrice. After 3 washes, the column was placed in a clean 1.5ml microcentrifuge tube and 100 µL of Nuclease-Free Water was added to the column and centrifuged at 14000 rpm for 1 minute. The extracted gDNA was then kept at 4 °C for 24 hours prior quality assessment and subsequently stored at -26 °C until amplification.

2.9.2 PCR amplification

To achieve a suitable DNA concentration for optimal results, the extracted DNA samples were amplified using the procedure recommended in the Celiac Strip test manual. To each PCR tube, 39 µL of PCR premix, 5 µL of primers and 1 µL of Taq were added to 5 µL of DNA, making a total volume of 50 µL per PCR tube. The DNA samples were amplified in a thermo cycler (Analytic Jena GMBH manufacturer and made in Germany) using the programme presented in Table 2.1

Table 2.9: PCR Thermo cycling condition for HLA DQ2\DQ8 gene amplification

Procedure	Temperature	Time	Number of cycles
Initial Denaturation	94 °C	5 Minutes	
Denaturation	94 °C	45 Seconds	
Annealing	57 °C	1 Minute	30 cycles
Extension	72 °C	45 Seconds	
Final Extension	72 °C	5 Minutes	
Hold	4 °C	∞	

2.9.3 Hybridization and development using Theromoshaker PSH-60HL

The preliminary steps for prepping the plate thermo shaker for analysis was conducted by strict adherence to the Opegen Thermo shaker Manual (Appendix B). Prior to analysis, the hybridization buffer, wash buffer 1 and wash buffer 2 were preheated to 42 °C in a water bath. Other reagents including denaturing solution, conjugate and substrate reagents were brought to room temperature and those with precipitates were shaken gently until they completely dissolved.

The hybridization strips were carefully removed from the aluminium pouch using tweezers to avoid touch and possible contamination. The strips were placed in the hybridization tray, one strip per channel, facing upward. The denaturation buffer (12.5 μL) was dispensed into the tray and 12.5 μL of the PCR product was also added carefully. The tray was then placed inside the plate of the thermo shaker, closed and incubated

for 10 minutes. After incubation, 2 mL of preheated hybridization buffer was quickly added to each channel and mixed slowly by moving the plate up and down. The thermo shaker was then closed and the plate was shaken at 42 °C and 450 rpm for 30 minutes incubation period (the incubation temperature of 42 °C and speed of 450 rpm was maintained throughout the hybridization process). The hybridization buffer was then removed by vacuum pump aspiration and 2 mL of preheated wash buffer 1 was added to each channel and washed for 10 minutes twice. After washing, 2 mL of conjugate was the added to each channel and incubated for 30 minutes after which the conjugate was aspirated and 2 mL preheated wash buffer 2 was added and the plate was incubated 5 minutes thrice. The wash buffer 2 was the aspirated after which 1 mL of substrate was added and incubated for 10 minutes. The plates were then removed and washed with 2 mL distilled water thrice after which the strips were removed and placedto dry in the dark.

2.9.4 Strip reading and interpretation

To interpret the strips after hybridization/development, they were placed on a paper and covered with cellophane. Interpretation was done manually using the evaluation chart included in the kit (Appendix B) by aligning the black and red lines of the strips with the lines on the chart and identifying the bands in accordance with their position on the strips.

2.10 Statistical analysis

Data analysis was conducted using SPSS (version 24.0). All quantitative variables or numbers were expressed in form of mean +/- standard deviation. Pearson's correlation matrix was used to observe association

between the HLA haplotypes and serum autoantibodies in relation to the bio demographical data.

Chapter Three

Results

3.1 Demographical characteristics of the study patients

The total number of patients recruited in this study was 135. The patients were categorized into 3 groups based on the nature of their disease condition. The first group tagged "Celiac Disease" comprised of 45 patients exclusively with Celiac disease, 18 of which were males while 27 were females. The patients in this group had mean age of 20.80±6.08 years, of which the males had mean age of 19.72±5.17years and the females, a mean age of 21.52±6.61 years. The mean body mass index (BMI) of the group was 26.31±0.67 Kg/m2with the male BMI at 26.58±0.67 Kg/m2 and that of the females at 26.14±0.63 Kg/m2.

The second group was tagged "T1DM" and comprised of 45 patients having type 1 diabetes mellitus exclusively, out of which 22 were males and 23 were females. The patients had mean age of 20.49±5.37 years, of which the males had mean age of 20.23±5.16years and the females, a mean age of 20.74±5.68 years. The mean body mass index (BMI) of the group was 26.70±0.84 Kg/m2with the male BMI at 26.81±0.83 Kg/m2and that of the females at 26.60±0.85 Kg/m2.

The third group was tagged "Celiac Disease + T1DM" and comprised of 45 patients having comorbidity of Celiac disease and type 1 diabetes mellitus, 21 of which were males and 24 were females. The patients had mean age of 21.80±5.47 years, of which the males had mean age of 21.67±6.00years and the females, a mean age of 21.92±5.09 years. The mean body mass index (BMI) of the group was 26.34±0.83 Kg/m2with the male BMI at 26.46±0.77 Kg/m2 and that of the females at 26.24±0.88 Kg/m2. The demographical characteristics of the patients are summarized in Table 3.1.

Table 3.1: Demographical characteristics of the study patients

		Celiac Disease (mean±SD)	T1DM (mean±SD)	Celiac Disease +T1DM (mean±SD)	*p*-value
Number of participants (N (%))	All	45 (100)	45 (100)	45 (100)	
	Male	18 (40)	22 (48.9)	21 (45.1)	
	Female	27 (60)	23 (51.1)	24 (53.3)	
Age (years)	All	20.80±6.08	20.49±5.37	21.80±5.47	0.51
	Male	19.72±5.17	20.23±5.16	21.67±6.00	
	Female	21.52±6.61	20.74±5.68	21.92±5.09	
BMI (Kg/m^2)	All	26.31±0.67	26.70±0.84	26.34±0.83	0.03
	Male	26.58±0.67	26.81±0.83	26.46±0.77	
	Female	26.14±0.63	26.60±0.85	26.24±0.88	

3.2 Biochemical characteristics of the study patient

The laboratory analysis of biochemical parameters in the serum samples obtained from the patients in each group revealed the following.

The levels of glucose in the serum of the patients with Celiac disease only was 94.82±3.89 mg/dL, of which the males had a mean serum glucose level of 94.94±4.26 mg/dL, and females had 94.72±3.71 mg/dL. The T1DM group had mean serum glucose level of 173.74±1.97 mg/dL, of which the males had a mean serum glucose level of 173.26±2.00 mg/dL, and females had 174.19±1.87 mg/dL. These values were significantly higher (at $p < 0.05$) compared to those of the Celiac Disease group. The Celiac Disease + T1DM group had a slightly higher mean serum glucose level of 174.92±2.76 mg/dL, of which the males had a mean serum glucose level of 175.15±2.79 mg/dL, and females had 174.72±2.78 mg/dL.

The mean levels of glycated hemoglobin (HbA1c) in the plasma of the patients with Celiac disease only was 5.43±0.66 %, of which the males had a mean plasma HbA1c of 5.48±0.48 %, and females had 5.40±0.76%. The T1DM group had mean plasma HbA1c 7.90±0.24 %, of which the males had a mean serum glucose level of 7.97±0.24 %, and females had 7.83±0.23 %. These values were significantly higher (at $p < 0.05$) compared to those of the Celiac Disease group. The Celiac Disease + T1DM group had a slightly lower mean plasma HbA1c of 7.81±0.40 %, of which the males had a mean plasma HbA1c of 7.77±0.40 %, and females had 7.85±0.41 %.

Other parameters analysed included; packed cell volume (PCV), which was 31.73±6.00 (Celiac Disease group), 32.98±5.49 (T1DM group) and 32.89±6.01 (Celiac Disease + T1DM group).) Haemoglobin, which was 10.59±2.44 % (Celiac Disease group), 10.48±1.81 % (T1DM group) and 10.26±1.76 % (Celiac Disease + T1DM group). White blood cell (WBC) count, which was 6.80±0.95 x103/L (Celiac Disease group), 6.91±0.61 x103/L (T1DM group) and 6.69±0.55 x103/L (Celiac Disease + T1DM group). Platelets count, which was 207.20±34.42 x103/L (Celiac Disease group), 213.44±40.42 x103/L (T1DM group) and 207.24±36.79 x103/L (Celiac Disease + T1DM group). Ferritin, which was 5.69±0.78 µg/L (Celiac Disease group), 6.31±0.83 µg/L (T1DM group) and 6.69±0.55 µg/L(Celiac Disease + T1DM group). Calcium, which was 7.38±0.93 mg/dL (Celiac Disease group), 7.66±0.09mg/dL (T1DM group) and 7.55±0.93mg/dL (Celiac Disease + T1DM group). Vitamin D3, which was 11.20±2.87 ng/mL (Celiac Disease group), 7.15±0.99 ng/mL (T1DM group) and 11.04±2.82 ng/mL (Celiac Disease + T1DM group). Urea, which was 27.15±6.85 mg/dL (Celiac Disease group), 35.23±4.36 mg/dL

(T1DM group) and 23.24±4.75 mg/dL (Celiac Disease + T1DM group). Creatinine, which was 0.77±0.04mg/dL (Celiac Disease group), 1.06±0.06mg/dL (T1DM group) and 0.61±0.01mg/dL (Celiac Disease + T1DM group). Statistical significant difference was observed between the T1DM group and Celiac Disease + T1DM group for serum concentration of Vitamin D3, Urea and Creatinine (at $p < 0.05$). The biochemical characteristics of the patients are summarized in Table 3.2.

Table 3.2: Biochemical characteristics of the study patient

	Group CD Mean±SD	Group T1DM Mean±SD	Group CD & T1DM Mean±SD	P value
Age	20.80±6.08	20.49±5.37	21.80±5.47	0.51
BMI	26.31±0.67	26.70±0.84	26.34±0.83	0.03
Glucose	94.82±3.89	173.74±1.97	174.92±2.76	0.001
HbA1C	5.43±0.66	7.90±0.24	7.81±0.40	0.001
Pcv	31.73±6.00	32.98±5.49	32.89±6.01	0.53
Hb	10.59±2.44	10.48±1.81	10.26±1.76	0.74
WBC	6.80±0.95	6.91±0.61	6.69±0.55	0.76
PLT	207.20±34.42	213.44±40.42	207.24±36.79	0.66
Ferritin	5.69±0.78	6.31±0.83	5.43±0. 31	0.09
Calcium	7.38±0.93	7.66±0.09	7.55±0.93	0.51
Vitamin D3	11.2±2.87	7.15±0.99	11.04±2.82	0.001
Urea	27.15±6.85	35.23±4.36	23.24±4.75	0.001
Creatinine	0.77±0.04	1.06±0.06	0.61±0.01	0.001

Table 3.3: Biochemical features of the patients according gender

	Gender	Group CD Mean±SD	P value	Group T1DM Mean±SD	P value	Group CD & T1DM Mean±SD	P value
Age	**Male**	19.72±5.17	0.19	20.23±5.16	0.45	21.67±6.00	0.56
	Female	21.52±6.61		20.74±5.68		21.92±5.09	
BMI	**Male**	26.58±0.67	0.29	26.81±0.83	0.84	26.46±0.77	0.12
	Female	26.14±0.63		26.60±0.85		26.24±0.88	
Glucose	**Male**	94.94±4.26	0.13	173.26±2.00	0.70	175.15±2.79	0.75
	Female	94.74±3.71		174.19±1.87		174.72±2.78	
HbA1C	**Male**	5.48±0.48	0.004	7.97±0.24	0.53	7.77±0.40	0.97
	Female	5.40±0.76		7.83±0.23		7.85±0.41	

3.3 Interrelation ships between BMI and biochemical characteristic of the study patients

The associations between BMI and some biochemical characteristics as well as the interrelationships between the parameters were determine using Pearson's correlation. Moderate positive correlation was observed between BMI and Urea as well as Creatinine with correlation coefficient of 0.44 and 0.37 respectively. The strongest associations between the biochemical parameters were between HbA1c and glucose level, with a strong positive correlation coefficient of 0.92, as well as creatinine and urea with a strong positive coefficient of 0.88. The significant weak negative associations were recorded between Vitamin D3 and urea (-0.24), creatinine (-0.28), glucose (-0.28) and HbA1c (-0.22).

As shown in Table 3.3, other significant interrelationships observed were between hemoglobin vs. PCV (0.58), PCV vs. platelets (0.22), PCV vs. Ferritin (0.45), hemoglobin vs. platelets (0.30), hemoglobin vs. ferritin (0.63) as well as ferritin vs. calcium (0.38).

Table 3.4: Pearson's correlation matrix showing interrelationships between BMI and biochemical characteristics of the patients

Pearson's Correlation Matrix					
	BMI	**Urea**	**Creatinine**	**Vitamin D3**	**Glucose**
Urea	0.44**				
Creatinine	0.37**	0.88**			
Vitamin D3	-0.14	-0.24**	-0.28**		
Glucose	0.14	0.07	0.05	-0.28**	
HbA1C	0.16	0.15	0.09	-0.22**	0.92**

	PCV	**Hemoglobin**	**WBC**	**Platelets**	**Ferritin**
Hemoglobin	0.58**				
WBC	0.003	0.03			
Platelets	0.22**	0.30**	-0.19		
Ferritin	0.45**	0.63**	0.19	0.21	
Calcium	0.16	0.19	0.09	0.11	0.38**

** indicates statistical significant correlation coefficient at $p < 0.05$

3.3 Frequency of celiac disease and T1DM associated HLA haplotypes (HLA DQ2 and HLA DQ8) among the study patients

The detection of the presence or absence of haplotypes that encode the HLA-DQ2 and HLA-DQ8 genes, which are the main HLA-haplotypes associated with Celiac diseases well as T1DM, unfolds the distribution of these haplotypes across the different study groups.

The most predominant HLA haplotype in the Celiac Disease group was HLA-DQ2, which was detected in 68.9 % of the patients, while 31.1 % of the Celiac disease patients had HLA-DQ8 gene as none of the patients had both HLA-DQ2 and DQ8 haplotypes. Amongst the T1DM group, 57.8 % of the patients had HLA-DQ8 haplotype while 11.1% had the HLA-DQ2 gene, with 31.1 % of the patients having both the HLA-DQ2 and DQ8 haplotypes. The majority of patients with comorbidity of Celiac disease and T1DM i.e. 80 %, had both HLA-DQ2 and DQ8 haplotypes,

While 20 % had HLA-DQ2 gene only with no patient having the HLA- DQ8 haplotype.

Table 3.5: The HLA-DQ haplotype of patients with the different groups of the patients

DQ type	Group CD No. (%)	Group T1MD No. (%)	Group CD & T1DM No. (%)	P value
DQ2	31 (68.9)	5 (11.1)	9 (20.0)	0.001
DQ8	14 (31.1)	26 (57.8)	0 (0.0)	
DQ2&8	0 (0.0)	14 (31.1)	36 (80.0)	
Total	45 (100.0)	45 (100.0)	45 (100.0)	

Table 3.6 :The HLA-DQ haplotype of patients with the gender

Gender	DQ	Group CD No. (%)	P value	Group T1DM No. (%)	P value	Group CD & T1DM No. (%)	P value
Male	**DQ2**	9 (50.0)	0.02	0 (0.0)	0.51	0 (0.0)	0.001
	DQ8	9 (50.0)		12 (54.5)		0 (0.0)	
	DQ2&8	0 (0.0)		10 (45.5)		21 (100.0)	
	Total	18 (100.0)		22 (100.0)		21 (100.0)	
Female	**DQ2**	22 (81.5)		5 (21.7)		9 (37.5)	
	DQ8	5 (18.5)		14 (60.9)		0 (0.0)	
	DQ2&8	0 (0.0)		4 (17.4)		15 (62.5)	
	Total	27 (100.0)		23 (100.0)		24 (100.0)	

Table 3.7: The HLA-DQ haplotype of patients with the different Marsh grading of duodenal biopsy

Marsh	DQ	Group CD No. (%)	P value	Group CD & T1DM No. (%)	P value
Marsh II	**DQ2**	9 (100.0)	0.001	0 (0.0)	0.03
	DQ8	0 (0.0)		0 (0.0)	
	DQ2&8	0 (0.0)		13 (100.0)	
	Total	9 (100.0)		13 (100.0)	
Marsh III	**DQ2**	31 (86.1)		9 (28.1)	
	DQ8	5 (13.9)		0 (0.0)	
	DQ2&8	0 (0.0)		23 (71.9)	
	Total	36 (100.0)		32 (100.0)	

Table 3.8:The HLA-DQ haplotype of patients with seropositivity to anti-tTG autoantibodies.

Type of DQ			Group CD No. (%)	Group CD & T1DM No. (%)	P value
DQ2	Anti- Tissue transglutaminase IgA	+ve	21 (67.7)	9 (100.0)	0.05
		-ve	10 (32.3)	0 (0.0)	
		Total	31 (100.0)	9 (100.0)	
	Anti- Tissue transglutaminase IgG	+ve	12 (38.7)	4 (44.4)	0.76
		-ve	19 (61.3)	5 (55.6)	
		Total	31 (100.0)	9 (100.0)	
DQ8	Anti- Tissue transglutaminase IgA	+ve	14 (100.0)	0 (0.0)	\
		-ve	0 (0.0)	0 (0.0)	
		Total	14 (100.0)	0 (0.0)	
	Anti- Tissue transglutaminase IgG	+ve	5 (35.7)	0 (0.0)	\
		-ve	9 (64.3)	0 (0.0)	
		Total	14 (100.0)	0 (0.0)	
DQ2&8	Anti- Tissue transglutaminase IgA	+ve	0 (0.0)	31 (86.1)	\
		-ve	0 (0.0)	5 (13.9)	
		Total	0 (0.0)	36 (100.0)	
	Anti- Tissue transglutaminase IgG	+ve	0 (0.0)	23 (63.9)	\
		-ve	0 (0.0)	13 (36.1)	
		Total	0 (0.0)	36 (100.0)	

Table 3.9: HLA-DQ haplotype of patients with seropositivity to anti-gliadin autoantibodies

Type of DQ			Group CD No. (%)	Group CD & T1DM No. (%)	P value
DQ2	Anti- gliadin antibody IgA	+ve	12 (38.7)	0 (0.0)	0.02
		-ve	19 (61.3)	9 (100.0)	
		Total	31 (100.0)	9 (100.0)	
	Anti- gliadin antibody IgG	+ve	17 (54.8)	9 (100.0)	0.01
		-ve	14 (45.2)	0 (0.0)	
		Total	31 (100.0)	9 (100.0)	
DQ8	Anti- gliadin antibody IgA	+ve	9 (64.3)	0 (100.0)	\
		-ve	5 (35.7)	0 (100.0)	
		Total	14 (100.0)	0 (100.0)	
	Anti- gliadin antibody IgG	+ve	9 (64.3)	0 (100.0)	\
		-ve	5 (35.7)	0 (100.0)	
		Total	14 (100.0)	0 (100.0)	
DQ2&8	Anti- gliadin antibody IgA	+ve	0 (100.0)	23 (63.9)	\
		-ve	0 (100.0)	13 (36.1)	
		Total	0 (100.0)	36 (100.0)	
	Anti- gliadin antibody IgG	+ve	0 (100.0)	36 (100.0)	\
		-ve	0 (100.0)	0 (0.0)	
		Total	0 (100.0)	36 100.0)	

3.5 Effect of T1DM on Celiac disease

To decipher the effect T1DM may have on Celiac disease, comparisons were made between the Celiac Disease group and the Celiac Disease + T1DM group for parameters associated with Celiac disease such as outcome of duodenal biopsy as well as serum levels of Celiac disease autoantibodies (anti-tTG and anti-gliadin).

3.5.1T1DM reduces the severity of Celiac disease associated duodenal aberrations.

The outcome of histological observation of duodenal biopsy conducted on the patients with Celiac disease and well as those with Celiac disease and T1DM was graded using the Marsh classification. Based on the severity of the findings, 80 % of patients in the Celiac Disease group scored Marsh III while the remaining 20 % scored Marsh II. In the Celiac Disease + T1DM group the proportion of patients with Marsh III level of lesions was 71 % which was lower than the percentage obtained for the Celiac Disease group. The remaining 28.9 % of patients in the Celiac Disease + T1DM group scored Marsh II. Figure 3.2 shows the graphical presentation of the outcome of duodenal biopsy.

Table 3.10: Marsh classification of duodenal biopsy of the patients in Celiac disease group and the Celiac Disease + T1DM group.

Type of marsh	Group CD No. (%)	Group CD & T1DM No. (%)	P value
Marsh II	9 (20.0)	13 (28.9)	0.001
Marsh III	36(80.0)	32 (71.1)	
Total	45(100.0)	45 (100.0)	

Table 3.11: Marsh classification of duodenal biopsy of the patients according gender

Type of marsh	Gender	Group CD No. (%)	Group CD & T1DM No. (%)	P value
Marsh II	Male	4 (44.4)	8 (61.5)	0.90
	Female	5 (55.6)	5 (38.5)	
	Total	9 100.0)	13 (100.0)	
Marsh III	Male	14 (38.9)	13 (40.6)	0.48
	Female	22(61.1)	19 (59.4)	
	Total	36 (100.0)	32 (100.0)	

Table 3.12: The serum concentration of anti-tTG antibodies across Marsh classification of duodenal biopsy of the patients

Type of marsh			Group CD No. (%)	Group CD & T1DM No. (%)	P value
Marsh II	Anti- Tissue transglutaminase IgA	+ve	9 (100.0)	8 (61.5)	0.24
		-ve	0 (0.0)	5 (38.4)	
		Total	9 (100.0)	13 (100.0)	
	Anti- Tissue transglutaminase IgG	+ve	0 (0.0)	8 (61.5)	0.13
		-ve	9 (100.0)	5 (38.4)	
		Total	9 (100.0)	13 (100.0)	
Marsh III	Anti- Tissue transglutaminase IgA	+ve	26 (72.2)	32 (100.0)	0.004
		-ve	10 (27.7)	0 (0.0)	
		Total	36 (100.0)	32 (100.0)	
	Anti- Tissue transglutaminase IgG	+ve	17 (47.2)	19 (59.3)	0.06
		-ve	19 (52.7)	13 (40.6)	
		Total	36 (100.0)	32 (100.0)	

3.5.2:T1DM increases the proportion of patients with positivity to Celiac Disease autoantibodies

The determination of the presence of serum antibodies associated with Celiac disease was conducted on serum samples from patients in the Celiac Disease group as well as those in the Celiac Disease + T1DM group. The frequency of the patients from both groups that showed positivity to anti-tTG (IgA), anti-tTG (IgG), anti-gliadin (IgA) and anti - gliadin (IgG) is presented in Figure 3.3. For the Celiac Disease group ,77.8 % showed positivity to anti-tTG (IgA) antibody and 37.8 % showed positivity to anti-tTG (IgG) antibody while 46.7 % were anti-gliadin (IgA) positive and 57.8 % showed positivity to anti-gliadin (IgG) antibody. In the Celiac Disease + T1DM group, a higher proportion of the patients showed positivity to anti-tTG antibodies with 88.9 % and 60 % positive for IgA and IgG respectively. Similarly, a higher proportion was observed for positivity to anti-gliadin IgA and IgG antibodies which were 51.1 % and 100 % respectively

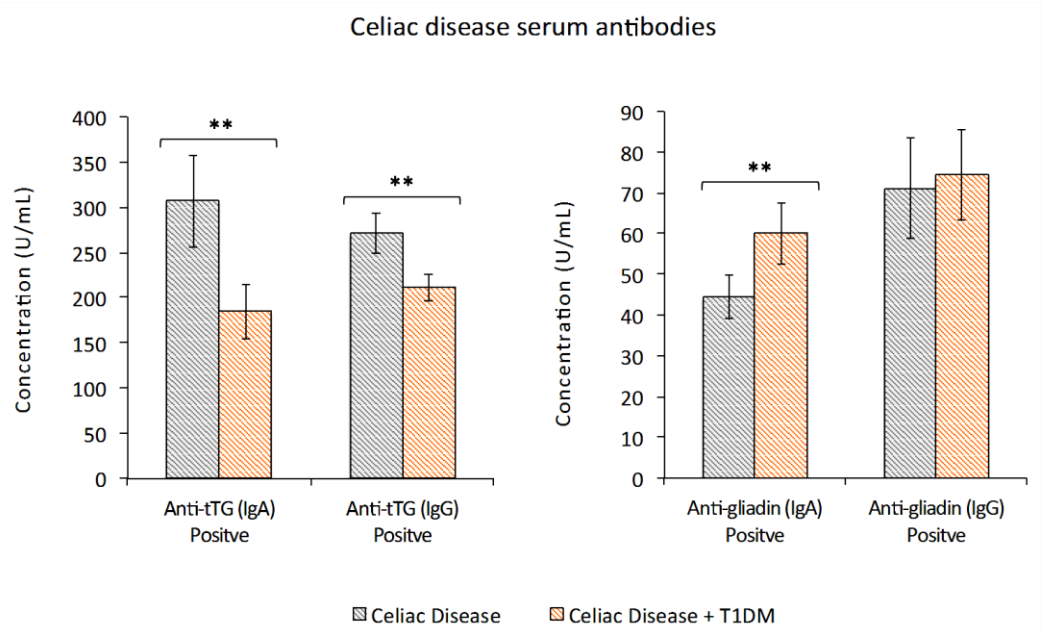
Table 3.13:The serum concentration of anti-tTG and anti-gliadin antibodies across different age groups of the patients

		Group CD No. (%)	Group CD & T1DM No. (%)	P value
Anti- Tissue transglutaminase IgA	**+ve**	35 (77.8)	40 (88.9)	0.16
	-ve	10 (22.2)	5 (11.1)	
	Total	45 (100.0)	45 (100.0)	
Anti- Tissue transglutaminase IgG	**+ve**	17 (37.8)	27 (60.0)	0.03
	-ve	28 (62.2)	18 (40.0)	
	Total	45 (100.0)	45 (100.0)	
Anti- gliadin antibody IgA	**+ve**	21 (46.7)	23 (51.1)	0.67
	-ve	24 (53.3)	22 (48.9)	
	Total	45 (100.0)	45 (100.0)	
Anti- gliadin antibody IgG	**+ve**	26 (57.8)	45 (100.0)	0.001
	-ve	19 (42.2)	0 (0.0)	
	Total	45 (100.0)	45 (100.0)	

3.5.2.1 T1DM alters serum levels of Celiac disease associated autoantibodies

The concentrations of Celiac disease associated antibodies in the serum of patients from the Celiac Disease group and the Celiac Disease + T1DM group were quantitatively determined. The mean serum levels of anti-tTG antibody in the Celiac Disease group was 306.83±51.00 U/mL (IgA) and 270.8±30.3 U/mL (IgG), which were significantly higher ($p > 0.05$) than the 184.26±21.85 U/mL (IgA) and 210.88±14.44 U/mL (IgG) recorded for the Celiac Disease + T1DM group. However, for anti-gliadin IgA antibodies, the mean levels of the Celiac Disease group was significantly lower ($p < 0.05$) compared to the Celiac Disease + T1DM group (i.e. 44±5.32 U/mL [Celiac Disease group] vs. 60.07±12.42 U/mL [Celiac disease + T1DM group]) and although the mean serum levels of anti-

gliadin IgG antibodies in the Celiac Disease group was slightly lower than Of the Celiac Disease + T1DM group (i.e. 71.06±7.54 U/mL [Celiac Disease group] vs. 74.41±11.21 U/mL [Celiac Disease + T1DM]), the difference was not statistically significant at $p < 0.05$. The graphical presentation of the concentration of serum autoantibodies is presented in Figure 3.1.



3.6Effect of Celiac disease on T1DM

To decipher the effect Celiac disease may have on T1DM, comparisons were made between the T1DM group and the Celiac Disease + T1DM group for parameters associated with T1DM such as serum levels of T1DM associated autoantibody i.e. anti-GAD antibody as well as serum levels of C-peptide.

3.6.1: Celiac disease alters proportion of patients with positivity to anti-GAD antibody and low C-peptide

The determination of the presence of serum antibodies associated with T1DM as well as levels of C-peptide was conducted on serum samples from patients in the T1DM group as well as those in the Celiac Disease + T1DM group. The proportion of the patients from both groups that showed positivity to anti-GAD antibodies as well as that of those with C-peptide levels below normal range is presented in Figure 3.5. For the T1DM group, 26.6 % showed positivity to anti-GAD antibody and while 77.7 % had C-peptide levels below the normal range. In the Celiac Disease + T1DM group, a higher proportion of the patients, i.e. 31.1 % showed positivity to anti-GAD antibody. However, the proportion of patients with below normal C-peptide levels in the Celiac Disease + T1DM group was 66.6 % which is lower than that recorded for the T1DM group.

Table 3.14: A serum concentration of Anti-GAD 65 antibody and Anti C- peptide across different groups of the patients

		Group T1DM No. (%)	Group CD & T1DM No. (%)	P value
Anti-GAD 65 antibody	**+ve**	12 (26.6)	14 (31.1)	0.64
	-ve	33 (73.3)	31 (68.8)	
	Total	45 (100.0)	45 (100.0)	
Anti C-peptide	**Normal**	10 (22.2)	15 (33.3)	0.24
	Low	35 (77.7)	30 (66.6)	
	Total	45 (100.0)	45 (100.0)	

3.6.1 Celiac disease elevates serum levels of anti-GAD antibody and reduces C-Peptide levels in T1DM

The concentrations of T1DMassociated antibodies in the serum of patients from the T1DM group and the Celiac Disease + T1DM group were quantitatively determined. The mean serum level of anti-GAD antibody in the T1DM group was125.38±18.92 U/mL which was significantly lower ($p > 0.05$) than the 191.74±25.42 U/mL recorded for the Celiac Disease + T1DM group.

However, for C-peptide, although the mean serum concentration obtained in the T1DM group was slightly higher than that of the Celiac Disease + T1DM group (i.e. 3.98±0.72nmol/L [T1DM group] vs. 3.45±0.67 nmol/L [Celiac Disease + T1DM]), the difference was not statistically significant at $p < 0.05$. The graphical presentation of the concentration of serum anti-GAD antibody and C-peptide is presented in Figure 3.6.

Table 3.15: The serum concentration of Anti-GAD 65 antibody and Anti C-peptide across different groups of the patients

		Group T1DM Mean±SD	Group CD & T1DM Mean±SD	P value
Anti-GAD 65 antibody	**+ve**	125.38±26.92	119.74±38.42	0.57
	-ve	7.63±0.81	6.97±0.92	
Anti C-peptide	**Normal**	3.78±0.72	3.910.67	0.84
	Low	0.02±0.001	0.01±0.001	

3.7 Interrelationship between Celiac disease antibodies, T1DM antibody and C-peptide

The interrelationships between serum antibodies anti-tTG, anti-gliadin, anti-GAD as well as C-peptide was analysed using Pearson's correlation. Strong positive association was observed between anti-tTG antibodies (IgG) vs. anti-gliadin (IgA) with a correlation coefficient of 0.64. Also, strong positive associations were observed between anti-gliadin (IgA) vs C-peptide with correlation coefficient of 0.69, anti-tTG (IgG) vs. C- peptide with correlation coefficient of 0.57 and anti-tTG (IgG) vs. anti- GAD with correlation coefficient of 0.54. Significant moderate positive interrelationships were observed between anti-tTG (IgG) vs. anti-tTG (IgA) with correlation coefficient of 0.43, anti-gliadin (IgA) vs. anti-tTG (IgA) with correlation coefficient of 0.43,anti-gliadin (IgG) vs. anti-tTG (IgA) with correlation coefficient of 0.49,anti-gliadin (IgG) vs. anti-tTG (IgG) with correlation coefficient of 0.50,and anti-gliadin (IgA) vs. anti-gliadin (IgA) also with correlation coefficient of 0.50. Weak positive association between anti-gliadin (IgA)vs. anti-GAD was also observed. The Pearson's correlation matrix showing Interrelationship between Celiac disease antibodies, T1DM antibody and C-peptide is presented in Table 3.15

Table 3.16: Pearson's correlation matrix showing Interrelationship between Celiac disease antibodies, T1DM antibody and C-peptide

Pearson's Correlation Matrix	anti-tTG (IgA)	anti-tTG (IgG)	anti-gliadin (IgA)
anti-tTG (IgG)	0.43**		
anti-gliadin (IgA)	0.43**	0.64**	
anti-gliadin (IgG)	0.49**	0.50**	0.50**
anti-GAD	0.23	0.54**	0.27**
C-Peptide	0.25	0.57**	0.69**

** indicates statistical significant correlation coefficient at $p < 0.05$

Table3.17: The HLA-DQ haplotype of patients with seropositivity to Anti-GAD 65 antibody and Anti-GAD 65 antibody autoantibodies

Type of DQ			Group T1DM No. (%)	Group CD & T1DM No. (%)	P value
DQ2	Anti-GAD 65 antibody	+ve	0 (0.0)	4 (44.4)	0.08
		-ve	5 (100.0)	5 (55.6)	
		Total	5 (100.0)	9 (100.0)	
	Anti C-peptide	Normal	0 (0.0)	0 (0.0)	\
		Low	5 (100.0)	9 (100.0)	
		Total	5 (100.0)	9 (100.0)	
DQ8	Anti-GAD 65 antibody	+ve	12 (46.2)	0 (100.0)	\
		-ve	14 (53.8)	0 (100.0)	
		Total	26 (100.0)	0 (100.0)	
	Anti C-peptide	Normal	5 (19.2)	0 (100.0)	\
		Low	21 (80.8)	0 (100.0)	
		Total	26 (100.0)	0 (100.0)	
DQ2&8	Anti-GAD 65 antibody	+ve	0 (0.0)	10 (27.8)	0.02
		-ve	14 (100.0)	26 (72.2)	
		Total	14 (100.0)	36 (100.0)	
	Anti C-peptide	Normal	5 (35.7)	15 (41.7)	0.70
		Low	9 (64.3)	21 (58.3)	
		Total	14 (100.0)	36 (100.0)	

Area Under the Curve

Test Result Variable(s): VAR00020

Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
		Lower Bound	Upper Bound
.047	.000	.722	.908

Table 3.18: The test result variable(s): VAR00020 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

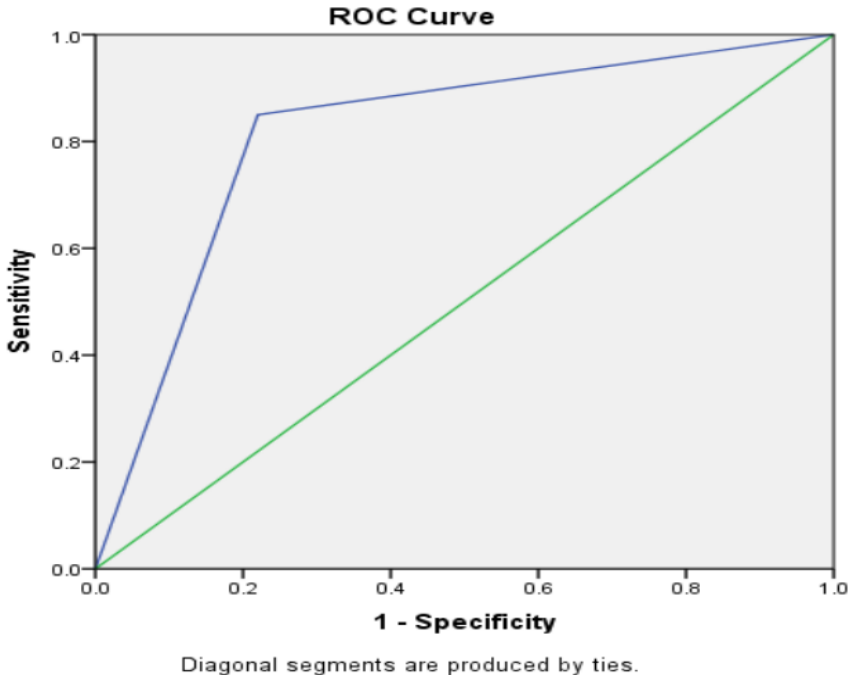


Figure3.2: Diagonal segments are produced by ties .

Chapter Four

Discussion

4. Discussion

4.1:Comparative demographic and biochemical characteristics of the patients

This study explores the general characteristics of celiac disease patients, compares these features with patients having T1DM as well as those with comorbidity of both disorders. With all the patients aged on average, between 20 – 21 years, their demographical and anthropometric disposition of the patients were observed to be similar, however, notable differences were observed in their biochemical characteristics. Specifically, glucose levels were not only expectedly higher in T1DM patients but were also observed to be slightly higher in patients having comorbidity with celiac disease. Although there is scarcity of research on biochemical features in celiac disease and T1DM comorbidity, few studies have reported increased blood glucose levels in T1DM patients with celiac disease (Scaramuzza *et al*., 2013). Interestingly however, HbA1c which is an index for measurement of glycemic control was slightly lower in patients with celiac disease and T1DM comorbidity. Studies conducted by Bakker et al, and Aljulifi et al also reported reduced

HbA1c in T1DM patients diagnosed with celiac disease compared with those having only T1DM (Bakker *et al*., 2013; Aljulifi *et al*., 2021).

These differences like in the case of this study, were not statistically significant, thus the observed reduction in HbA1c cannot be an indication of improvement in glycemic control. Other noteworthy differences observed between the groups were with respect to vitamin D3, urea and creatinine. Celiac disease and T1DM have both been associated with vitamin D deficiencies (Tavakkoli *et al*., 2013; Topal *et al*., 2015). This is because vitamin D plays peculiar roles in the onset of celiac disease as well as the pathogenesis and prevention of T1DM. Studies have identified vitamin D as a key modulator of immune mechanism and inflammation in the intestinal mucosa barrier (Infante *et al*., 2019; Assa *et al*., 2014). Also, vitamin D is known to suppress T-cell activation through binding with the vitamin D receptor (VDR). Polymorphisms in the VDR gene has been associated with celiac disease *(*Motohashi *et al., 2003;* Abd-Allah *et al*., 2014). Moreover, early in life supplementation with vitamin D protects against T1DM as vitamin D has been shown to modify T-cell differentiation, regulation action of dendritic cells and induce cytokine secretion, thereby shifting differentiation the balance to regulatory T cells (Topal *et al*., 2015). The serum levels of vitamin D3 recorded was generally low i.e. < 20 ng/mL, however, it was significantly lower in T1DM patients relative to those with celiac disease, suggesting that vitamin D may have a more detrimental effect on T1DM than on celiac disease. Also it was observed that serum urea and creatinine levels with were otherwise high in T1DM patients was significantly lower in patients with T1DM and celiac disease. Being useful biomarkers for assessing nephropathy in diabetic patients, elevations in urea and creatinine have

been correlated with poor glycemic control and indication of end stage renal disease (Chutani and Pande, 2017).

Interestingly, their reduction in patients with comorbidities as observed in this study could result from molecular-level interplay between the mediators two autoimmune disorders and as such, this is an area requiring further exploration.

4.2:Distribution of HLA-DQ2 and HLA-DQ8 haplotypes among the patients

As it is with many other autoimmune disorders, the relevance of the HLA class II heterodimers DQ8 and DQ2 in celiac disease and T1DM cannot be overemphasized. In this study, the prevalence of HLA-DQ8 and HLA-DQ2 expression among the study groups was analysed. Expression of homozygous HLA-DQ2 was skewed towards patients with celiac disease only and expression of homozygous HLA-DQ8 was skewed towards T1DM patients while, as expected, expression of heterozygous HLA-DQ2/ DQ8 was skewed towards patients having comorbidity of celiac disease and T1DM. The HLA-DQ2 homozygosity confers about 25 - 30 % risk of developing celiac disease in infants (Akar *et al*., 2015), whereas homozygous HLA-DQ8 has been implicated in $CD4^+$ T cell infiltration of islets cells in response against proinsulin thereby increasing susceptibility to autoimmune progression of T1DM (Pathiraja *et al*., 2015). Picot and McDermott reported 30 % prevalence of HLA-DQ2/DQ8 among patients with T1DM (Pociot and McDermott, 2002), a finding consistent with the result obtained from this study, as 14 out of 45 i.e. 31 % of the T1DM patients are HLA-DQ2/DQ8 heterozygotes. Moreover, combined positivity for HLA-DQ2/DQ8 is definitive of a population with

significantly high risk of developing T1DM (Decochez *et al*., 2005), and although 25 - 35 % of the general population have HLA-DQ2/DQ8 heterozygosity, only 3 % go on to develop celiac disease (Cecilio and Bonatto, 2015). Interestingly, no patients with celiac disease only had heterozygous HLA-DQ2/ DQ8 in this study.

The findings of this study are suggestive of the higher inclination of HLA-DQ2 homozygosity towards susceptibility to celiac disease relative to homozygous HLA-DQ8. Supporting this suggestion is the findings of Boa et al, where one third of T1DM patients with homozygous HLA-DQ2 expressed celiac disease-associated transglutaminase autoantibodies (Bao *et al*., 1999). It is therefore of great importance to understand the significant relevance of the interexpression of these genes in conferring genetic risk for celiac disease and T1DM as this may result to the identification of key pathways potentially involved in the pathogenesis of these diseases, that can be useful in development of therapeutic interventions.

4.3:T1DM influences celiac disease associated duodenal aberrations and serum autoantibodies

Another objective of this study was to determine the effect of T1DM on celiac disease, and this was achieved by exploring the differences in some clinical features between patients with only celiac disease in comparison with those having comorbidity with T1DM. Histological observation of duodenal biopsy, with is the gold-standard for the diagnosis of celiac disease (Villanacci *et al*., 2018), was the first feature analysed.

The grading of histological findings was done in accordance with the modified Marsh classification (Ensari and Marsh, 2019) and although majority of the patients both categories were graded Marsh III (characterized by > 40 jejunum intraepithelial lymphocytes [IEL] per 100 enterocytes; > 30 duodenum IEL per 100 enterocytes; increased crypt hyperplasia with mild to complete villi atrophy) while remaining were graded Marsh II (characterized by > 40 jejunum per 100 enterocytes; > 30 duodenum IEL per 100 enterocytes; increased crypt hyperplasia and no villi atrophy), the frequency of Marsh III was less in patients with T1DM. Whether the hyperglycemic conditioning T1DM condition contributes to ameliorating the development of celiac disease associated Duodenal and jejenal lesions, remains to be deciphered. However, the difference in number of patients with Marsh III among the group i.e. 6 patients, was not large enough to provoke further investigative exploration of the observed tendency. The detection of the presence of celiac disease associated autoantibodies provided another potent yardstick for identifying the possible effects of T1DM in celiac disease. The frequencies of patients that showed positivity to both IgA and IgG class of anti-tTG and anti-gliadin autoantibodies were higher in patients with T1DM comorbidity. Serological analysis of celiac disease autoantibodies were since the last 20 years used routinely to identify individuals with subclinical celiac disease prior to confirmation using histological evaluations (Bhatnagar and Bhan, 1999). However, the specificities of the autoantibodies are not the same. For example, anti-gliadin antibodies, despite being the first identified serological marker for screening celiac disease patients, have been reported to have very low specificity and hence is being confined for identifying individuals with wheat sensitivity (Caio *et al*., 2016). Although its usefulness in signaling abnormalities in gluten metabolism as well as early detection of celiac

disease susceptibility in children is still very much useful *(Chartrand et al*., 1997). On the other hand, anti-tTG antibodies are among the highly predictive and widely validated serological test for celiac disease diagnosis (Packova *et al*., 2020). The antibodies belong to both IgA and IgG classes with the IgA showing the largely superior specificity and sensitivity to celiac disease as the IgG class has been associated with high percentage of false positive and as a result, it is recommended for consideration in cases of patients with IgA deficiencies *(Werkstetter et al*., 2017, Villalta *et al.*, 2010). Therefore, the specificity and sensitivity of the celiac disease associated antibodies analysed on the study can be ranked thus; anti-tTG (IgA) > anti-tTG (IgG) > anti-gliadin (IgA) > anti-gliadin (IgG).

The low specificity and sensitivity of anti-gliadin antibody is consistent with the findings of this study as 100 % of patients with celiac disease and T1DM showed positivity for IgG class of anti-gliadin antibody despite only 71 % of the same group had Marsh III degree of duodenal aberrations. With a celiac disease sensitivity of 97 % and specificity of 91 %, anti-tTG (IgA) represents the most useful celiac disease serological marker as positivity to anti-tTG (IgA) alone especially at a high titer, is sufficient for diagnosis of celiac disease without the need for duodenal biopsy or assessing other specific antibodies such as anti-endomysial antibody (Vivas *et al*., 2009). This study observed higher prevalence of anti-tTG (IgA) antibody in celiac disease patients with T1DM- which is not farfetched as Boa et al in 1999, observed a one-third positivity to anti-tTG antibody in celiac disease patients having T1DM with 11.6 % having titer values higher than the control (Bao *et al*., 1999). The relationship between positivity to anti-tTG (IgA) with T1DM has been explored by previous studies. An anti-tTG (IgA) seroprevalence of 10.5 % has been

reported in T1DM adults with undiagnosed with celiac disease (Haladová *et al*., 2014), similarly, 11.1 % (Bhadada *et al*., 2011) and 21.2 % (Saadah *et al*., 2012) of T1DM patients were diagnosed to have celiac disease on basis of positivity to anti-tTG (IgA) antibody. Also 61 % of T1DM children showed positivity to anti-tTG (IgA) out of which 36 % developed celiac disease as identified by a follow-up study (Parkkola *et al*., 2018). These outcomes could imply on one hand, that the co-existence of T1DM in patients with celiac disease further aggravates the disease condition while on the other hand, T1DM increase susceptibility to development of celiac disease in already predisposed individuals. To further understand how T1DM affects serological profile of celiac disease patients the titer values of these antibodies were analysed.

The results obtained revealed a significantly higher anti-tTG (IgA) antibody titer in patients with celiac disease only compared to those having T1DM comorbidity. The lower antibody level observed in this study despite a higher prevalence of seropositivity, could indicate a tendency towards normalization of serum antibody levels in T1DM as previously reported by Waisbourd-Zinman et al where blood levels of anti-tTG antibody were spontaneously normalized in celiac disease patients with T1DM, recommending a 12-month serological follow-up rather than immediate duodenal biopsy (Waisbourd-Zinman *et al*., 2012). Nevertheless, the observed reduction in anti-tTG (IgA) antibody in celiac disease patients with T1DM supports the assertion that T1DM increase susceptibility of developing celiac disease and may not necessarily lead to aggravation of the disease condition, although further investigations are required to decipher the mutual exclusivity of both assertions.

4.4 The influence of celiac disease on T1DM-associated serum anti-GAD and c-peptide

This study also explored the possible effect of celiac disease on T1DM patients by analyzing the presence of T1DM associated antibody (anti-GAD) and C-peptide in patients with T1DM and comparing same with celiac disease patients having T1DM. Antibodies to glutamic acid decarboxylase have well established significance in diagnosis of T1DM even though its role in the pathophysiology of autoimmune diabetes still remains unclear (Jun *et al*., 2002). This enzyme catalyzes the rate-limiting step in the conversion of glutamic acid to gamma-amino butyric acid (GABA), a neurotransmitter in pancreatic islet β-cells as well as in the brain. Although other autoantibodies such as insulin autoantibody (IAA), anti-Zinc transport 8 antibodies (ZnT8A) and so on, have been associated with T1DM, anti-GAD autoantibodies is predominant in 70 - 80 % of T1DM patients and up to 90 % in T1DM children/ adolescents, and has been shown to persist over the years thus characterizing long standing disease duration (Graham *et al*., 2002).

Moreover, at the onset of T1DM, approximately 70 – 90 % of patients show positivity for anti-GAD, making it the autoantibody with the highest specificity to T1DM (Delic-Sarac *et al*., 2016). However, this study recorded just 21 % positivity to anti-GAD in T1DM patients which slightly increased to 31 % in those with celiac disease. The link between anti-GAD antibodies with sensitivity to gluten was investigated by Hadjivassiliou et al where they reported 40 % prevalence of anti-GAD positivity in patients with gluten ataxia (Hadjivassiliou *et al*., 2021). Another important serological marker analysed was C-peptide, a widely used indices for measuring pancreatic β cell function as well as

distinguishing between type 1 and type 2 diabetes mellitus. The diagnostic significance of C-peptide is based on its Equimolar production with endogenous insulin as it forms the part of pro-insulin cleaved prior to secretion of insulin. Additionally, C-peptide is being excreted at a more constant and slower rate relative to insulin and since insulin production is aberrant in T1DM, many studies have reported low C-peptide levels in T1DM patients (Kuhtreiber *et al*., 2015).

The majority of T1DM patients in this study i.e. 77 % had below normal range values for C-peptide with slightly lower prevalence recorded for those having comorbidity with celiac disease. Considering the absence of significant variation in prevalence of anti-GAD and C-peptide between patients with T1DM only and T1DM patients with celiac disease as observed in this study, the possible effect of celiac disease on pathophysiology of T1DM may occur at early onset of the disease rather than develop during disease progression. To investigate further, this study observed the serum concentration of these T1DM serological markers between the two groups. Despite observing no significant difference in C-peptide levels, the result of this study showed that serum concentration of anti-GAD autoantibodies was significantly higher in T1DM with CD.

Patients with celiac disease. Also, the titer of anti-GAD has been shown to be influenced by gluten sensitivity and the duration of gluten exposure was proposed to increase risk of development of T1DM in patients with gluten sensitive enteropathy (Hadjivassiliou *et al*., 2011; Hadjivassiliou et al., 2021). This clearly suggests that celiac disease contributes to anti-GAD seropositivity, which could be due to the central role of autoimmunity in the etiology of both diseases. The significance of anti-GAD in diagnosis of T1DM has not been directly attributed to its role in

the pathophysiology of T1DM, but rather resulted from precipitation of a65K protein autoantigen with GAD activity in sera of patients with T1DM(Velloso *et* al., 1993). However, many explorative studies have tried to unravel the possible mechanism through which GAD can trigger autoimmune diabetes. Pihoker *et al*, hypothesized that in the case of T1DM, the presentation of GAD to T-cells may unintentionally be the mechanism of initiating the breakdown of immunological tolerance to pancreatic β cells by altering the focus of T-cell response towards generation of pathogenic T-cell response that aggravates autoimmune attack on the pancreatic cells (Pihoker *et al*., 2005).

Interestingly, sensitivity to gluten has been shown to provoke the production of neurological disorder associated GAD antibody (Hadjivassiliou *et al*., 2011). Gluten ataxia mediated inducement of anti-GAD associated ataxia has been previously reported in mice (Boscolo et al., 2007), thus highlighting the possible connection between celiac disease and production of anti-GAD antibodies that could trigger the initiation of T1DM. Therefore, it can be suggested that although celiac disease may not affect the progression of T1DM, it plays a significant role in the pathogenesis of T1DM; hence, early diagnosis of celiac disease in children should warrant suspicion of subclinical T1DM.

Conclusions and Recommendations

4.5 conclusions

1-HLA DQ2 gene more common in patient with celiac disease and less than HLA DQ8.

2-HLA DQ8 gene more common in patient with Type 1 Diabetes mellitus and less than HLD2.

3-HLA DQ2 and DQ8 more common in patients type 1 Diabetes mellitus having Celiac disease and less than HLADQ2.

4-Anti –GAD Ab levels are highly in patient type 1 DM having Celiac disease compared with patient having.

4.6 Recommendations

Although the present study appreciably evaluated general characteristics as well as the interrelationship of celiac disease and T1DM, the following recommendations can be made.

i. Detailed molecular level assessment of the biochemical characteristics of the patients in the study groups could be conducted and the findings cross-referenced with expression of HLA-DQ haplotypes.
ii. Future studies can also investigate further, the observed tendency of T1DM to increase susceptibility to celiac disease using a larger sample size as well as conducting follow-up analyses.
iii. Deciphering the link between celiac disease and production of antibodies to GAD initiation of T1DM as well as elucidation of molecular pathways through which the Deciphering the link between celiac disease and production of antibodies to GAD initiation of T1DM as well as elucidation of molecular pathways through which the autoantibody can trigger the initiation of T1DM would lead to better understanding of the pathogenesis of both disease conditions and provide useful insights towards development of superior therapeutic interventions.
iv. Further research needed to investigate control and comparison with each groups takes in this study.
v. Research needed to investigate anti-GAD in patient celiac disease only without celiac disease

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.

Appendix

Appendix B: Type1 Diabetes mellitus patient's questionnaires

Patient Name: . . . . Phone number: . . . . Age .....

Gender male ☐ female ☐

address……

History Personal	**Yes**	**NO**
Type 1 DM:		
Increased thirst:		
Frequency urination:		
Extreme hunger:		
Blurred vision:		
Other Auto immune Disease:		

Laboratory tests:

FBS (fasting blood sugar):

HbA1C:

Anti _GAD

Anti_C peptide:

BMI (Body mass index)

RFT (Renal function test)

Ca+2

Vit D3

Ferritin

CBC (complete blood count)

Family history:

(Diabetes mellitus, Other autoimmune disease)

Appendix B: Celiac disease patient's questionnaires

Patient Name: . . . . . Phone number: . . . . Age: . . . . year…..

Gender: Male ☐ female ☐

History Personal **YES** **NO**

Celiac Disease:

Iron decency:

Decrease of calcium:

Decreased of Vit D3

Excessive of colon:

Diarrhea:

Weight

loss

Abdominal pain:

Constipation:

Fatigue:

Joint pain:

Mouth ulcer:

Blistering skin disease

Other auto immune disease:

Endoscopic examination:

Histological examination result:

Grade:

Laboratory tests

Anti-gliadin antibody (IgA\IgG):

Anti-tissue transglutaminase antibody (IgA\IgG):

BMI:

History family: Is there another member of the family is effected by celiac disease and another auto immune disease.

Appendix B: Strip reading and interpretation

The probes for the HLA alleles are distributed over the membrane in four different groups:

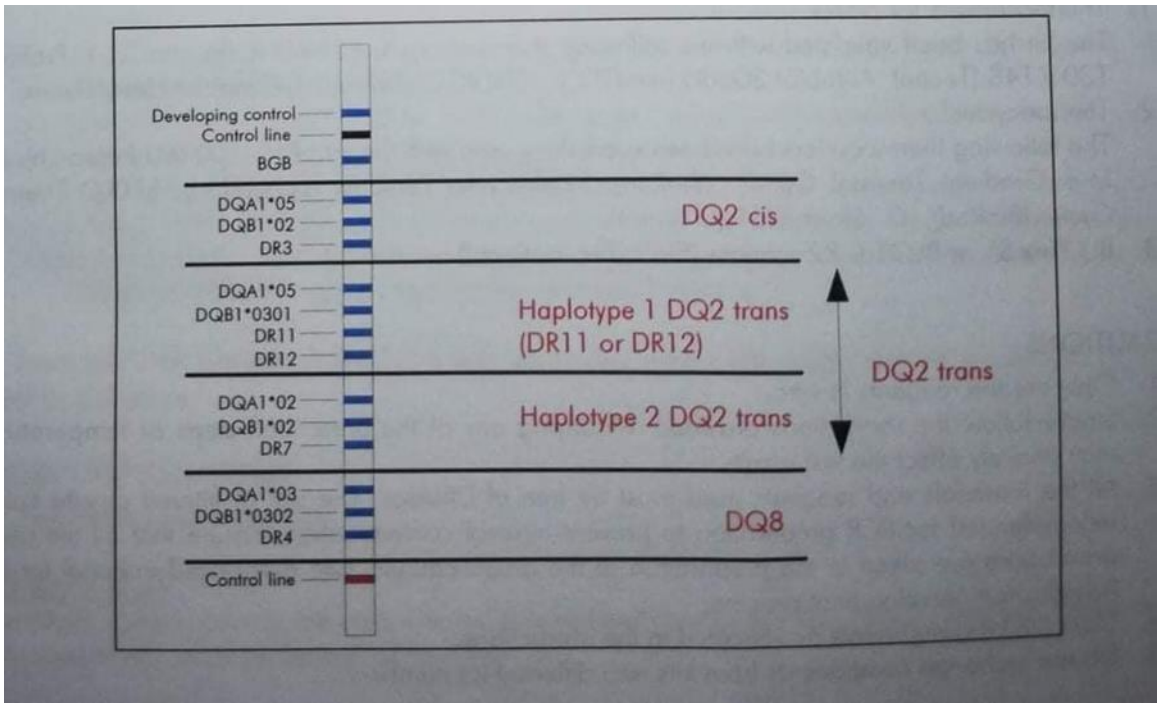
Alleles that constitute the DQ2 cis haplotype: DQA1*05-DQB1*02-DRB1*03.

Alleles that constitute the DQ2 Trans haplotype 1:DQA1*05-DQB1*0301-DRB1*11\DRB1*12.

Alleles that constitute the DQ2 Trans haplotype 2: DQA1*02-DQB1*02-DRB1*07.

Alleles that constitute the DQ8 haplotype: DQA1*03-DQB1*0302-DRB!*04.

The emergence on the membrane of groups of bands linked to each one of the various HLA haplotypes (DQA1*-DQB1*) associated with the celiac diseases leads to their rapid identification**.**



الخلاصة:

أن معدل أنتشار مرض داء حساسية القمح أعلى بشكل ملحوظ في مرضى السكري من النوع الأول مقارنة بعامة السكان ، ونتيجة لذلك ، جذبت العلاقة المتبادلة بين هذين الاضطرابين المناعين الذاتين أهتمام العديد من الباحثين على مر السنين .وكانت أكثر الأسئلة الملحة ما إذا كان وجود ما يصاحب ذلك من مرض حساسية القمح يشكل خطرا على مرضى السكري من النوع الاول أو ما إذا كان وجود نتائج مرض حساسية القمح يؤدي الى تنشيط المحفزات الجينية لمرضى السكري النوع الاول في الأفراد المعرضين بالفعل للأصابة. أيضا ، هناك مخاوف بشأن التأثيرات المحتملة لمرض السكري النوع الاول على الفيزيولوجيا المرضية والاستجابة لعلاج مرضى داء حساسية القمح في المرضى الذين يعانون من الاعتلال المشترك لكلا المرضين .هدفت هذه الدراسة إلى معالجة هذه الأسئلة من خلال تقييم الخصائص البيولوجية الديموغرافية ، والاستعداد الجيني وتوزيع المعلمات التشخيصية لمرض حساسية القمح و مرض السكري من النوع الاول في المرضى الذين لديهم تلك الامراض وكذلك الاعتلال المشترك لكلا الحالتين .تم أخذ 45 مريضا من مرضى السكري النوع الاول فقط ،و 45 مريضا من مرضى حساسية القمح فقط و 45 مريضا مع كلتا الحالتين لهذه الدراسة .تم قياس الخصائص الديموغرافية والقياسية البشرية والكيميائية الحيوية ، وكذلك توزيع جينات HLA-DQ2 و DQ8بين المرضى .أيضًا ، وتم تقييم خزعة الاثني عشر وكذلك فئات مصل IgA و IgG من مضادات الترانسجلوتاميناز(مضادات tTG) والأجسام المضادة الذاتية المضادة للجليادين للمرضى الذين لديهم مرض حساسية القمح فقط وأولئك الذين يعانون من الاعتلال المشترك مع مرض السكري من النوع الاول (CD + T1DM). تم تقييم مستويات مصل الجسم المضاد للكربوكسيلاز المضاد لحمض الجلوتاميك (GAD) والببتيد C أيضًا في المرضى الذين يعانون من مرضى السكري النوع الاول وأولئك الذين لديهم حساسية القمح مع مرض السكري.

أظهرت النتائج التي تم الحصول عليها اختلافات كبيرة في الجلوكوز 3.89 ± 94.82 (مجم/ ديسيلتر لمرضى حساسية القمح مقابل 1.97 ± 173.74 لمرضى السكر النوع الاول ، وكانت مستويات السكر التراكمي لمرضى حساسية الحنطة 0.66 ± 5.43 (مقابل(0.24 ± 7.90 لمرضى السكر النوع الاول ، فيتامين (0.99 ± 7.15) D3 نانوغرام / مل بالنسبة لمرضى السكري النوع الاول مقابل 2.82 ± 11.04 . للمرضى الذين لديهم اعتلال مشترك لكلا الحالتين ، اليوريا 4.36 ± 35.23 (مجم / ديسيلتر لمرضى السكري النوع الاول مقابل4.75 ± 23.24 مجم / ديسيلتر للمرضى الذين لديهم اعتلال مشترك لكلا الحالتين (والكرياتينين)1.06±0.06

مجم / ديسيلتر لمرضى السكر النوع الاول مقابل 0.01 ± 0.61 مجم / ديسيلتر للمرضى الذين لديهم اعتلال مشترك. كان HLA-DQ2 ينحرف نحو المرضى الذين يعانون من داء حساسية القمح بينما كان HLA-DQ8 يميل نحو المرضى الذين يعانون من السكر النوع الاول و80 ٪ من مرضى الذين لديهم اعتلال مشترك كانوا . HLA-DQ2 / DQ8 غالبية المرضى الذين يعانون من تصنيف Marsh III أي 71 ٪ ، إيجابية المصل لمضاد tTG IgA (88.9) ٪ (، IgG (60٪ (ومضاد الغلياديين IgA (51.1) ٪ (، (100) IgG كانوا مع مرضى الاعتلال المشترك على الرغم ان القيم الاحصائية أظهرت مستوى أقل بكثير مقارنة بمرضى داء حساسية القمح ($p < 0.05$) أيضا ، كانت مستويات مضادات GAD أعلى بشكل ملحوظ في المرضى الذين يعانون مرض السكري النوع الاول فقط مقارنة مع أولئك الذين لديهم اعتلال مشترك . ($p < 0.05$)من خلال النتائج ، تم الاستدلال على أن الوجود مرض داء حساسية القمح و داء السكر النوع الاول معا قد يؤدي إلى صعوبة في التحكم في نسبة السكر في الدم ولكن يبطئ تقدم أعراض مرض داء حساسية القمح أيضًا , في حين أن داء السكر النوع الاول قد يمنح القابلية للتأثر بالاصابة بمرض حساسية القمح ، فإن الوجود المسبق لداء حساسية القمح قد يؤدي إلى التسبب في / بدء بمرض داء السكر النوع الاول . لذلك ، توصي هذه الدراسة بأن التشخيص المبكر لمرض حساسية القمح أو داء السكر النوع الاول يجب أن يكون وافيا لتقليل الاشتباه في كون المرض داء السكر النوع الاول أو داء حساسية القمح وخاصة في المرضى الذين لاتظهر عليهم الاعراض.

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء إكلية الطب
فرع الاحياء المجهرية الطبية

تقييم مؤشرات المناعة الذاتية وتكرار HLA-DQ2 \ DQ8 في مرضى السكري من النوع ١ \ أو مرض الاضطرابات الهضمية

رسالة مقدمة الى مجلس كلية الطب جامعة كربلاء كجزء من متطلبات نيل شهادة الماجستير

في الأحياء المجهرية

من قبل

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