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Evaluation of HLA-DQ2 and DQ8 haplotypes in patients with Celiac Disease Correlation with Circulating Anti-Tissue Transglutaminase and Anti- Gliadin Antibodies

A thesis

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

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بسم الله الرحمن الرحيم

﴿وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ عَلَيْهِ

تَوَكَّلْتُ وَإِلَيْهِ أُنِيبُ﴾

سورة هود الآية (88)

DEDICATION

To

The Soul of My Father

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SUMMARY

The diagnosis of celiac disease (CD) remains one of the major challenges encountered with understanding the disease epidemiology, pathophysiology, management and development of therapeutic interventions. The atypical nature of majority of its symptoms coupled with late presentation of symptoms in early onset of disease among children and individuals with subclinical CD has been identified as the major culprits. While histological analysis of duodenal biopsy has been shown to have in capabilities of effectively diagnosing CD during early onset of disease in children due to absence of duodenal lesions, serological measurements of CD associated autoantibodies are useless in cases of seronegative patients with duodenal lesions or those with subclinical CD.

Genetic testing for the detection of HLADQ2 and HLA-DQ8 in patients suspected to have CD is gaining acceptance as a more effective method of CD diagnosis. The present study aimed to analyze the effectiveness of HLA-DQ genotyping of CD patients in predicting outcome of histologic and serological evaluations. The study recruited 90 patients clinically diagnosed as having CD and assessed their biodemographical characteristics of CD patients, their distribution with respect to histological analysis of duodenal biopsy, serological analysis of CD autoantibodies as well as presence of HLA-DQ2 and HLA-DQ8 haplotypes and their prediction of histological and serological profile of the patients. The results obtained indicated a female predominance of CD.

Genetic testing revealed that majority of the patients (77.8%) were HLA-DQ2 homozygotes while histologic analysis showed that 49 out of 90 patients had Marsh III grading of duodenal lesion with 25 patients having Marsh II. Seropositivity to anti-tissue glutaminase (tTG) IgA antibody was 41.1% with 63% of patients positive for anti-gliadin IgG antibody. Age-associated high antibody titer levels were observed in patients aged 33 – 39 years (390.04±67.21 U/mL) and 26 -32 years (352.62±50.12 U/mL) for anti-tTG IgA while 26-32 years (257.40±49.73 U/mL), 40 -47 years (220.04±24.35 U/mL) and then 33 – 39 years (214.55±32.40 U/mL) for anti-gliadin IgG. The prevalence of patients with HLA-DQ2 and HLA-DQ8 genotype was 100% as the predominant HLA-DQ2 predicted Marsh II outcome by 100%, Marsh III by 75%, anti-tTG IgA by 67.8%, anti-tTG IgG by 77.8%, anti-gliadin IgA by 82.9% and anti-gliadin IgG by 86%. These findings reinforce the assertion of the effectiveness of HLA-DQ genotyping CD diagnosis and recommends that genetic testing should be utilized on patients suspected to have CD since negative or positive results would rule out CD or prevent mal-diagnosis in patients with subclinical CD respectively.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

Abbreviations	Terms
AGA	anti- gliadin peptides antibodies
AIDS	Acquired immunodeficiency syndrome
Anti _tTG	Anti –tissue transglutaminase
Anti- GAD	Anti –glutamic acid decarboxylase
APC	antigen presenting cells
BMI	body mass index
BSG	British Society of Gastroenterology
CD	celiac disease
CD4	Cluster Differention 4
CD8	Cluster of Differention 8
DC	Dendetric Cell
DNA	deoxyribonucleic acid
DRC	dose response curve
EDTA	Ethylene Diamine tetra acetic acid
ELISA	Enzyme immunosorbents assay
EMA	endomysial antibody
GCD	gluten containing diet
GFD	Gluten free diet
HbA1c	Hemoglobin A 1c
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Ig	Immunoglobulin
IgA	immunoglobulin Alpha
IgG	immunoglobulin Gamma
IMGT	ImMunoGeneTi
K	potassium channels
LD	linkage disequilibrium
MHC	major histocompatibility complex
MIC-A	class I chain related A
NASPGHAN	North American Society for Pediatric Gastroenterology, Hepatology and Nutrition Guidelines

PCV	packed cell volume
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
TMB	Tetramethylbenzidine
tTG	tissue trans glutaminase
TTG	transglutaminase antibodies
WBC	white blood cell
WHO	World Health Organization

Chapter one
Introduction
and
Literature Review

1.1Introduction

Celiac disease (CD) also known as gluten-sensitive enteropathy, is a multifactorial immune-mediated disease that results from intolerance to gluten derived gliadin molecules by individuals with genetic predisposition. The human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 encodes the heterodimer major histocompatibility (MHC) class II molecules DQ2 and DQ8 on antigen presenting cells (APC) which bestows strong antigen interaction with gliadin-derived peptides which in turn leads to antigenic presentation to CD4+ T cells, thus triggering immune response. The resultant provocation of the immune response results in proliferation of intraepithelial lymphocytes, villous atrophy, cystic hyperplasia and eventually jejunal and duodenal lesions (Vigil, 2018).

With a global prevalence of 1.4%, many epidemiologists are of the opinion that CD is grossly underdiagnosed. This is because clinical presentation of CD-associated symptoms like severe chronic diarrhea, abdominal pain, fatigue, bloating, weight loss, vomiting and anemia occurs in most cases, at late disease onset and are not perculiar to CD exclusively *(Bradauskiene et al*., 2021). Since until patients present chronic gastrointestinal symptoms before CD is suspected, the major challenges faced in the management of CD remains under diagnosis, late diagnosis and maldiagnosis of the disease condition. Histological analysis of biopsy specimen from duodenum of patients suspected to have CD, for presence of enteropathy is considered the "gold standard" for diagnosis of CD (Villanacci *et al*., 2018). However, disease conditions such as Cohn's disease, microsopic enterocolitis, HIV/AIDS, Whipple disease, hypergamaglobulinemic sprue amongst many others have associated associated with enteropathies (Breitschwerdt, Halliwell,

Foley, Stark, & Corwin, 1980; KOTLER, GAETZ, LANGE, KLEIN, & HOLT, 1984; Malamut, Cerf-Bensussan, & Cellier, 2015; Sharma et al., 2018). Another alternative for diagnosis of CD is the serological detection of anti-tissue glutaminase (tTG) and anti- gliadin peptides antibodies as it provides a noninvasive method of detecting those with likelihood of having CD (Lerner, Ramesh, & Matthias, 2019). Seropositivity to these antibodies does not exclusively occur in CD as cases of CD patients that are seronegative to these antibodies have been previously reported (Krishnareddy & Lebwohl, 2021). Genetic testing for the presence of CD associated HLA-DQ2 and HLA-DQ8 haplotypes in patients suspected to have CD has over the past few years been included as a diagnostic method for CD (Al‐Hussaini et al., 2019; Martínez-Ojinaga, Molina, Polanco, Urcelay, & Núñez, 2018).

In this study, the diagnostic effectiveness of CD-directed HLA-DQ genotyping was evaluated by analyzing the biodemographical characteristics of CD patients, their distribution with respect to the three diagnostic methods vis-à-vis histological analysis of duodenal biopsy, serological analysis of CD autoantibodies as well as detection of HLA-DQ2 and HLA-DQ8 haplotypes. Comparisons were then made to ascertain whether HLA-DQ2/DQ8 genotyping predicts the outcome of histological and serological profile of the patients and can be a more effective method for CD diagnosis especially in cases when the other tests are useless.

1.1.1 Research questions

Does HLA-DQ2/DQ8 haplotype correlate with levels of anti-tTG and anti- gliadin peptides with respect to biodemographical factors in CD patients?

Can HLA-DQ2/DQ8 use to diagnose celiac disease particularly when the tests are useless?

1.1.2 Aim

To evaluate the presence of HLA-DQ2 and HLA-DQ8 haplotypes in patients diagnosed with CD and compare DQ2 and DQ8 ratio in CD patients with clinical symptoms, serological tests (anti-tissue transglutaminase, anti- gliadin peptides (AGA) antibodies) or intestinal biopsy.

1.1.3 General objective

To assess the presence of HLA-DQ testing (HLA-DQ2 and HLA-DQ8) in CD patients, and investigate their value for the prediction of celiac with respect to serological test (anti-tTG and anti- gliadin antibodies) relationship across biodemographic factors.

1.1.4 Specific objectives

i. To measure levels of anti-tTG and anti- gliadin antibodies in serum from patients with CD using ELISA techniques.
ii. To detect HLA-DQ2 and HLA-DQ8 haplotypes in CD patients.
iii. To correlate the HLA-DQ2 and HLA-DQ8 haplotypes obtained with those of anti-tTG and anti- gliadin antibodies across different collated biodemographical data using statistical analytic tools.

1.2 Literature review

1.2.1 Celiac disease

Celiac disease (CD) was simply defined by Hill et al, as a permanent sensitivity to gluten in wheat and related proteins found in barley and rye that occurs in genetically susceptible individuals and is manifest as an immune-mediated enteropathy as defined by characteristic changes seen on intestinal histology as recommended by North American Society for Pediatric Gastroenterology, Hepatology and Nutrition Guidelines (NASPGHAN) (Hill *et al*., 2005). The disease was also defined as an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals and characterized by the presence of a variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy (Green & Jabri, 2003; Husby *et al*., 2012; Jabri & Sollid, 2006).

1.2.1.1 Classification of CD

Celiac disease has been classified into five different categories, as follows;

Classic: which describes patients with the classic features of intestinal malabsorption who have fully developed gluten-induced villous atrophy and other classic histologic features.

Atypical: describing patients that generally have little to no gastrointestinal symptoms but come to medical attention because of other reasons such as iron deficiency, osteoporosis, short stature, or infertility. These patients generally have fully developed gluten induced villous atrophy.

Silent: which refers to asymptomatic patients who are discovered to have gluten-induced villous atrophy. These patients are clinically silent in that they do not manifest any clear gastrointestinal symptoms or associated atypical features of CD.

Latent: which describes patients with currently normal intestinal mucosa on a gluten containing diet (GCD) with positive serology test who will subsequently develop CD.

Refractory: Represents patients with true CD (i.e. not a misdiagnosis) who do not or no longer respond to a gluten free diet (GFD) (S. P. James, 2005; Ludvigsson, Leffler, et al., 2013; Rostom, Murray, & Kagnoff, 2006).

1.2.1.2 Prevalence of CD

In the last few years, considerable changes in the epidemiology of CD have been observed. A marked increase in CD prevalence and incidence has been reported, which can beat least partially explained by both the development of more sensitive serological tests and a high degree of disease suspicion (Green, Rostami, & Marsh, 2005).

Celiac disease is the result of both environmental, immune system and genetic factors. Distribution of these three components can probably be used to predict at risk areas of the world for gluten intolerance. In this respect, the world geographical distribution of CD seems to have followed the spread of wheat consumption and migratory flows of mankind (Cataldo & Montalto, 2007).

The prevalence of CD is difficult to be estimated because of the different forms of clinical picture and variety in presentation of the

disease itself, especially in patients who have mild form of the disease or those who have no apparent signs or symptoms (Ruby, Al-Khodary, Shubair, & Sirdah, 2014). However, the prevalence of CD is globally estimated at 1.4%, but large variations among countries have been shown (Bradauskiene et al., 2021; Lionetti & Catassi, 2011).

Despite the high prevalence, it has been reported that a ratio between diagnosed to undiagnosed is about 1:7 as reflected in CD iceberg presented in Figure 1.1.

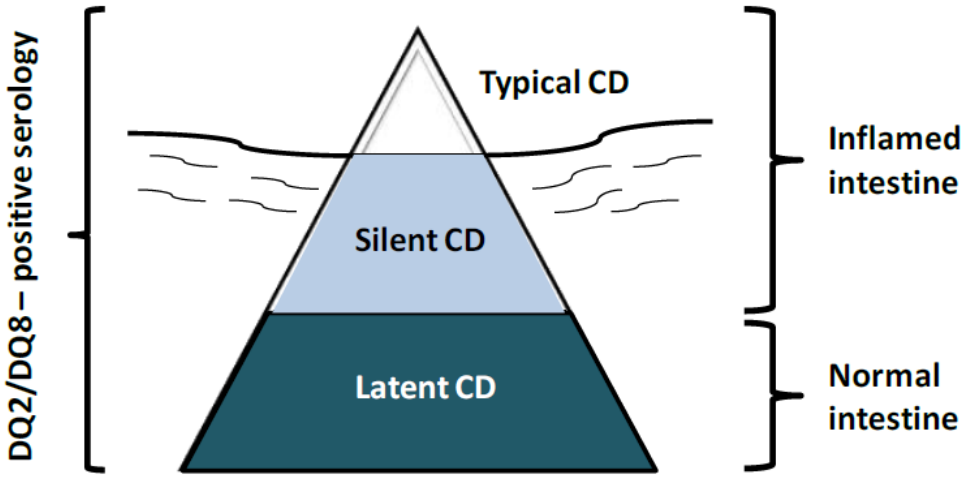


Figure 1.1: Iceberg model of celiac disease
The iceberg model of CD shows the ratio between diagnosed and undiagnosed CD. Patients with Typical CD are easily diagnosed as they present positive serology and inflamed intestine while those with Silent and Latent CD forms the vast majority but have normal intestine.
Sourced from: (Fasano & Catassi, 2001).

1.2.2 The Human leukocyte antigen

The human leukocyte antigen (HLA) system is a cluster of gene complex encoding the major histocompatibility complex (MHC) proteins known as antigens located on the cell membrane of leukocytes in humans from which its name was derived. The functions of these cell surface proteins are many like responsible for the regulation of the immune system whether humoral or cellular in humans. It is the most important area in the vertebrate genome regarding infection and autoimmunity, and is essential in adaptive and innate immunity. The HLA gene complex is located on a 3-Mbp stretch within short arm of chromosome number 6p21 as shown in Figure 1.2. The HLA genes are codominantly expressed and highly polymorphic, those have many diff erent alleles that modify the adaptive immune system that helps body to distinguish the body's own protein from foreign invaders protein like virus, bacteria, or any other pathogens (Horton et al., 2004; Trowsdale, 1993).

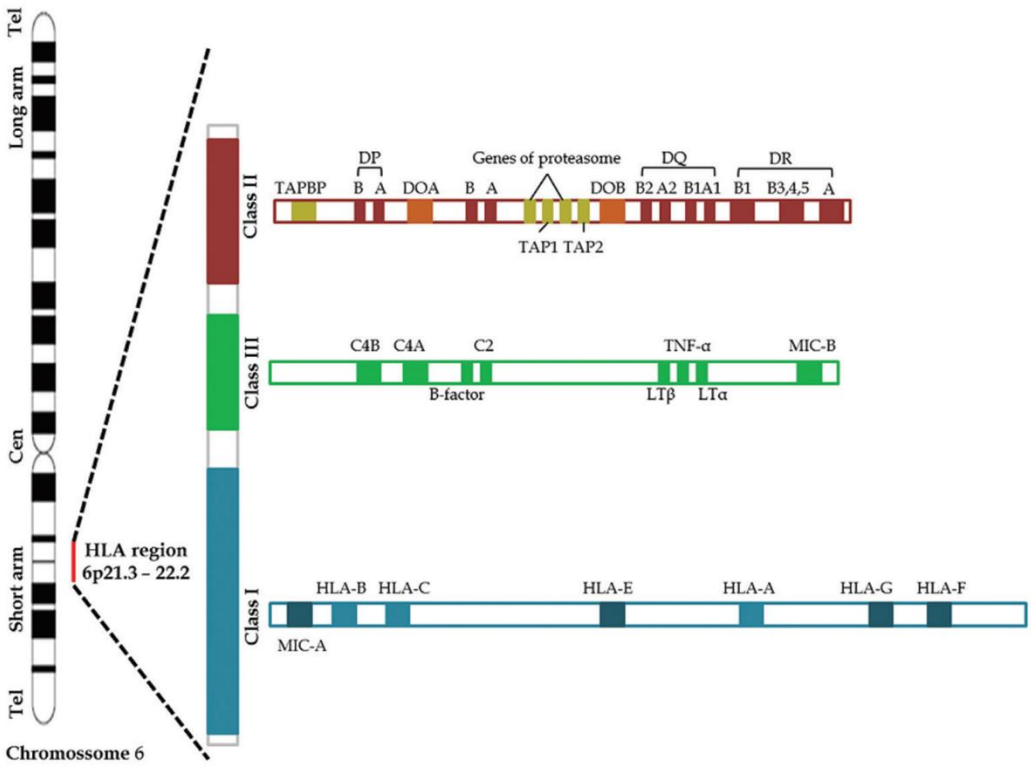


Figure 1.2: The location of HLA complex as found on human chromosome 6

The HLA gene complex is located on a 3-Mbp stretch within short arm of chromosome number 6p21.3-22.2. The class I (in blue), class III (in green) and class II which habours the DP, DQ and DR regions.

Sourced from: Shoeib, S., El-Shebiny, E., Efat, A., & El-Hady, K. A. (2019). Human leukocyte antigen in medicine. Menoufia Medical Journal, 32(4), 1197; Rhodes, D., & Trowsdale, J. (1999). Genetics and molecular genetics of the MHC. Reviews in immunogenetics, 1(1), 21-31 (Rhodes & Trowsdale, 1999; Shoeib, El-Shebiny, Efat, & El-Hady, 2019)

The human leukocyte antigen region can be divided into three different regions as shown in Figure 1.3. HLA class I loci (subdivided to HLA-A, B, C), class II loci (subdivided to HLA-DR, DQ, and DP), coded for cell surface glycoproteins important in the antigen presentation and self-recognition by immune cells, and HLA class III loci coded for molecules related to the complement system and cytokines which are not part of the HLA complex molecules but it's loci

are located in the region between HLA class I and class II on chromosome 6 (Megiorni & Pizzuti, 2012; Thorsby, 2011). Human leukocyte antigen class I and II genes are highly polymorphic and present strong linkage disequilibrium (LD) in which preferential combinations of alleles are inherited together in the genome more often than expected. The series of alleles at linked loci on a single chromosome is named "haplotype (Shiina, Hosomichi, Inoko, & Kulski, 2009). Human leukocyte antigen class I molecules are glycoproteins present on the surface of all nucleated cells in the body. Human leukocyte antigen class I consists of a heavy chain glycopeptide (α) encoded by HLA and linked non covalently to the β2-microglobulin light chain. The main function of class I is to present endogenous antigens to cytotoxic T-cells (CD8+ T Cell) (Neefjes, Jongsma, Paul, & Bakke, 2011).

The HLA class II molecules are present on the surface of antigen presenting cells (APCs) like dendritic cells, macrophages and B-cells and are composed of two transmembrane glycoproteins, Alpha (α) and Beta (β) chains as heterodimers. The key role of Class II molecules is to present exogenous antigens to helper T cells (CD4+ T cells) for initiation of the immune response (Ten Broeke, Wubbolts, & Stoorvogel, 2013).

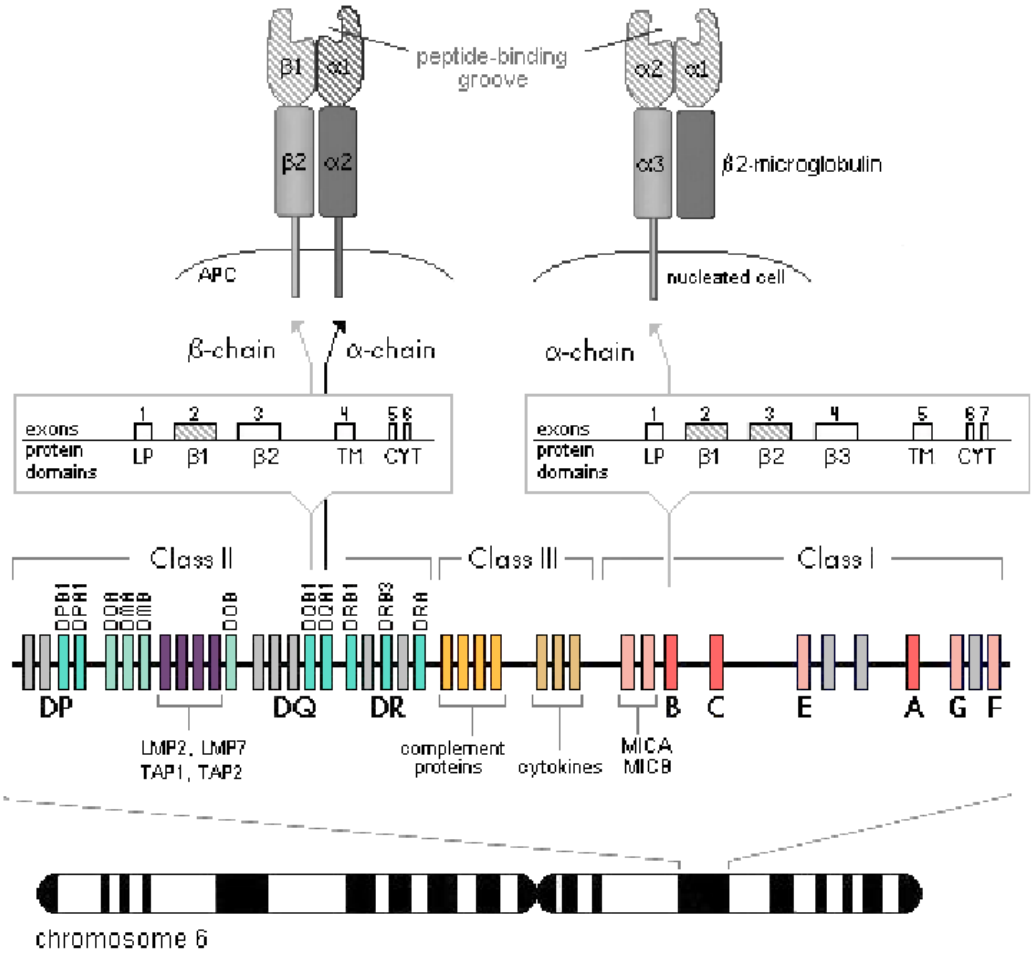


Figure 1.3: Genomic map of the location of the HLA class I and class II genes and its molecules

The HLA class I loci (subdivided to HLA-A, B, C), consists of a heavy chain glycopeptide (α) encoded by HLA and linked non covalently to the β2-microglobulin light chain. While class II loci (subdivided to HLA-DR, DQ, and DP), composed of two transmembrane glycoproteins, Alpha (α) and Beta (β) chains as heterodimers, coded for cell surface glycoproteins important in the antigen presentation and self-recognition by immune cells.

Sourced from: Shiina, T., Hosomichi, K., Inoko, H., & Kulski, J. K. (2009). The HLA genomic loci map: expression, interaction, diversity and disease. Journal of human genetics, 54(1), 15-39. (Shiina *et al.*, 2009).

1.2.2.1 HLA nomenclature

All alleles receive at least a four-digit name, which corresponds to the first two sets of digits, longer names are only assigned when necessary. HLA alleles defined at DNA level have the gene name followed by an asterisk (*). For molecular nomenclature, alleles name is initiated with the letters “HLA” that specifies the gene region, followed by letter/s (e.g. A, DR or DQA1) referring to the locus within the gene region. The first two digits define the allele group, usually equivalent to the serological antigen. The third and fourth digits used to assign the allele subtypes (Zaqout, 2016).

Numbers are defined to each allele in the order of which DNA sequences have been resolved. Alleles with different numbers in the first four digits have one or more different nucleotide substitutions that thus change the amino acid sequence of the encoded protein. The use of additional fifth and sixth digits was later implemented to specify alleles that differ only by synonymous nucleotide substitutions without a consequent change in amino acid (non-coding substitutions) (De Silva *et al*., 2014). Alleles that only differ by sequence polymorphisms in the introns or in the 5' or 3' untranslated regions that flank the exons and introns are distinguished by the use of the seventh and eighth digits. The number of new HLA alleles discovered every year increased rapidly until reaching the maximum possible current naming capacity. Hence, the need for a new nomenclature system was decided upon employs a new system using colons (:) in the allele names to act as delimiters of the separate fields and was introduced in 2010 ((Marsh *et al*., 2010). International ImMunoGeneTics (IMGT) database is responsible for

updating the information on HLA alleles (https://www.ebi.ac.uk/ipd/imgt/hla/).

The WHO established the most recent HLA naming system in 2010. The work was originally started in 1989 when a large number of HLA allele sequences were first analyzed and named as shown in Figure 1.4 (Nazahah & Koh, 2015).

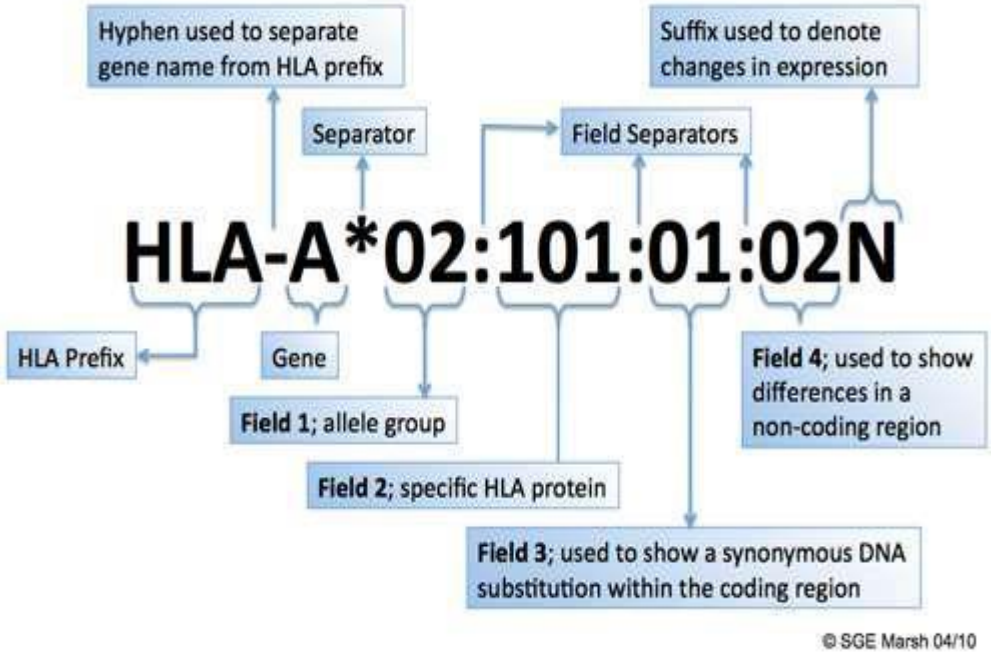


Figure 1.4: Schematic of human leukocyte antigen nomenclature system

The HLA prefix is separated form the gene by a hyphen, followed by an asterics, then numbers showing the allele group, specific HLA protein, synonymous DNA within substitution coding region, non-coding region all separated by colons and lastly suffix used to denote changes in expression.

Sourced from: Nazahah, M., & Koh, M. (2015). Tissue typing and its role in transplantation. ISBT Science Series, 10(S1), 115-123. (Nazahah & Koh, 2015).

1.2.2.2 HLA and disease association

The majority of the genes in the major histocompatibility complex region express high polymorphism, which is essential to their function. The most important function of HLA molecule is in the induction and regulation of immune responses. Many human diseases, including autoimmune, infectious, inflammatory, and malignant, are significantly more common among individuals carrying particular HLA alleles (Shoeib *et al*., 2019). The HLA region of the genome is associated with the greatest number of human diseases (L. M. James & Georgopoulos, 2018; Trowsdale & Knight, 2013). The association was found to be weak in some diseases and quite strong in other diseases, these latter diseases mainly being autoimmune diseases (Trowsdale, 2011). Most autoimmune diseases are associated with HLA class II genes and a few are associated with HLA class I genes. As presented in Figure 1.5, susceptibility to the diseases increase with specific HLA genes. Type 1 diabetes and CD are associated with DQB1 *02:01 and DQB1 *03:02 of HLA-class II allele (Viken *et al*., 2017). Other diseases associated with the regions include insulin dependent diabetes mellitus (Bratanic *et al*., 2010), rheumatoid arthritis (Coenen *et al*., 2009), multiple sclerosis (Lincoln *et al*., 2009), IgA deficiency (Ferreira *et al*., 2012), cancer susceptibility (Wu *et al*., 2007), idiopathic nephrotic syndrome (Debiec & Ronco, 2011) and sjogren‟s syndrome (Denham & Hill, 2013).

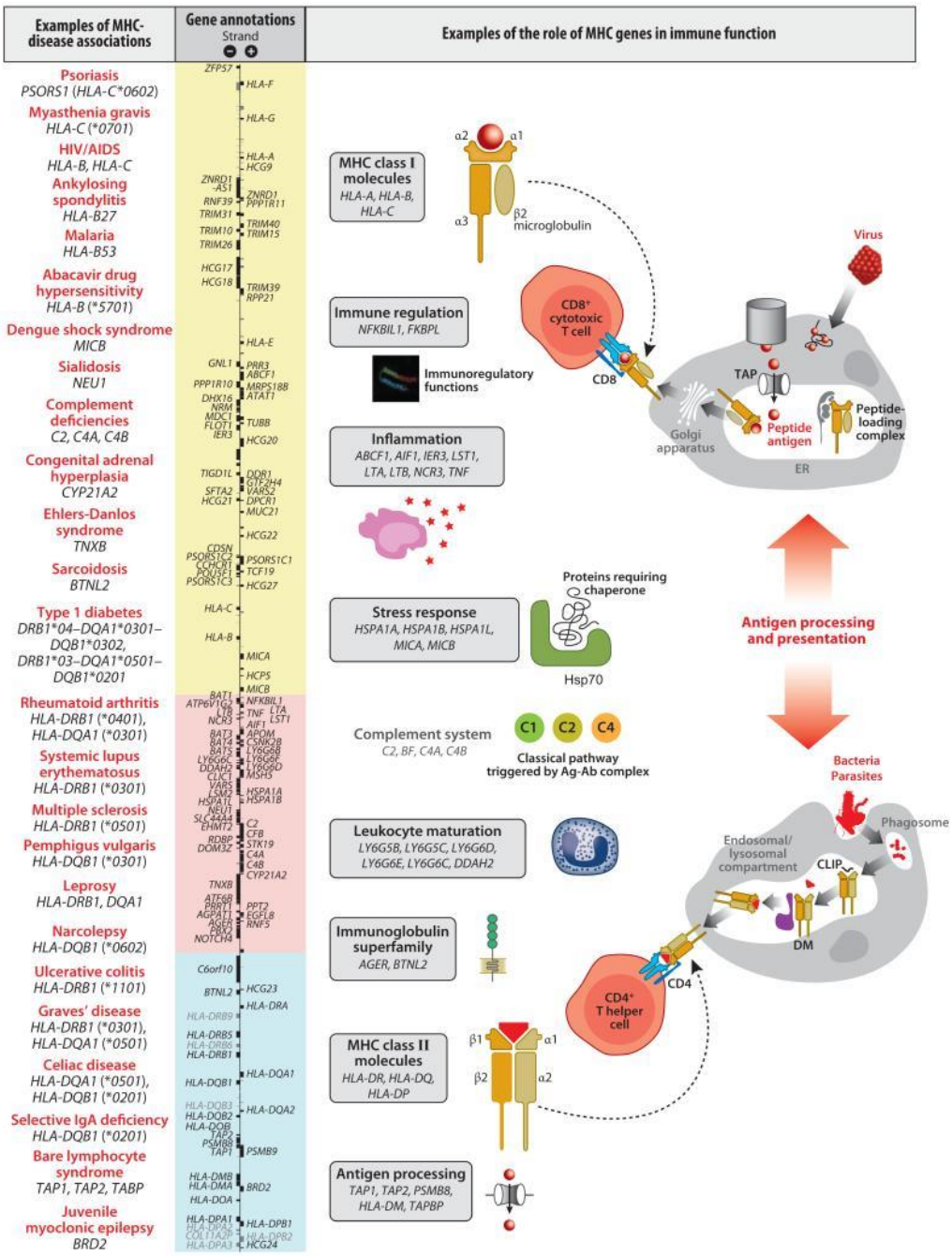


Figure 1.5: Diseases that found to be related with MHC region.
The different immune-related diseases that are associated with MHC region, their respective gene annotations and the roles of MHC region in immune function.
Sourced from: Trowsdale, J. (1993). Genomic structure and function in the MHC. Trends in Genetics, 9(4), 117-122. (Trowsdale & Knight, 2013)

1.2.2.3 CD and the HLA region

The strongest and best characterized genetic susceptibility factors in celiac disease are (HLA) class II genes known as HLA-DQ2 and HLA-DQ8, approximately 95% of the patients express HLA-DQ2 and the remainder is mostly HLA-DQ8 positive (Kupfer & Jabri, 2012a). Nevertheless, although some 40% of the population express one or both of these HLA-DQ haplotypes, only 1% of the population develops CD, implying that HLA is a necessary but not sufficient factor for CD development. In the absence of additional predisposing factors the disease will never develop (Almeida et al., 2016; Megiorni et al., 2009). HLA-DQ2 heterodimers, hereafter called DQ2.5, encoded by DQA*05 and DQB*02 alleles, which may be inherited together on the same chromosome (cis configuration) or separately on the two homologous chromosomes (trans configuration).

Generally, DQA*05 and DQB*02 are present in cis on DR3 haplotype or in trans on DR5/DR7 haplotypes. Almost all DQ2 negative patients (5-10%) carry DQ8 heterodimers encoded by DQA*03 and DQB*03:02 alleles, generally in cis positionon on DR4 haplotype as described in figure 1.5 (Abadie, Sollid, Barreiro, & Jabri, 2011; Megiorni & Pizzuti, 2012). Those CD patients who are neither DQ2 nor DQ8 carry DQB*02 together with DQA alleles other than DQA*05, or they carry DQA*05 together with DQB alleles other than DQB*02. In other words, they carry genes coding for only one of the chains of the DQ2 encoded heterodimer (DQA*05 or DQB*02 allele) (Heyman, 2014; Karell et al., 2003) .

Moreover, CD susceptibility depends on the dosage effect of the DQ2 haplotype. Homozygous individuals for the DQ2 haplotype express the highest levels of DQ2 heterodimers. This genotype is associated with the highest risk of CD (Liu *et al*., 2014). Also individuals who are homozygous for only the β-chains of the HLA-DQ2 (DQB*02 allele), have an increased risk for CD (Biagi *et al*., 2014)). Table 1.1 describes HLA-DQ risk gradient to CD.

Table1.1 HLA-DQ risk gradient to celiac disease

	Haplotype 1		Haplotype 2			
S/N	**DQA1**	**DQB1**	**DQA1**	**DQB1**		**Risk**
1	05:01	02:01	03:01	03:02	DQ2 cis and DQ8	1:7
2	05:01	02:01	X[a]	02:01[b]	DQ2 cis, β2	1:10
3	03:01	03:02	X[a]	02:01[b]	DQ8, β2	1:24
4	X[a]	02:01[b]	X[a]	02:01[b]	β2/ β2	1:26
5	05:01	02:01		02:01[b] neg	DQ2 cis	1:35
6	02:01	02:02	05:05	03:01	DQ2 trans	1:35
7	03:01	03:02		02:01[b] neg	DQ8	1:89
8	X[a]	02:01[b]		02:01[b] neg	β2	1:210
9	05:01	02:01 neg		02:01[b] neg	α5	1:1842
10	Other				Others	1:2518

β2: *DQB*02* in the absence of *DQA*05*. α5: *DQA*05* in the absence of *DQB*02*.
X[a]: different from *DQA*05*. 02:01b: either *DQB*02:01* or *DQB*02:02*.
Sourced from: Lavant, E. H., & Carlson, J. A. (2009). A new automated human leukocyte antigen genotyping strategy to identify DR-DQ risk alleles for celiac disease and type 1 diabetes mellitus. Clinical chemistry and laboratory medicine, 47(12), 1489-1495. (Lavant & Carlson, 2009).

1.2.3 Risk factors for CD

Gastrointestinal infections in children with genetic susceptibility to celiac disease increase the risk of this autoimmunity. The risk is modified by HLA genotype, infant gluten consumption, breastfeeding, and rotavirus vaccination, indicating complex interactions among infections, genetic factors, and diet in the etiology of celiac disease in early childhood (Kemppainen *et al*., 2017).

1.2.3.1 Infant feeding

Continuing breastfeeding at time of gluten introduction has been suggested to limit the amount of gluten the child receives and thereby decreasing the chance of developing CD (A. Akobeng, Ramanan, & Basude, 2005; A. K. Akobeng, Ramanan, Buchan, & Heller, 2006). Another possible explanation is that the milk in itself protects against gastrointestinal infections (Selimoglu & Karabiber, 2010). Commercial milk formulas are based on altered cow's milk that does not contain the same antibodies found in breast milk. In addition differences in gut microbiota were found between breastfed and formula fed infants (Nadal, Donant, Ribes-Koninckx, Calabuig, & Sanz, 2007). Breast-feeding reduces the genotype-related differences in microbiota composition, which could partly explain the protective role attributed to breast milk against CD (Sánchez *et al*., 2011). First time of gluten introduction also considered as a risk of CD as early introduction of solid foods (i.e. before the intestinal immune system reaches a certain level of maturation) may lead to the development of intolerance (Cummins & Thompson, 2002). However, the overlap of gluten introduction with breast-feeding may be a more important protective factor in minimizing the risk of celiac disease (Selimoglu & Karabiber, 2010).

1.2.3.2 Child birth

The sterile protected intestinal mucosa of the fetus becomes exposed to and subsequently colonized by a complex and diverse bacterial community shortly after delivery. Both, the exposure to microbial ligands and the bacterial colonization have been described to differ between neonates born vaginally or by cesarean delivery, which leads later to alteration of gut microbiota (Biasucci, Benenati, Morelli, Bessi, & Boehm, 2008; Dominguez-Bello et al., 2010). These differences might influence the development of the mucosal immune system and ultimately contribute to the risk of autoimmune diseases such as CD later in life (Decker, Hornef, & Stockinger, 2011). The season of birth could potentially affect both the microbiota and the innate immune system of individuals at risk of CD through different exposures to infectious disease. Summer birth would be associated with later CD as children born in summer months are more likely to have initial exposure to gluten during winter months, when concurrent viral infection is more probable (Lebwohl, Green, Murray, & Ludvigsson, 2013; Lewy, Meirson, & Laron, 2009). Another potential cause of seasonal variations in CD is lack of vitamin D. Low levels of vitamin D during winter time predisposes to certain immune-mediated diseases (Munger, Levin, Hollis, Howard, & Ascherio, 2006).

1.2.3.3 Gastrointestinal infection

Infections (either viral or bacterial) could potentially increase the risk of CD in several ways. They may influence the microbiota, thereby compromising the mucosal barrier function (Sanz, Palma, & Laparra, 2011). Infections may also lead to the release of γ-interferon, increasing

HLA expression (Koning, 2012). Importantly, some infections seem to influence TTG release, and lead to increased intestinal permeability which permits absorption of intact gliadin molecules and may initiate the immune process leading to CD (Koning, Schuppan, Cerf-Bensussan, & Sollid, 2005); (Sarmiento *et al*., 2012; Stene *et al*., 2006). These are illustrated in Figure 1.6.

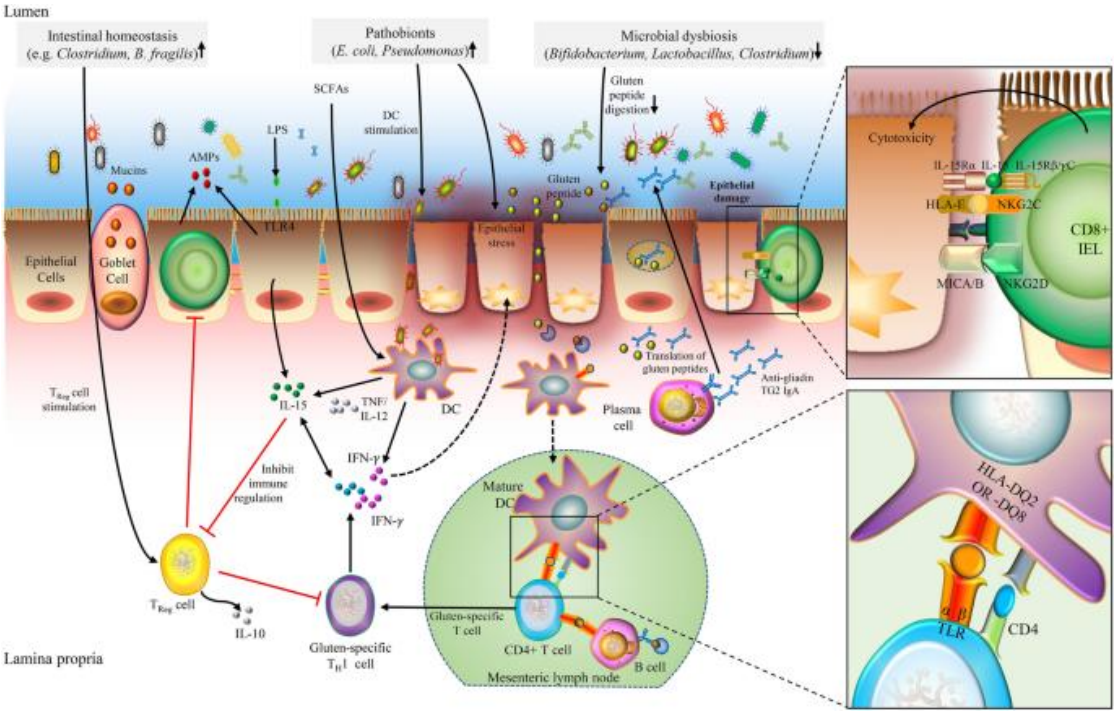


Figure 1.6: The immune response in CD pathogenesis is mediated by both B cell and T cell response.
The intestinal microbiota, both commensals microbes and pathobionts, might contribute to the development of celiac disease by influencing the gluten peptide digestion, stimulation of DC and TReg cells, epithelial cell stress, intestinal permeability modulation, and pro-inflammatory cytokines production.
Sourced from: Akobeng, A. K., Ramanan, A. V., Buchan, I., & Heller, R. F. (2006). Effect of breast feeding on risk of coeliac disease: a systematic review and meta-analysis of observational studies. Archives of disease in childhood, 91(1), 39-43. (A. K. Akobeng, Singh, Kumar, & Al Khodor, 2020).

1.2.4 Pathophysiology of CD

Celiac disease results from the interaction between immune, genetic, and environmental factors (Heap & van Heel, 2009). Celiac disease is induced by the ingestion of gluten, which is derived from wheat, barley, and rye. The gluten protein is enriched in glutamine and proline and is poorly digested in the human upper gastrointestinal tract. The term gluten refers to its alcohol-soluble fraction "gliadin" that contains the bulk of the toxic components.

Undigested molecules of gliadin, such as a peptide from an α-gliadin fraction made up of 33 amino acids, are resistant to degradation by gastric, pancreatic, and intestinal brush-border membrane proteases in the human intestine and thus remain in the intestinal lumen after gluten ingestion. These peptides pass through the epithelial barrier of the intestine, possibly during intestinal infections or when there is an increase in intestinal permeability, and interact with (APCs) in the lamina propria (Denham & Hill, 2013; Green & Cellier, 2007).

١.2.4.1 Mucosal immune responses

In patients with CD, immune responses to gliadin fractions promote an inflammatory reaction, primarily in the upper small intestine, characterized by infiltration of the lamina propria and the epithelium with chronic inflammatory cells and villous atrophy. This is illustrated in Figure 1.7. The response is mediated by both the innate and the adaptive immune systems. The adaptive response is mediated by tTG an enzyme in the intestine that deamidates gliadin peptides, thereby forming a negatively charged glutamic acid residue, the resulting peptide can bind in the binding groove of the DQ2 or DQ8 molecules on

APCs with a higher affinity increasing the immunogenicity. Gliadin reactive CD4+ T cells in the lamina propria then recognize deamidated gliadin peptides, and subsequently produce proinflammatory cytokines, particularly γ- interferon.

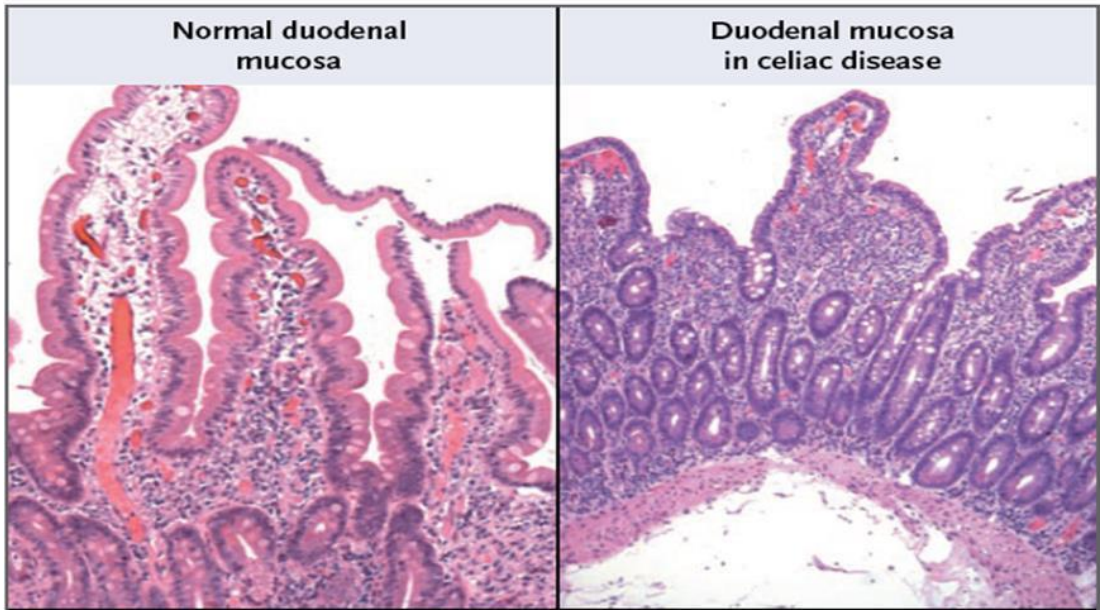


Figure 1.7: Histology of the small intestine (left) normal (right) celiac disease affected small intestine
The infiltration of the lamina propria and the epithelium with chronic inflammatory cells and villous atrophy can be observed.
Sourced from: Walker, M. M., & Murray, J. A. (2011). An update in the diagnosis of coeliac disease. Histopathology, 59(2), 166-179. (Walker & Murray, 2011).

The ensuing inflammatory cascade releases metalloproteinases and other tissue-damaging mediators that induce crypt hyperplasia and villous injury, as well as the activation and expansion of B cells that produce antibodies. Gliadin peptides also activate an innate immune response in the intestinal epithelium that is characterized by increased expression of interleukin-15 by enterocytes, resulting in the activation of intraepithelial lymphocytes. These activated cells become cytotoxic and

kill enterocytes that express (MHC) class I chain related A (MIC-A) (a stress protein) on their surface (Green & Cellier, 2007; Kupfer & Jabri, 2012b; Trynka, Wijmenga, & van Heel, 2010), as shown in Figure 1.8.

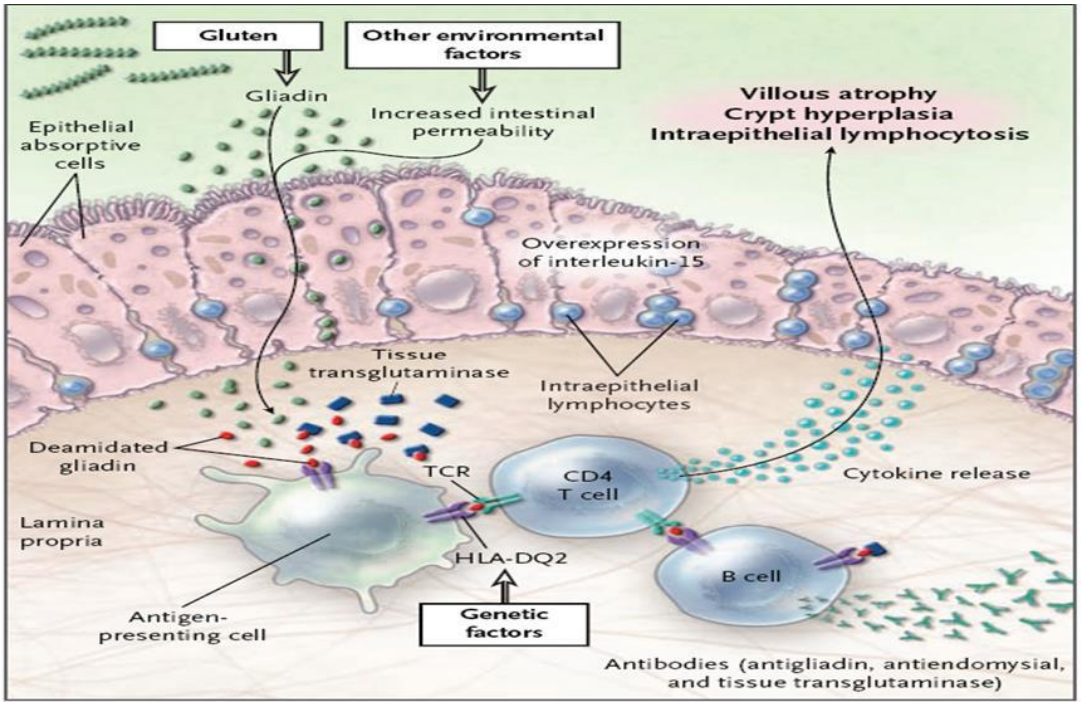


Figure 1.8: Pathophysiology of celiac disease
Gluten is aided other environmental factors across intestinal absorptive cells into the lamina propia where it gets converted into deamidated gliadin by tissue transglutaminase. Deamidated gliadin peptides interacts with HLA-DQ2 MHC class II on antigen presenting cells leading to antigenic presentation of activation of CD4+ Tcells which the provokes immune response that culminates to villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis.
Sourced from: Green, P. H., & Cellier, C. (2007). Celiac disease. New England Journal of Medicine, 357(17), 1731-1743. (Green & Cellier, 2007).

1.2.5 Clinical manifestations of CD

Typical symptoms of CD includes abdominal pain, diarrhea, constipation, weight loss, muscle weakness, malabsorption, bloating, failure to thrive, dyspepsia and gastritis (Reilly, Fasano, & Green, 2012). While atypical symptoms are anemia, dermatitis herpetiformis, osteoporosis, food allergy, neurological disorders, menstrual abnormality, asthenia, chronic fatigue, dental defects, hair loss, short stature, infertility, recurrent miscarriages, late puberty, early menopause, depression and low B12/folic acid (Tack, Verbeek, Schreurs, & Mulder, 2010)).

The associated conditions that are possibly gluten dependent include; autoimmune thyroiditis, rheumatoid, autoimmune hepatitis, type 1 diabetes mellitus, Sjogren‟s syndrome, Addison disease, autoimmune atrophic gastritis and psoriasis. While those that are gluten independent include; Down's syndrome, Turner syndrome, Williams syndrome, congenital heart defects and IgA deficiency (Lionetti & Catassi, 2011).

1.2.6 Diagnosis of CD

The variety of clinical manifestations which CD may present complicates its recognition. A correct diagnosis cannot rely on a single test, but requires a precise reconstruction of a puzzle, whose pieces are represented by the clinical, serological, genetic and histological aspects (Ludvigsson *et al.,* 2014). The diagnosis of CD according to NASPGHAN, ESPGHAN and British Society of Gastroenterology (BSG) international guidelines, is based on the results of two serological tests endomysial antibody (EMA) and/or tTG antibodies during GCD. GFD changes the clinical, serological and histological pattern, making it

impossible to recognize the characteristic aspects of disease. The diagnosis must be confirmed by histopathological examination of multiple intestinal biopsies obtained from the second or more distal part of duodenum because the histologic changes in CD may be patchy. In circumstances where the diagnosis is uncertain, typing for HLA-DQ2 and HLA-DQ8 is consider a useful tool to exclude CD or to make the diagnosis unlikely in the case of a negative test result for both markers. The diagnosis of CD is considered definitive when there was complete symptom resolution and the positive serological and histological tests revert to negative after treatment with a strict GFD (Hill *et al*., 2005; Husby *et al*., 2012; Ludvigsson *et al*., 2014).

1.2.6.1 Antibody markers for CD diagnosis

The different antibody markers utilized for CD diagnosis include;

Total IgA: Serological screening methods for CD are predominantly immunoglobulin A (IgA) class assays, as the vast majority of CD patients produce IgA auto-antibodies. Patients with IgA deficiency are however not resistant to the development of CD and can develop immunoglobulin G (IgG) celiac antibodies. Two to three percent of biopsy proven CD patients are IgA deficient, with positive IgG antibodies. Additionally children under the age of 2 who have not yet developed IgA may also develop IgG celiac antibodies (Hill *et al*., 2005).

IgA class transglutaminase antibodies (TTG): anti transglutaminase antibodies are the tests with the highest sensitivity for CD (98%) with specificity estimated at around 90%. Because of its simplicity, overall good diagnostic accuracy, ease of interpretation and availability of

results within minutes detection of tTG is the serologic test of choice for the diagnosis of CD (Rostom *et al*., 2006).

IgA class endomysial antibodies (EMA): antiendomysial antibodies while having a lower sensitivity (90%) compared to IgA class tTG, show an almost absolute specificity for CD. Despite the high specificity of this antibody, there are several test-related issues that may limit its use in clinical practice. It is semiquantitative, time consuming, operator-dependent, and expensive. However, IgA EMA testing could be clinically useful if the result of the IgA tTG test is equivocal (Asaad Assiri & Newland, 2012).

IgA class-anti-gliadin antibodies: anti gliadin antibodies are now an obsolete test with levels of sensitivity and specificity significantly lower than tTG and EMA, and the search for their presence is useful only in early childhood (children aged<2 years); because they are the first antibodies to appear, they show a higher sensitivity than other tests in this age group. Positivity for IgA AGA associated with negativity for EMA and tTG is almost denial an expression of CD in adults and in children aged >2 years (Leffler & Schuppan, 2010).

1.2.6.2 Intestinal biopsy for CD diagnosis

Intestinal biopsies taken from the first and second duodenal portion remain an essential means of confirming the diagnosis of CD showing the typical histologic abnormalities (villous atrophy, crypt hyperplasia, and leukocyte infiltration). Duodenal biopsy for the diagnosis of CD is most commonly performed after a patient is found to have a positive serologic test. But diagnostic biopsy may also be performed in the

seronegative individual with signs and symptoms highly suspicious for CD (Asaad Assiri & Newland, 2012).

1.2.6.3 Genetic testing for HLA-DQ2 and HLA-DQ8

Since CD is closely associated with histocompatibility antigens HLA-DQ2 and -DQ8, practically all patients with CD are positive for one or both of these HLAs or for a fraction of the DQ2 heterodimer. The diagnostic value of HLA genotyping in patients who may have CD revolves around its high negative predictive value, meaning that patients who lack the appropriate HLA genotypes described above are extreme rare to have CD and the proportion of false negatives is very small (Karell *et al*., 2003). However, the positive predictive value of the HLA genotyping for CD susceptibility is very low as a large proportion of individuals without CD carry either HLA-DQ2 or -DQ8; the prevalence of DQ2 in the general population varies between 0% and40% while that of DQ8 varies between 0% and 20% between countries (Abadie *et al*., 2011). A positive result only means a genetic predisposition for celiac autoimmunity. Likewise, the proportion of individuals with false-positive results is large (Sollid & Lie, 2005). The main clinical significance of genetic testing is to exclude a diagnosis of CD in the absence of HLA-DQ2 (and its fractions) and -DQ8 in cases of diagnostic doubt (Rubio–Tapia *et al*., 2009). Furthermore, to exclude the predisposition to CD in family members of celiac patients and other high-risk individuals in the absence of HLA-DQ2 (and its fractions) and -DQ8 (Villanacci, Ceppa, Tavani, Vindigni, & Volta, 2011).

Therefore, genetic test should be considered and performed under the following situations;

- Patients with discrepant celiac-specific serology and histology.

- When there has been failure to improve on a GFD (non-responsive CD).
- To evaluate patients on a GFD in whom no testing for CD was done before GFD and unable to undertake an oral gluten prior to investigation.
- In screening high-risk individuals such as first-degree relatives. CD has a strong genetic component, and is one of the hereditary complex disorders, with a prevalence of 5–15% in first-degree relatives of a CD patient and a strikingly high-concordance rate among monozygotic twins as compared with dizygotic twins. The strong genetic influence in CD is apparent, as the concordance between monozygotic twins is 80%, whereas in dizygotic twins, is 11% (Anderson et al., 2013; Jones & Warner, 2010; Nisticò et al., 2006; Vaquero et al., 2014).
- In individuals with autoimmune diseases, IgA deficiency, with Down, Turner or Williams syndromes to assess the genetic predisposition to CD.
- When the diagnosis was performed by serogenetic approach only (Testing for HLA-DQ alleles and confirmatory serology) which reduce the numbers of unnecessary gastroscopies. Most test for detection of celiac disease require consuming a gluten containing diet by the patients at the time of testing(Lewis, Haridy, & Newnham, 2017)

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Study population and design

A total of 90 patients clinically diagnosed as having Celiac disease at the Advisory Clinic for Digestive Center in Karbala through evaluation of clinical symptoms, serological examinations and small bowel biopsy, were recruited for a cross-sectional study between December 2020 and February 2021.

Ethical approval (Appendix M) was sort and obtained from the appropriate regulatory body and the written informed consent was obtained from the subjects or parents/ guardians (for minors) prior to recruitment into the study.

2.1.2 Sample collection

Information about biodemographical characteristics (such as age, name, gender, medical history, family history, endoscopic results and treatment type) of the subjects were obtained through questionnaire interviews (Appendix N) as well as from patients' medical records.

Venous blood samples were collected using venipuncture after disinfecting the antecubital fossa with 70% ethanol. Two millilitres of the blood sample were dispensed into EDTA containing tubes for whole blood analysis while 3 mL were dispensed into gel tube for serum preparation. Serum was prepared by centrifugation of the blood samples at 3000 rpm for 15 minutes and the supernatant was dispensed into Eppendorf tubes and stored at -20^{o}C until use.

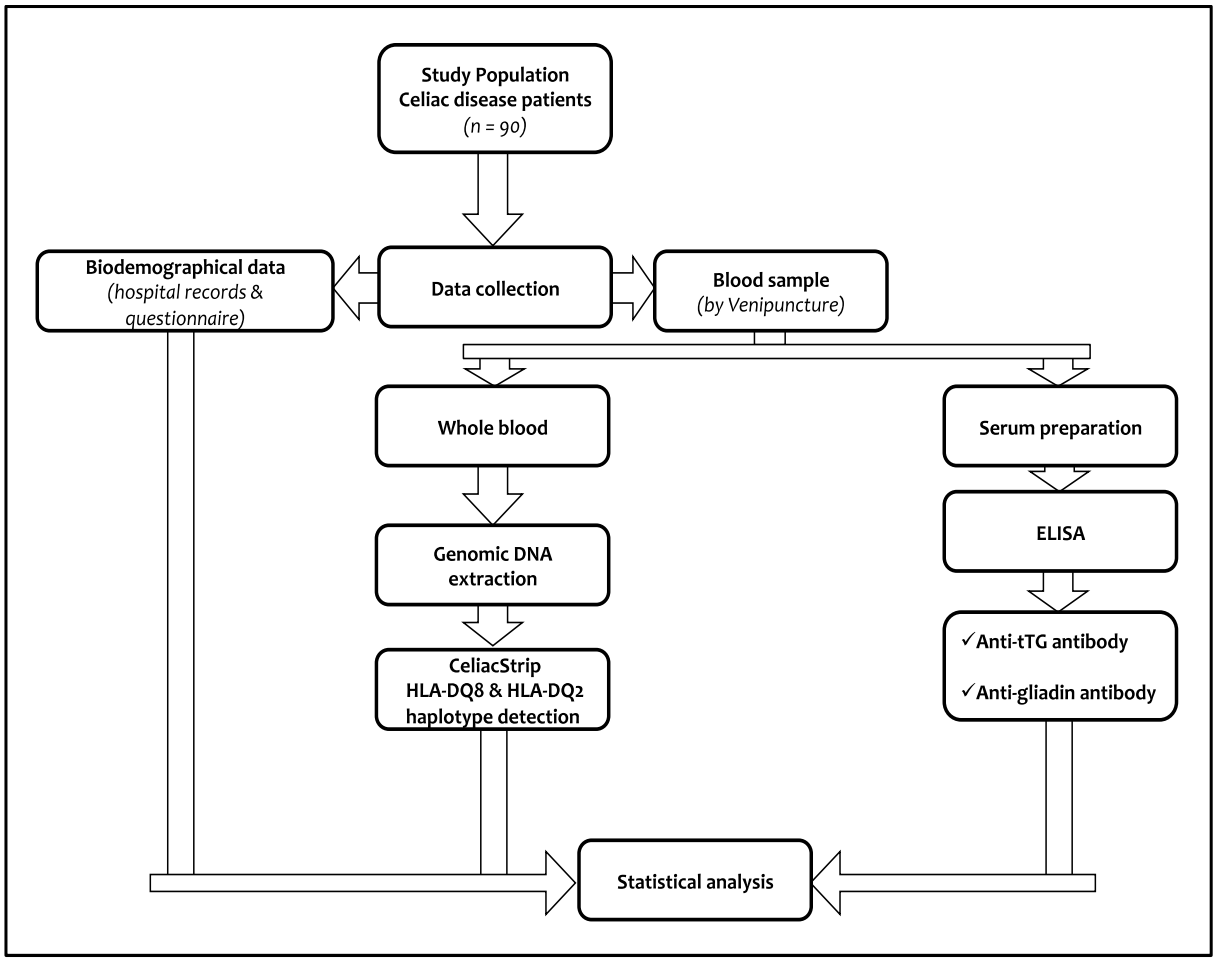


Figure 2.1: Schematic presentation showing details of the materials and methods employed in the study

Laboratory instruments in the study

The table show all of laboratory instruments used during this study .

Table 2.1 Instrument and manufacturer origin

Instrument	**Manufacturer origin**
Disposable syringe (5ml)	China
EDTA tube	China
Gel and eppendorff tubes	China
Micropipettes 10M,100 M, 1000M	Germany
Plastic rack	China
Centrifuge	Japan
Deep freezer	Japan
ELISA reader and printer	USA
Micro centrifuge	
Thermo shaker	
ELISA automated washer	USA
Vortex	USA
70% alcohol for sterilization	China
Pipettes	Germany

2.2.1 DNA extraction kit

Table 2.2 Kit of DNA extraction

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		

Component of celiac strip kit

Table 2.3 Kit of celiac strip

Component		Quantity
Strips	Strip s	16
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

Elisa Kit Content of Tissue trans glutamines Antibody IgA

Table 2.4 Elisa kit TtG antibody IgA

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	

Table 2.5 Elisa kit content of Anti gliadin antibody

Symbol	Components	Volume \Qty.
SORB Mt	Galidin 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

2.2 Methods

2.2.1 Gene expression analysis of HLA haplotypes (HLA-DQ8 and HLA-DQ2) associated with Celiac disease using CeliacStrip

The expression HLA genes in the samples were carried out using the CeliacStrip kit (Operon Immuno & Molecular diagnostics, Spain). The kit employs RT-PCR technology to enable 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 Celaic disease. The specific haplotypes detectable by the CeliacStrip kit used in this study are presented in Table 2.6.

Table 2.6: The main HLA haplotypes associated with Celiac disease detectable by the CeliacStrip test

S/N	HLA region	Specific Celiac disease associated haplotype
1	HLA-DQ8	DQA1*03 – DQA1*0302 – DRB1*04
2	HLA-DQ2 [cis]	DQA1*05 – DQB1*02 – DRB1*03
3	HLA-DQ2 [trans]	DQA1*05 – DQB1*0301 – DRB1*11/DRB1*12

The test was conducted in three stages. First, genomic DNA was extracted from nucleated cells in the whole blood sample, then the extract gDNA was then amplified using PCR techniques, and the final stage involved hybridization and development of PCR product to enable gene identification and evaluation. All procedures were conducted using strict adherence to manufacturer's guidelines.

2.2.1.1 Extraction of genomic DNA

The whole blood samples were prepared for genomic DNA extraction by placing them under aseptic condition in accordance to the protocol of ReliaPrep™ Blood gDNA Miniprep System (Promega, U.S.A.). The blood sample was allowed to acclimatize to the room temperature prior to the analysis. The tube was shaken thoroughly for 10 minutes to ensure even fluid distribution and 200 µL was dispensed into a 1.5 mL centrifuge tube containing 20 µL of a protein-ase K (PK) solution provided in the kit. The mixture was briefly mixed and 200 µL of Cell Lysis Buffer was added. The tube was then place on a vortex for at least 10 seconds and incubated at 56 °C for 10 minutes. The tube was removed after the incubation and its content was transferred into a collection tube, after which 250 µL Binding Buffer was dispensed into the collection tube and spun briefly at 14,000 rpm. Tweezers were then used to gently remove the ReliaPrep™ Binding Column to prevent contamination resulting from touch, and column placed into a tube rack after which the content of the collection tube was transferred into the column and centrifuged at 14000 rpm for 1 minute. After centrifugation, the column was checked to make sure that all the lysate has completely passed through the membrane before discarding the liquid. The column was then removed, placed into a fresh collection tube and washed thrice by addition of 500 µL of Column Wash Solution and subsequent centrifugation at 14,000 rpm for 1 minute. After 3 washes, 100 µL of Nuclease-Free Water was added to the column and it was placed into a clean 1.5 mL centrifuge spun 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.

2.2.1.2 Amplification of genomic DNA using PCR

The extracted genomic DNA was subjected to amplification to improve concentration desired regions by PCR using the procedure recommended in the CeliacStrip test manual. Five microlitres of the DNA sample was added to a PCR tube containing 39 µL of PCR premix, 5 µL of primers and 1 µL of Taq to make a total volume of 50 µL per PCR tube. The DNA samples were amplified in a thermocycler (Analytic Jena GMBH, Germany) using the programme presented in Table 2.7.

Table 2.7: 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	∞	

2.2.1.3 PCR product Hybridization and development using Theromoshaker PSH-60HL

First the thermoshaker PSH-60HL was set up by conducting a series of calibration analysis following strict adherence to the Opegen Thermoshaker Manual (Appendix O). Before commencing the hybridization, the reagents to be used such as the hybridization buffer, wash buffer 1 and wash buffer 2 were first preheated to 42 °C in a water bath while the denaturing solution, conjugate and substrate reagents were placed in racks to attain ambient temperature. All reagents were shaken gently to ensure that precipitates were completely dissolved.

Tweezers were used to carefully remove the hybridization strips from its aluminium pouch in order to prevent possible contamination and they were placed faced up in the hybridization tray with one strip in one channel. The hybridization buffer dispensed first after which PCR product were added gently into the tray both of which were 12.5 µL, making a total mixture volume of 25 µL per channel. The tray was incubated for 10 minutes inside the thermoshaker. Subsequently, the tray was removed and 2 mL of preheated hybridization buffer was quickly added to each channel and mixed slowly by moving the plate up and down. The tray was then returned into the thermoshaker and spun at 450 rpm for 30 minutes at 42 °C (this incubation condition was maintained throughout the hybridization process). After incubation the hybridization buffer was then removed using vacuum pump aspiration and subsequently, each channel was washed twice using 2 mL of preheated wash buffer over duration of 10 minutes each. The conjugate (2 mL) was then added and incubated for 30 minutes. Afterwards, the conjugate was removed and the channels were washed thrice as

previously described. After washing, 1 mL substrate was added to the channels and the tray was incubated for 10 minutes. After which the plates were then removed and washed with 2 mL distilled water thrice after which the strips were removed and placed to dry in the dark.

2.2.1.4 Reading and interpretation of strip

After hybridization and development of PCR product, the strips were read an interpreted using the manual technique. The strips were placed on a paper, covered with cellophane and the black and red lines on the strip were aligned with the evaluation chart included in the kit (Appendix P). The bands were identified in accordance with their position on the strips(Appendix R ,S,T,W).

2.2.2 Serological antibody analysis using ELISA

Serological analysis was carried out on the serum samples to quantitatively measure the presence of circulating autoantibodies to tissue trans glutaminase (tTG) antigen as well as IgA/IgG antibodies to deamidated gliadin by the indirect enzyme linked immunosorbent assay technique using antibody specific ELISA kits. The general assay principle entails the interaction between antigens coated on the surface of the microwells and specific antibodies in the patients' serum sample. The incubation period allows strong antibody-antigen interactions as washing steps after incubation removes unbound protein component. Subsequent addition enzyme conjugate binds to the immobilized antibody -antigen- complexes, while a second washing after incubation removes unbound enzyme conjugate. Addition of substrate solution to the enzyme-bound conjugate hydrolyses the substrate forming a coloured product. The stop solution which is an acid stops the reaction

generating a coloured end product which can be measured spectrophometrically; the intensity of which correlates with the concentration of the antibody-antigen complex. The positive and negative controls serve as an internal quality control to validate the results obtained.

2.2.2.1 Determination of Anti-tTG antibody

The Anti-tTG Ab ELISA Kit (Euroimmun, Germany) was used to measure the presence of circulating transglutaminase autoantibody as per manufacturer's procedures. The micro wells were labelled control, calibrator and sample before dispensing 100 µL of the appropriate fluid into each well. The 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. Subsequently, 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 to the immobilized antibody -antigen-complexes and the plate was covered and incubated for 1 hour at room temperature. Afterwards, 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 which generated a blue coloured product. Afterwards, 50 µL of the stop solution was added into each well at a rapid, steady pace without interruption, during which the formation of a bright yellow product was observed. The absorbance of the plates where then read at 450 nm from (Human, Germany). 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.

2.2.2.2 Determination of Deamidated gliadin antibody

The quantitative measurement of IgA/IgG antibodies to deamidated gliadin antigen in the serum samples, by the indirect enzyme linked immune reaction was carried out using the DGP IgA/IgG ELISA Kit (Euroimmun, Germany) was used as per manufacturer's procedures. The micro wells were labelled control, calibrator and sample before dispensing 100 µL of the appropriate fluid into each well. The 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. Subsequently, 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 anti- human lgA peroxidase conjugate was added and the plate was covered and incubated for 1 hour at room temperature. Subsequently, the micro wells were washed three times and 100 µL of tetramethylbenzidine (TMB) 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 which generated a blue dye product. Afterwards, 50 µL of the stop solution was added into each well at a rapid, steady pace without interruption, during which the formation of a bright yellow product was observed.

The absorbance of the plates where then read at 450 nm from (Human, Germany). 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 gliadin lgA value of each serum sample was determined using its absorbance value and extrapolating from the DRC on the X-axis.

2.3 Statistical analysis

Statistical analysis was performed using SPSS (version 24.0). the values were logarithmically transformed to obtain a normal distribution . the normality test was also utilized for monitoring the variables' distribution and the missing variables and the outliers were excluded from the data.

All numerical variables were presented in form of mean +/- standard deviation .Evaluation of the relationship among variables and analysis of variance were determined using t-test crosstabs and general linear model (ANOVA).The p-value ≤ 0.05 were considered as significant . Pearson's correlation matrix was used to observe association between the HLA haplotypes and serum autoantibodies in relation to the biodemographical data. Statistical analysis was carried out using SPSS (version 24.0).

The techniques and procedures used in this study are schematically summarised in Figure 2.1.

Chapter Three

Results

3. Results

3.1 Patients' biodemographical characteristics

This study recruited a total of 90 celiac disease patients out of which 36 i.e. 40% were males and 54 i.e. 60% were females. The age of the patients ranged from 5 to 47 years with the mean age for all the patients at 22.37±10.05 years. The males had a mean age of 20.14±7.10 years and the females 21.62±6.50 years. Measurement of body mass index (BMI) showed that the mean BMI for all the patients was 26.29±0.66 with was not different from mean BMI obtained when grouped based on gender (i.e. males 26.23±0.69 kg/m^2 and females 26.33±0.65 kg/m^2). The demographic and anthropometric features of the patients is presented in Table 3.1.(Appendix A,B,C)

Table 3.1 Demographical and anthropometric features of the patients

			95% confidence interval
		mean±SD	*Lower - Upper*
Number of participants *(n (%))*	All	90 (100)	
	Male	36 (40)	
	Female	54 (60)	
Age *(years)*	All	22.37±10.05	20.30 - 24.45
	Male	20.14±7.10	
	Female	21.62±6.50	
BMI *(Kg/m^2)*	All	26.29±0.66	26.15 - 26.43
	Male	26.23±0.69	
	Female	26.33±0.65	

Analysis of biochemical parameters in the serum samples obtained from the patients indicated that the mean serum glucose level for all the patients was 94.65±3.89 mmol/dL while for males and females, the mean glucose levels were 94.88±3.97 mmol/dL and 94.50±3.86 mmol/dL respectively. Mean glycated hemoglobin (HbA1c) was 5.47±0.66 % which did not differ significantly when grouped based on gender (males 5.57±0.66 % and females 5.41±0.65 %) while packed cell volume (PCV) showed a mean value of 31.68±6.03 with males having mean PCV of 31.88±6.31 and females 31.55±5.89. Mean hemoglobin values for all the patients was 10.56±2.42 % with males having 10.92±2.52 % and females 10.32±2.34 %. The mean white blood cell (WBC) count for all the patients was 6.84±0.91 $x10^9$/L which was slightly higher for males i.e. 7.00±0.81 $x10^9$/L compared to females i.e. 6.74±0.97 $x10^9$/L. Similarly, platelet count which showed a mean cell count of 206.63±34.27 $x10^9$/L for all the patients was slightly higher in males i.e. 208.61±36.02 $x10^9$/L than females i.e. 205.31±33.3 $x10^9$/L. These data are presented in Table 3.2(Appendix C,D)

Table 3.2 Biochemical features of the patients

			95% confidence interval
		mean±SD	*Lower - Upper*
Glucose *(mmol/dL)*	All	94.65±3.89	93.83 - 95.47
	Male	94.88±3.97	
	Female	94.50±3.86	
HbA1c *(%)*	All	5.47±0.66	5.33 - 5.61
	Male	5.57±0.66	
	Female	5.41±0.65	
PCV	All	31.68±6.03	30.42 - 32.94
	Male	31.88±6.31	
	Female	31.55±5.89	
Haemoglobin *(%)*	All	10.56±2.42	10.05 - 11.07
	Male	10.92±2.52	
	Female	10.32±2.34	
WBC *(x 10^9/L)*	All	6.84±0.91	6.65 - 7.03
	Male	7.00±0.81	
	Female	6.74±0.97	
Platelets *(x 10^9/L)*	All	206.63±34.27	199.45 - 213.81
	Male	208.61±36.02	
	Female	205.31±33.3	

Other biochemical parameters analyzed were serum ferritin, calcium, vitamin D3, urea and creatinine. The results obtained indicated a mean ferritin of 5.68±2.01 µg/L for all the patients while males had 5.80±2.12 µg/L and the females 5.60±1.94 µg/L. Calcium showed mean values of 7.39±1.32 mg/dL for all the patients while males and females had mean values of 7.24±1.33 mg/dL and 7.48±1.32 mg/dL respectively. Vitamin D3 was 11.29±3.83 ng/mL for all patients as males recorded 11.17±4.04

ng/mL and females had 11.37±3.73 ng/mL. The mean values obtained for urea and creatinine for all the patients were 26.88±11.37 mg/dL and 0.76±0.43 mg/dL respectively while gender grouping yielded 27.61±11.58 mg/dL (urea, males), 26.40±11.32 mg/dL (urea, females), 0.76±0.43 mg/dL (creatinine, males) and 0.75±0.44 mg/dL (creatinine, females). These data are presented in Table 3.3. (Appendix C, D)

Table 3.3 Other biochemical parameters of the patients

			95% confidence interval
		mean±SD	*Lower – Upper*
Ferritin *(µg/L)*	All	5.68±2.01	5.26 - 6.10
	Male	5.80±2.12	
	Female	5.60±1.94	
Calcium *(mg/dL)*	All	7.39±1.32	7.11 - 7.66
	Male	7.24±1.33	
	Female	7.48±1.32	
Vitamin D3 *(ng/mL)*	All	11.29±3.83	10.49 - 12.10
	Male	11.17±4.04	
	Female	11.37±3.73	
Urea *(mg/dL)*	All	26.88±11.37	24.50 - 29.27
	Male	27.61±11.58	
	Female	26.40±11.32	
Creatinine *(mg/dL)*	All	0.76±0.43	0.66 - 0.85
	Male	0.76±0.43	
	Female	0.75±0.44	

3.2 Celiac disease diagnostic parameters of the patients

Since all the patients have already been clinically diagnosed celiac disease, the different indices used for diagnosis of celiac disease

specifically, genetic testing for human leukocyte antigens HLA-DQ2 and DQ8, histological analysis of duodenal biopsy and serological measurement of celiac disease autoantibodies i.e. IgA and IgG classes of anti-tissue transglutaminase (anti-tTG) and anti-gliadin antibodies were analyzed. This is to enable reliable and statistical comparison of the different diagnostic parameters in line the objectives of the study.

3.2.1 Distribution of celiac disease-associated HLA haplotypes (HLA-DQ2 and HLA-DQ8) among the patients

The genetic profile of the patients with respect to the celiac disease associated HLA-DQ2 or HLA-DQ8 homozygosity as well as HLA-DQ2/DQ8 heterozygosity was analyzed.

From the results obtained, 70 patients representing 77.8% of all patients were HLA-DQ2 homozygotes, out of which 30 male patients representing 83.3% of the male population and 40 female patients representing 74.1% of the female population were recorded. The HLA-DQ8 homozygous patients were 13, representing 14.4% of all the patients out of which 4 male patients representing 11.1% of males and 9 females representing 16.7% of the females were recorded.

Seven patients representing 7.8% of all patients were HLA-DQ2/DQ8 heterozygous of which 2 male patients (5.6% of male population) and 5 females (9.3% of female population) were recorded. The graphical presentation of the distribution of HLA haplotypes of the patients is presented in Figure 3.1(Appendix K)

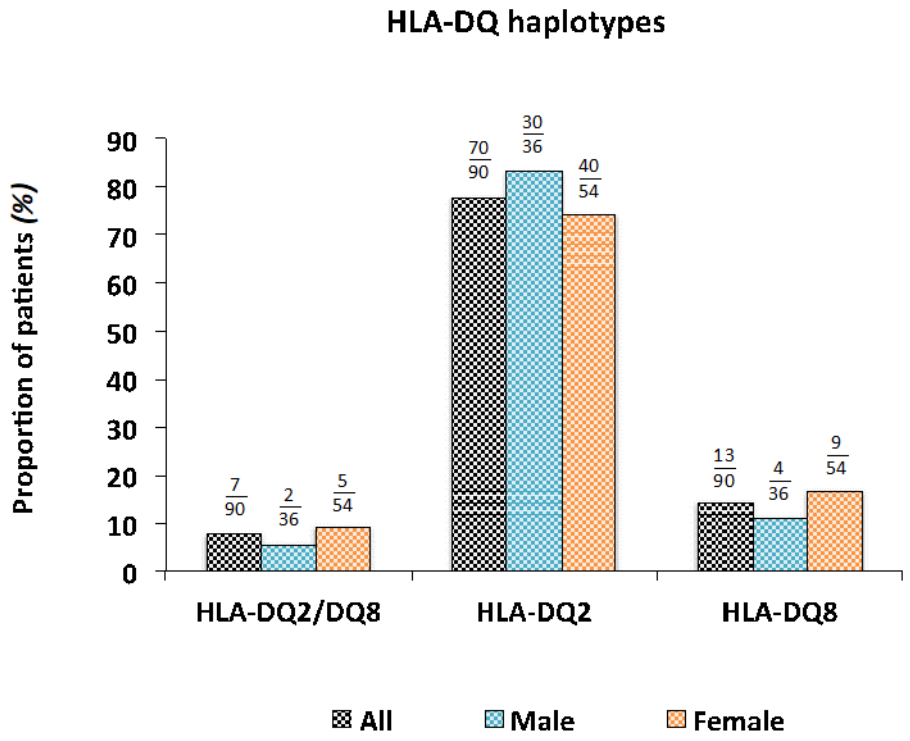


Figure 3.1 Distribution of celiac disease HLA-DQ haplotypes among the patients

The graph shows the percentage of patients with HLA-DQ2, HLA-DQ8 and HLA-DQ2/DQ8 genes among all the patients as well as across gender-based grouping. For each group, the fraction of the patients with each haplotype in the sub-group population is written on the respective bar. Majority of the patients were HLA-DQ2 homozygotes as 70 patients out of the total 90 had HLA-DQ2 genes only; of which 30 were males and 40 were females.

3.2.2 Histological findings of the patients' duodenal biopsy using Marsh classification

The findings of histological observation of duodenal biopsy of the patients were graded using the Marsh classification. The most severe grading of duodenal lesions, Marsh III was observed in 49 patients, which represented 54.4% of all the patients. The frequency of Marsh III was higher in females (i.e. 32 patients representing 59.3% of the female population) than in the males (i.e. 17 patients representing 47.2% of the male population). For Marsh II, 25 patients representing 27.8% of all the

patients recorded out of which the males were 11 (representing 30.6% of male population and the females were 14 (representing 25.9% of the female population). The number of patients graded Marsh I were 16, which represented 17.8% of all the patients of which 8 male patients (22.2% of male population) and 8 females (14.8% of female population) were recorded. The graphical presentation of Marsh classification of the patients is presented in Figure 3.2. (Appendix H)

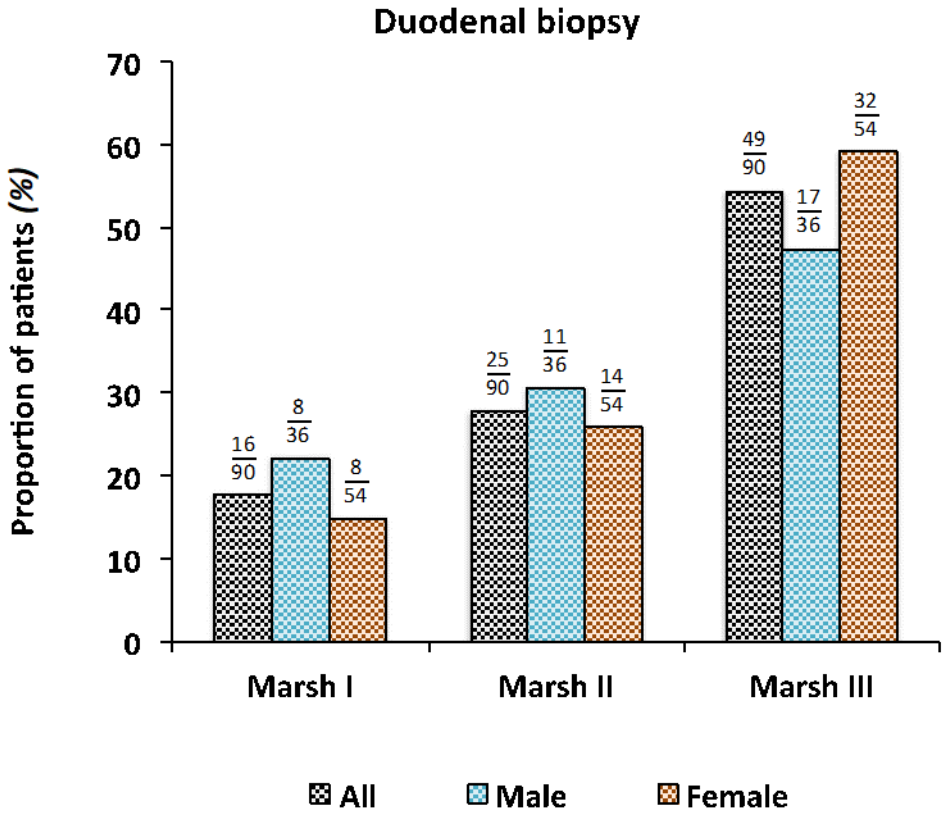


Figure 3.2 Marsh classification of duodenal biopsy of the patients
The graph shows the percentage of patients with Marsh I, Marsh II and Marsh III findings of histological duodenal biopsy among all the patients as well as across gender-based grouping. For each group, the fraction of the patients with each Marsh classification in the sub-group population is written on the respective bar. Majority of the patients were graded Marsh III i.e. 49 patients out of the total 90; of which 17 were males and 32 were females.

3.2.3 Seropositivity of the patients to celiac disease associated autoantibodies

Analysis of serum autoantibodies to transglutaminase and gliadin, which are antibodies associated with celiac disease showed the number of patients that were positive for these antibodies. Seropositivity to IgA and IgG classes of anti-tTG was observed in 37 patients (representing 41.1% of all patients) and 18 patients (representing 20% of all patients) respectively.

Based on gender, 14 male patients (representing 38.9% of the male population) and 7 male patients (representing 19.4% of the male population) tested positive for anti-tTG IgA and anti-tTG IgG antibodies respectively, while 23 female patients (representing 42.6% of the female population) and 11 female patients (representing 20.4% of the female population) tested positive for anti-tTG IgA and anti-tTG IgG antibodies respectively.

Likewise, seropositivity to IgA and IgG classes of anti-gliadin was observed in 35 patients (representing 38.9% of all patients) and 57 patients (representing 63.3% of all patients) respectively.

Based on gender, 16 male patients (representing 44.4% of the male population) and 21 male patients (representing 58.3% of the male population) tested positive for anti-gliadin IgA and anti-gliadin IgG antibodies respectively, while 19 female patients (representing 35.2% of the female population) and 36 female patients (representing 66.7% of the female population) tested positive for anti-gliadin IgA and anti-gliadin IgG antibodies respectively.

The frequency distribution of the patients that tested positive to IgA and IgG classes of anti-tTG and anti-gliadin is presented in Figure 3.3(Appendix F)

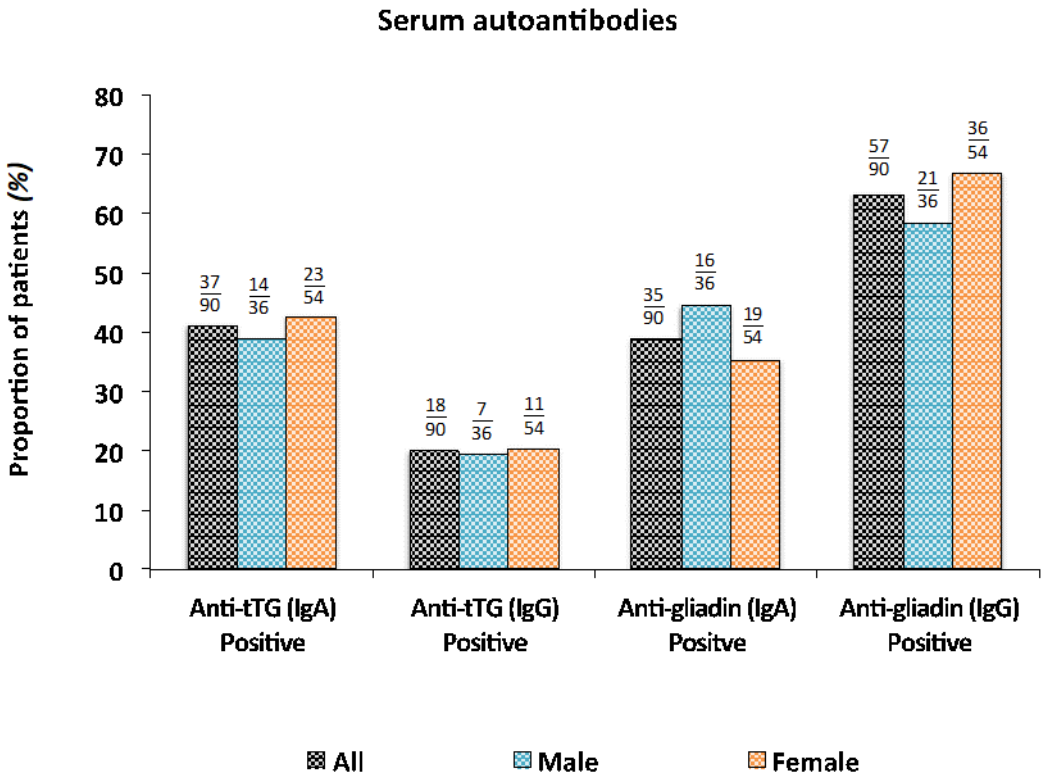


Figure 3.3: Distribution of the patients with positivity to celiac disease associated serum antibodies.

The graph shows the percentage of patients with seropositivity to IgA and IgG classes of anti-tTG and anti-gliadin antibodies among all the patients as well as across gender-based grouping. For each group, the fraction of the patients with positivity for each autoantibody in the sub-group population is written on the respective bar. Majority of the patients showed positivity to anti-gliadin IgG antibody i.e. 57 patients out of the total 90; of which 21 were males and 36 were females.

3.2.3.1 Effect of patient age on titer values of celiac disease associated serum autoantibodies.

The results obtained from measurement of the serum anti-tTG and anti-gliadin antibody titer of the patients revealed age-dependent difference in antibody concentration, which prompted their categorization into different age groups. Table 3.٤(Appendix E ,G) shows the concentration of serum antibodies across different age groups of the patients.

For anti-tTG, the findings indicated that the highest mean IgA antibody concentration was observed in patients within the age group of 33 – 39 years (390.04±67.21 U/mL), followed by 26 -32 years (352.62±50.12 U/mL) and then 19-25 years (167.60±47.33 U/mL) while the highest mean IgG antibody concentration was observed in patients within the age group of 19-25 years (138.92±27.25 U/mL), followed by 12-18 years (32.83±6.44 U/mL) and then 26 – 32 years (29.40±6.41 U/mL). The 17 patients aged between 5 – 11 years and 5 patients aged between 40-47 years did not show positivity to anti-tTG IgG antibody.

For anti-gliadin, the findings indicated that the highest mean IgA antibody concentration was observed in patients within the age group of 19 – 25 years (106.61±21.70 U/mL), followed by 26 -32 years (38.82±6.20 U/mL) and then 12-18 years (38.62±6.00 U/mL) while the highest mean IgG antibody concentration was observed in patients within the age group of 26-32 years (257.40±49.73 U/mL), followed by 40 -47 years (220.04±24.35 U/mL) and then 33 – 39 years (214.55±32.40 U/mL).

Table 3.٤: The serum concentration of anti-tTG and anti-gliadin antibodies across different age groups of the patients

Age ***(years)***	**n** ***(%)***	**Anti-tTG** ***(U/mL)***		**Anti-gliadin** ***(U/mL)***	
		IgA^+ (*mean±SD)*	IgG^+ (*mean±SD)*	IgA^+ (*mean±SD)*	IgG^+ (*mean±SD)*
5-11	17 (18.9)	74.52±5.50	-	21.80±4.04	93.04±6.84
12-18	12 (13.3)	66.81±10.43	32.83±6.44	38.62±6.00	185.53±5.70
19-25	21 (23.3)	167.60±47.33	138.92±27.25	106.61±21.70	116.25±5.72
26-32	30 (33.3)	352.62±50.12	29.40±6.41	38.82±6.20	257.40±49.73
33-39	5 (5.6)	390.04±67.21	21.93±4.22	10.51±0.44	214.55±32.40
40-47	5 (5.6)	37.33±6.01	-	15.63±1.21	220.04±24.35

3.3 HLA-DQ haplotypes predicts histological and serological diagnosis of celiac disease.

Since all the patients in this study as highlighted in section 3.2.1, were either HLA-DQ2 homozygotes, HLA-DQ8 homozygotes or HLA-DQ2/DQ8 heterozygotes, the effectiveness of HLA-DQ haplotype genotyping in predicting the outcome of diagnosis of celiac disease through histological observation of duodenal biopsy and serological measurements of celiac disease autoantibodies was assessed. The HLA-DQ haplotype of the patients categorized based on Marsh grading of histological findings as well as that of those with seropositivity of IgA and IgG classes of anti-tTG and anti-gliadin, was evaluated.

3.3.1 HLA-DQ haplotypes prediction of duodenal biopsy histological findings

Comparing the Marsh grading of the patients with their respective HLA-DQ haplotype revealed that homozygosity to HLA-DQ2 has the highest predictability of celiac disease diagnosis by histological observation of duodenal biopsy. The distribution of the patients based on Marsh grading of duodenal biopsy across the HLA-DQ haplotypes is presented in Figure 3.4(Appendix J).

From the findings, all the patients with Marsh II grading (100%), were HLA-DQ2 homozygotes.

For Marsh III, 75.5% of the patients (i.e. 37 out of 49 patients) were HLA-DQ2 homozygotes, while 18.4% (i.e. 9 out of 49 patients) were HLA-DQ8 homozygotes with the remaining 6.1% (i.e. 3 out of 49 patients) having HLA-DQ2/DQ8 heterozygosity.

Similarly, 50% of patients with Marsh I grading (i.e. 8 out of 16 patients) were HLA-DQ2 homozygotes, while 25% (i.e. 4 out of 16 patients) were HLA-DQ8 homozygotes with the remaining 25% (i.e. 4 out of 16 patients) having HLA-DQ2/DQ8 heterozygosity.

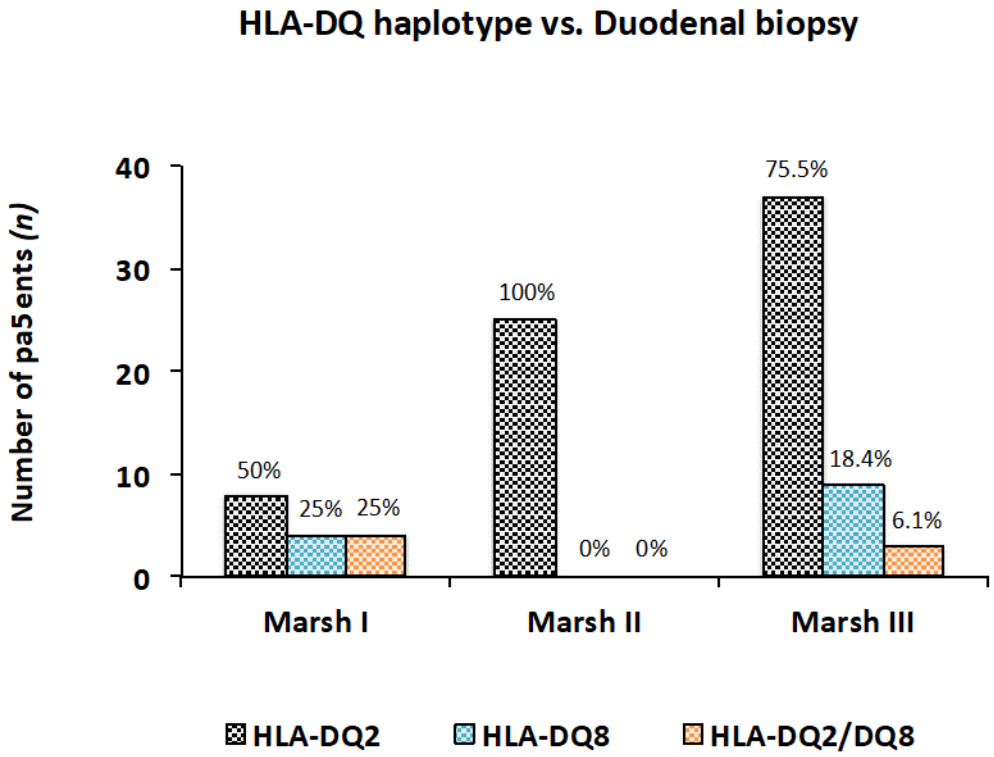


Figure 3.4 The HLA-DQ haplotype of patients with the different Marsh grading of duodenal biopsy.
The graph shows the number of patients with HLA-DQ2, HLA-DQ8 and HLA-DQ2/DQ8 genotype across the Marsh-based grouping. For each group, the percentage of the patients with a particular genotype among the respective Marsh classification is written on the respective bar. All the patients with Marsh II grading as well as the majority of the patients with Marsh III and Marsh I were HLA-DQ2 homozygotes (i.e. 100% for Marsh II, 75.5% for Marsh III and 50% for Marsh I).

3.3.2 HLA-DQ haplotypes prediction of seropositivity to celiac disease associated autoantibodies

The patients' seropositivity to IgA and IgG classes of anti-tTG and anti-gliadin autoantibodies was compared with their respective HLA-DQ haplotype. The findings also revealed that homozygosity to HLA-DQ2 has the highest predictability of celiac disease diagnosis by seropositivity to celiac disease associated autoantibodies as majority of patients were HLA-DQ2 homozygotes. The distribution of the patients

based on seropositivity to anti-tTG and anti-gliadin across the HLA-DQ haplotypes is presented in Figure 3.5(Appendix L). For anti-tTG IgA, 67.6% of the patients (i.e. 25 out of 37 patients) were HLA-DQ2 homozygotes, while 18.9% (i.e. 7 out of 37 patients) were HLA-DQ8 homozygotes with the remaining 13.5% (i.e. 5 out of 37 patients) having HLA-DQ2/DQ8 heterozygosity. Likewise, 77.8% of anti-tTG IgG positive patients (i.e. 14 out of 18 patients) were HLA-DQ2 homozygotes, while 11.1% (i.e. 2 out of 18 patients) were HLA-DQ8 homozygotes with the remaining 11.1% (i.e. 2 out of 18 patients) having HLA-DQ2/DQ8 heterozygosity.

For anti-gliadin IgA, 82.9% of the patients (i.e. 29 out of 35 patients) were HLA-DQ2 homozygotes, while 11.4% (i.e. 4 out of 35 patients) were HLA-DQ8 homozygotes with the remaining 5.7% (i.e. 2 out of 35 patients) having HLA-DQ2/DQ8 heterozygosity. Likewise, 86% of anti-gliadin IgG positive patients (i.e. 49 out of 57 patients) were HLA-DQ2 homozygotes, while 7% (i.e. 4 out of 57 patients) were HLA-DQ8 homozygotes with the remaining 7% (i.e. 4 out of 57 patients) having HLA-DQ2/DQ8 heterozygosity.

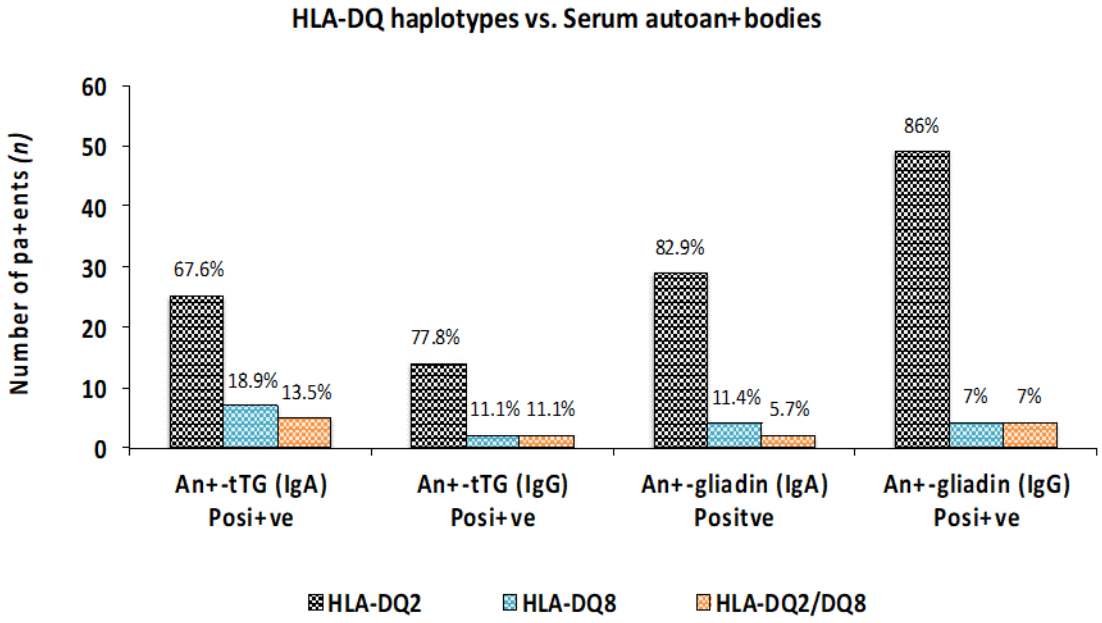


Figure 3.5 The HLA-DQ haplotype of patients with seropositivity to anti-tTG and anti-gliadin autoantibodies

The graph shows the number of patients with HLA-DQ2, HLA-DQ8 and HLA-DQ2/DQ8 genotype across anti-tTG and anti-gliadin seropositivity-based grouping. For each group, the percentage of the patients with a particular genotype among the respective autoantibody is written on the respective bar. The majority of the patients seropositive to all the autoantibodies were HLA-DQ2 homozygotes (i.e. 67.6% for anti-tTG IgA, 77.8% for anti-tTG IgG, 82.9% for anti-gliadin IgA and 86% for anti-gliadin IgG).

Chapter Four

Discussion

4. Discussion

Central to understanding the pathophysiology of CD is the close examination of the biological features of CD patients. In this study, the first goal was to identify patients that have been clinically diagnosed as having CD, evaluate their general biological characteristics and observe the aberrations that are apparent.

4.1 Demographic, anthropometric and biochemical characteristics of the CD patients

The first observation in this study was the gender distribution of the patients. Although random sampling technique was used during patients' recruitment, the study population was found to be comprised of 60% females; of which the possible explanation for this occurrence is the reported female predominance in CD.

In a study conducted by Dixit et al involving 1,682 patients diagnosed with CD, 68% were found to be females (Dixit *et al*., 2014). Other studies have also reported similar predominance with for example, a female population of 410 females in 581 patients (Ciacci *et al*., 2003) and 157 females in 249 patients (Ludvigsson, Rubio-Tapia, et al., 2013). However, delay in presentation of clinical symptoms, short span of clinical manifestation as well as less frequency of diagnosis in males especially in early adulthood has been identified as the possible reasons for the observed female predominance (Dixit *et al*., 2014). Also, despite the patients in this study were of ages spanning from 5 years to 47 years- an indication that age-related bias was avoided during the patient recruitment, the mean and mode age for all the patients as well as for each gender were between 20 to 30 years while the mean BMI was 26.29, thus demographically, the study population comprised

of mostly young adults who are slighly overweight. Interestingly, this fits with demography having the highest prevalence of CD as reported by previous studies (Ludvigsson & Murray, 2019; Yuan et al., 2017). Analysis of biochemical parameters revealed some abnormalities that are attributable to the CD condition. While serum levels of glycemic indices (i.e.glucose and glycated hemoglobin [HbA1c]) were within the normal range, the values obtained were thresholds values. Also considering the relatively young age of the patients, the glycemic status of the suggest the onset of prediabetes.

Diabetes is the most common comorbidity of CD (Jalilian & Jalali, 2021) and as highlighted by previous studies, metabolic challenges associated with CD result to the development of type 2 diabetes mellitus (Mostowy *et al.*, 2016), while similarities in etiological and genetic factors confers susceptibility to type 1 diabetes in CD patients (Camarca *et al*., 2012; Oujamaa *et al*., 2019). Moreover, several studies have reported poor glycemic control marked by high HbA1c levels in patients having the concomitant existence of diabetes and CD (Amin *et al.*, 2002).

The serum hemoglobin and PCV recorded in this study were below normal range. Studies have reported low hemoglobin levels and anemia in CD patients and have associated same with reports of persistent tiredness (Jelsness-Jørgensen, Bernklev, & Lundin, 2018; Jordá & Vivancos, 2010), nutritional deficiencies and CD associated inflammation (Berry et al., 2018; Harper, Holleran, Ramakrishnan, Bhagat, & Green, 2007). Other aberrations observed in the biochemical indices analysed were low serum ferritin and vitamin D3 levels as well as high serum urea levels. Iron deficiency has been recognized as one the hallmarks of CD with nutritional malabsorption resulting from CD-associated Villous atrophy identified and the culprit (Thompson, Lewis,

& Booth, 1966; Vici, Camilletti, & Polzonetti, 2020). Thus similar to the findings of this study, previous studies have reported low ferritin levels in adult and pediatric CD patients (Bogaert *et al.*, 2020). Also due to chronic inflammation-mediated intestinal malabsorption of fat soluble vitamins like vitamin D3, CD patients especially children reduced bone mineralization (Björck, Brundin, Karlsson, & Agardh, 2017), while adults with CD have displayed symptoms of osteoporosis (Legroux-Gérot *et al.*, 2009). Generally, high levels of urea in blood signals possibility of renal failure. Studies have reported similar high levels of urea in patients with CD with cases of CD-associated membranous nephropathy in adults (Pestana, Vida, Vieira, Durães, & Silva, 2021)as well as mebranoproliferative glomerulonephritis (Boonpheng, Cheungpasitporn, & Wijarnpreecha, 2017).

4.2 Evaluation of CD diagnostic parameters in the patients

This study also evaluated the distribution of CD associated HLA haplotypes among the patients. The HLA-D2 and HLA-DQ8 are alleles of the major histocompatibility complex (MHC) II that confers genetic susceptibility to CD (Cecilio & Bonatto, 2015). Antigen presenting cells (APCs) possessing MHC class II of the HLA-DQ2 or HLA-DQ8 genotype have the ability to recognize the macromolecular complex formed when gliadin interacts with tissue transglutaminase, thus bringing about antigenic stimulation of immune response. This forms the basis of the pathophysiology of CD (Falcigno *et al.*, 2020). Studies have shown that HLA-DQ2 homozygosity is the genotype with the highest risk as it confers between 25 - 30% risk of developing early onset CD in children, which is 5 times higher than the lower risk

genotype of HLA-DQ8 homozygosity ((Liu et al., 2014; Lopes, Muniz, Oliveira, & Sdepanian, 2019).

The HLA-DQ2/DQ8 heterozygotes have been shown to have the lowest risk of developing CD as despite having a prevalence of 25 - 35% in the general population, the eventuality of developing CD in individuals with this genotype is only 3% (Mazzilli *et al*., 1992). Therefore, the HLA-DQ2 homozygosity forms the most predominant genotype in CD patients (Lopes *et al*., 2019; Mansouri *et al*., 2021). This in line with the findings of this study as the majority of the patients (77.8%) were HLA-DQ2 homozygotes while 14.4% were HLA-DQ8 homozygotes with only 7.8% having HLA-DQ2/DQ8 heterozygosity. A previous study conducted on 74 CD patients reported prevalence of 79.7% for HLA-DQ2 and 8.1% for HLA-DQ8 homozygosity while HLA-DQ2/DQ8 had the prevalence of 10.8% (Cecilio & Bonatto, 2015). Although the intricate molecular mechanism behind the strong association between HLA-DQ2 homozygosity and development of CD is yet to be elucidated, it has been suggested that the HLA-DQ2 confers strong antigenic recognition epitopes for gliadin antigens and a higher ability to bind to high range of gluten peptides, on the MHC class II of APCs in individuals with this genotype and hence a higher risk of developing gluten associated enteropathy (Falcigno *et al*., 2020).

The histological observation and evaluation of the morphology of duodenal biopsy has been of critical importance in the diagnosis of CD and as such histology is still considered the ‘gold standard’ for CD diagnosis (Caio *et al*., 2019). This study utilized the modified Marsh classification to grade the histological findings obtained. According to the classification, Marsh III is characterized by > 40 jejunum intraepithelial lymphocytes [IEL] per 100 enterocytes, > 30 duodenum

IEL per 100 enterocytes and increased crypt hyperplasia with mild to complete villous atrophy, while Marsh II is characterised by > 40 jejunum per 100 enterocytes, > 30 duodenum IEL per 100 enterocytes and increased crypt hyperplasia and no villous atrophy. However, when the jejunal IEL per 100 enterocytes is > 40 and duodenal IEL per 100 enterocytes is > 30, but there is normal crypt hyperplasia and no villous atrophy, it is classified as Marsh I (Oberhuber, 2000). The most severe grading of duodenal lesions, Marsh III was observed in 54.4% of the patients with a slight female predominance. Similar to this finding, Marsh III was recorded in 133 out of 153 CD patients, i.e. in 86.9% of the patients (Rahmati *et al*., 2014). Also, Kalhan et al reported a similar female predominance among CD patients with Marsh I, II and III (Kalhan *et al*., 2011). However, in this study, the prevalence of Marsh I and II was higher in males relative to female patients with Marsh II having a lower prevalence and Marsh I the least (i.e. 27.8% and 17.8% respectively). Conversely, Marsh I and II were reported to be predominant (56 out of 142 patients) compared to Marsh III (15 out of 142 patients) (Thijs, Van Baarlen, Kleibeuker, & Kolkman, 2004). Nevertheless, the graded nature of the Marsh classification based on severity of duodenal lesions questions its usefulness in the diagnosis of early onset or subclinical CD.

The serum levels of CD associated antibodies anti-tTG and anti-gliadin in the patients were also analyzed in this study and patients with seropositivity to these antibodies were identified. For over the past two decades, analysis of serology of CD patients for presence as well as titer levels of CD autoantibodies have been used for routine diagnosis (Bhatnagar & Bhan, 1999). The diagnostic prowess of these antibodies are however, not the same. While anti-tTG antibodies has been shown to

have relatively high levels of specificity and sensitivity in diagnosing CD and as a result it is widely accepted as a valid serological test for CD, anti-gliadin have been associated with low specificity with its usefulness confined to identifying individuals with wheat sensitivity, abnormalities in gluten metabolism and detection of early onset of CD in children (Chartrand *et al.*, 1997; (Caio, Riegler, Patturelli, Facchiano, & Sapone, 2016; Chartrand et al., 1997; Packova et al., 2020). Also, the IgA antibody class have been shown to possess the superior specificity and sensitivity to celiac disease compared to the IgG class which have been associated with high percentage of false positive and is useful in cases of patients with IgA deficiencies (Villalta *et al*., 2010; Werkstetter *et al*., 2017). Thus, based on the specificity and sensitivity the antibodies can be ranked in the order; anti-tTG (IgA) > anti-tTG (IgG) > anti-gliadin (IgA) > anti-gliadin (IgG). The high frequency of patients with seropisitivity to antigliadin IgG i.e. 63.3% can be attributed to its low specificity thus undermining its diagnostic potency.

However, positivity to the highly specific IgA class of anti-tTG was observed in 41.1% of all the patients. Teresi et al also observed a similar anti-tTG IgA seropositivity of 42.2% in CD patients of Saharawi population of Arab origin (Teresi *et al.*, 2010), while 31.8% seropositivity was reported in another study (Bayrama *et al*., 2015). It is important to highlight however, that in serological analysis of CD autoantibodies exclusively for diagnosis of CD has raised many controversies and researchers are worried about the diagnostic accuracy of this parameter (Turner, 2018; Vahedi et al., 2003).

During course of this study, it was observed that the antibody titer levels were strongly associated with the age group of the patients. While

patients of young age (less than 12 years old) and those above 39 years have low titer levels, antibody titers were significantly higher is patients aged between 12 to 39 years. Previous studies have reported higher titer values of CD antibodies which have been associated with disease severity in adults compared to pediatric population, and the amelioration of symptoms in elderly patients above 60 years of age (Vivas, Vaquero, Rodríguez-Martín, & Caminero, 2015; Zanini et al., 2012).

4.3 Diagnostic superiority of HLA-DQ genotyping in CD patients

Although the criteria for diagnosing CD appears to be clear, challenges are still being faced with respect to the diagnosis of subclinical CD and early onset of CD in children as 80% of children with CD are asymptomatic (Bhattacharya, Lomash, Sakhuja, Dubey, & Kapoor, 2014). Also the identification of a superior testing parameters that encompasses all possible forms and stage of disease progression has proven to be useful in mitigating the risks of CD maldiagnosis.

For histological analysis of duodenal biopsy, studies involving pediatric population of CD patients have recorded very low cases of Marsh III and Marsh II (Bhattacharya *et al.*, 2014; Meena *et al*., 2019) and villous atrophy has been shown to be absent in subclinical CD among adults (Nejad, Hogg-Kollars, Ishaq, & Rostami, 2011).

Similarly, in many studies, seropositivity to CD autoantibodies occurs in less than 60% of CD patients, thereby indicating an active seronegative population of CD patients (Volta *et al*., 2016). In this study, 17.7% of the patients had no villous atrophy while 58% of the patients were seronegative to anti-tTG IgA antibody and as high as 80%

were seronegative to anti-tTG IgG. These findings undermines the diagnostic effectiveness and coverage of the parameters.

However, analysis of genetic predisposition to CD through detection of HLA-DQ haplotypes showed that 100% of the patients in this study had either homozygous or heterozygous HLA-DQ2 and HLA-DQ8 genotype. This supports the high predictive prowess of HLA-DQ genotyping in diagnosis of CD as different studies have reported frequency of HLD-DQ haplotypes in more than 90% of CD patients (Alhabbal & Abou Khamis, 2021; Martínez-Ojinaga et al., 2018; Mashayekhi et al., 2018; Murad, Jazairi, Khansaa, Olabi, & Khouri, 2018; Rostami-Nejad et al., 2014).

Interestingly, the findings of this study suggest that HLA-DQ2 has the highest predictive ability for Marsh II and Marsh III grading of duodenal biopsy as all the patients with Marsh II and 75.5% of those with Marsh II were HLA-DQ2 homozygotes. Similarly, vast majority (above 65% in all cases) of patients with seropositivity to the IgA and IgG classes of anti-tTG and anti-gliadin were HLA-DQ2 homozygotes. Therefore, it can be inferred that the HLA-DQ2 gene has the highest predictive ability for CD autoantibodies seropositivity as well.

Although many studies have made comparison between outcome of duodenal biopsy and serum levels of CD autoantibodies, there is limitation of studies highlighting the relationship of these indices with HLA-DQ haplotype. A few of these studies have observed an increasing population of CD patients with HLADQ2 and HLA-DQ8 genes that are positive to anti-tTG but with no evidence of villous atrophy. These patients have been categorized as “potential CD patients” (Ludvigsson, Leffler, et al., 2013). This group of patients coupled with those have seronegative CD are at a higher risk of developing refractory disease and CD associated morbidity resulting from late diagnosis (Ludvigsson,

Leffler, et al., 2013; Volta et al., 2016). Therefore, the fundamental step for decisive diagnosis of CD is carrying out a genetic test since a negative result most likely eliminates CD suspicion so clinicians can seek for other causes of symptoms that are identical to those of CD.

Chapter Five

Conclusion

and

Recommendations

5.1 Conclusion

- **Universality of HLA-testing:** All CD patients had either HLA-DQ2 or HLA-DQ8 genes or both.
- The coverage of Marsh classification of duodenal biospsy histological evaluation was not universal as a few patients fell below the Marsh-guided diagnosis of CD despite being previously clinically diagnosed.
- Similarly, the shortfalls in the universality of seropositivity to anti-tTG and anti-gliadin autoantibodies among CD patients was highlighted as about half of the patients were seronegative to the otherwise highly revered anti-tTG IgA antibody.
- These findings reinforce the superiority of HLA-DQ genotyping for effective CD diagnosis

5.2 Recommendations

The present study hereby makes the following recommendations.

i. Young male adults aged between 18 to 40 years with family history of CD and/ or presentation of symptoms of gluten intolerance should be tested for CD to aid early diagnosis.
ii. Emphasis should be laid on nutritional deficiencies that can arise from malabsortion due to CD-associated villous deterioration during administration of treatment for the disease.
iii. The importance of genetic testing for HLA-DQ haplotypes should be recognized and accepted as a routine test for CD diagnosis especially in cases where serological analyses are useless.
iv. Future studies should trace the detailed molecular footprints that bestows strong susceptibility of CD in individuals with HLA-DQ2 genotype.
v. Also, studies involving large sample size can be conducted to evaluate the HLA-DQ haplotypes in pediatric population which will be succeeded by follow-up studies to assess their possibility of developing CD later in life.

Reference

References

Abadie, V., Sollid, L. M., Barreiro, L. B., & Jabri, B. (2011). Integration of genetic and immunological insights into a model of celiac disease pathogenesis. *Annual review of immunology, 29*, 493-525.

Akobeng, A., Ramanan, A., & Basude, D. (2005). EFFECT OF BREAST-FEEDING ON RISK OF COELIAC DISEASE: A SYSTEMATIC REVIEW AND META-ANALYSIS OF OBSERVATIONAL STUDIES: PG1-18. *Journal of pediatric gastroenterology and nutrition, 40*(5), 638-639.

Akobeng, A. K., Ramanan, A. V., Buchan, I., & Heller, R. F. (2006). Effect of breast feeding on risk of coeliac disease: a systematic review and meta-analysis of observational studies. *Archives of disease in childhood, 91*(1), 39-43.

Akobeng, A. K., Singh, P., Kumar, M., & Al Khodor, S. (2020). Role of the gut microbiota in the pathogenesis of coeliac disease and potential therapeutic implications. *European Journal of Nutrition*, 1-22.

Al-Hussaini, A., Eltayeb-Elsheikh, N., Alharthi, H., Osman, A., Alshahrani, M., Sandogji, I., . . . Bashir, M. S. (2019). HLA-DQ genotypes relative risks for celiac disease in Arabs: A case-control study. *Journal of digestive diseases, 20*(11), 602-608.

Alhabbal, A., & Abou Khamis, I. (2021). HLA-DQ Genotyping of Celiac Disease among Syrian patients. *Annals of the Romanian Society for Cell Biology*, 4926-4936.

Almeida, L. M., Gandolfi, L., Pratesi, R., Uenishi, R. H., Almeida, F. C. d., Selleski, N., & Nóbrega, Y. K. d. M. (2016). Presence of DQ2. 2 associated with DQ2. 5 increases the risk for celiac disease. *Autoimmune diseases, 2016*.

Amin, R., Murphy, N., Edge, J., Ahmed, M. L., Acerini, C. L., & Dunger, D. B. (2002). A longitudinal study of the effects of a gluten-free diet on glycemic control and weight gain in subjects with type 1 diabetes and celiac disease. *Diabetes Care, 25*(7), 1117-1122.

Anderson, R. P., Henry, M. J., Taylor, R., Duncan, E. L., Danoy, P., Costa, M. J., . . . Knight, R. E. (2013). A novel serogenetic approach determines the community prevalence of celiac disease and informs improved diagnostic pathways. *BMC medicine, 11*(1), 1-13.

Asaad Assiri, M., & Newland, C. (2012). Diagnosis of Celiac Disease. *Celiac Disease, An Issue of Gastrointestinal Endoscopy Clinics-E-Book, 22*(4), 661.

Bayrama, Y., Parlaka, M., Aypakb, C., Bayramc, I., Yılmazc, D., & Çıkmand, A. (2015). Diagnostic accuracy of IgA anti-tissue transglutaminase in celiac disease in Van-Turkey. *East J Med, 20*(2015), 20-23.

Berry, N., Basha, J., Varma, N., Varma, S., Prasad, K. K., Vaiphei, K., . . . Kochhar, R. (2018). Anemia in celiac disease is multifactorial in etiology: A prospective study from India. *JGH Open, 2*(5), 196-200.

Bhatnagar, S., & Bhan, M. (1999). Serological diagnosis of celiac disease. *Indian journal of pediatrics, 66*(1 Suppl), S26-31.

Bhattacharya, M., Lomash, A., Sakhuja, P., Dubey, A. P., & Kapoor, S. (2014). Clinical and histopathological correlation of duodenal biopsy with IgA anti-tissue transglutaminase titers in children with celiac disease. *Indian Journal of Gastroenterology, 33*(4), 350-354.

Biagi, F., Schiepatti, A., Malamut, G., Marchese, A., Cellier, C., Bakker, S. F., . . . Ciacci, C. (2014). PROgnosticating COeliac patieNts SUrvivaL: the PROCONSUL score. *PloS one, 9*(1), e84163.

Biasucci, G., Benenati, B., Morelli, L., Bessi, E., & Boehm, G. n. (2008). Cesarean delivery may affect the early biodiversity of intestinal bacteria. *The Journal of nutrition, 138*(9), 1796S-1800S.

Björck, S., Brundin, C., Karlsson, M., & Agardh, D. (2017). Reduced bone mineral density in children with screening-detected celiac disease. *Journal of pediatric gastroenterology and nutrition, 65*(5), 526-532.

Bogaert, L., Cauchie, M., Van Hoovels, L., Vermeersch, P., Fierz, W., De Hertogh, G., . . . Bossuyt, X. (2020). Optimization of serologic diagnosis of celiac disease in the pediatric setting. *Autoimmunity reviews, 19*(5), 102513.

Boonpheng, B., Cheungpasitporn, W., & Wijarnpreecha, K. (2017). Renal disease in patients with celiac disease. *Minerva medica, 109*(2), 126-140.

Bradauskiene, V., Vaiciulyte-Funk, L., Martinaitiene, D., Andruskiene, J., Verma, A. K., Lima, J. P., . . . Catassi, C. (2021). Wheat consumption and prevalence of celiac disease: Correlation from a multilevel analysis. *Critical Reviews in Food Science and Nutrition*, 1-15.

Bratanic, N., Smigoc Schweiger, D., Mendez, A., Bratina, N., Battelino, T., & Vidan-Jeras, B. (2010). An influence of HLA-A, B, DR, DQ,

and MICA on the occurrence of Celiac disease in patients with type 1 diabetes. *Tissue Antigens, 76*(3), 208-215.

Breitschwerdt, E., Halliwell, W., Foley, C., Stark, D., & Corwin, L. (1980). hereditary diarrhetic syndrome in the Basenji characterized by malabsorption, protein losing enteropathy and hypergammaglobulinemia. *Journal of the American Animal Hospital Association*.

Caio, G., Riegler, G., Patturelli, M., Facchiano, A., & Sapone, A. (2016). Pathophysiology of non-celiac gluten sensitivity: where are we now? *Minerva gastroenterologica e dietologica, 63*(1), 16-21.

Caio, G., Volta, U., Sapone, A., Leffler, D. A., De Giorgio, R., Catassi, C., & Fasano, A. (2019). Celiac disease: a comprehensive current review. *BMC medicine, 17*(1), 1-20.

Camarca, M. E., Mozzillo, E., Nugnes, R., Zito, E., Falco, M., Fattorusso, V., . . . Troncone, R. (2012). Celiac disease in type 1 diabetes mellitus. *Italian Journal of Pediatrics, 38*(1), 1-7.

Cataldo, F., & Montalto, G. (2007). Celiac disease in the developing countries: a new and challenging public health problem. *World journal of gastroenterology: WJG, 13*(15), 2153.

Cecilio, L. A., & Bonatto, M. W. (2015). The prevalence of HLA DQ2 and DQ8 in patients with celiac disease, in family and in general population. *ABCD. Arquivos Brasileiros de Cirurgia Digestiva (São Paulo), 28*, 183-185.

Chartrand, L. J., Agulnik, J., Vanounou, T., Russo, P. A., Baehler, P., & Seidman, E. G. (1997). Effectiveness of antigliadin antibodies as a screening test for celiac disease in children. *CMAJ, 157*(5), 527-533.

Ciacci, C., D'agate, C., De Rosa, A., Franzese, C., Errichiello, S., Gasperi, V., . . . Greco, L. (2003). Self-rated quality of life in celiac disease. *Digestive diseases and sciences, 48*(11), 2216-2220.

Coenen, M. J., Trynka, G., Heskamp, S., Franke, B., van Diemen, C. C., Smolonska, J., . . . Postma, D. S. (2009). Common and different genetic background for rheumatoid arthritis and coeliac disease. *Human molecular genetics, 18*(21), 4195-4203.

Cummins, A., & Thompson, F. (2002). Effect of breast milk and weaning on epithelial growth of the small intestine in humans. *Gut, 51*(5), 748-754.

Debiec, H., & Ronco, P. (2011). Nephrotic syndrome: A new specific test for idiopathic membranous nephropathy. *Nature Reviews Nephrology, 7*(9), 496.

Decker, E., Hornef, M., & Stockinger, S. (2011). Cesarean delivery is associated with celiac disease but not inflammatory bowel disease in children. *Gut microbes, 2*(2), 91-98.

Denham, J. M., & Hill, I. D. (2013). Celiac disease and autoimmunity: review and controversies. *Current allergy and asthma reports, 13*(4), 347-353.

Dixit, R., Lebwohl, B., Ludvigsson, J. F., Lewis, S. K., Rizkalla-Reilly, N., & Green, P. H. (2014). Celiac disease is diagnosed less frequently in young adult males. *Digestive diseases and sciences, 59*(7), 1509-1512.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences, 107*(26), 11971-11975.

Falcigno, L., Calvanese, L., Conte, M., Nanayakkara, M., Barone, M. V., & D'Auria, G. (2020). Structural perspective of gliadin peptides active in celiac disease. *International journal of molecular sciences, 21*(23), 9301.

Fasano, A., & Catassi, C. (2001). Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology, 120*(3), 636-651.

Ferreira, R. C., Pan-Hammarström, Q., Graham, R. R., Fontán, G., Lee, A. T., Ortmann, W., . . . Núñez, C. (2012). High-density SNP mapping of the HLA region identifies multiple independent susceptibility loci associated with selective IgA deficiency. *PLoS Genet, 8*(1), e1002476.

Green, P. H., & Cellier, C. (2007). Celiac disease. *New England Journal of Medicine, 357*(17), 1731-1743.

Green, P. H., & Jabri, B. (2003). Coeliac disease. *The Lancet, 362*(9381), 383-391.

Green, P. H., Rostami, K., & Marsh, M. N. (2005). Diagnosis of coeliac disease. *Best Practice & Research Clinical Gastroenterology, 19*(3), 389-400.

Harper, J. W., Holleran, S. F., Ramakrishnan, R., Bhagat, G., & Green, P. H. (2007). Anemia in celiac disease is multifactorial in etiology. *American journal of hematology, 82*(11), 996-1000.

Heap, G. A., & van Heel, D. A. (2009). *Genetics and pathogenesis of coeliac disease.* Paper presented at the Seminars in immunology.

Heyman, M. B. (2014). Celiac Disease: Past, Present, and Future Challenges: Dedicated to the Memory of Our Friend and Colleague, Prof David Branski (1944-2013). *Journal of pediatric gastroenterology and nutrition, 59*, S1.

Hill, I. D., Dirks, M. H., Liptak, G. S., Colletti, R. B., Fasano, A., Guandalini, S., . . . Pivor, M. (2005). Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *Journal of pediatric gastroenterology and nutrition, 40*(1), 1-19.

Horton, R., Wilming, L., Rand, V., Lovering, R. C., Bruford, E. A., Khodiyar, V. K., . . . Wright, M. W. (2004). Gene map of the extended human MHC. *Nature Reviews Genetics, 5*(12), 889-899.

Husby, S., Koletzko, S., Korponay-Szabó, I., Mearin, M., Phillips, A., Shamir, R., . . . Catassi, C. (2012). European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *Journal of pediatric gastroenterology and nutrition, 54*(1), 136-160.

Jabri, B., & Sollid, L. M. (2006). Mechanisms of disease: immunopathogenesis of celiac disease. *Nature clinical practice Gastroenterology & hepatology, 3*(9), 516-525.

Jalilian, M., & Jalali, R. (2021). Prevalence of celiac disease in children with type 1 diabetes: A review. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*.

James, L. M., & Georgopoulos, A. P. (2018). Persistent antigens hypothesis: the human leukocyte antigen (HLA) connection. *Journal of Neurology & Neuromedicine, 3*(6).

James, S. P. (2005). National Institutes of Health consensus development conference statement on celiac disease, June 28–30, 2004. In: Elsevier.

Jelsness-Jørgensen, L.-P., Bernklev, T., & Lundin, K. E. (2018). Fatigue as an extra-intestinal manifestation of celiac disease: A systematic review. *Nutrients, 10*(11), 1652.

Jones, H. J., & Warner, J. T. (2010). NICE clinical guideline 86. Coeliac disease: recognition and assessment of coeliac disease. *Archives of disease in childhood, 95*(4), 312-313.

Jordá, F. C., & Vivancos, J. L. (2010). Fatigue as a determinant of health in patients with celiac disease. *Journal of clinical gastroenterology, 44*(6), 423-427.

Kalhan, S., Joseph, P., Sharma, S., Dubey, S., Dudani, S., & Dixit, M. (2011). Comparative study of histopathological Marsh grading with clinical and serological parameters in celiac iceberg of north India. *Indian Journal of Pathology and Microbiology, 54*(2), 279.

Karell, K., Louka, A. S., Moodie, S. J., Ascher, H., Clot, F., Greco, L., . . . Disease, E. G. C. o. C. (2003). HLA types in celiac disease patients not carrying the DQA1* 05-DQB1* 02 (DQ2) heterodimer: results from the European Genetics Cluster on Celiac Disease. *Human immunology, 64*(4), 469-477.

Kemppainen, K. M., Lynch, K. F., Liu, E., Lönnrot, M., Simell, V., Briese, T., . . . She, J.-X. (2017). Factors that increase risk of celiac disease autoimmunity after a gastrointestinal infection in early life. *Clinical Gastroenterology and Hepatology, 15*(5), 694-702. e695.

Koning, F. (2012). *Celiac disease: quantity matters.* Paper presented at the Seminars in immunopathology.

Koning, F., Schuppan, D., Cerf-Bensussan, N., & Sollid, L. M. (2005). Pathomechanisms in celiac disease. *Best Practice & Research Clinical Gastroenterology, 19*(3), 373-387.

KOTLER, D. P., GAETZ, H. P., LANGE, M., KLEIN, E. B., & HOLT, P. R. (1984). Enteropathy associated with the acquired immunodeficiency syndrome. *Annals of internal medicine, 101*(4), 421-428.

Krishnareddy, S., & Lebwohl, B. (2021). Seronegative Celiac Disease. In *Diagnosis and Management of Gluten-Associated Disorders* (pp. 89-98): Springer.

Kupfer, S. S., & Jabri, B. (2012a). Celiac disease pathophysiology. *Gastrointestinal endoscopy clinics of North America, 22*(4).

Kupfer, S. S., & Jabri, B. (2012b). Pathophysiology of celiac disease. *Gastrointestinal Endoscopy Clinics, 22*(4), 639-660.

Lavant, E. H., & Carlson, J. A. (2009). A new automated human leukocyte antigen genotyping strategy to identify DR-DQ risk alleles for celiac disease and type 1 diabetes mellitus. *Clinical chemistry and laboratory medicine, 47*(12), 1489-1495.

Lebwohl, B., Green, P. H., Murray, J. A., & Ludvigsson, J. F. (2013). Season of birth in a nationwide cohort of coeliac disease patients. *Archives of disease in childhood, 98*(1), 48-51.

Leffler, D. A., & Schuppan, D. (2010). Update on serologic testing in celiac disease. *American Journal of Gastroenterology, 105*(12), 2520-2524.

Legroux-Gérot, I., Leloire, O., Blanckaert, F., Tonnel, F., Grardel, B., Ducrocq, J.-L., & Cortet, B. (2009). Screening for celiac disease in patients with osteoporosis. *Joint Bone Spine, 76*(2), 162-165.

Lerner, A., Ramesh, A., & Matthias, T. (2019). Serologic diagnosis of celiac disease: new biomarkers. *Gastroenterology Clinics, 48*(2), 307-317.

Lewis, D., Haridy, J., & Newnham, E. D. (2017). Testing for coeliac disease. *Australian prescriber, 40*(3), 105.

Lewy, H., Meirson, H., & Laron, Z. (2009). Seasonality of birth month of children with celiac disease differs from that in the general population and between sexes and is linked to family history and environmental factors. *Journal of pediatric gastroenterology and nutrition, 48*(2), 181-185.

Lincoln, M. R., Ramagopalan, S. V., Chao, M. J., Herrera, B. M., DeLuca, G. C., Orton, S.-M., . . . Ebers, G. C. (2009). Epistasis among HLA-DRB1, HLA-DQA1, and HLA-DQB1 loci determines multiple sclerosis susceptibility. *Proceedings of the National Academy of Sciences, 106*(18), 7542-7547.

Lionetti, E., & Catassi, C. (2011). New clues in celiac disease epidemiology, pathogenesis, clinical manifestations, and treatment. *International reviews of immunology, 30*(4), 219-231.

Liu, E., Lee, H.-S., Aronsson, C. A., Hagopian, W. A., Koletzko, S., Rewers, M. J., . . . Simell, V. (2014). Risk of pediatric celiac disease according to HLA haplotype and country. *New England Journal of Medicine, 371*(1), 42-49.

Lopes, L. H., Muniz, J. G., Oliveira, R. P., & Sdepanian, V. L. (2019). Celiac disease in Brazilian first-degree relatives: the odds are five times greater for HLA DQ2 homozygous. *Journal of pediatric gastroenterology and nutrition, 68*(5), e77-e80.

Ludvigsson, J. F., Bai, J. C., Biagi, F., Card, T. R., Ciacci, C., Ciclitira, P. J., . . . Van Heel, D. A. (2014). Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. *Gut, 63*(8), 1210-1228.

Ludvigsson, J. F., Leffler, D. A., Bai, J. C., Biagi, F., Fasano, A., Green, P. H., . . . Leonard, J. N. (2013). The Oslo definitions for coeliac disease and related terms. *Gut, 62*(1), 43-52.

Ludvigsson, J. F., & Murray, J. A. (2019). Epidemiology of celiac disease. *Gastroenterology Clinics, 48*(1), 1-18.

Ludvigsson, J. F., Rubio-Tapia, A., Van Dyke, C. T., Melton III, L. J., Zinsmeister, A. R., Lahr, B. D., & Murray, J. A. (2013). Increasing

incidence of celiac disease in a North American population. *The American journal of gastroenterology, 108*(5), 818.

Malamut, G., Cerf-Bensussan, N., & Cellier, C. (2015). Identification of new cases of severe enteropathy has recently increased the spectrum of intestinal non-celiac villous atrophy. *Expert review of gastroenterology & hepatology, 9*(6), 719-721.

Mansouri, M., Dadfar, M., Rostami-Nejad, M., Ekhlasi, G., Shahbazkhani, A., & Shahbazkhani, B. (2021). The frequency of HLA-DQ2/DQ8 haplotypes and celiac disease among the first-degree relatives of patients with celiac disease. *Gastroenterology and Hepatology From Bed to Bench, 14*(1), 36.

Marsh, S. G., Albert, E., Bodmer, W., Bontrop, R., Dupont, B., Erlich, H., . . . Hurley, C. (2010). An update to HLA nomenclature, 2010. *Bone marrow transplantation, 45*(5), 846-848.

Martínez-Ojinaga, E., Molina, M., Polanco, I., Urcelay, E., & Núñez, C. (2018). HLA-DQ distribution and risk assessment of celiac disease in a Spanish center. *Revista Española de Enfermedades Digestivas, 110*(7), 421-426.

Mashayekhi, K., Rostami-Nejad, M., Amani, D., Rezaei-Tavirani, M., Mohaghegh-Shalmani, H., & Zali, M. R. (2018). A rapid and sensitive assay to identify HLA-DQ2/8 risk alleles for celiac disease using real-time PCR method. *Gastroenterology and hepatology from bed to bench, 11*(3), 250.

Mazzilli, M. C., Ferrante, P., Mariani, P., Martone, E., Petronzelli, F., Triglione, P., & Bonamico, M. (1992). A study of Italian pediatric celiac disease patients confirms that the primary HLA association is to the DQ (α1∗ 0501, β1∗ 0201) heterodimer. *Human immunology, 33*(2), 133-139.

Meena, D. K., Akunuri, S., Meena, P., Bhramer, A., Sharma, S. D., & Gupta, R. (2019). Tissue transglutaminase antibody and its association with duodenal biopsy in diagnosis of pediatric celiac disease. *Pediatric gastroenterology, hepatology & nutrition, 22*(4), 350-357.

Megiorni, F., Mora, B., Bonamico, M., Barbato, M., Nenna, R., Maiella, G., . . . Mazzilli, M. C. (2009). HLA-DQ and risk gradient for celiac disease. *Human immunology, 70*(1), 55-59.

Megiorni, F., & Pizzuti, A. (2012). HLA-DQA1 and HLA-DQB1 in Celiac disease predisposition: practical implications of the HLA molecular typing. *Journal of biomedical science, 19*(1), 1-5.

Mostowy, J., Montén, C., Gudjonsdottir, A. H., Arnell, H., Browaldh, L., Nilsson, S., . . . Torinsson Naluai, Å. (2016). Shared genetic factors involved in celiac disease, type 2 diabetes and anorexia nervosa suggest common molecular pathways for chronic diseases. *PloS one, 11*(8), e0159593.

Munger, K. L., Levin, L. I., Hollis, B. W., Howard, N. S., & Ascherio, A. (2006). Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *Jama, 296*(23), 2832-2838.

Murad, H., Jazairi, B., Khansaa, I., Olabi, D., & Khouri, L. (2018). HLA-DQ2 and-DQ8 genotype frequency in Syrian celiac disease children: HLA-DQ relative risks evaluation. *BMC gastroenterology, 18*(1), 1-4.

Nadal, I., Donant, E., Ribes-Koninckx, C., Calabuig, M., & Sanz, Y. (2007). Imbalance in the composition of the duodenal microbiota of children with coeliac disease. *Journal of medical microbiology, 56*(12), 1669-1674.

Nazahah, M., & Koh, M. (2015). Tissue typing and its role in transplantation. *ISBT Science Series, 10*(S1), 115-123.

Neefjes, J., Jongsma, M. L., Paul, P., & Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews immunology, 11*(12), 823-836.

Nejad, M. R., Hogg-Kollars, S., Ishaq, S., & Rostami, K. (2011). Subclinical celiac disease and gluten sensitivity. *Gastroenterology and Hepatology From Bed to Bench, 4*(3), 102.

Nisticò, L., Fagnani, C., Coto, I., Percopo, S., Cotichini, R., Limongelli, M. G., . . . Sferlazzas, C. (2006). Concordance, disease progression, and heritability of coeliac disease in Italian twins. *Gut, 55*(6), 803-808.

Oberhuber, G. (2000). Histopathology of celiac disease. *Biomedicine & Pharmacotherapy, 54*(7), 368-372.

Oujamaa, I., Sebbani, M., Elmoumou, L., Bourrahouate, A., El Qadiry, R., El Moussaoui, S., . . . Mghari-Tabib, E. (2019). The prevalence of celiac disease-specific auto-antibodies in type 1 diabetes in a Moroccan population. *International journal of endocrinology, 2019*.

Packova, B., Kovalcikova, P., Pavlovsky, Z., Bartusek, D., Prokesova, J., Dolina, J., & Kroupa, R. (2020). Non-invasive prediction of persistent villous atrophy in celiac disease. *World journal of gastroenterology, 26*(26), 3780.

Pestana, N., Vida, C., Vieira, P., Durães, J., & Silva, G. (2021). Celiac Disease as a Rare Cause of Membranous Nephropathy: A Case Report. *Cureus, 13*(2).

Rahmati, A., Shakeri, R., Sohrabi, M., Alipour, A., Boghratian, A., Setareh, M., & Zamani, F. (2014). Correlation of tissue transglutaminase antibody with duodenal histologic marsh grading. *Middle East journal of digestive diseases, 6*(3), 131.

Reilly, N. R., Fasano, A., & Green, P. H. (2012). Presentation of celiac disease. *Gastrointestinal Endoscopy Clinics, 22*(4), 613-621.

Rhodes, D., & Trowsdale, J. (1999). Genetics and molecular genetics of the MHC. *Reviews in immunogenetics, 1*(1), 21-31.

Rostami-Nejad, M., Romanos, J., Rostami, K., Ganji, A., Ehsani-Ardakani, M. J., Bakhshipour, A.-R., . . . Wijmenga, C. (2014). Allele and haplotype frequencies for HLA-DQ in Iranian celiac disease patients. *World journal of gastroenterology: WJG, 20*(20), 6302.

Rostom, A., Murray, J. A., & Kagnoff, M. F. (2006). American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. *Gastroenterology, 131*(6), 1981-2002.

Ruby, A. M., Al-Khodary, R., Shubair, M., & Sirdah, M. (2014). Immunodiagnosis of celiac disease among children with chronic diarrhea in Gaza Strip, Palestine. *Am J BioSci, 2*(6), 4.

Sánchez, E., De Palma, G., Capilla, A., Nova, E., Pozo, T., Castillejo, G., . . . Polanco, I. (2011). Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by Bacteroides species. *Applied and environmental microbiology, 77*(15), 5316-5323.

Sanz, Y., Palma, G. D., & Laparra, M. (2011). Unraveling the ties between celiac disease and intestinal microbiota. *International reviews of immunology, 30*(4), 207-218.

Sarmiento, L., Galvan, J. A., Cabrera-Rode, E., Aira, L., Correa, C., Sariego, S., . . . Resik, S. (2012). Type 1 diabetes associated and tissue transglutaminase autoantibodies in patients without type 1 diabetes and coeliac disease with confirmed viral infections. *Journal of medical virology, 84*(7), 1049-1053.

Selimoglu, M. A., & Karabiber, H. (2010). Celiac disease: prevention and treatment. *Journal of clinical gastroenterology, 44*(1), 4-8.

Sharma, A., Wang, X. J., Russo, P. A., Wu, T.-T., Nehra, V., & Murray, J. A. (2018). Features of adult autoimmune enteropathy compared

with refractory celiac disease. *Clinical Gastroenterology and Hepatology, 16*(6), 877-883. e871.
Shiina, T., Hosomichi, K., Inoko, H., & Kulski, J. K. (2009). The HLA genomic loci map: expression, interaction, diversity and disease. *Journal of human genetics, 54*(1), 15-39.
Shoeib, S., El-Shebiny, E., Efat, A., & El-Hady, K. A. (2019). Human leukocyte antigen in medicine. *Menoufia Medical Journal, 32*(4), 1197.
Sollid, L. M., & Lie, B. A. (2005). Celiac disease genetics: current concepts and practical applications. *Clinical Gastroenterology and Hepatology, 3*(9), 843-851.
Stene, L. C., Honeyman, M. C., Hoffenberg, E. J., Haas, J. E., Sokol, R. J., Emery, L., . . . Eisenbarth, G. S. (2006). Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. *American Journal of Gastroenterology, 101*(10), 2333-2340.
Tack, G. J., Verbeek, W. H., Schreurs, M. W., & Mulder, C. J. (2010). The spectrum of celiac disease: epidemiology, clinical aspects and treatment. *Nature reviews Gastroenterology & hepatology, 7*(4), 204.
Ten Broeke, T., Wubbolts, R., & Stoorvogel, W. (2013). MHC class II antigen presentation by dendritic cells regulated through endosomal sorting. *Cold Spring Harbor perspectives in biology, 5*(12), a016873.
Teresi, S., Crapisi, M., Vallejo, M. D. C., Castellaneta, S. P., Francavilla, R., Iacono, G., . . . Catassi, C. (2010). Celiac disease seropositivity in Saharawi children: a follow-up and family study. *Journal of pediatric gastroenterology and nutrition, 50*(5), 506-509.
Thijs, W., Van Baarlen, J., Kleibeuker, J., & Kolkman, J. (2004). Duodenal versus jejunal biopsies in suspected celiac disease. *Endoscopy, 36*(11), 993-996.
Thompson, G., Lewis, B., & Booth, C. (1966). Absorption of vitamin D3-3H in control subjects and patients with intestinal malabsorption. *The Journal of clinical investigation, 45*(1), 94-102.
Thorsby, E. (2011). On the future of HLA. *Tissue Antigens, 78*(4), 229-240.
Trowsdale, J. (1993). Genomic structure and function in the MHC. *Trends in Genetics, 9*(4), 117-122.
Trowsdale, J. (2011). The MHC, disease and selection. *Immunology letters, 137*(1-2), 1-8.

Trowsdale, J., & Knight, J. C. (2013). Major histocompatibility complex genomics and human disease. *Annual review of genomics and human genetics, 14*, 301-323.
Trynka, G., Wijmenga, C., & van Heel, D. A. (2010). A genetic perspective on coeliac disease. *Trends in molecular medicine, 16*(11), 537-550.
Turner, J. M. (2018). Diagnosis of celiac disease: taking a bite out of the controversy. *Digestive diseases and sciences, 63*(6), 1384-1391.
Vahedi, K., Mascart, F., Mary, J.-Y., Laberenne, J.-E., Bouhnik, Y., Morin, M.-C., . . . Matuchansky, C. (2003). Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *The American journal of gastroenterology, 98*(5), 1079-1087.
Vaquero, L., Caminero, A., Nuñez, A., Hernando, M., Iglesias, C., Casqueiro, J., & Vivas, S. (2014). Coeliac disease screening in first-degree relatives on the basis of biopsy and genetic risk. *European journal of gastroenterology & hepatology, 26*(3), 263-267.
Vici, G., Camilletti, D., & Polzonetti, V. (2020). Possible role of vitamin D in celiac disease onset. *Nutrients, 12*(4), 1051.
Vigil, A. (2018). Pathophysiology of Celiac Disease.
Viken, M., Flåm, S., Skrivarhaug, T., Amundsen, S., Sollid, L., Drivvoll, A., . . . Lie, B. (2017). HLA class II alleles in Norwegian patients with coexisting type 1 diabetes and celiac disease. *Hla, 89*(5), 278-284.
Villalta, D., Tonutti, E., Prause, C., Koletzko, S., Uhlig, H. H., Vermeersch, P., . . . Ellis, J. H. (2010). IgG antibodies against deamidated gliadin peptides for diagnosis of celiac disease in patients with IgA deficiency. *Clinical chemistry, 56*(3), 464-468.
Villanacci, V., Ceppa, P., Tavani, E., Vindigni, C., & Volta, U. (2011). Coeliac disease: the histology report. *Digestive and Liver Disease, 43*, S385-S395.
Villanacci, V., Lorenzi, L., Donato, F., Auricchio, R., Dziechciarz, P., Gyimesi, J., . . . Polanco, I. (2018). Histopathological evaluation of duodenal biopsy in the PreventCD project. An observational interobserver agreement study. *Apmis, 126*(3), 208-214.
Vivas, S., Vaquero, L., Rodríguez-Martín, L., & Caminero, A. (2015). Age-related differences in celiac disease: Specific characteristics of adult presentation. *World journal of gastrointestinal pharmacology and therapeutics, 6*(4), 207.
Volta, U., Caio, G., Boschetti, E., Giancola, F., Rhoden, K. J., Ruggeri, E., . . . De Giorgio, R. (2016). Seronegative celiac disease: Shedding

light on an obscure clinical entity. *Digestive and Liver Disease, 48*(9), 1018-1022.

Walker, M. M., & Murray, J. A. (2011). An update in the diagnosis of coeliac disease. *Histopathology, 59*(2), 166-179.

Werkstetter, K. J., Korponay-Szabó, I. R., Popp, A., Villanacci, V., Salemme, M., Heilig, G., . . . Thomas, A. (2017). Accuracy in diagnosis of celiac disease without biopsies in clinical practice. *Gastroenterology, 153*(4), 924-935.

Wu, Y., Liu, B., Lin, W., Xu, Y., Li, L., Zhang, Y., . . . Xu, A. (2007). Human leukocyte antigen class II alleles and risk of cervical cancer in China. *Human immunology, 68*(3), 192-200.

Yuan, J., Zhou, C., Gao, J., Li, J., Yu, F., Lu, J., . . . Wu, Z. (2017). Prevalence of celiac disease autoimmunity among adolescents and young adults in China. *Clinical Gastroenterology and Hepatology, 15*(10), 1572-1579. e1571.

Zanini, B., Magni, A., Caselani, F., Lanzarotto, F., Carabellese, N., Villanacci, V., . . . Lanzini, A. (2012). High tissue-transglutaminase antibody level predicts small intestinal villous atrophy in adult patients at high risk of celiac disease. *Digestive and Liver Disease, 44*(4), 280-285.

Appendices

Appendix A:-

The patient category according to age groups

Age(years)	No.(%)
5-11	17(18.9)
12-18	12(13.3)
19-25	21(23.3)
26-32	30(33.3)
33-39	5(5.6)
40-47	5(5.6)
Total	90(100.0)

Appendix B:-

Distribution of gender according to age of patient

Age (years)	Male No. (%)	Female No. (%)	p.value
5-11	8(22.2)	9(16.7)	0.003
12-18	6(16.7)	6(11.1)	
19-25	6(16.7)	15(27.8)	
26-32	8(22.2)	22(40.7)	
33-39	3(8.3)	2(3.7)	
40-47	5(13.9)	0(0.0)	
Total	36(100.0)	54(100.0)	

Appendix C:-

Biochemical features of the patients

	mean±SD	95% Confidence Interval of the Difference	
		Lower	Upper
BMI	26.29±0.66	26.15-26.43	
Glucose	94.65±3.89	93.83-95.47	
HbA1C	5.47±0.66	5.33-5.61	
Pcv	31.68±6.03	30.42-32.94	
Hb	10.56±2.42	10.05-11.07	
WBC	6.84±0.91	6.65-7.03	
PLT	206.63±34.27	199.45-213.81	
Ferritin	5.68±2.01	5.26-6.10	
Calcium	7.39±1.32	7.11-7.66	
Vitamin D3	11.29±3.83	10.49-12.10	
Urea	26.88±11.37	24.50-29.27	
Creatinine	0.76±0.43	0.66-0.85	

Appendix D:-

Biochemical features of the patients according gender

	Male mean±SD	Female mean±SD
BMI	26.23±0.69	26.33±0.65
Glucose	94.88±3.97	94.50±3.86
HbA1C	5.57±0.66	5.41±0.65
Pcv	31.88±6.31	31.55±5.89
Hb	10.92±2.52	10.32±2.34
WBC	7.00±0.81	6.74±0.97
PLT	208.61±36.02	205.31±33.3
Ferritin	5.80±2.12	5.60±1.94
Calcium	7.24±1.33	7.48±1.32
Vitamin D3	11.17±4.04	11.37±3.73
Urea	27.61±11.58	26.40±11.32
Creatinine	0.76±0.43	0.75±0.44

Appendix E:-

The serum concentration of anti-tTG and anti-gliadin antibodies

			Age(years)					
			5-11 No.(17) (mean±SD)	**12-18 No.(12) (mean±SD)**	**19-25 No.(21) (mean±SD)**	**26-32 No.(30) (mean±SD)**	**33-39 No.(5) (mean±SD)**	**40-47 No.(5) (mean±SD)**
TtG	**IgA**	**+ve**	74.5(5.5)	66.8(10.4)	167.6(47.3)	352.6(50.1)	390.0(0.0)	37.3(0.0)
		-ve	5.0(0.7)	4.9(0.4)	3.9(0.5)	3.0(0.7)	5.2(0.0)	2.6(0.0)
	IgG	**+ve**	0.0(0.0)	32.8(6.4)	138.9(0.0)	29.4(6.4)	21.9(0.0)	0.0(0.0)
		-ve	1.9(0.5)	8.8(0.5)	3.8(0.4)	3.3(0.4)	3.4(0.0)	5.1(0.39)
Anti-G	**IgA**	**+ve**	21.8(4.0)	38.6(6.0)	106.6(0.0)	38.8(6.2)	10.5(0.4)	15.6(0.0)
		-ve	9.4(0.2)	7.0(0.9)	8.3(0.5)	9.0(0.6)	0.0(0.0)	4.2(0.0)
	IgG	**+ve**	93.0(6.8)	185.5(5.7)	116.2(5.7)	257.4(49.7)	214.5(0.0)	220(0.0)
		-ve	1.4(0.0)	0.0(0.0)	4.9(0.5)	8.3(0.1)	8.4(0.0)	5.3(0.0)

across different age groups of the patients

Appendix F:-

The serum concentration of anti-tTG and anti-gliadin antibodies across gender

			Male No.(%)	Female No.(%)
TtG	**IgA**	**+ve**	14(38.9)	23(42.6)
		-ve	22(61.1)	31(57.4)
		Total	36(100.0)	54(100.0)
	IgG	**+ve**	7(19.4)	11(20.4)
		-ve	29(80.6)	43(79.6)
		Total	36(100.0)	54(100.0)
Anti-G	**IgA**	**+ve**	16(44.4)	19(35.2)
		-ve	20(55.6)	35(64.8)
		Total	36(100.0)	54(100.0)
	IgG	**+ve**	21(58.3)	36(66.7)
		-ve	15(41.7)	18(33.3)
		Total	36(100.0)	54(100.0)

Appendix G:-

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

			Age(years)						
			5-11 No. (%)	**12-18 No. (%)**	**19-25 No. (%)**	**26-32 No. (%)**	**33-39 No. (%)**	**40-47 No. (%)**	**Total No. (%)**
TtG	**IgA**	**+ve**	2(11.8)	8(66.7)	9(42.9)	12(40.0)	3(60.0)	3(60.0)	37(41.1)
		-ve	15(88.2)	4(33.3)	12(57.1)	18(60.0)	2(40.0)	2(40.0)	53(58.9)
		Total	17(100.0)	12(100.0)	21(100.0)	30(100.0)	5(100.0)	5(100.0)	90(100.0)
	IgG	**+ve**	0(0.0)	6(50.0)	2(9.5)	7(23.3)	3(60.0)	0(0.0)	18(20.0)
		-ve	17(100.0)	6(50.0)	19(90.5)	23(76.7)	2(40.0)	5(100.0)	72(80.0)
		Total	17(100.0)	12(100.0)	21(100.0)	30(100.0)	5(100.0)	5(100.0)	90(100.0)
Anti-G	**IgA**	**+ve**	8(47.1)	8(66.7)	2(9.5)	9(30.0)	5(100.0)	3(60.0)	35(38.9)
		-ve	9(52.9)	4(33.3)	19(90.5)	21(70.0)	0(0.0)	2(40.0)	55(61.1)
		Total	17(100.0)	12(100.0)	21(100.0)	30(100.0)	5(100.0)	5(100.0)	90(100.0)
	IgG	**+ve**	15(88.2)	12(100.0)	12(57.1)	13(43.3)	2(40.0)	3(60.0)	57(63.3)
		-ve	2(11.8)	0(0.0)	9(42.9)	17(56.7)	3(60.0)	2(40.0)	33(36.7)
		Total	17(100.0)	12(100.0)	21(100.0)	30(100.0)	5(100.0)	5(100.0)	90(100.0)

Appendix H:-

Marsh classification of duodenal biopsy of the patients according gender

Gender	Marsh I No. (%)	Marsh II No. (%)	Marsh III No. (%)	Total No. (%)
Male	8(22.2)	11(30.6)	17(47.2)	36(100.0)
Female	8(14.8)	14(25.9)	32(59.3)	54(100.0)
Total	16(17.8)	25(27.8)	49(54.4)	90(100.0)

Appendix I:-

The serum concentration of anti-tTG and anti-gliadin antibodies across Marsh classification of duodenal biopsy of the patients

			Marsh I No. (%)	Marsh II No. (%)	Marsh III No. (%)	Total No. (%)
TtG	**IgA**	**+ve**	2(12.5)	9(36.0)	26(53.1)	37(41.1)
		-ve	14(87.5)	16(64.0)	23(46.9)	53(58.9)
		Total	16(100.0)	25(100.0)	49(100.0)	90(100.0)
	IgG	**+ve**	2(12.5)	2(8.0)	14(28.6)	18(20.0)
		-ve	14(87.5)	23(92.0)	35(71.4)	72(80.0)
		Total	16(100.0)	25(100.0)	49(100.0)	90(100.0)
Anti-G	**IgA**	**+ve**	2(12.5)	7(28.0)	26(53.1)	35(38.9)
		-ve	14(87.5)	18(72.0)	23(46.9)	55(61.1)
		Total	16(100.0)	25(100.0)	49(100.0)	90(100.0)
	IgG	**+ve**	10(62.5)	17(68.0)	30(61.2)	57(63.3)
		-ve	6(37.5)	8(32.0)	19(38.8)	33(36.7)
		Total	16(100.0)	25(100.0)	49(100.0)	90(100.0)

Appendix J:-

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

	Marsh I No. (%)	Marsh II No. (%)	Marsh III No. (%)	Total No. (%)
DQ2&DQ8	4(25.0)	0(0.0)	3(6.1)	7(7.8)
DQ2	8(50.0)	25(100.0)	37(75.5)	70(77.8)
DQ8	4(25.0)	0(0.0)	9(18.4)	13(14.4)
Total	16(100.0)	25(100.0)	49(100.0)	90(100.0)

Appendix K:-

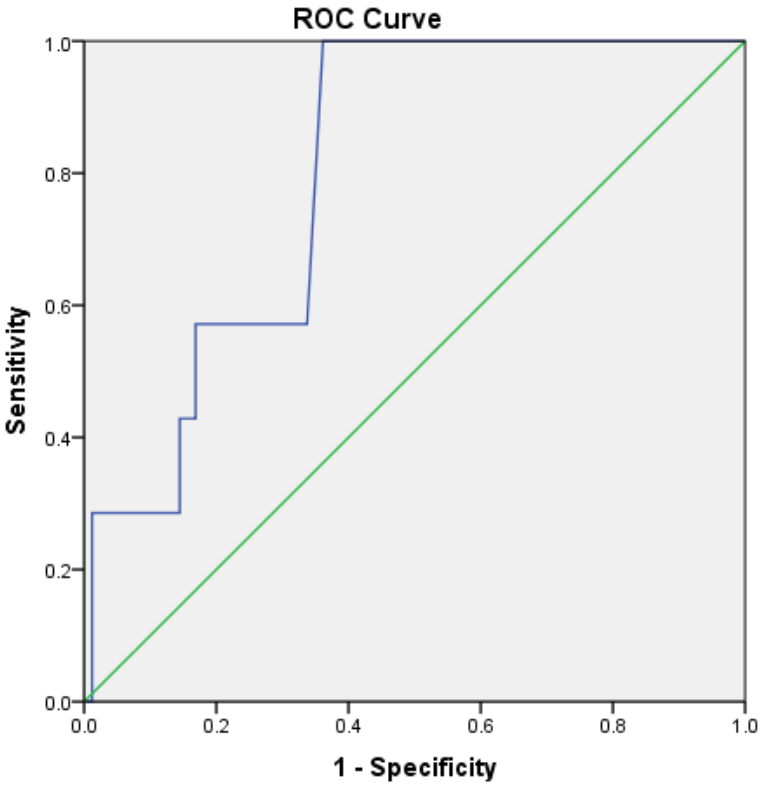
The HLA-DQ haplotype of patients with the gender

Gender	DQ2&DQ8 No. (%)	DQ2 No. (%)	DQ8 No. (%)	Total No. (%)
Male	2(5.6)	30(83.3)	4(11.1)	36(100.0)
Female	5(9.3)	40(74.1)	9(16.7)	54(100.0)
Total	7(7.8)	70(77.8)	13(14.4)	90(100.0)

Appendix L:-

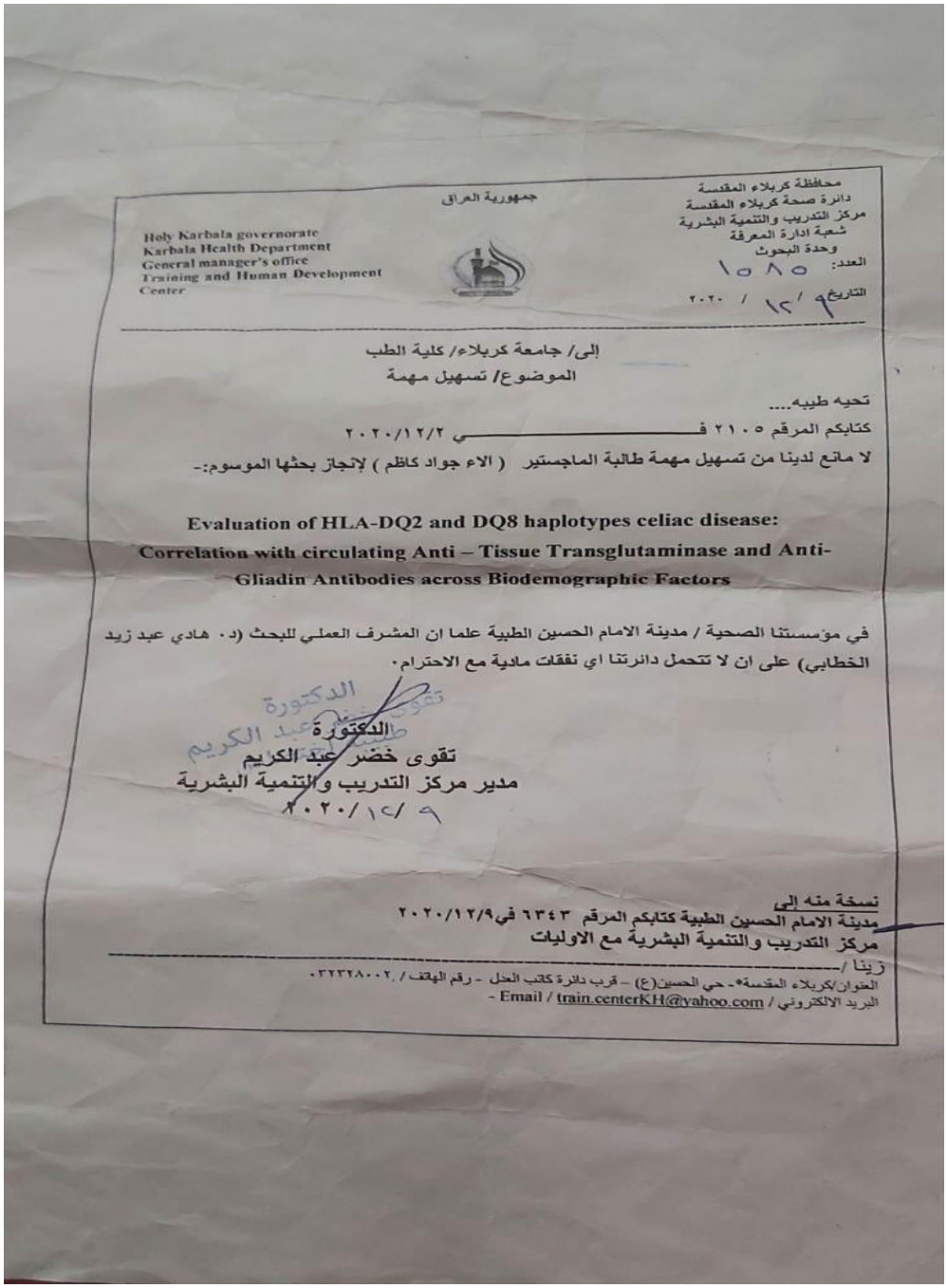
The HLA-DQ haplotype of patients with seropositivity to anti-tTG and anti-gliadin autoantibodies

			DQ2&DQ8 No. (%)	DQ2 No. (%)	DQ8 No. (%)	Total No. (%)
TtG	**IgA**	**+ve**	5(13.5)	25(67.6)	7(18.9)	37(100.0)
		-ve	2(3.8)	45(84.9)	6(11.3)	53(100.0)
	IgG	**+ve**	2(11.1)	14(77.8)	2(11.1)	18(100.0)
		-ve	5(6.9)	56(77.8)	11(15.3)	72(100.0)
Anti-G	**IgA**	**+ve**	2(5.7)	29(82.9)	4(11.4)	35(100.0)
		-ve	5(9.1)	41(74.5)	9(16.4)	55(100.0)
	IgG	**+ve**	4(7.0)	49(86.0)	4(7.0)	57(100.0)
		-ve	3(9.1)	21(63.6)	9(27.3)	33(100.0)



Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.802	.063	.008	.679	.926

Appendix M: Ethical approval was obtained from the appropriate regulatory bodies in the Imam Hussein medical city.



جمهورية العراق

Holy Karbala governorate
Karbala Health Department
General manager's office
Training and Human Development Center

محافظة كربلاء المقدسة
دائرة صحة كربلاء المقدسة
مركز التدريب والتنمية البشرية
شعبة ادارة المعرفة
وحدة البحوث
العدد: ١٥٨٥
التاريخ: ٩ / ١٢ / ٢٠٢٠

إلى/ جامعة كربلاء/ كلية الطب
الموضوع/ تسهيل مهمة

تحيه طيبه....

كتابكم المرقم ٢١٠٥ في ٢٠٢٠/١٢/٢

لا مانع لدينا من تسهيل مهمة طالبة الماجستير (الاء جواد كاظم) لإنجاز بحثها الموسوم:-

Evaluation of HLA-DQ2 and DQ8 haplotypes celiac disease: Correlation with circulating Anti – Tissue Transglutaminase and Anti-Gliadin Antibodies across Biodemographic Factors

في مؤسستنا الصحية / مدينة الامام الحسين الطبية علما ان المشرف العملي للبحث (د. هادي عبد زيد الخطابي) على ان لا تتحمل دائرتنا اي نفقات مادية مع الاحترام.

الدكتورة
تقوى خضر عبد الكريم
مدير مركز التدريب والتنمية البشرية
٢٠٢٠/١٢/٩

نسخة منه إلى
مدينة الامام الحسين الطبية كتابكم المرقم ٦٣٤٣ في ٢٠٢٠/١٢/٩
مركز التدريب والتنمية البشرية مع الاوليات
زينا /

العنوان/كربلاء المقدسة- حي الحسين(ع) – قرب دائرة كاتب العدل - رقم الهاتف / ٠٣٢٣٢٨٠٠٢
البريد الالكتروني / train.centerKH@yahoo.com - Email /

Appendix N: Questionnaire form (Celiac disease)

Demographical

1- Age: 2- Gender:

Anthropometric:

1-BMI (Kg/m^2

Family history:

Medical history:

1- Duration of celiac diseases:

2- Other autoimmune disease: 2- Gluten free diet (GFD):

2- Date of Gastroscopy(OGD-Oesophago-gastro-duodenoscopy)

Pathological finding:

Marsh classification: grade 1: grade 2: grade 3:

Appendix O: PCR product Hybridization and development using Theromoshaker PSH-60HL

Specific binding for PCR amplified DNA fragments take place during this step, using a series of probes that are covalently bound to a nylon membrane.

- Probes for the different HLA allege groups detected by the test, two of which (DQA18*033 and DQB1*02) are lined in duplicate on two membrane locations,

- A probe for the *B*-globin fragment, used as an internal PCR control,

- A development control probe.

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

- Alleles that constitute the DQ2cis haplotype DQA1*05-DQB1*02-DRB1*03.

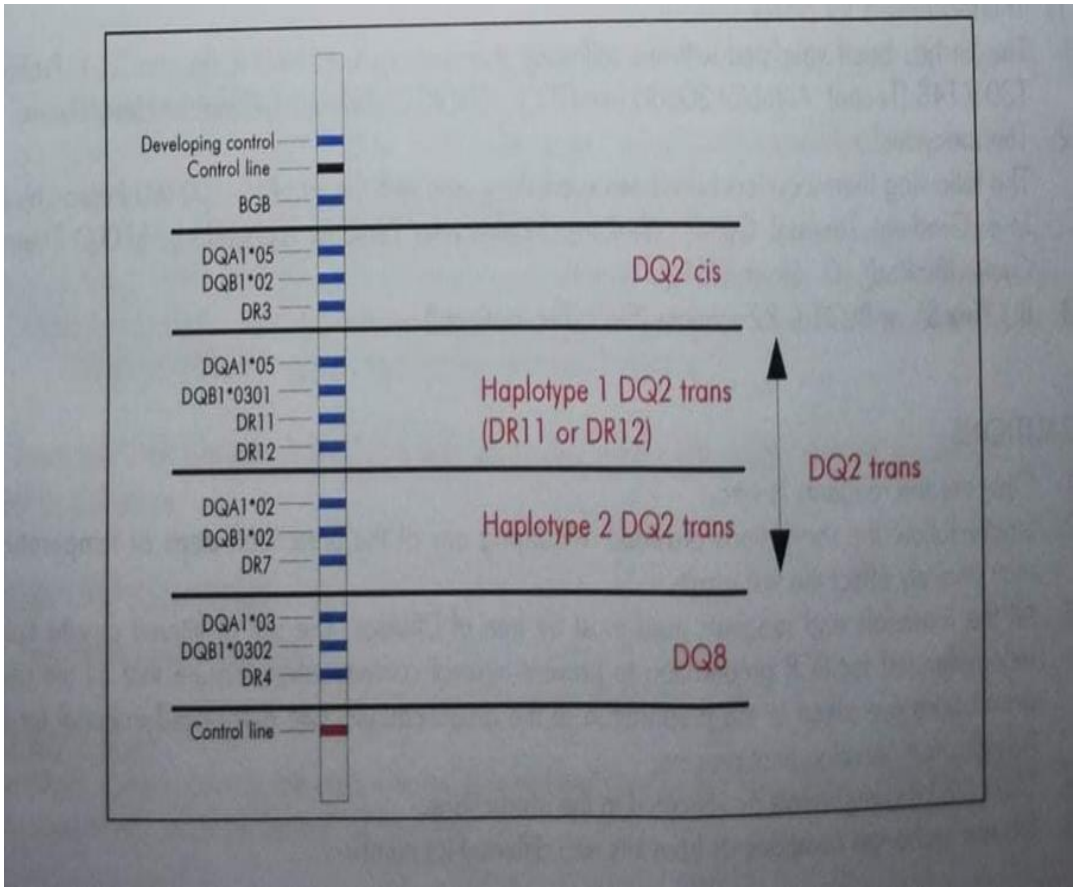
- Alleles that constitute the DQ2trans haplotype 1: DQA1*O5-DQB1*0301-DRB1*11/DRB1*12

- Alleles that constitute the DQ2trans haplotype2: DQA1*02-DQB*02-DRB1*07

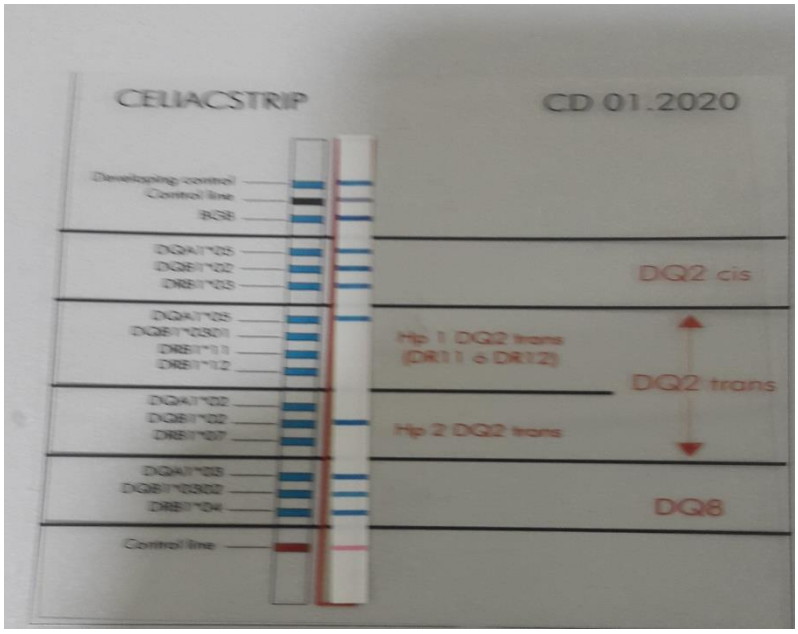
- Alleles that constitute the DQ8 haplotype: - DQA1*03-DQB1*0302-DRB1*04

Appendix P: Reading and interpretation strip: -

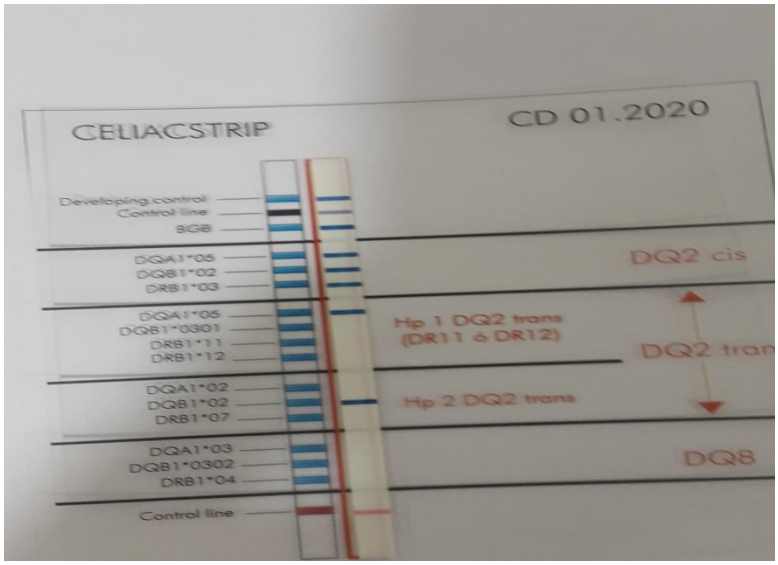
The presence of some of the haplotypes associated with celiac disease will be determined by the presence of group of bands the identified the alleles that form each one of the haplotype.



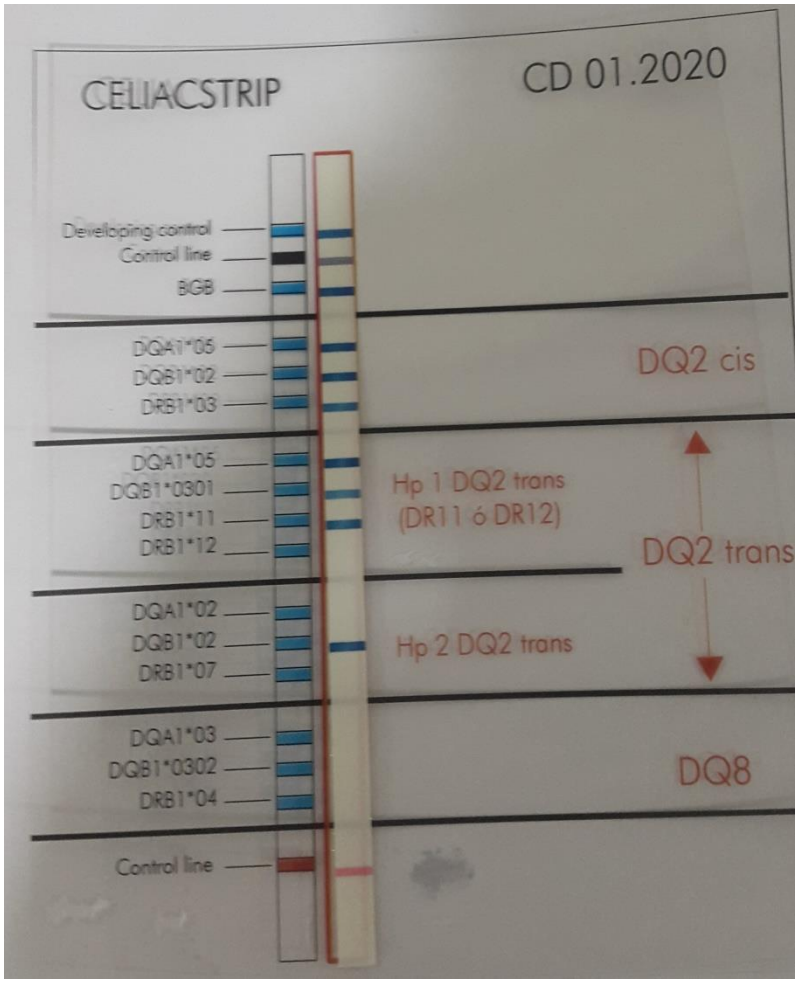
Appendix R



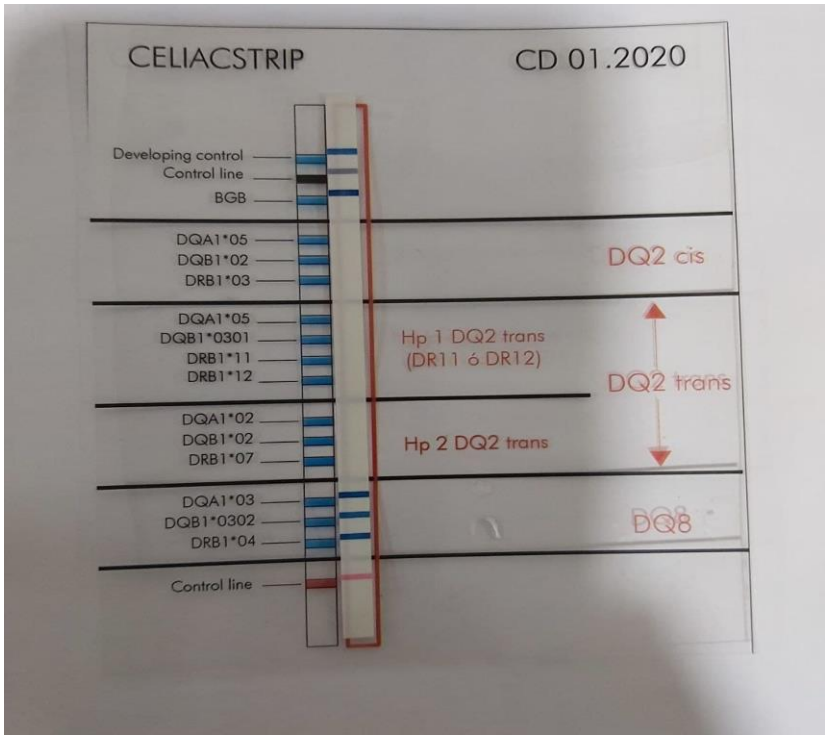
Appendix S



Appendix T


CELIACSTRIP
CD 01.2020
Developing control
Control line
BGB
DQA1*05
DQB1*02
DRB1*03
DQ2 cis
DQA1*05
DQB1*0301
DRB1*11
DRB1*12
Hp 1 DQ2 trans
(DR11 ó DR12)
DQ2 trans
DQA1*02
DQB1*02
DRB1*07
Hp 2 DQ2 trans
DQA1*03
DQB1*0302
DRB1*04
DQ8
Control line

Appendix w


CELIACSTRIP
CD 01.2020
Developing control
Control line
BGB
DQA1*05
DQB1*02
DRB1*03
DQ2 cis
DQA1*05
DQB1*0301
DRB1*11
DRB1*12
Hp 1 DQ2 trans
(DR11 ó DR12)
DQ2 trans
DQA1*02
DQB1*02
DRB1*07
Hp 2 DQ2 trans
DQA1*03
DQB1*0302
DRB1*04
DQ8
Control line

الخلاصة:

يظل تشخيص مرض داء حساسية القمح أحد التحديات الرئيسية التي تواجه فهم وبائيات المرض، والفيزيولوجيا المرضية، وإدارة وتطوير التدخلات العلاجية. تم تحديد الطبيعة غير النمطية لغالبية الاعراض إلى جانب الكشف المتأخر للأعراض في البداية المبكرة للمرض بين الأطفال والأفراد المصابين بمرض حساسية القمح وليس لديهم اعراض المرض على أنها المسبب الرئيسي. في حين تبين أن التحليل النسيجي لخزعة الاثني عشر لديه عجز في التشخيص الفعال مرض حساسية القمح أثناء الظهور المبكر للمرض عند الأطفال بسبب عدم وجود فحوصات لافات الاثني عشر، فإن القياسات المصلية للأجسام المضادة الذاتية المرتبطة بمرض حساسية القمح غير مجدية في حالات المرضى المصابين بآفات الاثني عشر أو المصابين بمرض حساسية القمح وليس لديهم اعراض المرض. يكتسب الاختبار الجيني للكشف عن HLADQ2 وHLA-DQ8 في المرضى المشتبه في وجود مرض حساسية القمح قبولًا كطريقة أكثر فعالية لتشخيص مرض حساسية القمح. هدفت الدراسة الحالية إلى تحليل فعالية التنميط الجيني HLA-DQ لمرضى حساسية القمح في التنبؤ بنتائج التقييمات النسيجية والمصلية. شملت الدراسة ٩٠ مريضًا تم تشخيصهم سريريًا على أنهم يمتلكون مرض حساسية القمح وقيمت الخصائص الديموغرافية الحيوية لديهم، وتوزيعهم فيما يتعلق بالتحليل النسيجي لخزعة الاثني عشر، والتحليل المصلي للأجسام المضادة الذاتية لمرض حساسية القمح بالإضافة إلى وجود الأنماط الفردية HLA-DQ2 وHLA-DQ8 التنبؤ بالمظهر النسيجي والمصلي للمرضى. أشارت النتائج التي تم الحصول عليها إلى هيمنة الإناث على مرض حساسية القمح. كشفت الاختبارات الجينية أن غالبية المرضى (٧٧.٨٪) كانوا متجانسة الزيجوت HLA-DQ2 بينما أظهر التحليل النسيجي أن ٤٩ من ٩٠ مريضًا لديهم تصنيف Marsh III لضرر الاثني عشر مع ٢٥ مريضًا لديهم Marsh II .كانت الإيجابية المصلية للأجسام المضادة للجلوتاميناز (tTG)المضاد للأنسجة 41.1٪ مع ٦٣٪ من المرضى إيجابيين للأجسام المضادة IgG المضادة للجليدين(anti-gliadin) . لوحظ ارتفاع مستويات عيار الأجسام المضادة المرتبطة بالعمر في المرضى الذين تتراوح أعمارهم بين ٣٣ و٣٩ عامًا (٣٩٠.٠٤ ± ٦٧.٢١ وحدة / مل) و٢٦-٣٢ عامًا (٣٥٢.٦٢ ± ٥٠.١٢ وحدة / مل) لمضاد tTG IgA بينما ٢٦-٣٢ عامًا (٢٥٧.٤٠ ± ٤٩.٧٣ وحدة / مل)، ٤٠-٤٧ عامًا (٢٢٠.٠٤ ± ٢٤.٣٥ وحدة / مل) ثم ٣٣-٣٩ عامًا (٢١٤.٥٥ ± ٣٢.٤٠ وحدة / مل) لمضاد الغليادين (anti-gliadin).IgGكان معدل انتشار المرضى الذين يعانون من النمط الوراثي HLA-

DQ2 و HLA-DQ8 100٪ حيث تنبأت HLA-DQ2 السائدة بنتيجة Marsh II بنسبة ١٠٠٪ ، و Marsh III بنسبة ٧٥٪ ، ومضادة لـ tTG IgA بنسبة ٦٧.٨٪ ، ومضادة لـ tTG IgG بنسبة ٧٧.٨. ٪، مضاد الغليادين IgA بنسبة ٨٢.٩٪، ومضاد الغليادين IgG بنسبة ٨٦٪. تعزز هذه النتائج تأكيد فعالية تشخيص مرض حساسية القمح للتنميط الجيني HLA-DQ وتوصي باستخدام الاختبارات الجينية على المرضى المشتبه في إصابتهم بمرض حساسية القمح نظرًا لأن النتائج السلبية أو الإيجابية تستبعد مرض حساسية القمح أو تمنع التشخيص الخاطئ للمرضى المصابين بمرض حساسية القمح وليس لديهم اعراض المرض على التوالي.

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تقييم الأنماط الجينية HLA-DQ2 / DQ8 في المرضى الذين يعانون من مرض حساسية القمح وارتباطها بالأجسام المضادة للأنسجة ترانسكلوتامنيز والأجسام المضادة للكليادين

رسالة مقدمة الى مجلس كلية الطب \جامعة كربلاء كجزء من متطلبات نيل شهادة الماجستير في الأحياء المجهرية الطبية

من قبل

الاء جواد كاظم

بكالوريوس علوم حياة/١٩٩٩

دبلوم عالي علوم حياة/٢٠١٠

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2021 ميلادي

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