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Correlation of Serum Soluble Interleukin-2 Receptor and Interleukin-18 with Auto-antibody Profile In Patients With Celiac Disease In Karbala Province

A Thesis

Submitted to the Council of the College of Medicine and the Committee of Post Graduate Studies / University of Kerbala, as Partial Fulfillment of the Requirements for the Master Degree of Science in Medical Microbiology/Clinical Immunity

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صَدَقَ اللَّهُ الْعَلِيُّ الْعَظِيمُ

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We certify that this M.Sc. thesis titled

“Correlation of Serum Soluble Interleukin-2 Receptor and Interleukin-18 with Auto-antibody Profile In Patients With Celiac Disease In Karbala Province”

Was prepared under our supervision in the College of Medicine/ University of Kerbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology/Clinical Immunity.

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“ Correlation of Serum Soluble Interleukin-2 Receptor and Interleukin-18 with Auto-antibody Profile In Patients With Celiac Disease In Karbala Province ”

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DEDICATION

To our time Imam ; Al-Hujjah Ibn Al- Hassan "God accelerated his appearance".

To the soul of my father ….Who I hope he is happy for my graduation.

To my faithful husband, Assistant Prof. Dr. Ali Fadhil Alrammahi ....Who always standing next to me in every moment of this journey, his limitless support and encouragement made this work come into existence.

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SUMMARY

Celiac disease is an autoimmune chronic disease involving mainly the digestive system with variable degree of severity and type of presenting symptoms. It's triggered by ingesting gluten-containig food, found mainly in wheat, barley, and rye in the genetically predisposed people of all ages. For decades, the pathogenesis of celiac disease has been focused on and tried to connect with other human autoimmune disorders. Several factors are stimulating the onset of the disease including environmental, genetic and local immune disorders.

The aim of this study is to comparatively assess serum soluble interleukin-2receptor (sIL-2R) and interleukin 18 (IL-18) with anti-tissue transglutaminase (anti- tTG) and anti-deamidated gliadin peptide (anti-DGP) antibodies across biodemographic factors in patients with celiac disease.

The tissue transglutaminase(tTG) enzyme and deamidated gliadin peptide (DGP) are involved in the series of the immune processes associated with the celiac disease .The sIL-2R together with IL-18 are predictive markers for inflammatory process of small intestine in adult Iraqi people with celiac disease since both markers are upregulated in the disease. Histological examination for duodenal tissue biopsies which was considered as a cornerstone in the diagnosis of celiac disease for several decades was used in this study and by applying MARSH grading system in this examination.

Method: cross sectional study was carried out to test 45 patients with celiac disease of adult age group of both genders for serum level of the four markers using an ELISA technique then to be compared with 45 healthy controls comparable for age and gender. Endoscopic obtained biopsies from patient's duodenal tissues were examined histologically (by applying hematoxylin and eosin protocol) with a subsequent MARSH grading.

Results: High serum anti-tTG, DGP , IL-18 and sIL-2R concentrations were noticed in the majority of the patients with celiac disease, which was probably attributed to consumption of gluten in the diet of most of the candidates. This study demonstrates a significant statistical difference in serum levels of the above four markers among those patients in comparison to controls group ($p< 0.05$).There were significant correlations of anti-tTG,IL-18,and sIL-2R markers level with the different histological grades($p< 0.05$).

Conclusion: The inflammatory process associated with CD can be monitored by detecting the serum level of pro-inflammatory cytokines of the patients and so the physicians can predict the clinical outcome. This review also calls for investigating persons who have refractory anemia or unusual extraintestinal features. The usage of the serological inflammatory markers may reduce the need for application of the oesophageogastroduodenoscopy (OGD).

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List of abbreviations

Symbols	Descriptions
AGA	Anti gliadin antibody
AID	Autoimmune disease
Anti-DGP	Anti –deamidated gliadin peptide antibody
Anti-tTG	Anti-tissue transglutaminase
Anti-EMA	Anti endomysial antibody
APC	Antigen presenting cell
ATIs	Alpha-amylase/Trypsin inhibitors
ACG	American College of Gastroenterology
BCR	B-cell receptor
BACH2	BTB Domain And CNC Homolog 2 gene
BSG	British Society of Gastroenterology
CD	Celiac disease
CXCR3	Chemokine receptor 3
CFTR	Cystic fibrosis transmembrane conductance regulator
DGP	Deamidated gliadin peptide
DM	Diabetes Mellitus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor Receptor
EoE	Eosinophilic esophagitis
EoG	Eosinophilic gastritis
ESPGHAN	European Society of Pediatric Gastronterology Hepatology &Nutrition
FODMAPS	Fermentable oligo,di,monosaccharides and polyols
FasL	Fas ligand
FOXP3	Fork head box transcription factor P3
GA	Gluten ataxia
GAF-3X	Gliadin-analogous fusion peptide; consisting of 3 repetitive sequences
GFD	Glutamine free diet
GC	Gluten challenge test
GRND	Gluten related neurological disorders
Hb	Hemoglobin
HLA	Human leukocyte antigen

HMW	High molecular weight
HP	haptoglobin
HRP	Horseradish peroxidase enzyme
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL-2R	Interleukin -2 Receptor
IL-18	Interleukin-18
IL-8	Interleukin-8
INF γ	Interferon gamma
IEL	Intra-epithelial lymphocytes
IGIF	Interferon gamma inducing factor
IIFA	Indirect immunoflourscent assay
IDA	Iron deficiency anemia
ITP	Idiopathic thrombocytopenic purpura
JAK	Janus Associated Tyrosine Kinase
kDa	Kilo dalton
LMW	Low molecular weight
MAPK	Mitogen Activated Protein Kinase
MARSH	Mucosal Algorithm Rules for Scoring Histology
MYO9B	Myosin IX B gene
MYD-88	Myeloid differentiation-88 gene
MHC	Major histocompatibility complex
µL	Microliter
mL	Milliliter
MICA	MHC class I chain associated molecule
MS	Multiple Sclerosis
NCGS	Non celiac gluten sensitivty
ncRNA	Non coding Ribonucleic acid
NF-KB	Nuclear factor-KB
NK cells	Natural killer cells
NLRP	Node like receptor protein
NSAID	Non steroidal anti-inflammatory drugs
OGD	Oesophageogastroduodenoscopy
OD	Optical density
PARS 2	Protease activated receptors
Pi3K	Phosphatidyl inositol-3 Kinase
RCD	Refractory celiac disease
SS	Sjogren syndrome
SLE	Systemic lupus erythematosus
sIL-2R	Soluble Interleukin –2 Receptor
SHC	Src homologue and collagen protien

STAT	Signal transducer& activator of transcription
SYK	Spleen Tyrosine Kinase
TG2	Tissue transglutaminase 2 enzyme
TJ	Tight junction
TCR	T-cell recptor
TH1	T-helper cell type-1
TH-2	T-helper cell type 2
TNF	Tumor necrosis factor
TNF α	Tumor necrosis factor alpha
UNL	Upper normal limit
WGO	World gastroenterology organization
WDEIA	Wheat dependent exercise induced anaphylaxis
ZOT	Zonula occludens toxin
ZO-1	Zonula occludens -1 protien

CHAPTER ONE

INTRODUCTION AND LITERATURES REVIEW

Chapter One

1.1 Introduction

Celiac disease (CD) is an autoimmune gastrointestinal disease. It results from exposure to gluten, which is of high content of gliadin peptide. In the genetically susceptible persons, the intestinal enzymes are unable to digest the gliadin in the food. This may evoke an intestinal inflammatory reaction(Aronsson et al., 2019, Caio et al., 2019a).

Gluten is an ethanol soluble protein with resistance to digestion and constituted from several immunogenic peptides. Gliadins, the main component of gluten are complex proteins, rich in glutamines and prolines. Both of these two components are resistant to the intestinal digestive enzymes and consequently can trigger an intestinal inflammatory process that can be very harmful with a long standing complications. Gluten is found in several types of grains, including wheat, barley, rye and kamut (Caio et al., 2020) .

The incidence of celiac disease raised over the last few decades with a world prevalence of about 1-1.5 %,partially due to the increased sensitivity of the diagnosing tools and the change in the environmental response to the dietary gluten (King et al., 2020b, King et al., 2020a).The incidence of CD in the Arabian countries is about equal with the highest ratio in Saudi Arabia up to 3.2% (El-Metwally et al., 2020) .

Coordination of both genetic and environmental factors are important in the pathogenesis of CD. The well-studied inherited predisposition in gluten enteropathy disease is HLA-DQ2 and/or HLA-DQ8(Poddighe et al., 2020).This genetic specification is essential in the differentiation between the sporadic and familial celiac disease(Airaksinen et al., 2020). The identification of CD at early stage of the disease may prevent its complications which may be

serious like iron deficiency anemia and bone growth disturbances and infertility(Al-Toma et al., 2019) .Gluten free diet may prevent the development of small bowel adenocarcinoma as a late sequel (Caio et al., 2019b) .

The diagnosis of CD depends on clinical, serological and small bowel biopsy interpretation, while in suspected cases of CD the diagnosis needs HLA typing(Rubio-Tapia et al., 2013). Serological identification of autoantibodies against tissue transglutaminases (anti-tTG), gliadin (AGA) and other targets are considered as non-invasive effective methods of detection (Ylönen et al., 2020, Rossin and Piacentini, 2020). However, these antibodies have also been associated with other disease conditions such as liver dysfunction (Lauret and Rodrigo, 2013), inflammatory bowel disease, in Crohn's disease and ulcerative colitis(Alper et al., 2018) , peripheral and motor neuropathies (Hadjivassiliou and Croall, 2021), type 1 diabetes(Liu et al., 2014) , arthritis(Abbasi et al., 2017) , exercise induced anaphylaxis(Geller, 2020), and alcohol abuse(Gili et al., 2013). For these reasons, histopathological analysis of biopsy specimen (Duodenal /or jejunal biopsy) from the small intestine remains the gold standard for detection of CD enteropathy inspite of the progress of the serological tests. Villous atrophy, increased intraepithelial lymphocytes and crypt hyperplasia are the main histological changes of small bowel in CD(Al-Toma et al., 2019, Alina et al., 2020, Husby et al., 2020). Recently,the antigliadin antibody (AGA) has poor sensitivity and specificity for celiac disease and is no longer used. However, the sensitivity, specificity, and accuracy of anti-deamidated gliadin peptide (anti-DGP) antibodies has been reported as a better diagnostic test for celiac disease than the conventional gliadin antibody testing(Rubio-Tapia et al., 2013). Native peptides or peptides deamidated by tissue transglutaminase (tTG) bind to the positively charged human leukocyte antigen (HLA)-DQ2 or DQ8 molecules expressed on the outer membrane of antigen-presenting cells (APC)and are recognized by CD4+

T cells. T-cell activation results in release of interferon gamma (INF- γ)and destruction of the epithelium.

Cytokines play a significant role in the pathogenesis of CD as the activation of T-cells leads to the production of interleukin (IL)-2, its receptor IL-2R and IL-18 amongst many other soluble factors, which drives the proliferation of Th2 cells as well as activation of B cells, natural killer (NK) cells and macrophages(Wosen et al., 2018) . Moreover, the predictive usefulness of serum cytokines in distinguishing between CD and non- celiac gluten sensitivity have been highlighted in a recent study(Masaebi et al., 2020) , thus implying that these cytokines could be potent markers of CD with diagnostic significance and can be used for monitoring the activity of the disease and the status of gluten free diet of patients. IL-2 and its receptor (IL-2R) together with other inflammatory markers were elevated in the CD, especially in the non GFD cases (Tye-Din et al., 2020, Scherf et al., 2020).

Interleukin-18 is a pro inflammatory mediator and was studied thoroughly in cases of infection, autoimmune diseases (AID) and tumor immunity. It's expressed in several cell types (Rex et al., 2019, Dixon and Kuchroo, 2020). Recently,IL-18 was seen to promote T-helper cell(Th1) interferon-gamma and affects the Th1 cell polarization (Delbue et al., 2019) (Domsa et al., 2020).
The inclusion of serological detection of sIL-2R and IL-18 would be advantageous in strengthening the diagnostic and follow-up evaluation of patients with CD in clinical settings and minimize the need for intestinal biopsy.

1.1.1 Hypothesis

i. Do the serum levels of sIL-2R and IL-18 correlate with levels anti-tTG and anti-DGP antibodies with respect to biodemographical factors in patients with CD?

ii. Can serological assessment of sIL-2R and IL-18 augment the measurement of anti-tTG and anti-DGP antibodies in the diagnosis and monitoring the severity of CD in those patients?

1.2 The aim of this study

To comparatively assess serum sIL-2R and IL-18 with anti-tissue transglutaminase (anti- tTG) and anti-deamidated gliadin peptide(anti-DGP) antibodies across biodemographic factors in patients with celiac disease.

1.2.1 Study objectives

This study is carried out to achieve the following objectives:

i. To measure levels of sIL-2R and IL-18 in serum from patients with CD and control participants using ELISA techniques.

ii. To measure levels of anti-tTG and anti-DGP antibodies in serum from patients with CD and control participants using ELISA techniques.

iii. To correlate the levels of sIL-2R and IL-18 obtained with those of anti-tTG and anti-DGP antibodies across different collated biodemographical data using statistical analytic tools.

1.3 Literature Review

1.3.1 General view of Celiac disease

Celiac disease(CD) is a chronic immune mediated gastroenteropathy affecting genetically susceptible individuals (Tye-Din et al., 2018, Valitutti et al., 2019). It happens when the susceptible person ingest foods or drinks containing gluten material (Elli et al., 2019, Sollid et al., 2020) .Such relationship was discovered too early since 1950s(Ludvigsson et al., 2014).

The rapid development of the modern life and the economic improvement was noticed, especially in the last half of the last century. This was associated with a shift in the human life style, including the type of food. Such change in the diet habits of the humanity was observed to be cooperated with the a rise of many health problems, including obesity, hypertension, autoimmune disorders (AID), cardiac diseases and cancers (Goyal et al., 2015, d'Angelo et al., 2019).

Nutritional therapy is one of the strategies to manage several diseases, including celiac disease, so prohibiting the ingestion of such foods play a central role in treating the disease and preventing the rise of complications. This needs a good planning to produce healthy foods for patients having CD with a focus on the age of such individuals whether they were elderly, young or infants(Jnawali et al., 2016, Wei et al., 2020).

The ingestion of gluten present in the wheat, rye, barley kamut and other products induces an autoimmune reaction in the genetically predisposed person especially with HLA-DQ2 and HLA-DQ8.This response exaggerates both intestinal and extraintestinal changes (Werkstetter et al., 2017, Wei et al., 2020). Serological tests, like Anti-tissue transglutaminase (tTG) and Anti-deamidated gliadin peptide (DGP) antibodies were discovered to diagnose CD(Ylönen et al., 2020).

1.3.2 Epidemiology

Celiac disease is a global health disaster with a prevalence of small bowel biopsy proved cases reaches up to 1%(Stefanelli et al., 2020).The prevalence is variable among different countries ,partly due to the controversy in the health system of these countries and the number of the diagnosing investigations done(Fasano and Catassi, 2012, Du et al., 2018) .

There is wide variation in the geographical distribution. The general idea is that CD is more common in western countries with a prevalence1% of general population, with a noticeable increase in the annually recorded cases especially in pediatric age group, partly due to, the increasing tests sensitivity, change in the type of feeding and the increased rate of associated gastrointestinal manifestation(Namatovu et al., 2014).

Western countries are considered as the historical sites of celiac disease cases due to the ingestion of the gluten staple food. Among European countries, there is unexplained variation in the prevalence of the disease in spite of the similarities in the rate of gluten ingestion and genetic susceptibility.(Catassi et al., 2014).

In the United States of America ,the observed incidence still ranges 0.4-1% of the general population, with some differences from the European qualifications of diagnosing CD (Badizadegan et al., 2020).

In Asia, which represents 60% of earth population ,there is an increasing incidence of CD (with differences among the Asian countries) and it's believed to be a large reservoir of undiagnosed cases(Singh et al., 2016, Agarwal et al., 2020) .

At first ,the countries of the middle East were considered as low prevalent for the celiac disease, but with the introduction of the s serological test(anti tissue transglutaminase (tTG) and the anti-endomyseal antibodies),the incidence

raised to a level similar to that observed in the western countries(Parzanese et al., 2017).

In Saudi Arabia, the prevalence is about 1% in biopsy proved cases and as in other areas of world, the real fact can be higher because of the underestimated cases(Al Hatlani, 2015, Al-Ajlan, 2016).

In Iraq limited Researches were done and showed that celiac disease ranges between 10% to 11.2% in type 1 diabetic children(Mansour and Najeeb, 2011). Another study illustrated that CD records 12.1% in Iraqi patients with irritable bowel syndrome (Ibrahim, 2020).

The general speaking is that CD cases are represented by an iceberg where the symptomatic discovered patients are above the sea level and the hidden undiscovered, which are the majority, locate below the water line, as illustrated in Figure 1.1(Nenna et al., 2013).

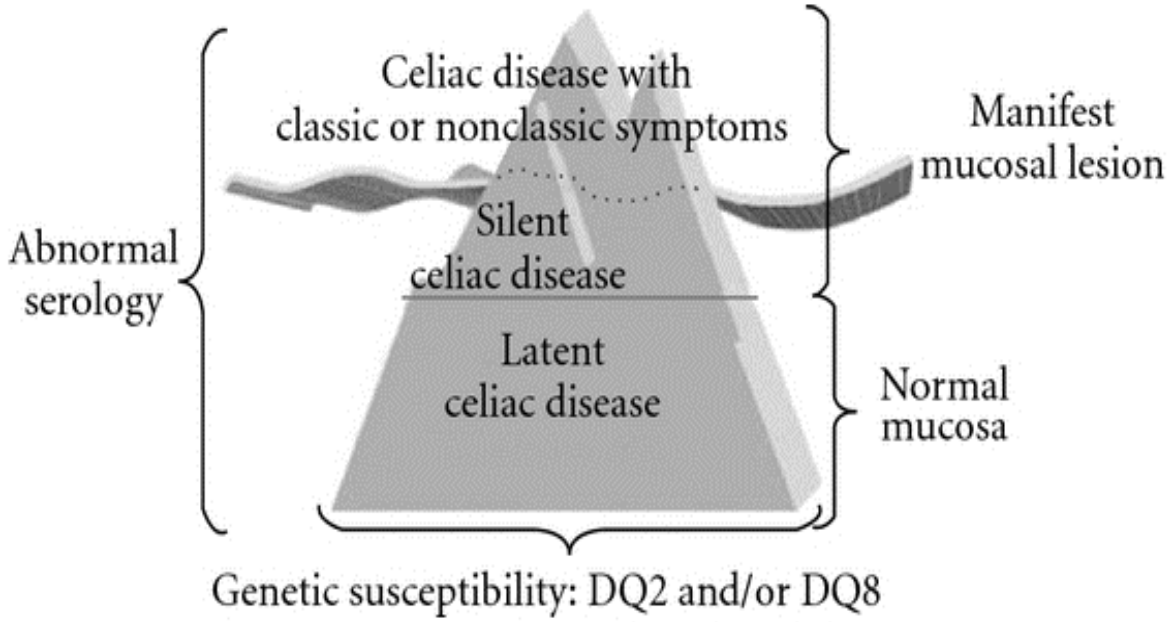

Figure 1.1: Celiac disease is represented as an iceberg with the most cases are underdiagnosed (Admou et al., 2012).

Regarding age incidence, CD can be seen at any age but most cases are diagnosed during the childhood after the 2nd year of age (King et al., 2020a)or

in the third decade of life (adulthood)(Śmigielski and Gmitrowicz, 2014, Pastore et al., 2019) .The progress in the diagnosing serological and biopsy tools increased the number of the cases, especially those with subtle manifestations. This fact also changed the percentage of cases diagnosed in the adult and elderly persons. The cases detected in the elderly group rises a contemplating question whether these cases are because of late diagnosis or truly developed in this late age group and the discovery of such cases may be attributed to the late diagnosis or being the person of little or no frank gastrointestinal features(Ludvigsson et al., 2014, Dumic et al., 2019) .Studies have shown that genetically-susceptible infants ingesting foods with even small amounts of gluten rises the chance of developing of symptomatic celiac disease at later life(Vriezinga et al., 2014b).In Iraq the incidence of celiac disease is higher in children(Hameed et al., 2016).

Concerning the gender differences in celiac cases, the celiac disease is registered more in women with a female to male ratio reaches up to 3:1(Pulido et al., 2013).Hameed and his team also documented that CD is more commen in women than men (Hameed et al., 2016).Other articles reported female to male ratio 2.7:1(Abadie et al., 2011).

1.3.3 Etiology of celiac disease

The association between the malabsorption and the ingestion of dietary gluten was noticed since the 19th century. At first, the disease was named as Gee-Herter disease in relation to the two scientists who displayed some features of the disease in the middle of the 20th century(Makharia et al., 2012).It's well known that oral gluten intake is not the only blamed factor but association with immune disorders and genetic susceptibility(Ontiveros et al., 2015).

1.3.3.1 Environmental factors

The ingestion of gluten is the principal environmental pathogenic operator the development of celiac disease. Some facts were noticed regarding the possible effect of the environmental agents:

1-The increase of the western countries prevalence of CD during the last two decades of the last century can't be explained alone by the progress of the diagnosis methods (Singh et al., 2018) .

2-The better care methods of the infants has reduced the incidence of the gastrointestinal infection which reflected on the better control of CD (Gujral et al., 2012).

3-About the time of having gluten in infants, Observational studies concluded that timing and dosing of gluten ingestion for the first time of life may affect the onset of symptoms and the rise of complications the celiac disease, mainly in the period of the first 16-24 weeks of life, especially in those with positive family history (Vriezinga et al., 2014a).

4- Although the great beneficial role of the breast feeding to the newborn baby, including the passive introduction of probiotic but there is still controversy about whether the breast feeding have a reducing influence on the development of celiac disease especially those who faced low gluten diet in the first 6 months of life (Størdal et al., 2013, Lionetti and Catassi, 2015) .

5-Infections: in the early infancy, mostly gastrointestinal or respiratory type may have a pertinent relationship with the triggering or accelerating the development of celiac disease. This can be explained by the increased level of interferon-1 level, destruction of intestinal epithelial barrier and the similarity between the microbial and the gluten peptide with an alteration in the gene expression of intestinal mucosa (Canova et al., 2014, Beyerlein et al., 2017) .

6-Intestinal Microbiota status: The normal gut microbiota has specific job in human food metabolism, drug metabolism, keeping of structural safety of the gut mucosal barrier, immunomodulation, and protection against ingested microbial infection(Jandhyala et al., 2015).The change in the intestinal commensal microbiota had been reported both in the children and adult CD cases (Nistal et al., 2012, Wacklin et al., 2013) .

7-Bottle feeding: The colonization of the human intestine has a variable physiological effects and has modulation role of the intestinal immunity.In the infants with breast feeding, the microbiota is less diverse than the bottle-fed babies and is more predominant with *Bacteroides uniformis*(which shows a greatest degree of intestinal protection) at 1 and 4 months of age, while formula-fed infants had a higher prevalence of *B. intestinalis* (Sánchez et al., 2011).

1.3.3.2 Certain cereal ingestion

Different wheat (and other seeds) components can induce immune-mediated disorders in certain population.

1.3.3.2.1 Gluten

Starch comprises about 60-75% of grains while the protein accounts for only 8-15%, in spite of that the wheat protein plays an important role in getting the dough properties. Gluten a mixture of gliadins and glutenins, is considered major storage protein in the cereals like wheat, rye and barely. The general composition of wheat is demonstrated in Figure 1.2 (Biesiekierski, 2017). Gliadins are alcohol-soluble single polypeptides consisting from 250-650 amino acids with a molecular weight ranges from 30000 to 75000.There are four electrophoretic subtypes of gliadin;α,β,γ and ω . These subtypes are with

similar epitopic characters leading to form undifferentiated antibodies (Ozuna et al., 2015).

Gluten is responsible for the viscoelasticity of the dough made from the wheat(Barker and Pilbeam, 2015).

The protein portion of the seed constitutes of about 8-15% of flour weight and it comprises different types like albumins, globulins, gliadins and glutenins, according to their solubility basis (Sharma et al., 2020). Glutenin provides dough extensibility and presents a polymer bound by disulphide bonds and has two subtypes HMW and LMW glutenin. Wheat proteins are also names as prolamins because a of the high content of prolamin and glutamic acid types of amino acids(García-Molina and Barro, 2017). Generally speaking, gluten can comprise all grain proteins (prolamins) and can refer to the material that triggers the immunotoxicity in certain persons with gluten-sensitive disorders that include: allergic(wheat allergy),autoimmune(celiac disease, dermatitis herpitiformis and gluten ataxia) and immune-mediated(non-celiac gluten sensitivity)(Sollid et al., 2012).

Different strategies have been postulated to reduce the antigenicity of the gluten including reducing the CD epitope of bread (ZHANG et al., 2018) or other mechanisms like manipulating the molecular biology, probiotic ingestion or nano technology (Scherf et al., 2018, Yoosuf and Makharia, 2019).

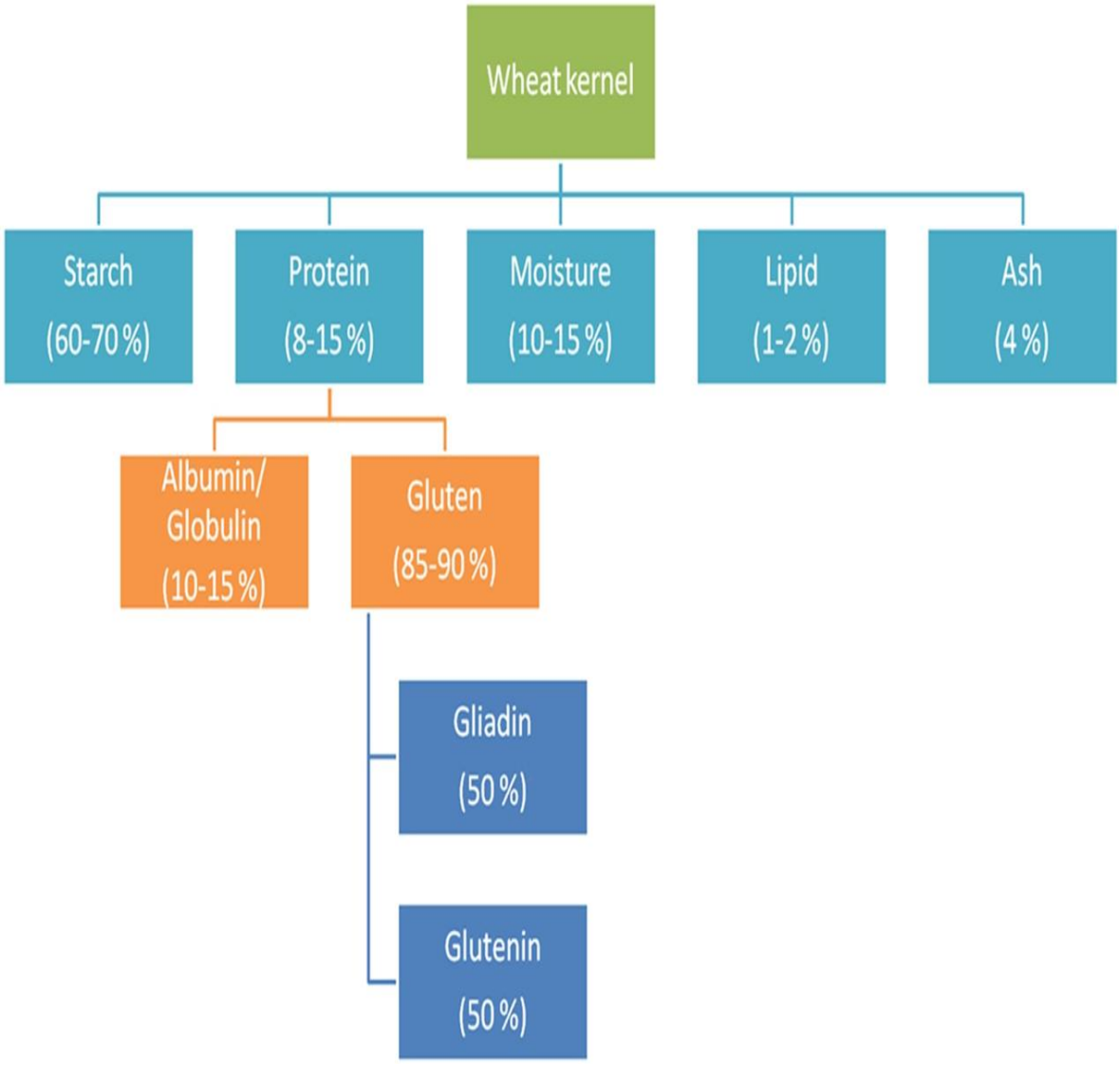

Figure 1.2: The composition of wheat (Biesiekierski, 2017).

The widely- studied wheat proteins;gliadin and glutenin have specific portions that are blamed to induce the process of celiac autoimmunity.These specific amino acid sequences are the epitopics for CD, they are called immunogenic(toxic) peptides and are resistable to digestion by the gastrointestinal tract enzymes (Singh et al., 2018), mapping of α-gliadin polypeptide is shown in the below Figure 1.3 (Fasano, 2011).Wheat,barley and rye are the most well-konown crops that contain the gluten proteins and thus can induce the process of CD(Scherf et al., 2016).

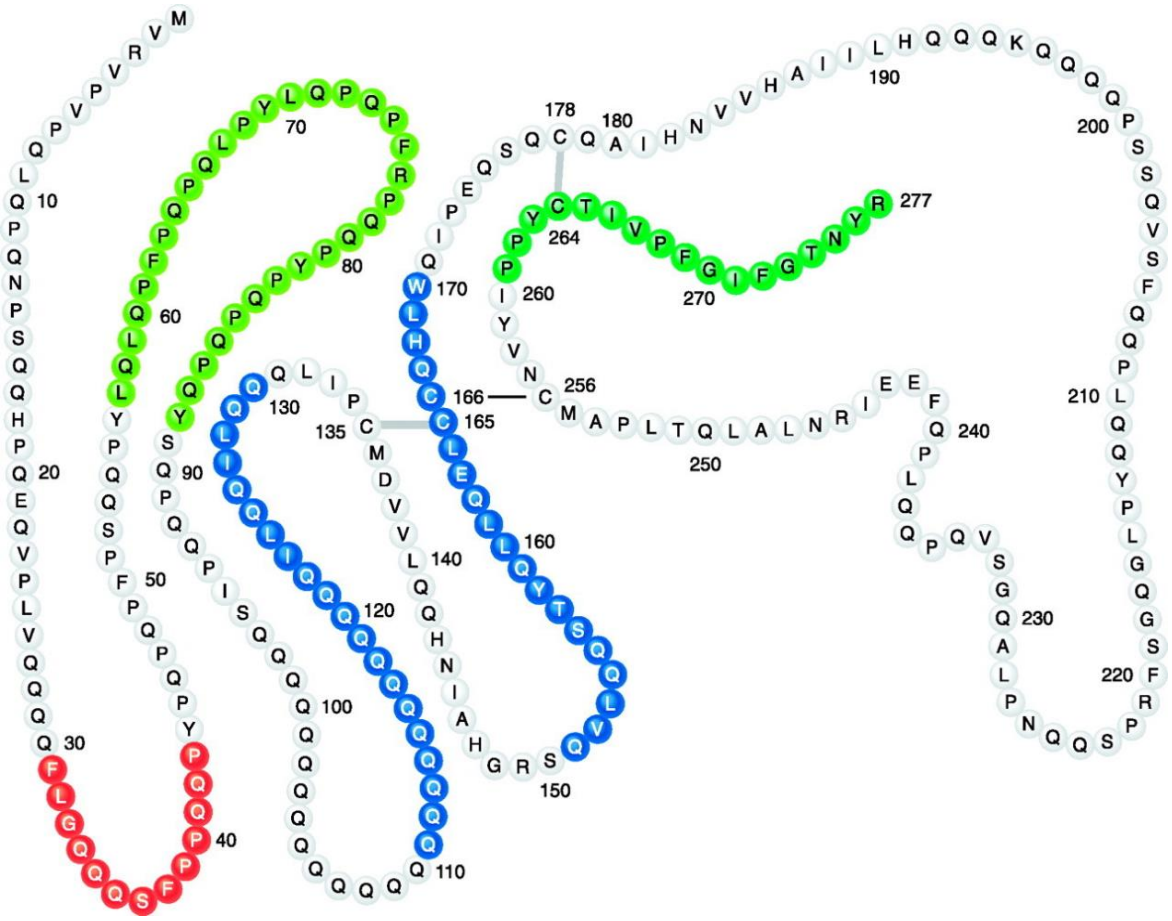

Figure 1.3: Mapping of α-gliadin polypeptide: red part=cytotoxic activity ,light green part = immunomodulatory portion, blue chain= zonulin release and gut-permeating activity , and dark green chain=CXCR3-IL-8 release in CD patients (Fasano, 2011).

There is gluten in the oats, instead have other prolamins called avenins. Oats have few CD toxic peptides that may induce intolerance in some persons when having little oats amount(Gilissen et al., 2016). **2**

1.3.3.2.2 Alpha-amylase/Trypsin Inhibitors(ATIs) and Lectins

ATIs and Lectins are compact albumin proteins and constitutes about 2-4% of total wheat protein(Zevallos et al., 2017).Recently ATIs have been discovered to be involved in the wheat allergy,since they can activate the antigen presenting cell (APC) in the intestinal mucosa with a subsequent release of inflammatory mediators like interlukine IL-8 and tumor necrosis

factor(TNF).ATIs are implicated mainly in the development of extra-intestinal symptoms of non celiac gluten sensititivty(NCGS) and in Baker's asthma(Fasano et al., 2015, Leccioli et al., 2017).

Lectins induce intestinal enterocytes damage with a subsequent decrease of nutrient absorption(Biesiekierski et al., 2013).

1.3.3.2.3 FODMAPS

Fermentable olig, di,monosaccharides and polyols (FODMAPS) are short chain carbohydate and there are no human gut enzymes to digest the FODMAPS so they pass to the large intestine where they are fermentated by the colonic bacteria,producing gases.Removal of these materials from food was shown to improve patients with NCGS and irritable bowel syndrome(Altobelli et al., 2017).

1.3.3.3 Genetic Susceptibility

It’s well known that CD is an autoimmune disease with an environmental participation that occurs only in the genetically susceptible persons.CD runs in families, the first-degree relatives have a chance to get CD about 7.5% (Singh et al., 2015), with elevated compact constructed in monozygotic twins of 83.3% for CD emergeance, a percentage greater than that of other AID (Bogdanos et al., 2012) .While for the second-degree relatives a lower incidence might be present (Ludvigsson et al., 2014).

Celiac disease is an immunological disorder that has a complicated way of heredity represented by MHC and non-MHC loci. The MHC region presents on chromosome 6. Heterodimeric class II human leukocyte antigen(HLA) genes, especially HLA-DR and DQ are more linked with CD (Farina et al., 2019) .

About 90% - 95% of patient with celiac disease have one or two copies of HLA-DQ2 (variant HLA-DQ2.5).The remaining affected patients about 5-10%

possess DR4-DQ8 and express DQ8 molecules(Aggarwal et al., 2012, Kurppa et al., 2012, Sollid et al., 2012).Celiac disease has increased association with other autoimmune diseases like type I diabetes Mellitus(DM),such genetic association was demonstrated in figure 1.4 .

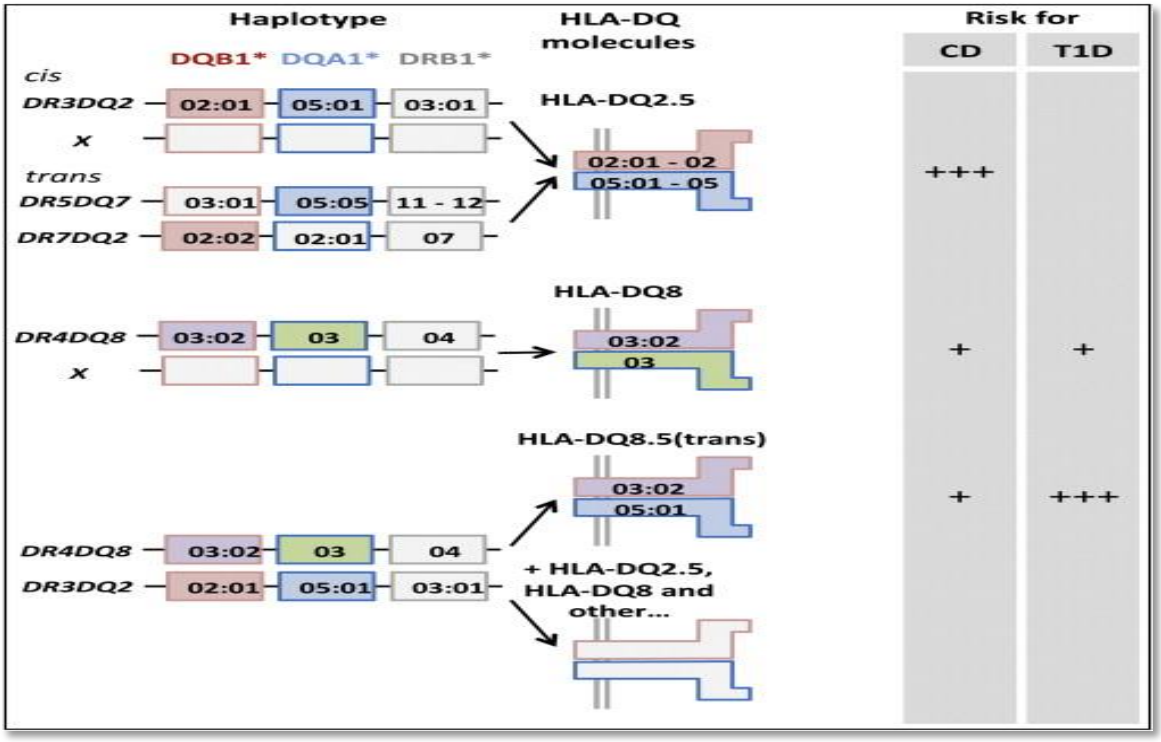

Figure 1.4: Class II HLA association with celiac disease and type I DM (Aggarwal et al., 2012).

The encoded proteins, α- and β-heterodimers are present as surface receptors on antigen presenting cells(APC) ,forming clefts that bind gliadin particles found in the food as shown in Figure 1.5 (Al-Hussaini et al., 2018).

The presence of HLA-DQ2 and /or –DQ8 favorite the diagnosis of celiac disease in the suspected cases(Catassi and Catassi, 2018).It's believed that the frequency risky celiac –related HLA genotyping is about 30% but only 1-3% will develop the celiac disease (Parzanese et al., 2017).

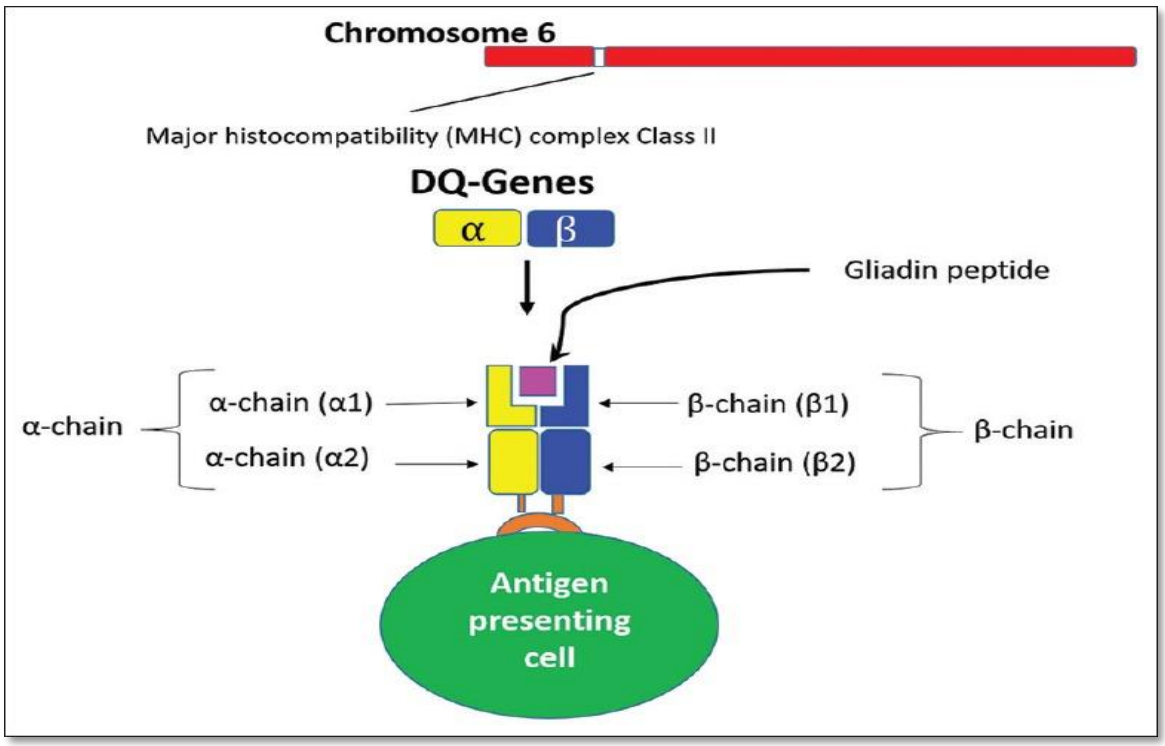

Figure 1.5: DQ-genes and their molecules expression on the APC (Al-Hussaini et al., 2018).

In Iraqi CD cases, HLA- DQ2 genotype is the most prevalent genes. On the other hand, DRB1*04 alleles were shown to have a turn in celiac disease evolvement, therefore negative results of DQ2 and /or DQ8 don't eliminate celiac disease(Hameed et al., 2016).

Concerning MHC class I , HLA-A25 was declared only in Turkish children(Barada et al., 2012, Gujral et al., 2012). While HLA B8 had been connected to CD in Iraq , Turkey and Algeria.Other MICA allele was recorded to be over-expressed in atypical CD Saharawi people (Gujral et al., 2012, Singh et al., 2016).

Recently, it was demonstrated that 39 non-HLA loci can also predispose to celiac disease (Trynka et al., 2011).One of these non-HLA effective genes locates on chromosome 19, is the myosin IXB gene (*i.e.*, MYO9B).This can predict the responsiveness to a gluten-free diet (GFD)(Gagliardi et al., 2021).

More recent, a novel emerging scope is developed, which explains that the alterations in gene expression or cellular phenotype can be evoked by epigenetic modifications, such as methylation or ncRNAs (transcribed non-translated RNA molecules). The two share in the regulation of gene expression next to transcription (Remely et al., 2014, Perry et al., 2015). Other article has clarified an influence of microbiota on the level of methylation of the free fatty acid receptor 3 gene participated in metabolic process in addition to inflammatory response (Remely et al., 2014).

1.3.4 Pathophysiology of Celiac Disease

There is a complex interplay between the patient polygenic status and the ingestion of gluten which leads to a disturbance in the intestinal epithelial barrier with a subsequent induction of gut inflammatory process (Gujral et al., 2012) .

The pathogenesis of CD starts when there is an intestinal immune response evoked by the permeation of the ingested toxic gluten particles through the barrier of the small intestine,either by the paracellular pathway controlled by the tight junctions,or trancellularly by the action of endocytosis(Zimmermann et al., 2014).

The digestion of dietary protein is accomplished by a cascade of digestive processes, begins by the gastric and pancreatic proteases.Consequently the peptides are hydrolized by the intestinal peptidase located on the enterocytes brush border.Since proline are resistant for digestion by proteases,proline-rich glutens are inaccessible to proteolytic beakdown (Fasano, 2011) .Subsequently, after metabolism by the enzymes,the peptides (antigens) can cross the intestinal epithelial layer either paracellularly or trancellularly.The inter-epithelial tight junction regulates the paracellular pathway and allows only the escape of the

small molecular weight peptides(not more than 5.5K Da)(Semova et al., 2012) .Alternatively,larger molecules can pass transcellulary by the transcytosis method (Perrier and Corthesy, 2011) .

All the types of gliadin (α, β, ω and γ) are considered to be immunogenic for CD but the 33-mer peptide of α 2-gliadin is known to be the most powerful stimulator of mucosal T-lymphocytes after deamidation by the enzyme of tissue transglutaminase2(TG2)(Ludvigsson et al., 2014). Alpha-gliadin copy number is about 25-150 genomic copies(α-gliadins from the D-genome are the most potent immunogenic(Scherf et al., 2016). γ-gliadins is in the 2nd rank of immunogenicity with a lower number of copies,15-40(Gil-Humanes et al., 2012).

An absolute fact is that stimulation of CD occurs with having gluten in individuals who carry specific MHC haplotype DQ2&DQ8 in the HLA.The HLA-DQ2 &DQ8 are cell surface molecules present on the APC (antigen-presenting cells) that found in the intestinal mucosa. These cells have positively charged pockets with preferable joining to negatively charged molecules; and to the gluten particles which carry negative charges (Kupfer and Jabri, 2012). The negativity of the gluten particles is achieved when these undigested gluten fragments pass through the mucosal barrier into the lamina propria where they are deaminated by the enzyme TG2 which changes the glutamine into glutamate and so acquiring the negative charge of the gluten particles(De Re et al., 2017), the highly ubiquitous enzyme TG2 catalyses the irreversible crosslinking of glutamine residue with a lysine residue as well as hydrolysis of peptide bound glutamic acid or glutamine on the fragment coverting it into glutamate, both being processes that enhances the immunogenicity of the gliadin peptides (Martucciello et al., 2020).

The binding of the negatively charged gluten peptides created by the action of TG2 bind it to the positively charged amino acid on HLA-DQ2 and DQ8

,thereby stimulating both adaptive and innate immune response in the CD patients (Sollid et al., 2012) .

After binding the native peptides or peptides deamidated by TG2 to the positively charged HLA-DQ2 or DQ8 molecules expressed on the outer membrane of the APC (macrophages ,dendritic or B-cells) and are recognized by CD4+ T cells causing T-cell activation that results in release or production of different types of cytokines mainly interferon-γ. These cytokines can affect the scenario of events in CD in two pathways:

First, they activate T-helper 1(TH-1) cells to produce IL-15 and IL-21 which subsequently induce the intra-epithelial(IEL) cytotoxic CD8+ lymphocytes leading to intestinal inflammation and damage. The increased activity and number of these IEL is one of the important histological features accompanying the CD(Pozo-Rubio et al., 2012, Quinn et al., 2015) ,also activation of TH-1 results in production of IL-2, its receptor IL-2R and IL-18 amongst many other soluble factors, which drives the proliferation of Th2 cells as well as activation of B cells, natural killer (NK) cells and macrophages (Wosen et al., 2018).The second lane is the activation of the T-helper 2(TH-2) cells causing B-cell differentiation with a following production of the anti-gliadin or anti-DGP, anti-endomysium and anti-tTG autoantibodies that are useful in the serological diagnosis of CD(Hardy and Tye-Din, 2016, Torsten and Aaron, 2018).

Studies on the pathophysiology of CD have provided increasing evidence of CD4+T cell mediated hyperactivity in tissue injury. Activation of CD4+ T cells from the lamina propria induces the synthesis of IL-2 as well as the expression of its specific receptor, the IL-2R. The binding of IL-2 to IL-2R leads to stimulation of T cell proliferation, and cascades of immunological responses including activation of B lymphocytes and macrophages (Jabri and Sollid, 2017). Apparently, sIL-2R has been identified as the marker of immune

activation as studies have provided evidences of heightened mucosal lymphocyte activity in patients with untreated CD with high serum levels of sIL-2R pointed as the major culprit (Abbas et al., 2018).

Another cytokine highly associated with CD pathophysiology is the IL-18, a proinflammatory cytokine produced by antigen presenting cells. Recent studies have shown that IL-18 specifically binds to receptors preferentially expressed on T and NK cells where it acts as a co-stimulant for Th1 cells to augment IL-2, granulocyte macrophage-colony stimulating factor (GM-CSF) and soluble IL-2R production, induce cell proliferation and enhance T and natural killer (NK) cell maturation and cytotoxicity (Wosen et al., 2018). Also, the mucosa of patients with CD have been shown to contain high levels of IL-18 and its associated Th1 factors, which are reduced after a period of gluten-free diet(Mormile and Vittori, 2013, Mormile, 2016b).

The pathogenesis of CD is simply summarized by the Figures(1.6,1.7and1.8).

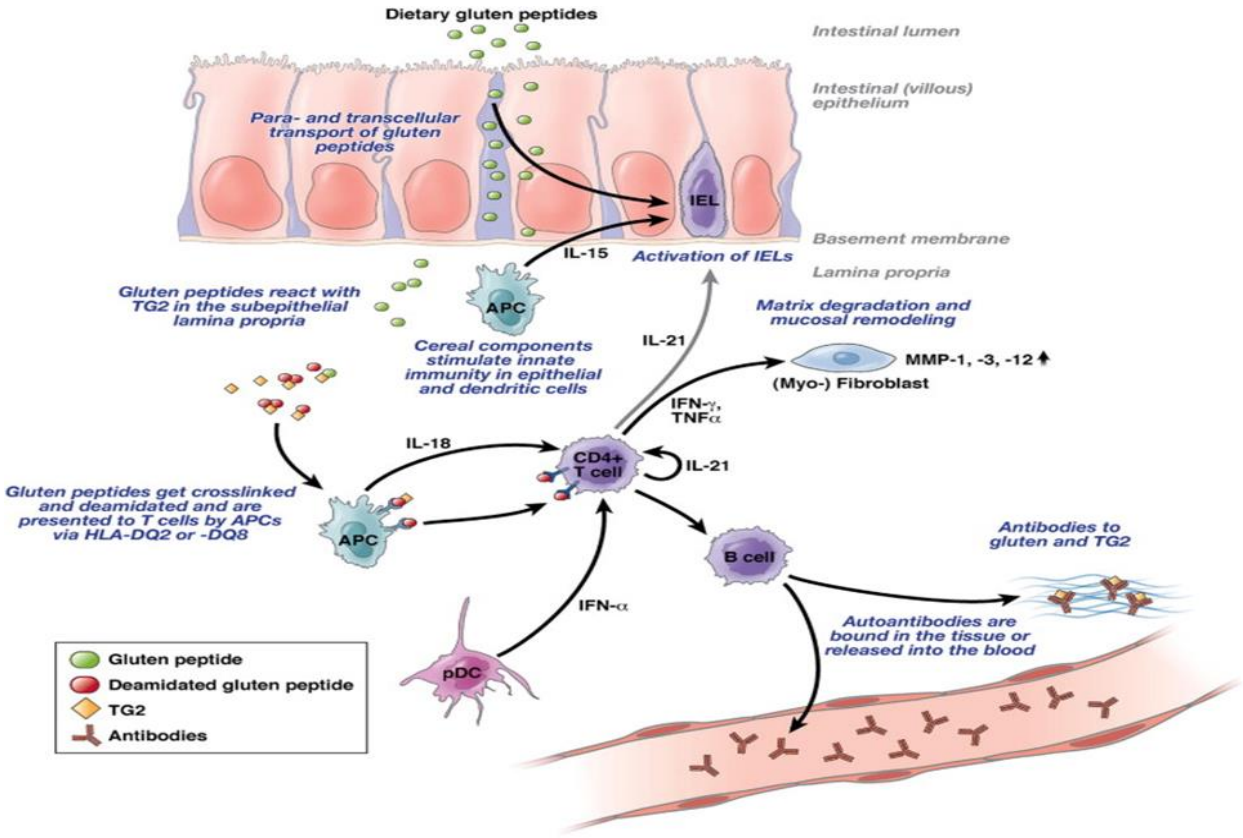

Figure 1.6 : Celiac disease is caused by an abnormal immune response to the gluten peptides in the upper small intestine (Schuppan et al., 2009).

The new advance is the discovery of the pivotal role of BACH 2 gene in the manipulation of the inflammatory process of celiac disease as shown in the Figure 1.7. BTB Domain And CNC Homolog 2 gene (BACH2) is a transcription factor that is important in the germinal center formation and control of both B- and T-cells immune balance in the intestinal wall to counterstain the excessive immune stimulation and inflammation. Recently, it was illustrated that γ-INF can reduce the activity of BACH2(Roychoudhuri et al., 2013).

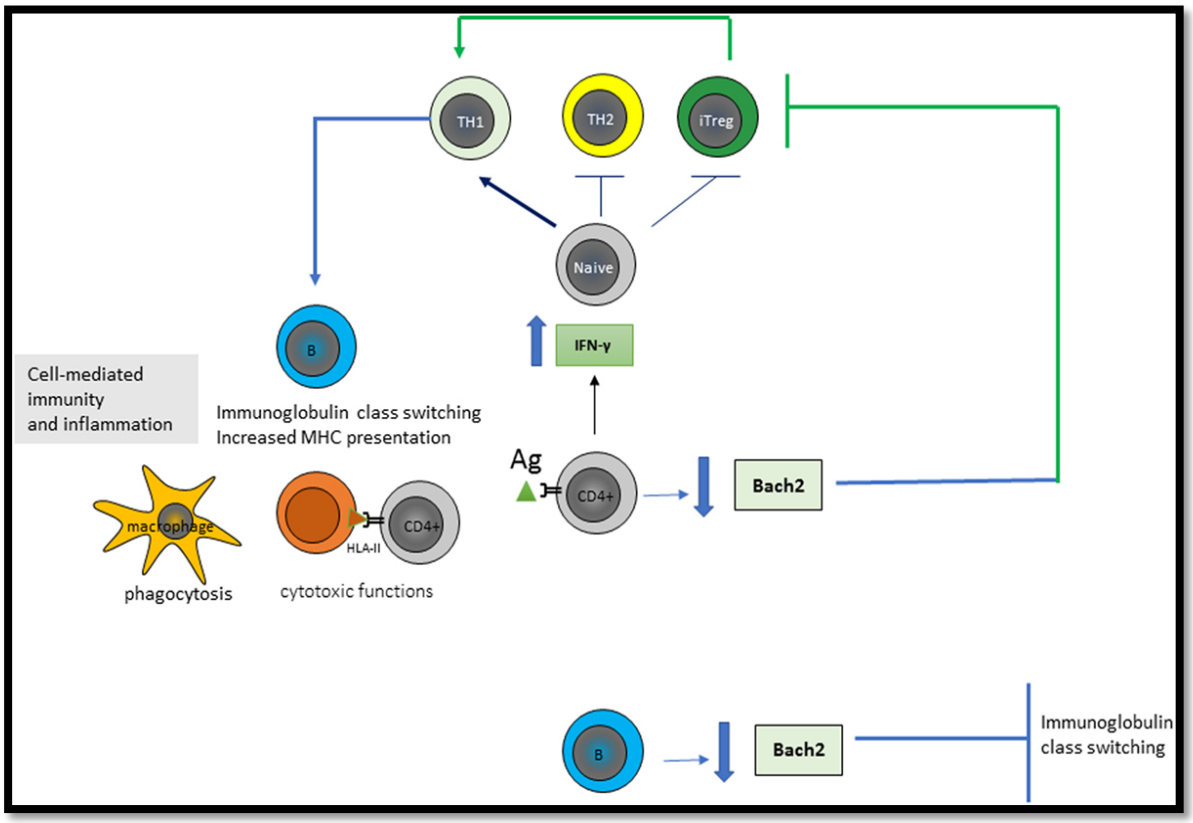

Figure 1.7: The main steps in the immune stimulation of CD (De Re et al., 2017).

It is important to mention some facts about the tissue transglutaminase 2 enzyme.TG2 is abundant both intracellulary and extracellulary. Intra- cellularly ,it's enzymatically inactive ,but it becomes active when moves to the extracellular compartment. It's a Ca^{+2} dependent enzyme and has a role in the deamidation of the gliadin particles in the small intestine.Tissue transglutaminase 2 enzyme(TG2) is regulated by several factors, and γ-INF is a

well-known stimulator, hence contributing to the exacerbation of CD. High level of autoantibody against TG2 is not specific to CD and can be detected in other disease like inflammatory bowel diseases(Alper et al., 2018), liver dysfunction (Lauret and Rodrigo, 2013) ,type 1 diabetes (Liu et al., 2014) and others. TheTG2-gliadin complex will bind to B-cell receptor(BCR) and then present this combination to the T-cell.T-cell activates the B-cell to transform into plasma cell with production of anti-TG2 IgA and anti-TG-2 IgG immunoglobulin (Chaudhary and Wesemann, 2018) as illustrated in the Figure 1.8.

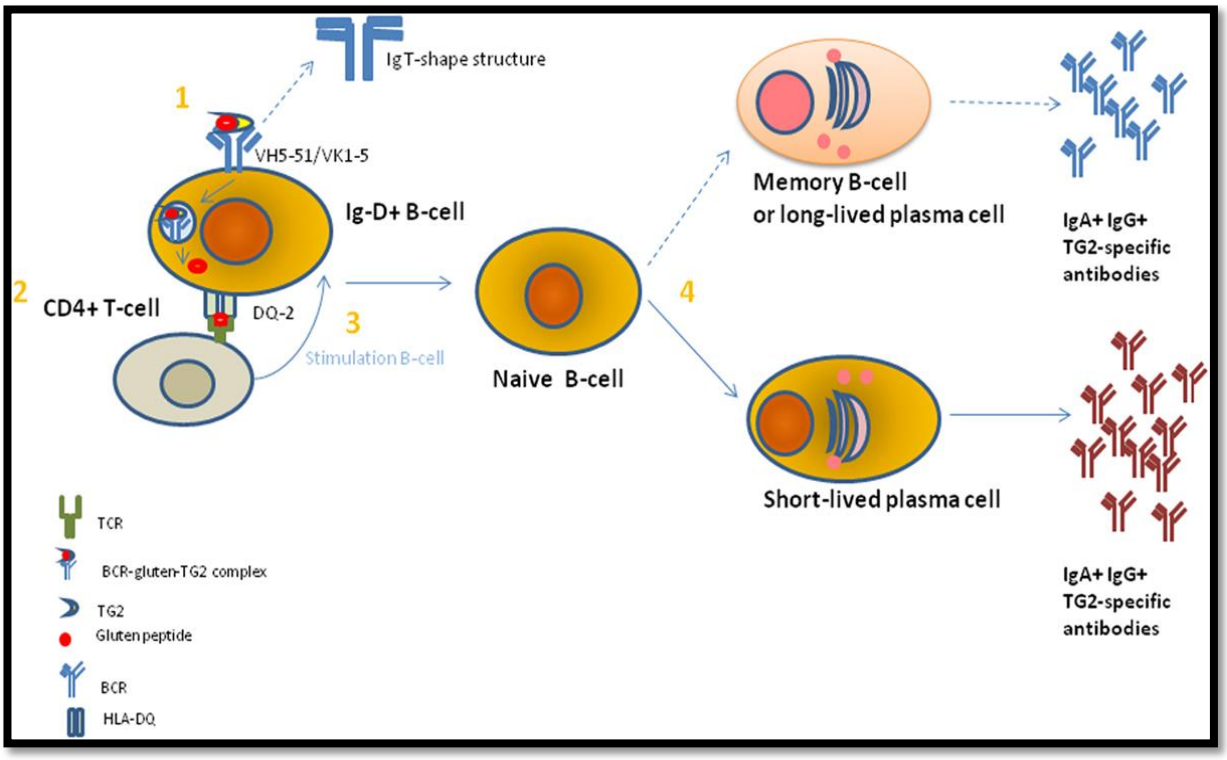

Figure 1.8 : Production of TG2 autoantibody in relation to gliadin (De Re et al., 2017).

It was observed that the B-cells will be reduced in number if the CD patients were on gluten free diet and B-cell depletion is one of the expected novel therapy in this disease(Nikiphorou and Hall, 2014).

It's obligatory to mention few words about the intestinal physical barrier and permeability since these two terms play a pivotal role in the pathogenesis of CD and other inflammatory bowel disease.The intestinal barriers extends over

a surface area about 400 m^2 and this costs the body about 40% of facts to it's energy.All these facts are to prevent the loss of water and electrolyes and the entrance of food and germs antigens into the body(Brandtzaeg, 2011) .There is a robust correlation between the intestinal barrier and the intestinal permeability,so to reach a peacefully state with the gut symbionts without creating chronic inflammation and to create a defensive mechanisms against the entrance of microorganism and their toxins (Hooper et al., 2012). Figure 1.9 summerize the relationship between the heads of the triangle (intestinal permeability, intestinal microbiota and the mucosal immunology).

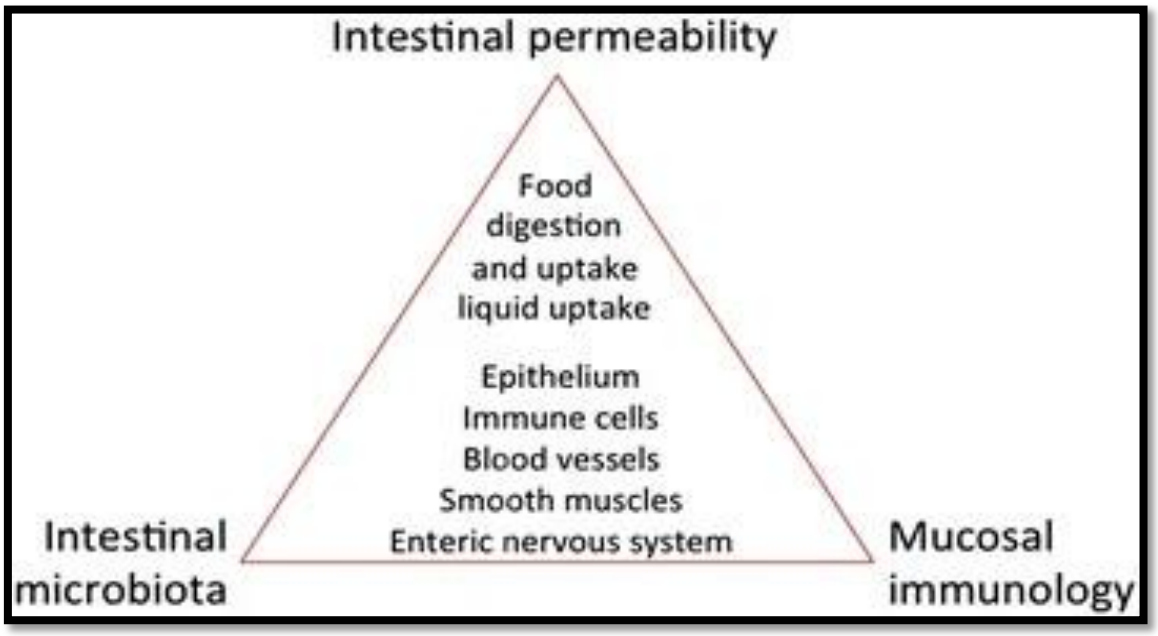

Figure 1.9: The three elements of keeping good intestinal health(Bischoff et al., 2014).

Intestinal barrier is composed of different types of protecting systems (Baxter et al., 2019)including: **1-**The mucosal barrier(including its intercellular tight junction(TJ). **2-**Physical barrier; including the cellular and stromal components of mucosa. **3-**The chemical barrier(consisting from the digestive enzymes, antimicrobial peptides and other inflammatory cytokines). **4-** gut microbiota .

5-The Intestinal immune system.

The intercellular tight junction(TJ) acts as barrier that regulates the passage of small water-soluble molecules and ions and it's considered as a marker of

absorptive and secretory epithelia. The components are in dynamic status and may show changes according to physiological or immunological conditions (Liu et al., 2013) and as shown in Figure 1.10.

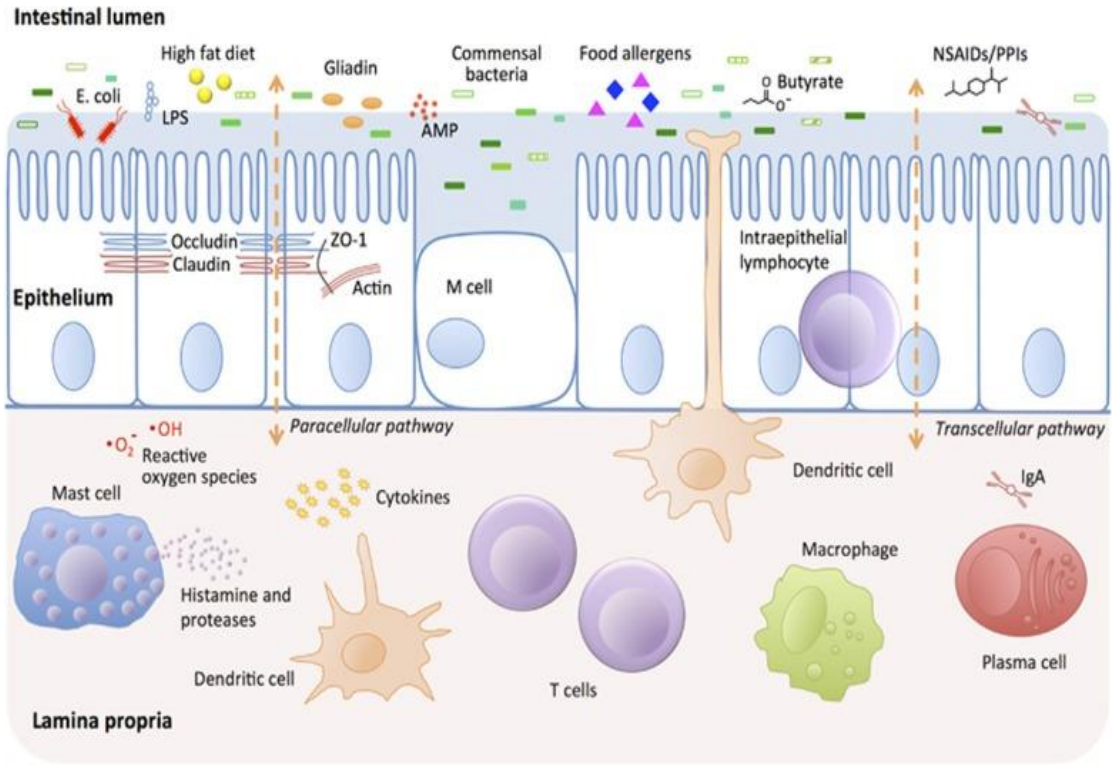

Figure 1.10: Intestinal barrier and the influencing factors (König et al., 2016).

The discovery of the Zot, an enteroxin released by *Vibrio Cholera* which reversibly loosens the TJ, led to more focusing on the effect of the TJ in the immunological and infectious conditions of the GIT(Bruno et al., 2013).

It's well known that intestinal TJ structure are abnormal in CD patients with a consequent increased permeability(Heyman et al., 2012).In CD gliadin passes through the intestinal enterocytes into the lamina propria where it evokes an immune and inflammatory reaction while the intact intestinal lining is impermeable to gliadin(König et al., 2016). Gliadin itself upgrades the gut permeability by stimulation the release of zonulin by binding to CXCR3 receptors on the apical surface of the enterocytes with a subsequent MYD-88 dependent release of zonulin (Junker et al., 2012).Zonulin is a protein that

disorders the intestinal barrier function by modifying TJ proteins(like zo-1) and it's increased in the submucosa of CD patients six times more than that of healthy individuals(Samsel and Seneff, 2013). Accordingly, it was seen that zo-1 is reduced in the duodenal biopsies of these patients and this action can be reversed with the gluten free diet(Pérez et al., 2017).

Zonulin, 47 kDa particle is an endogenous analogue to zonula occludens toxins(zot) which is enterotoxin liberated from *Vibrio Cholera*(Hollon et al., 2015).Zonulin is able to increase the intestinal permeability in the small intestine(Moreno-Navarrete et al., 2012).In CD patients there is increased serum level of zonulin antibodies as measured by ELISA as an indicator of increased intestinal permeability in this disease (Ulluwishewa et al., 2011, Józefczuk et al., 2018) .

Zonulin is a pre-Haptoglobin(HP)-2 . After cleavage , zonulin converts into the inactivated form,α and β form of haptoglobin(HP),hence at this point the zonulin loses it's ability to increase the paracellular permeability (Camilleri et al., 2012).

It was shown that zonulin exerts it's action by EGF stimulation(dependent on PAR2 stimulation) since it was illustrated that EGF can modulate the cytoskeleton actin(Liu et al., 2010, Heisenberg and Bellaïche, 2013).Laboratory studies showed that EGFR phosphorylation by zonulin promotes intestinal permeability and this action can be inhibited by EGFR blockers(Sellitto et al., 2012).

1.3.5 Histological changes of celiac disease

The study of histological changes of a biopsy from the small intestine is considered as cornerstone in the diagnosis of CD 50 years ago in spite of the progress in the serological tests (Robert et al., 2018).The development of the duodenal tissue changes occurs gradually .The changes detected by

microscopical evaluation of the duodenal tissue focuses on two important spectra first the inflammation reflected by the observation and counting of the intraepithelial lymphocytes and second the damage of the mucosa interpreted by the villous atrophy and the crypt hyperplasia . Different classifications were established to evaluate the histological changes of the small bowel ,the most famous was Mucosal Algorithmic Rules for Scoring Histology (MARSH) (Taavela et al., 2013).

The biopsy evaluation and not the disappearance of the symptoms is considered as a prerequisite for well-being of patient with CD. Massive clinical response is seen after applying the gluten free Diet(GFD) in patients with CD only within several weeks. However intestinal repair does not occur in all patients in whom complete clinical recovery was achieved. In some cases there are sever mucosal damage in asymptomatic patients (Rubio-Tapia et al., 2010, Ludvigsson et al., 2014).

The variability and the discrepancy of the biopsy evaluators can be partially due to the incorrect orientation of the biopsy leading to tangential cutting which affects the reproducibility of the results and of course this influences the diagnosis especially in the borderline cases(Ludvigsson et al., 2014).The biopsy is recommended to confirm the diagnosis in patients with positive serological tests or in cases with seronegative but with high suspicious clinincal findings(Lebwohl et al., 2014).

By using the endoscopy, the clinician will observe fissuring, scalloping and reducing villi folding of the duodenal mucosa while the characteristic histological features include increased intra-epithelial lymphocytosis (IEL>25/100enterocytes), shortening of the villi height, and the crypt hyperplasia. To analyze the changes histologically, (MARSH) is preferred but other systems are used like Marsh-Oberhuber and Corazza (Ludvigsson et al.,

2013) and the main differences among these three systems are explained in the Table 1.1.

Table 1.1: The Different Histological classification of CD (Ludvigsson et al., 2013).

Morphology of duodenal mucosal biopsy	Classification		
	Marsh	**Marsh-Oberhuber**	**Corazza**
Normal	Type 0	Type 0	Normal
Normal architecture and increased intraepithelial lymphocytes ≥25/100 enterocytes	Type 0	Type 0	Grade A
Normal architecture and increased intraepithelial lymphocytes ≥30/100 enterocytes	Type 1	Type 1	Grade A
Normal architecture and increased intraepithelial lymphocytes ≥30/100 enterocytes with crypt hyperplasia	Type 2	Type 2	Grade A
Partial villous atrophy and increased intraepithelial lymphocytes ≥30/≥25/100enterocytes	Type 2 hyperplastic lesion Crypt hyperplasia, increased crypt height and influx of intraepithelial lymphocytes	Type 3 destructive	Grade B1 atrophic, villous to crypt ratio <3:1
		Type 3a partial villous atrophy; villi blunt and shortened with a villous:crypt ratio,1:1	
		Type 3b subtotal villous atrophy; villi atrophic but still separate and recognisable	
Villous atrophy intraepithelial lymphocytes ≥30/≥25/100 enterocytes	Type 3 destructive severe inflammation, flat villi; hyperplastic crypts	Type 3c total villous atrophy; villi rudimentary or absent; mucosa resembles colonic mucosa	Grade B2 atrophic, villi are no longer detectable
Atrophic hypoplastic lesion: flat mucosa, normal crypt height, no inflammation with normal intraepithelial lymphocyte counts	No equivalent	Type 4	No equival

The histological changes with the CD are variable according to the time of diagnosis and the degree of gluten free diet. This gives different grades of MARSH histological system which were illustrated as photos in the below Figure 1.11 (Bao et al., 2012).

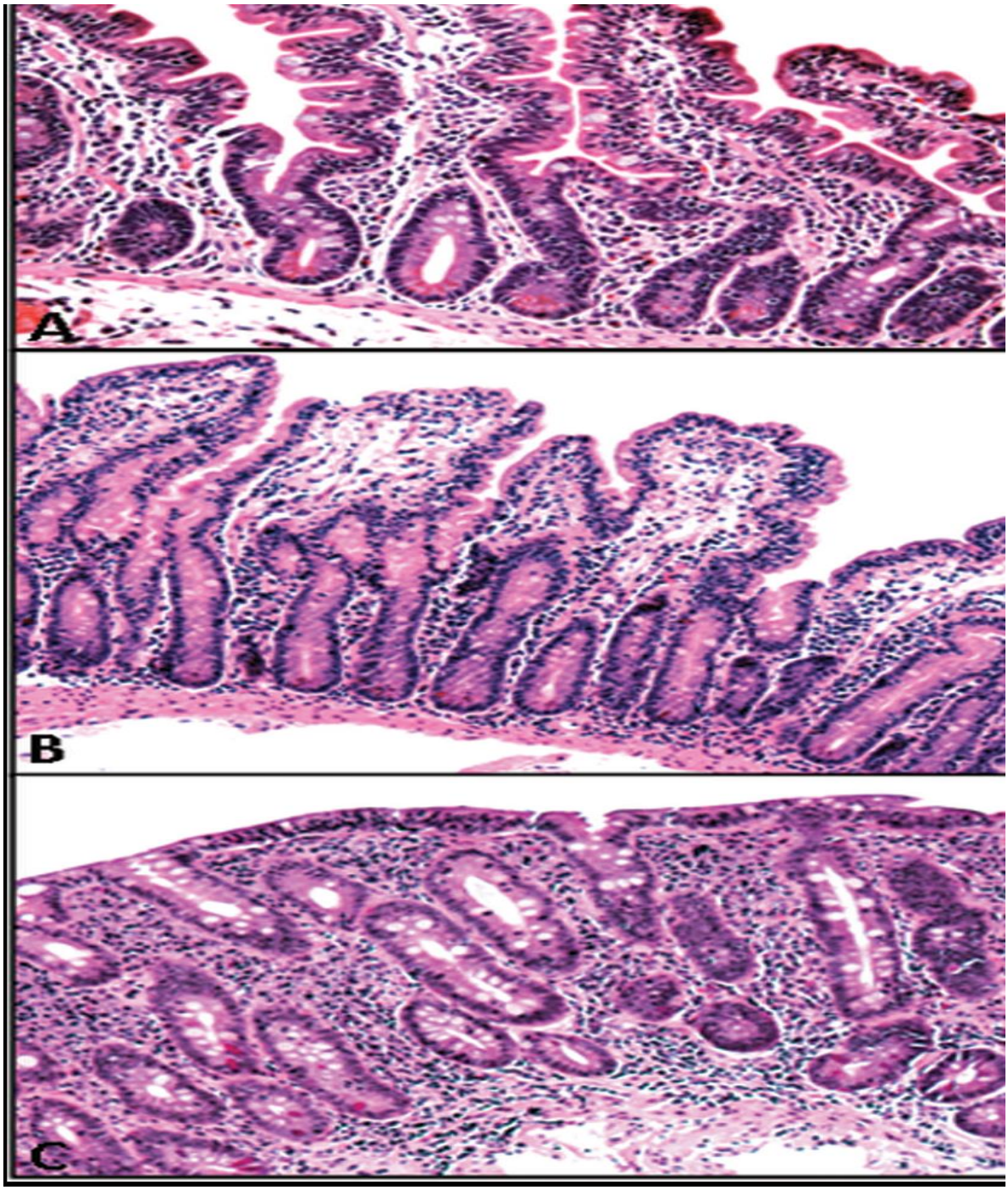

Figure 1.11: Celiac disease MARSH grading of A-grade 1, B-grade 3b , C-grade 3c .

It's well known that the traditional diagnosis of CD depends on the combination of serological tests and the histopathological examination, but some pitfalls may affect the diagnosis of the disease depending on the biopsy taking and interpretation (Ravelli and Villanacci, 2012), as follows:

1-Endoscopic procedure: attention should be paid to the next points:

A-The common macroscopic appearance of upper small intestine in CD is tubular surface with scalloped and ridged folds, but these are not specific to celiac disease because they can be showed in other disorders like Crohn's disease. In addition the lesion in the duodenum/jejunum may be patchy(Bai et al., 2013), It's better to repeat the endoscopy in negative histology but with positive serological auto-antibodies(Husby et al., 2012). **B-**Type of endoscopy instrument can affect the results since the combination of both conventional endoscopy and the capsule is more useful and the usage of double-ballooned is more informative (Pennazio et al., 2015). **C-**The site of biopsy is essential since duodenal bulb may normally show shortened villi (pseudoflattened mucosa) and It's better to get multiple(4-6) biopsies from different sites since the changes can be focal(Lebwohl et al., 2011, Rostami-Nejad et al., 2013). **D-** Duodenal and jejunal biopsy gives tissue interpretation better than ileum. **E-**The bigger biopsy specimens give more details than the small ones(Kelly et al., 2015).

2-Histological processing and examination: The endoscopic duodenal biopsies should be examined meticulously because of their small sizes. The biopsy of CD from duodenal bulb may not show the crypt hyperplasia. The associated chronic inflammation may be seen in other conditions like Crohn's disease or the chronic usage of non-steroidal anti-inflammatory drugs(NSAIDs).During processing the multiple biopsies should be kept in separated cassettes .Tangential sectioning of the paraffin embedded blocks

may give a false interpretation of short or atrophied villi. It's better to use CD 3 immunohistochemical staining for better evaluation of the IELs (Lonardi et al., 2013, Robert et al., 2018) .

1.3.6 Clinical Presentation of Celiac disease

There is more predilection for female with a female:male ratio about 2.8:1(Abadie et al., 2011, Hameed et al., 2016) with two peaks(Igbinedion et al., 2017) ,first after the infant weaning and second wave during the second to third decade of age(Kabbani et al., 2012).

Celiac disease is an autoimmune disease stimulated by ingestion of gluten in the genetically susceptible persons. It's prevalence about 1% worldwide. The diagnosis of CD was improved over the last few years yielding increase in the diagnosis of cases especially in the subclinical type of the disease. The clinical presentation can be very variable and different presentation modes were proposed .Intestinal, extra-intestinal, potential and refractory(Caio et al., 2019a).

The intestinal type is more detected in the pediatric age group starting with age below 3 years ,as diarrhea, loss of appetite ,failure to thrive, and abdominal bloating(Meijer et al., 2021).

In adult CD, the intestinal manifestations are presented as abdominal distension , diarrhea ,abdominal colic or constipation (Reilly et al., 2011).

The extra-intestinal manifestations are variable and occur both in children and adult cases of celiac disease as follows:

A-Hematological manifestations: Iron deficiency anemia(IDA) is the most important hematological manifestation both in the fully established cases and in the subclinical asymptomatic cases. The causes of IDA in CD are multifactorial, loss duodenal absorptive surface is the most important etiology

but other factors are also blamed like the gastric and duodenal bleeding, intestinal inflammation and neoplastic lesions such as lymphoma. Reduced expression of the regulatory proteins involved in the iron metabolism is an additional factor in the IDA in celiac disease.(Freeman, 2015, Stefanelli et al., 2020). Other types of anemia can also present like hemolytic anemia and macrocytic anemia due to folic acid deficiency .The anemia can persist for a variable period after the initiation of the GFD(Martín-Masot et al., 2019).

B-Bone density changes: including osteopenia and osteoporosis (detected in about 70% of patients at the time of diagnosis. This can occur partly due to vitamin D and minerals malabsorption (Kamycheva et al., 2017).

C-Growth retardation and short stature in children and can be corrected on the GFD.(Soliman et al., 2019).

D-Oral Manifestations: recurrent aphthus stomatitis is a painful well demarcated ulceration inside the mouth. It's not clear whether this can happen due to immune disorder or a nutritional deficiency. Second oral problem is the dental enamel defects that are manifested as teeth color defects, pitting, grooving and even total losing of enamel.(Therrien et al., 2020).

E-Neurological manifestations: the prevalence is up to 10% and may be detected in 42% of untreated individuals. The pathogenesis of neurological disorders in CD is multifactorial, can be related to immunoglobulin cross-reactivity, immune complex deposition or minerals deficiencies (Hadjivassiliou et al., 2019).Recurrent headache (including tension, idiopathic or migraine types) can be a marker of CD(Zis et al., 2018).

There is an increased rate (1.5-2 folds) of different variants of neuropathy in CD patients with an elevated detection of Anti -ganglioside antibodies in the serum of these patients. The symptoms of this neuronal disorders may be manifested even before the diagnosis of the CD (Yu et al., 2018).

Gluten ataxia is a neurological disorder that can be triggered by gliadin ingestion with or without associated CD. Anti-gliadin antibodies are usually detected serologically but rarely anti t TG autoantibodies (Rashtak et al., 2011).

Gluten-induced cognitive disorder is expressed by patients as lack of concentration, diminished attentiveness, short-term memory defects and confusions, all these occur after gluten ingestion(Lichtwark et al., 2014).

Celiac-induced epilepsy cases are increasingly discovered among CD patients. The etiology is hypothesized to be due to folic acid deficiency or autoimmune mechanism. Focal seizures are the predominant type with or without brain calcification(Ludvigsson et al., 2012b).

F-Psychological disorders: different psychological disorders were prescribed with the diagnosis of CD. They include anxiety, depression, attention –defects and abnormal eating habits. Most of these will decrease with the GFD(Smith et al., 2017).

G-Ocular manifestation: vitamin deficiencies and hypocalcemia could be the main etiology of the Celiac ocular disorders. These include uveitis, cataracts and vitamin-A deficiency retinopathy which can be reduced by the patient compliance with GFD (Mollazadegan et al., 2011, Therrien et al., 2020).

H-Dermatological manifestations: several dermatological pathologies can be seen in patient with CD. These include eczema, psoriasis , atopic dermatitis and urticaria with variable hazardous ratio(Jelsness-Jørgensen et al., 2018, Therrien et al., 2020).

I-Fatigue: up to 37% of CD may complain from chronic fatigability and chronic fatigue can be an indicator of screening for CD(Jelsness-Jørgensen et al., 2018).

J-Musculoskeletal manifestations:

Osteoporosis , defined as reduced bone density(decreased bone mass & microarchitecture) (Rachner et al., 2011) , occurs with variable ratio(26-72%) of CD patients with risk of bone fractures. Different hypothesis of etiology of osteoporosis were assumed. Secondary hyperparathyorodism ,gonadal atrophy , increased cytokines and autoantibodies against osteoprotegerin were the main possible causes(Larussa et al., 2012).

Osteomalacia is reduced bone mineralization and bone mass, with low bone mineral to bone matrix ratio, due to decreased vitamin D mainly. It accounts for about 18% of celiac patients, it cause symptomatic hypocalcaemia, muscle weakness, bone pain ,tenderness(musculoskeletal pain), skeletal deformity or even bone fracture(Frikha et al., 2012).

Arthralgia and osteoarthritis are also common Extraintestinal manifestations with an incidences reaches up to 30% at time of CD diagnosis(Popp and Mäki, 2019).

K-Cardiopulmonary manifestations: pericarditits, pericardial effusion and autoimmune myocarditits are recorded at the time of CD detection (Ciaccio et al., 2017).

L-Reproductive system: Amenorrhea ,delayed menarche, early menopause and infertility, and abortion all were recorded patients in with CD (Beksaç et al., 2017, Glimberg et al., 2021).

1.3.7 Clinical types of Celiac Disease

Different terminology systems have been developed to summarize the variable clinical conditions that are related to the ingestion of gluten. The Oslo Classification of CD in 2011 (Ludvigsson et al., 2013) identified the variants of the disease as classical, non-classical, subclinical, potential and refractory

CD. The terms symptomatic and overt CD were omitted. In the **classical type**; the patients are present with the intestinal manifestations like diarrhea, malabsorption and weight loss. The **non-classical** group show no malabsorption signs or symptoms but have non-gastrointestinal manifistations. In the **subclinical variant** , the patients have clinical or laboratory changes(like IDA and reduced ferritin level) but with no symptoms. G.Caio and his colleagues published an article in 2019 and they preferred to change the term classical into intestinal and the non-classical term to extra-intestinal(Caio et al., 2019a).The **refractory type** is present when the patients have persistent or recurrent malabsorptive symptoms with histologically-illustrated villous atrophy and increased IELs even with rigorous GFD (Rubio-Tapia and Murray, 2010, Ludvigsson et al., 2014). While the **potential** variant of CD denotes that the patient have normal intestinal mucosa but with increased risk for developing CD as shown by positive serology markers and positive HLA-DQ2&DQ8 (Ludvigsson et al., 2013). The term potential is sometimes used interchangeably with the word **latent**. In spite of minor gastrointestinal symptoms, the patients with potential CD are advised to be kept on GFD since some researches approved that gluten-containing food will induce future villous atrophy in some of these patients(Sperandeo et al., 2011).

Seronegative variant represents only 2-3% of cases of CD and it's diagnosis represents a diagnosing challenge since there are negativity for the serological markers but with positive Genetic mapping and villous atrophy specific for CD. Considering the other causes of villous atrophy like infections, tumors, Crohn's disease and reassessment of the patient on GFD after 1 year will be fundamental in specifying this condition (Greenson, 2015).

1.3.8 Non celiac Gluten-Related Disorders

Wheat is a major cereal food for billions of human and considered as an important source for carbohydrates(Lafiandra et al., 2014).Gluten is a seed storage protein and it was the most famous wheat particles in inducing the celiac disease and non-celiac gluten sensitivity(NCGS) causing a lot of global impaired life quality with morbidity and mortality in the susceptible patients(Truzzi et al., 2021).Wheat allergy(WA) (including Baker's asthma, atopic dermatitis , urticarial and anaphylaxis) is another wheat-related condition. Wheat Allergy occurs due to ingestion, inhalation or contact with wheat gliadin, glutenin , amylase trypsin inhibitors(ATIs) and lipid transfer protein (albumin/globulin) fraction(Kucek et al., 2015). Figure 1.12 is a schematic presentation of wheat related disorders.

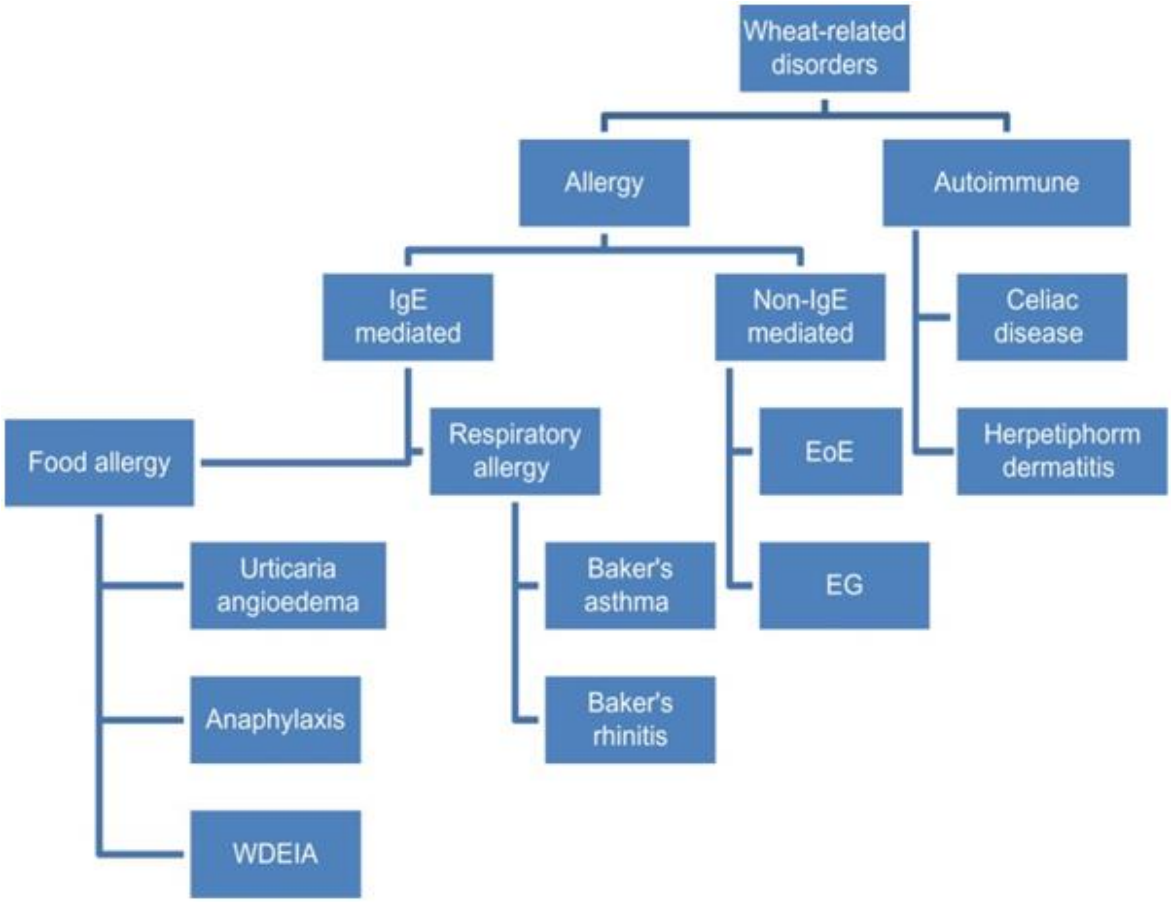

Figure 1.12: Wheat related disorders .EoE=eosinophilic esophagitis ,EG=eosinophilic gastritis, WDEIA=wheat dependent exercise-induced anaphylaxis(Cianferoni, 2016).

1.3.8.1 Non Celiac Gluten Sensitivity (NCGS)

Non Celiac Gluten Sensitivity is caused by ingestion of gluten, wheat carbohydrates (FODMAPs), ATIs and wheat agglutinin. Since that time the term NCGS was applied .The patients suffer from abdominal distension and pain, diarrhea aphthus stomatitis. The extra-intestinal symptoms include fatigue, foggy mind, headache, depression ,anxiety , joint/ muscle pain and skin rash(Sapone et al., 2012) ,so symptomatically NCGS may resemble CD but differs according the immunological basis, serological investigation, HLA typing and of course, the intestinal histology(Czaja-Bulsa, 2015).

The pathogenesis of NCGS is different from the CD in that it's not autoimmune disease. It's related to innate immune response(instead of adaptive type in CD) as evident by the increase in TLR2,INF-α,IL-8 and IL-12 with normal intestinal permeability(Caio et al., 2020) .It was observed that there is increased serum level of intestinal fatty acids and lipopolysaccharide-binding protein(Catassi et al., 2017).It was reported that in 66% of NCGS patients there is activation of mucosal basophils associated with increased duodenal IELs and eosinophilic infiltration in duodenum and colon(Carroccio et al., 2012).

1.3.8.2 Wheat Allergy

Wheat allergy (WA) is a disordered immune reaction in response to wheat proteins. WA symptoms depends on the route of allergen entry (ingestion or inhalation) and the mechanism of the immune response (IgE dependent or independent mechanism).Other cereals like oat or barley which induce CD may not be harmful for WA persons. The IgE-dependent WA occurring with having the Wheat proteins include GIT symptoms like abdominal colic and vomiting, angioedema /urticaria , atopic dermatitis, wheat-dependent exercise-induced anaphylaxis (WDEIA)(Cianferoni et al., 2013). Respiratory WA includes Baker's asthma and rhinitis. The allergy comes from different components of

wheat, like ATIs, lipid transfer proteins, gliadin and LMWGS. The skin-prick test(SPT) and in vitro specific IgE immune assay(sIgE) is the first step of diagnosis (Pahr et al., 2014) .

The well-known non-IgE dependent WA disorders are the eosinophilic esophagitis and eosinophilic gastritis. The mechanism of the two condition is still unknown and can occur both in children and adults. The detection can be done by clinical ,histopathological and the history on non response to 8-weeks course of proton pump inhibitor drugs.SPT and sIgE have limited role in the diagnosis (Cianferoni and Spergel, 2015).

Wheat dependent exercise-induced anaphylaxis**(WDEIA**) is allergic condition exacerbated by ingestion of wheat-rich food followed by a physical exercise. This condition can be caused by other etiology like infection, alcohol and NSAIDs. The symptoms include uticaria, angioedema , hypotension and shock.(Jiang et al., 2018).The mechanism is still unclear in spite of this it was thought that exercise causing reduction of gastric acid secretion (GAS) somewhat due to stimulation of sympathetic innervation resulting in more catecholamine secretion, diminished GAS lead to reduction of allergen digestion, physical activity stimulates the intestinal osmolarity and permeability of the intestine and increases the phosphorylation of tight junctions leading to the passage of more intact allergen causing degranulation of the IgE-induced mast cells (Jiang et al., 2018).

Baker's asthma is considered as the most common occupational-cereals (wheat comprises about 70% of cases) induced asthma. It's an IgE-dependent process in addition to genetic predisposition like TLR4 and beta-2 adrenergic genes mutations. The condition is usually associated with allergic rhinitis(Elli et al., 2015).

1.3.9 Dermatitis Herpitiformis

Dermatitis herpitiformis(DH) is an extra-intestinal variant of celiac disease, associated with itchy and blistering skin lesions ,mostly seen in elbows, knees and buttocks. DH is a reversible on GFD which should be strict and life long(Rubio-Tapia et al., 2013).Gut symptoms and family history of celiac may not be present at the time of detection of DH. The corner stone in the diagnosis of DH is the perilesional skin immunofluorescent IgA granular deposit in the dermal papillae, Figure 1.13 shows clinical and immunofluorescent findings.

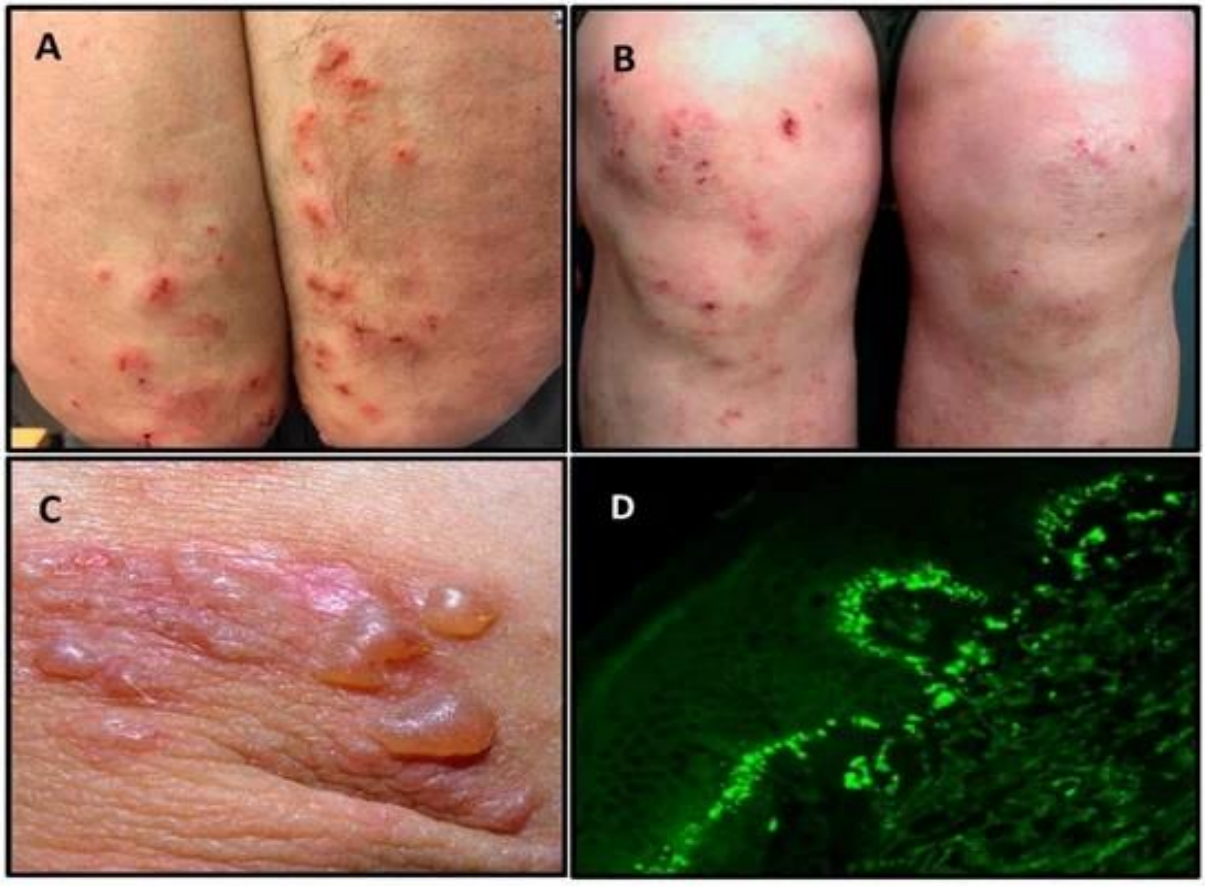

Figure 1.13: Skin blisters and papules in DH (A-C).IgA immunofluorescent deposit in the dermal papillae(D)(Reunala et al., 2018).

The pitfalls in the diagnosis include the negative immunoflourscence test (5%) especially if the biopsy from the blisters themselves , the negative serological tTG test and normal looking duodenal biopsy which occurs in

about 30% of cases. HLA typing is applied in the ambiguous cases when the clinical diagnosis is highly suspected but other tools are negative(Al-Toma et al., 2019).

1.3.10 Gluten Related Neurological Disorders

Gluten related Neurological Disorders (GRNDs) are neurological manifestation related to ingestion of gluten. Gluten Ataxia (Cerebellar Ataxia) GA and peripheral neuropathies are the most common. The pathogenesis is obscured and these disorders can occur with an associated gastrointestinal manifestations of CD(Hadjivassiliou et al., 2016).It was observed that 50% of GA(proved by MRI) cases are not associated with enteropathy but are correlated with increased serum anti gliadin antibodies (AGA) and they are improved on strict GFD (Hadjivassiliou et al., 2018).

1.3.11 Diagnosis of Celiac Disease

The diagnosis of CD has changed dramatically since 1950s when the detection based on the observation for the malabsorptive disorder. After the creation of the endoscopy, the histological assessment became the cornerstone of diagnosis, while the serological tests of CD showed more sensitive results in 1980s. The first step is the serological markers tests in patients with clinical presentations (signs &symptoms) and can be confirmed by application of oesophageogastrodudenoscopy(OGD)(Ludvigsson et al., 2013, Husby et al., 2020).

Synchronized employment of serologic tools and biopsy for CD diagnosis is preferred by the four guidelines promulgated by gastrointestinal organizations. European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) agreement guideline \2012 , American College of Gastroenterology (ACG) guideline \2013, the British Society of

Gastroenterology (BSG), and recently the World Gastroenterological Organization(WGO) guideline for adult CD patients(Kaswala et al., 2015) . A fundamental overlapping among these guidelines with the central difference is that ACG and BSG necessitate biopsy and histological examination for definite diagnosis , on the other hand ESPGHAN and WGO allow CD diagnosis without histological resluts in documented cases (Kaswala et al., 2015, Castillo et al., 2015). The WGO for CD diagnosis of adult patients advocate serological tests inclusive "anti-tTG and/or anti-EMA, or anti-DGP" antibodies and propose histology which is regarded not mandatory. This is appropriate in regions where healthcare facilities are limited (Ludvigsson et al., 2014). While the ESPGHAN guideline delegate for CD diagnosis without biopsies in children existing characteristic clinical manifestations and levels of TG2-IgA antibodies 10-fold or more the upper normal limit (UNL), recognition of anti-endomysial antibody in another test plus genetic study (HLA-DQ2/DQ8 typing) for verification the diagnosis [triple test " ESPGHAN criteria] (Husby et al., 2012).Anyway the diagnostic Approaches of celiac disease as follows (Husby et al., 2012, Husby et al., 2020):

a- Clinical findings(signs & symptoms).
b- Serological Tests.
c- Genetic study (HLA-DQ2/DQ8 genotyping).
d- Biopsy of small intestinal and histological examination.

1.3.11.1 Serologic tests for CD diagnosis

No anyone of serological tests is definitely specified for CD and detection of antibodies often support the diagnosis in spite of the dramatic difference in the diagnostic accuracy between laboratories (Kelly et al., 2015).

Serological tests include: AGA , anti-tTG, anti-EMA, and anti-DGP antibodies are celiac-specific and often the first step while screening for CD (Bai et al.,

2013). All serologic tests are automated except for the EMA test, since it is observer-dependent and requires longer time in comparison to other tests. Generally, CD diagnosis should be accomplished before gluten being restricted from diet since both circulating antibody levels\ small intestine pathology can be improved and even resolved when restrict gluten (Rubio-Tapia et al., 2013).

Regarding Anti-gliadin antibody **(AGA),** the native gliadin is the target for antibodies (IgA and IgG) and hence AGA test was the primary serologic tool that signs the requirement of small intestinal biopsy in celiac patients. AGA test is of restricted accuracy (Karnsakul et al., 2012), in general it fell out of priority in the 1990s after a wide availability of more precise tests (Di Simone et al., 2013, Rubio-Tapia et al., 2013) comparing to the lesser accuracy and low sensitivity / specificity of this test , except in younger children (Rubio-Tapia et al., 2013).

While anti-tissue transglutaminase antibody (tTG-IgA) measurement is the former testing recommended for individuals in whom associated IgA deficiency is not expected due to its superior standardization and being a high sensitive and specific test (Rubio-Tapia et al., 2013),for IgA-deficient CD patients tTG (IgG) antibodies are used previously (Rubio-Tapia et al., 2013, Absah et al., 2017). The greater the antibody titer, the higher the probability of a valid positive value(Van der Windt et al., 2010, Husby et al., 2020). Anti-tTG antibody test is mainly done by an ELISA technique and less commonly by radioimmunoassay(Tonutti and Bizzaro, 2014).

Concerning anti-endomysial antibodies (EMA), recognition of EMA by indirect immunofluorescent assay (IIFA) using either human umbilical cord or monkey esophageal tissue, with results being recorded as semiquantitative titers (Picarelli et al., 2013), EMA is regarded an important indicator for CD with high specificity and sensitivity (Kneepkens and von Blomberg, 2012). However a less sensitive EMA has been suggested, in particular young

children < 2 years (Wolf et al., 2014) and in elderly patients (Fasano and Catassi, 2012, Cappello et al., 2016) and false negative results in children aged < 2 years and in patients of IgA deficiency are foreseeable (Rubio-Tapia et al., 2013).

Whilst EMA still is the most sensitive CD test in a reference lab, the test technical disadvantages giving a significant inter-observer and inter-site differences have directed the effort toward the replacement of EMA by a recent ELISA based assays (Husby et al., 2012, Fasano and Catassi, 2012).

Recently anti-deamidated gliadin derived peptides (DGP) antibody test which is against DGP has become clinically attainable. The ability of intestinal tTG to transform gliadin into deamidated one provides the bases for DGP test , and these antibodies offers a highly specific and sensitive test for CD than previous AGAs (Di Simone et al., 2013, Rubio-Tapia et al., 2013) . Anti- DGP antibody test is highly beneficial in young children < 2years of age (Wolf et al., 2014).

Furthermore, it has been announced that measurement of DGP (IgG) class is superior to tTG (IgG) antibodies on screening of CD cases with IgA-deficiency (Fasano and Catassi, 2012, Rubio-Tapia et al., 2013).Inaddition, a retrospective study demonstrated that IgG class of anti-DGP antibodies is specifically preceding the tTG (IgA) antibodies mostly in young children. This finding refers to a possibility of early CD detection by regular follow-up using DGP test in some children with a genetic risk for CD(Lammi et al., 2015).

Moreover, anti-DGP assays proved to be of value in monitoring dietary adherence to GFD in children (Monzani et al., 2011, Rubio-Tapia et al., 2013) and more substantial test in monitoring celiac autoimmunity (Lammi et al., 2015).

1.3.11.1.1 The role of combined serological tests:

The combined testing of CD will increase the diagnostic accuracy (Parzanese et al., 2017). Since no single test is absolutely sensitive \ specific for CD, collection of individual tests in a commercial panel is more beneficial as they have additive role in exploring celiac patients with IgA deficiency(Cappello et al., 2016). However DGP (IgG) can detect some of CD patients with adequate IgA who are sero-negative for tTG IgA antibodies (Rubio-Tapia et al., 2013). The simultaneous testing of tTG (IgA) and DGP (IgG) as screening tests having a better specificity in different age groups (adults and children)(Villalta et al., 2010, Rubio-Tapia et al., 2013).

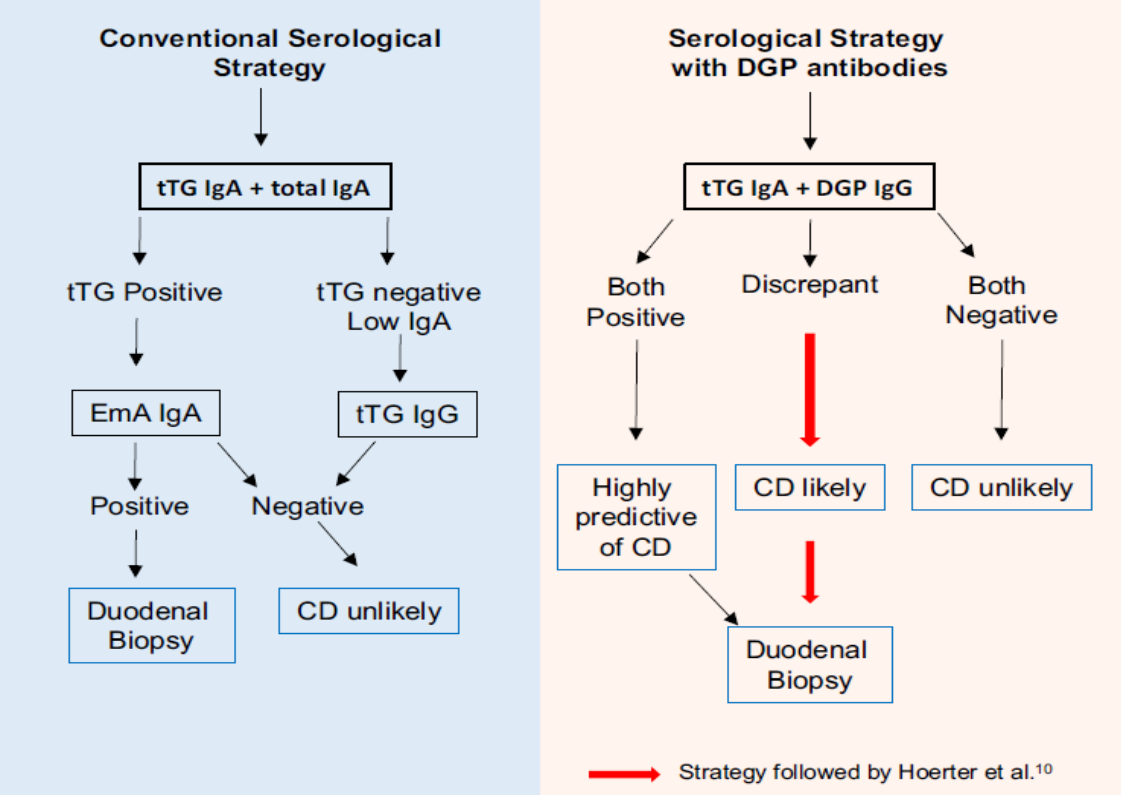

Figure 1.14: The comparison of first serological strategies, with and without DGP antibodies in the diagnosis of celiac disease(Hoerter et al., 2017).

Figure 1.14 above shows the comparison of the serological strategies, with and without DGP antibodies in the diagnosis of celiac disease; as shown in the study by Hoerter and his team (Hoerter et al., 2017), the serum concentration of DGP antibodies can not discriminate between DGP-positive CD patients and

cases regarded as false positives .Therefore, patients with discrepant results (tTG negative and DGP positive, or vice versa) should undergo duodenal biopsy (Bai and Verdú, 2017). Moreover, antibodies to DGP may be the only serological marker in newborn, as anti-tTg and EMA autoantibodies are not present at this age (Wolf et al., 2014, Assandri and Montanelli, 2019).

1.3.11.1.2 The role of total IgA level in serological tests:

False negative values may occur in IgA-deficient cases; such results could be seen at time in which measuring IgA of (anti-tTG, EMA and DGP) antibodies which are the standard for the CD immunoassays (Rubio-Tapia et al., 2013, Cappello et al., 2016).

When CD is predominantly suggestive even before screening, so measuring of total IgA level is suggested in such category, specially when serological tests of IgA-class are negative. A clinical approach is to start with total IgA measurement to detecte IgA level sufficiency and, in case of deficiency, so to incorporate testing of IgG-class into the serology testing , where the preferred tests would be DGPs (IgG) and / or tTG (IgG)(Rubio-Tapia et al., 2013, Hoerter et al., 2017).

1.3.11.2 Intestinal endoscopy and biopsy

The histolopathlogical presentations are also significant in determining the reasones of malabsorption (differential diagnosis)(Yantiss and Odze, 2009, Cappello et al., 2016, Husby et al., 2020).

According to CD guidelines, histologic picture of small bowel is required to confirm diagnosis. Endoscopy and sampling of small intestinal biopsies is the corner stone to diagnose adulthood CD(Villanacci et al., 2011, Mills and Murray, 2016).

Various constructions to be perfectly examined in the histology of small intestine, including: infiltration of inflammatory cells, low-height enterocytes, crypt hyperplasia, and blunt\ atrophied villi. The intestinal histological lesions can be classified into four distinct diagnostic grades (scoring system) according to the Marsh classification (Villanacci et al., 2011), later Oberhuber offered modifications of Marsh grading where M III grade subdivided into a, b, and c scores (modified Marsh-Oberhuber)(Mills and Murray, 2016), other is the Corazza classification; these classifications illustrated in (Table 2.1) (Ludvigsson et al., 2013).

1.3.11.3 Genetic testing

The remarkable role of HLA(DQ) alleles genotyping is for excluding the presence of /or susceptibility to CD (Hadithi et al., 2007, Husby et al., 2012) ,and HLA recording has important role in CD diagnosis (Hadithi et al., 2007, Rubio-Tapia et al., 2013) . According to the 2012 ESPGHAN guidelines, the value of genetic testing in CD is due to the fact that the disease is improbable when HLA-DQ2 and HLA-DQ8 haplotypes are disproved, so HLA –typing was regarded the first tool in screening individuals at risk of CD(Husby et al., 2020).

Recently, about 39 non-HLA loci can also make liable to celiac disease as mention previously, One of these non-HLA effective genes locates on chromosome 19, is the myosin IXB gene (*i.e.*, MYO9B)(Dubois et al., 2010, Gagliardi et al., 2021).

HLA genetic testing at birth have been argued as an approach of mass population screening to detect those with a genetic predisposition for CD before the clinical manifestations occurrence and consequent health issues (Rubio-Tapia et al., 2013).

1.3.11.4 Gluten challenge test (GC)

Administration of a gluten-containing diet, along with medical observation for the clinically suspected patient, whom were formerly managed by gluten restriction and returned to usual; known as Gluten challenge test. This challenge enabled the diagnosis of celiac disease (Leffler et al., 2013). It was routinely applied in the past, however uncommonly used nowadays because of the availability of highly precise serological tests (Mendo-Lopez et al., 2021).

GC is still the preferable test for the CD diagnosis in those patients with positive HLA-genotyping, but normal findings for both serology and biopsy as they are done on gluten restriction. However only a few data was available about the diagnostic adequacy of the test, the optimum duration, and the dose of gluten (Rujner et al., 2002, Leffler et al., 2013). A recent study notified that the diagnostic alterations are presented in the majority of the patients within 2 weeks of gluten ingestion even if a patient could not tolerate > 3 g of gluten per day (Leffler et al., 2013). Other studies documented that normal histology preserved when <10 mg gluten intake per day, whereas 50 mg daily gluten intake show a raised ratio of villous height-to-crypt depth and significant abnormalities (increased IEL and villous atrophy) with 100 to 500 mg daily ingestions of gluten (Akobeng and Thomas, 2008, Lebwohl et al., 2018). In addition, the gluten challenge has enabled experts in designing a diagnostic algorithms and monitoring clinical responses in clinical trials. Also the test presents a beneficial role in improving the incoming therapies as an adjuvant therapy to GFD (Castillo et al., 2015).

For the time being, plentiful data proposed that for celiac disease, the immune reaction against gluten could be evoked *in vitro* by utilizing culture (mucosal) cells obtained from intestinal biopsies (Khalesi et al., 2016).

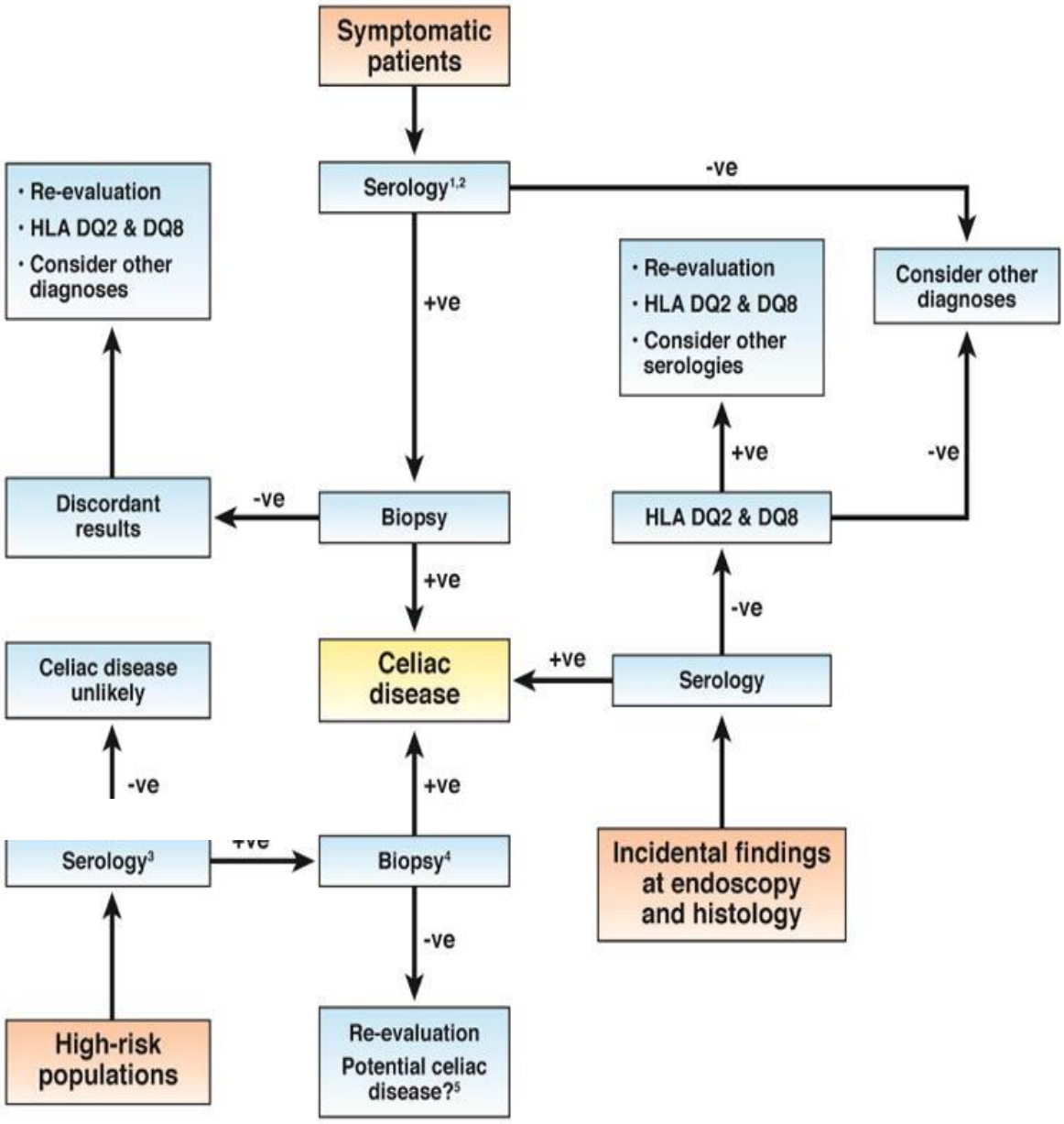

Figure 1.15: (Kelly et al., 2015). The schematic plan of the diagnosis of celiac disease. Serology is the first step in the diagnosis· most reliable serological tests are anti-DGP, anti- tTG(IgG&IgA) and anti-endomysial antibody. negative serological patients are confirmed by biopsy. negative HLA-DQ2&DQ8 in the high risk population are permanently excluded from the diagnosis. biopsy can be delayed in symptomless cases with only mild increased serological tests. Potential CD is associated with normal duodenal biopsy but with increased serology.

Serological tests have been developed over the last three decades with progress in their sensitivity and specificity. The classical anti-gliadin antibody test was replaced by the more accurate test anti-deamidated gliadin together with the classical anti tTG IgA(Husby et al., 2012).According to the last European guidelines,the diagnosis of CD can be achieved by the application of anti-tissue transglutaminase(more than ten folds the upper normal limit) in symptomatic and genetically susceptible person together with positive EMA and response to gluten free diet. This can be done without the need to apply the endoscopic procedure(Husby et al., 2012).The high limit of the serological tests enable the clinician to predict the grade of the villous atrophy(Tortora et al., 2014).

In 1970s, three duodenal tissue biopsy with subsequent histopathological assessment, one from the flat mucosa, second after GFD and the third is from the upper small intestine after gluten food challenge. With the progress in the development of highly specific and sensitive serological tests, one small biopsy from duodenum ,followed by convenient clinical and serological follow up for the GFD is nowadays considered as perfect for the diagnosis of CD(Gujral et al., 2012). The histopathology assessment of the duodenal tissue remains a cornerstone in the diagnosis of CD(Popp et al., 2019).

It's well known that CD is a multigenic disease with the interplay of environmental factors, autoimmunity and Genetic predisposition. CD is associated(as mentioned above in the text) with HLA-DQ2 and DQ8 especially if the person had 2 copies of HLA-DQ 2.5 genes) .In addition other non-HL A genes are also involved in the pathogenesis of CD like IL-18R ,CCR9 and TAGAP genes(Sharma et al., 2016).

As illustrated in Figure 1.15, Genetic study is indicated in the suspected cases when the clinical or the endoscopic findings are not clear. The HLA-study can be applied in relatives of patient with celiac disease(Liu et al., 2014).

1.3.12 Differential Diagnosis of Celiac Disease

The conditions that may simulate CD are related to the age of patient. For instance ,the children CD should be differentiated from lactose intolerance& fructose intolerance (Schuppan and Zimmer, 2013),so variable disorders cause villous atrophy with subsequent malabsorption syndrome that can be summarized as following (Rubio-Tapia and Murray, 2010, Leonard et al., 2017) :

1-autoimmune enteritis, where there is production of anti-enterocytes auto-antibodies leading to mucosal and villous atrophy, giving a picture similar to CD but the difference is of little IELs. Serology and genetic tests will resolve the problem(Lucia et al., 2018).

2-Infectious gastroenteritis with similar clinical presentation to CD. Giardiasis, a parasitic infection caused by *G.Lambliae* causes villous atrophy with signs of malabsorption and iron deficiency(Hashim, 2018).Other parasites is called cryptosporidiosis ,waterborne infection invades the duodenal wall(Hashim et al.). Bacterial (including Intestinal tuberculosis) and viral gut infections can be a source of confusion with the CD(Diaconescu et al., 2016).

3-cystic fibrosis: a congenital infection due to a mutation in CFTR gene with occasional gastrointestinal manifestations(Sanderson, 2020).

4-Inflammatory bowel diseases (IBD): including crohn's disease and ulcerative colitis; are chronic inflammation that can involve any part of GIT, with clinical feature similar to that of CD, like diarrhea and weight loss. Endoscopy with

subsequent histopathological application and serology will be helpful in the differentiation between the two conditions(Kotze et al., 2010, Bai et al., 2013).

5-Irritable bowel syndrome (IBS): a dysfunctional disorder with intermittent symptoms of diarrhea or constipation and abdominal bloating (Girbovan et al., 2017).

6-Eosinophilic gastroenteritis: where there is infiltration of intestinal wall by eosinophils ,giving features of diarrhea and abdominal cramps(Ingle and Hinge, 2013).

7- Food protein hypersensitivity (Muraro et al., 2017, Rael et al., 2020).

8- Whipple disease (Günther et al., 2015).

9-Intestinal lymphoma (Zhang et al., 2016).

10-Collagenous sprue (Nielsen et al., 2013).

All the above conditions are differentiated from celiac disease using serology, endoscopy and biopsy interpretation and genetic counseling in addition to specific tests applied for each disease.

1.3.13 Complications of Celiac Disease

The complications of celiac disease are more in cases where the diagnosis is late ,detection in elderly, or those with homozygote DQ2 who are not on strict GFD (Caio et al., 2019a) .

A- Refractory CD (RCD): represents only 1.5% of total CD cases. It presents as persistent villous atrophy and malabsorption in spite of strict GFD and with negative serology(Dewar et al., 2012).

B- Intestinal lymphoma: The relation between celiac disease and cancers were noticed since about five decades. The development of the intestinal non –Hodgkin T-cell lymphoma (and to less extent B-cell lymphoma) in

CD patients could be 6-9 times more than the general population, especially in the late diagnosed cases. It was seen that the refractory CD may progress into lymphoma in 33-50% of cases within 5 years (Caio et al., 2019a).

C- **Adenocarcinoma :** Small intestinal adenocarcinoma is less common than intestinal lymphoma in it's association with CD but still more incidence than in general population. Sudden intestinal occlusion or malena in CD patient can a warning sign of tumor development(Biagi et al., 2014, Caio et al., 2019a).

D- **hyposlenism:** includes both functional and anatomical, presents in about 30% of adult CD and rises up to 80% in the complicated cases. Small sized spleen discovered by the abdominal ultrasound and Howell-Jolly bodies detection in peripheral blood should guide the doctor for such CD complication. This status will make the patient more susceptible for bacterial infection ,autoimmunity problems and thromboembolism risk(Di Sabatino et al., 2013).

1.3.14 Associated Disorders with Celiac Disease

Celiac disease is an autoimmune disease and there is a risk (together with their first degree relatives)of association with other disorders especially the other AID in up to 15% of CD patients(Spijkerman et al., 2016),these are:

1.3.14.1 Diabetes Mellitus (DM):

The association between DM and CD is one of the most extensively illustrated simultaneous AID. The detection of both disorders can be at the same time or CD may be discovered subsequent to DM. The prevalence of CD among diabetic patients type I averages 2-11%(so tests for CD should be carried out in the DM type 1)(Aggarwal et al., 2012).It was also illustrated that DM type 1 can be subsequent to CD before age 20 years(Hazard ratio=2.4%)(Elfström et al., 2014).

There is a second peak incidence of CD among diabetic patients type 1 around age 45 years. Both CD and DM share the same risk HLA-type ,hence DM type 1 patients have either HLA-DQ2 or DQ8 in 90% of cases(Bakker et al., 2013).

Gluten free diet will improve both the diabetic metabolic control and growth status in CD and can delay or prevent the osteopenic and vascular complications (Camarca et al., 2012).

The incidence of type 2 DM in CD was comparable to general population but the bad glycemic control predisposes the celiac patients to co-morbidity(Kylökäs et al., 2016).

1.3.14.2 Autoimmune Thyroiditis:

It has been illustrated that 2-7% of autoimmune thyroid diseased patients (Hashimotos thyroiditis and Graves disease) have CD and in the reverse manner, 26% of individuals with CD show serological markers of autoimmune thyroid diseases gland with impaired function(Mehrdad et al., 2012).

Food Gluten withdrawal will not stop the impairment of thyroid function; Other studies documented that after 1 year of GFD regimen there was decrease in the serological thyroid autoantibodies (Metso et al., 2012).

The pathogenesis of the association between the 2 conditions is not so clear but it can be explained by the fact that both disorders have similar genetic predisposition including gene of cytotoxic-T-lymphocytes associated antigen-4 (CLTA-4) which confers susceptibility to thyroid autoimmunity. It has been found that tTG antibody can bind to thyroid tissue which may deregulate it's biological processes(Caja et al., 2011).

1.3.14.3 Addison's Disease:

It has been found that CD could be a risk group in Addison's disease with an incidence up to 12% of CD in Addison disease patients(Dalin et al., 2017) and in a reverse manner, Addison's disease is found in about 11.4% of CD individuals. Unfortunately, GFD doesn't improve Addison's disease patients(Freeman, 2016).

1.3.14.4 Dermatological Disorders:

Skin manifestations like Dermatitis herpetiformis(already mentioned previously),alopecia areata, vitiligo, psoriasis and dermatomyositis have been demarcated as the most important associated skin diseases in the CD patients(Abenavoli et al., 2019).

1.3.14.5 Rheumatoid Diseases:

Sjogren syndrome(SS),an autoimmune disease with lymphocyte infiltration and dysfunction of the exocrine glands and female :male ratio about 9:1 ,has been found to be associated with CD with a rate of association about 4.5% to 15%(Parisis et al., 2020).

Systemic lupus erythematosus(SLE) is an autoimmen and multisystemic disease. It has been found that there is 3-folded increased risk of developing SLE in CD patients more than the general population. There are elevated anti-gliadin antibodies but with no histological changes (Ludvigsson et al., 2012a).

1.3.14.6 Hematological Manifestations:

The association of Idiopathic thrombocytopenic purpura(ITP) with CD was discovered about 40 years ago with a possible similar genetic predisposition. Other hematological manifestations including hypercoagulability state, with

secondary abortion, thromboembolism and variable cardiovascular disorders(Balaban et al., 2019).

1.3.14.7 Hepatic Disorders:

Variable hepatobiliary disorders were discovered to be associated with CD. Some of these can be mild with just increased of liver enzymes that can be modified with GFD and others are serious with no response to the gluten modification treatment of CD. The studied diseases can be of autoimmune mechanism (primary biliary cirrhosis, autoimmune hepatitis and primary sclerosing cholangitis).The non-immune mediated group include non alcoholic fatty liver disease, Wilson disease and Budd-Chiari Syndrome(Balaban et al., 2019).

1.3.15 Soluble Interleukin-2 Receptor (sIL-2 R)

1.3.15.1 Introduction:

Rubin and his colleagues discovered in 1984 the presence of soluble IL-2 receptor in cultured T-lymphocytes infected with Human T-cell leukemia virus-1(HTLV-1).Since that time serum measurement of (sIL-2R) was considered as marker of T-cell activity and as a tool of assessing the immune system activation (Goldsmith et al., 2016).

1.3.15.2 Structure:

The soluble molecule of the IL-2R represents the extracellular part of the membrane bound receptor, it has the capacity to bind IL-2 molecule & it presents on the surface of different haemopoietic cells including T-cell, Nk, monocytes, eosinophils and in some tumor cells. IL2-R is available in 3 different forms; alpha variant(IL-2Rα, CD25, M=55 KDa) which is considered as the most powerful type. Beta type(IL-2Rβ,CD122, M=75 KDa) and gamma IL-2R(IL-2Rγ,CD132, M=74 KDa). Structurally, IL-2Rα is 256 amino acid-units polypeptide with an extracellular(soluble) part is of 219 residues(Witkowska, 2005, Eurelings et al., 2019).

1.3.15.3 Biological action of sIL-2R:

The IL2 -IL-2R pathway was focused in the immune stimulation and it was found to have pivotal role in the tolerance and in immune system activation. The action of the IL-2R depends on it's structure whether mono-'di-or tri-compartments and the dose of the available IL-2 as shown in the Figure 1.16 down. The IL-2Rα can be cleaved by the protease enzyme producing sIL-2R molecule(Damoiseaux, 2020).

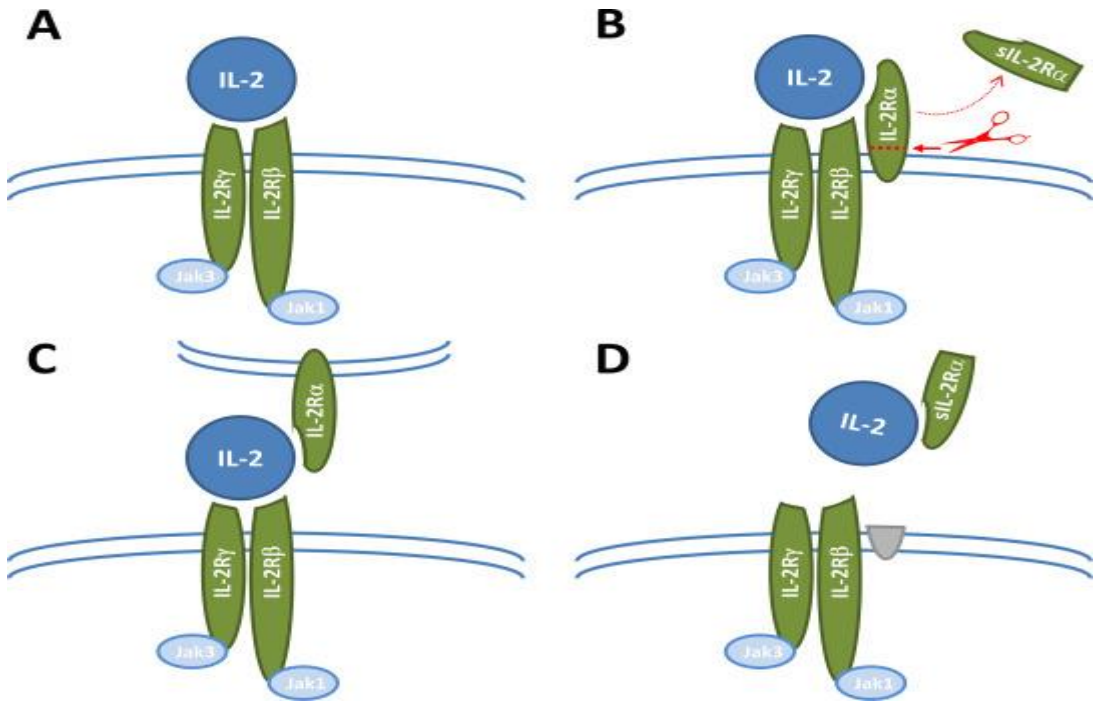

Figure 1.16: The action of sIL-2 Receptor (Damoiseaux, 2020).

Interestingly IL-2Rβ and IL-2Rγ are not unique for IL-2 and can bind to other cytokine family factor like IL-15. The IL-2Rα is considered as the more specific receptor for IL-2 than the other counterparts(Budagian et al., 2006, Yu et al., 2013).

Upon T-cell activation & sIL-2R is produced, the IL-2R will change to a low affinity receptor. Several variants of IL-2Rα gene exist and these contribute to some autoimmune diseases like DM and MS (multiple sclerosis) and this is

ascribed to reduced IL-2R signaling in the regulatory type of T-lymphocytes(Treg) (Cerosaletti et al., 2013).

The IL-2R exerts it's action through a cascade of intracellular signaling factors and genes including the Janus tyrosine kinase(JAK),signal transducer and activator of transcription(STAT),Src homologue and collagen(SHC protien) and phosphatidylinositol-3-kinase(Pi3K) as demonstrated in Figure 1.17 below (Doersch et al., 2017) .Finally the result of IL-2R activation depends on the target cell, examples in T-cell there will be T cell stimulation, in Treg cells induction results in tolerance (Cerosaletti et al., 2013). While the influence of IL-2 &IL-2R signaling on fibroblasts and skin cells is wound healing (Doersch et al., 2017).

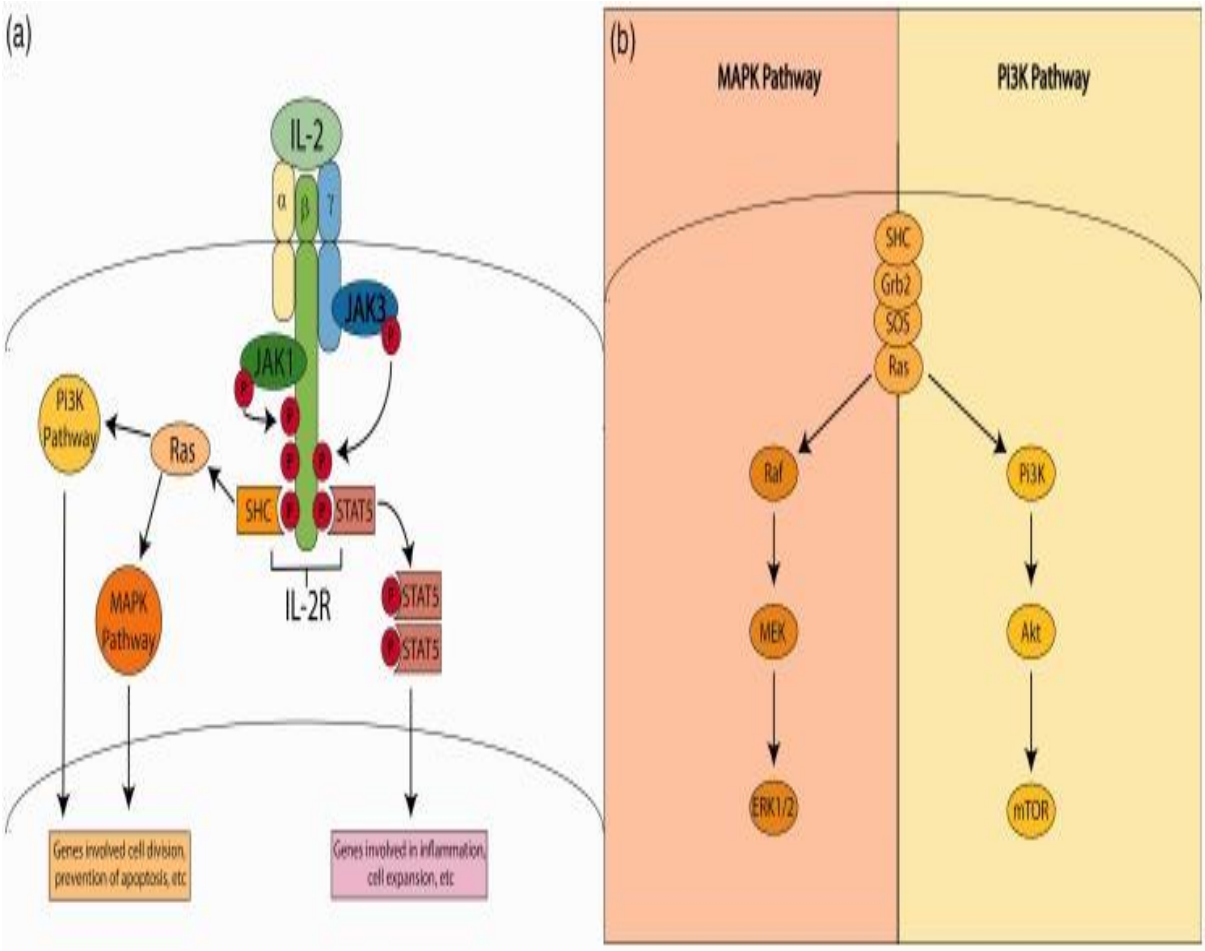

Figure 1.17: Intracellular processes of IL-2 stimulation (Doersch et al., 2017)

On the other hand the defect in the IL-2R signaling was illustrated in some disorders like of defect in the wound healing or some of the AID such as SLE (Doersch et al., 2017), Diabetes Mellitus(DM) and MS(multiple sclerosis) (Cerosaletti et al., 2013).

There are different scenarios of the action of sIL-2R in the pathogenesis of inflammatory diseases. One of the presumed effect is that sIL-2R competes with the T-cell for IL-2 factor and hence inhibiting T-cell proliferation. The second hypothesis is that sIL-2R binds IL-2 and therefore increasing the half life of IL-2 and hence exaggerating it's immune-inducing effect. The third action is that sIL-2R-IL-2 complex presents to T-helper lymphocytes and thus upregulating FOXP3 expression leading to differentiation into T-regulatory cells helping in controlling the immune activity. This effect was noticed in different inflammatory conditions like SLE, rheumatorid arithritis and granulomatous diseases like tuberculosis and sarcoidosis (Eurelings et al., 2019).

1.3.15.4 Role of sIL-2R in Celiac Disease:

It's well known that the increased concentration of sIL-2R with a subsequent stimulation of IL-2 occurs within 2 hours in the onset of gluten ingestion, together with other inflammatory mediators(Vorobjova et al., 2019).

Soluble IL-2R is considered as a marker of T-helper lymphocytes activation, which plays the central role in the pathogenesis of CD. The sIL-2R showed an increase it's plasma concentration in comparison to control group and was correlated with the grade of intestinal damage according to the MARSH grading system of the histological assessment of CD(Vorobjova et al., 2019).

1.3.16 Interleukin-18 (IL-18)

1.3.16.1 History:

The first discovery of IL-18 was in 1989 and originally named interferon-gamma(INF-γ)-inducing factor(IGIF). This cytokine belongs to IL-1 family and has tremendous role in inflammation and immune reaction. It's manufactured by variable cellular types like monocytes, Kupffer cells, T and B-lymphocytes, microglial cells dendritic cells, epithelial cells and induced macrophages(Wang et al., 2018).

1.3.16.2 Mechanism of Action of IL-18:

The gene of IL-18 locates on chromosome 11q22.2-q22.3 and at least there are six polymorphism of this gene. Such polymorphism has an inflammatory and tumorigenic effect (Dondeti et al., 2016, Al-Shobaili et al., 2016).

IL-18 exists in an inactive form(24 KDa) and it's converted into the active form, a protein of 18 KDa (193 amino acids) by the action of IL-1β converting enzyme mediated by post-translational regulation (Al-Shobaili et al., 2016, Kaneko et al., 2019).

In addition to promotion of INF-γ production, IL-18 also stimulates the release of IL-2 and expression of IL-2 receptor(IL-2R) and Fas ligand (FasL) on TH1 cell in addition to the stimulation of natural killer (NK) cells. The stimulation of INF-γ production occurs due to the action of IL-18 on different target cells like TH1, Nk, B-cells, dendritic cells and macrophages. It also stimulates (together with IL-12) TH2 cell cytokines production. Together with IL-3, IL-18 stimulates the mast cells and basophils to produce IL-4 and IL-13.All the above points refer to that IL-18 participates in both innate and acquired immune systems (Nakanishi, 2018, Yasuda et al., 2019).

There are two types of IL-18 receptors(IL-18R), IL-18R α and IL-18R β, the cytoplasmic domain of both contain domain termed the Toll-like receptor(TLR).The stimulation of IL-18R will activate nuclear factor NF-kB and mitogen activated protein kinase(MAPK) via the association of other factors like tumor necrosis factor(TNF)-activated receptor that eventually leading to expression of genes like FasL and TNFa (Yasuda et al., 2019).

1.3.16.3 Biological function of IL-18:

Recent data suggest pivotal role of IL-18 both in physiological and pathological conditions, which were illustrated by a recent article (Yasuda et al., 2019) and as shown in Figure 1.18 :

1.3.16.3.1 Physiological role of IL-18:

Several physiological functions of IL-18 were illustrated in the following points (Rathinam and Chan, 2018) :

1- Protective mechanism against both intra-and extra-cellular bacteria, viruses and helminthes through the induction of both innate and acquired immune systems.

2- Metabolic Homeostasis through regulation of food intake and energy expenditure, activation of lipid oxidation in skeletal muscles and the protective role against metabolic syndrome.

3- Regulation of gut homeostasis through the protective role of IL-18 in keeping healthy intestinal epithelial barrier in conjunction with NLRP inflammasome and the effect of the intestinal microbiota.

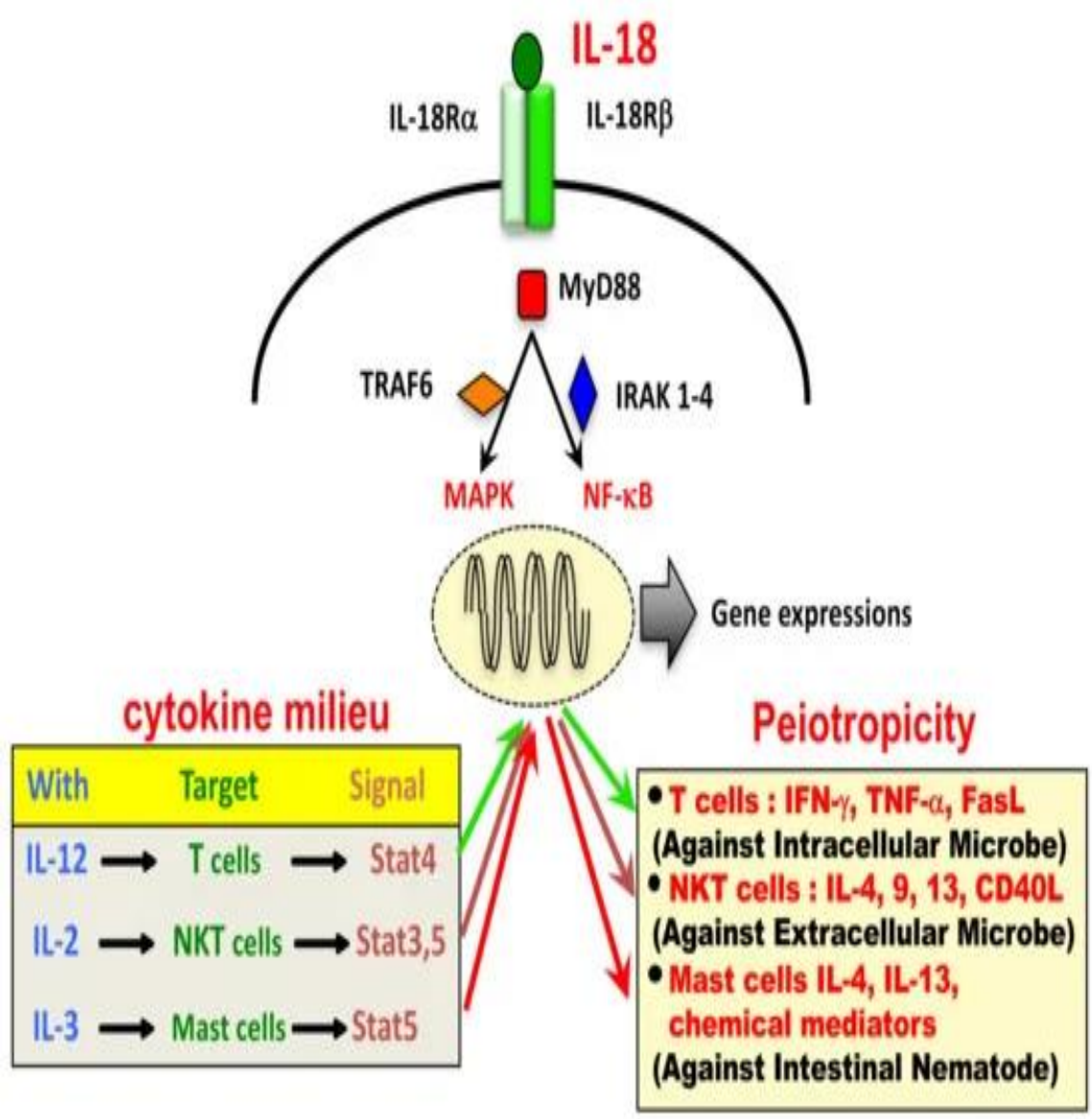

Figure 1.18: The Biological function of IL-18 (Yasuda et al., 2019)

1.3.16.3.2 Pathological role of IL-18:

The role of IL-18 was found in several diseases including the sepsis shock, allergy and asthma, arterial atheroma formation, predicting the course of renal diseases and have protecting effect in cancer (Nakanishi, 2018, Yasuda et al., 2019).

1.3.16.4 Role of IL-18 in Celiac Disease:

The proinflammatory cytokine ,IL-18 is a potent stimulator of γ-INF production which plays a crucial role in the regulation of TH1 response.The cell TH1 plays central role in the pathogenesis of CD.IL-18 has been detected together with it's mRNA in the duodenal mucosa of celiac patients.In addition Celiac patients expressed high serum level of IL-18 ,the level of which was reduced after application of gluten free diet regimen and kept high level following implementation of gluten challenge test (Attarwala, 2016).

The serum measurement of IL-18 was correlated with the intestinal villous atrophy in association with other Celiac markers like anti-tTG antibodies.IL-18 was found to be higher in the non-treated individuals and can be applied as a marker for detecting the activity of celiac disease or discovering the non compliant patients (Mormile, 2016a).

CHAPTER TWO

MATERIALS AND METHODS

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Study design:

The present study is a cross-sectional study designed to analyze the expression levels of selected cytokines and serum auto-antibodies in patients with Celiac disease and compare the same with those of non-Celiac participants recruited from three different hospitals/ clinical centers.

2.1.2 Study population:

A total of 45 patients clinically diagnosed as having Celiac disease, aged 17 years and above were recruited from three hospitals/ clinical centers namely; Imam Al-Hussein Medical City, Imam Zain Al Abidine Hospital and Alkafeel Super Specialty Hospital, all located in the city of Karbala, Iraq. The non-Celiac participants consisted of 45 individuals aged 17 years and above, certified as Celiac negative through serological test.

Informed consent was obtained from all patients and participants and ethical approval was sort and obtained from the research ethics committee of the College of Medicine, University of Kerbala as well as the appropriate approval bodies of the hospitals from which the samples were taken (Appendix A).

2.1.3 Descriptive variables of the study groups:

2.1.3.1. Inclusion criteria:

a-For patients: The rule 'four out of five ' was a quantitative approach and was suggested for the last few years where celiac disease diagnosis in a patient is definite as he fulfills at minimum 4of 5criteria (Catassi and Fasano, 2010) :

1. Clinical manifestations typically suggesting for CD.
2. Positive titer of serum celiac markers (IgA) class autoantibodies.
3. Positive HLA-(DQ2 and/or DQ8) genotyping.
4. Celiac histologic picture on duodenal biopsy.
5. Improvement on restricting dietary gluten (Catassi and Fasano, 2010).

* The criteria for the diagnosis of celiac disease in adults still depends on the presence of histological changes including increased mucosal IEL, crypt hyperplasia and duodenal villous atrophy while the patient is on a gluten-containing diet, along with findings from serology analysis (Kaukinen et al., 2001)

So for the test group:

***** Patients 17 years old and above were included.

*Patients attended GIT (gastrointestinal tract) centers whom diagnosed to have CD by specialists. The diagnosis was established depending on a combination of clinical features, serological markers, and histopathological pictures of CD in duodenal mucosa according to current international guidelines (Husby et al., 2012).

*Positive serological markers for CD.

*Confirmation by biopsy in adults.

*With a few exceptions of some patients who diagnosed depending on serology only when tTG IgA> 10 folds upper limit of normal according to revised criteria of ESPGHAN 2012 and the biopsy was done for them to evaluate the severity of the disease.

b-The controls who enrolled in the study were healthy persons of 17 years old and above.

2.1.3.2 Exclusion criteria:

Exclusion criteria for recruitment of patients with Celiac disease into the study were;

i. Suspicion but not confirming presence of Celiac disease through biopsy or serological test.
ii. Not consenting to submit self for endoscopy with duodenal biopsy, for histological examination.
iii. Presence of other autoimmune disorders such as diabetes mellitus, autoimmune thyroiditis amongst others, or associated chronic inflammatory bowel disease such as Crohn's disease or ulcerative colitis as well as forms of chronic renal and liver diseases or bowel tumors.
iv. Individuals aged below 17 years.

Exclusion criteria for recruitment of non-Celiac disease participants (control group) into the study were;

i. Family history of Celiac disease.
ii. Presence of other autoimmune disorders such as diabetes mellitus, autoimmune thyroiditis amongst others, or associated chronic inflammatory bowel disease such as Crohn's disease or ulcerative colitis as well as forms of chronic renal and liver diseases or bowel tumors.
iii. Obesity.
iv. Presence of cardiovascular disease.
v. Individuals aged below 17 years.

2.1.4 Sample collection:

Sociodemographical data were extracted from the patients' hospital records while the non-Celiac participant underwent a questionnaire-based interview.

Blood sample was collected by venipuncture using disposable syringes at the Aspiration Unit of the hospitals. Approximately 4 mL of blood were collected in gel tubes, barcoded and sent to the Immunology Unit for onward processing. The samples were centrifuged at 3000 rpm for 20 minutes and 1 mL of supernatant (serum) was aspirated and placed in labeled Eppendorf tubes, and stored at -20^{o}C until use.

Duodenal biopsy from the patients was carried out by the oesophageo-gastroduodenoscopy (OGD) and the tissue was kept in a container with 10% formalin liquid, labeled and then sent to Histopathological Unit of the hospitals for staining and result interpretation (Zahary et al., Peate, 2017).

2.2 Methods:

The laboratory analyses conducted in this study comprised of histological analysis of duodenal biopsy samples from the patients, serological determination of Celiac disease associated cytokines i.e. interleukin 18 (IL-18) and soluble interleukin 2 receptor (sIL-2R) as well as serological determination of Celiac disease associated antibodies i.e. anti-tissue transglutaminase (tTG) IgA antibodies and anti-deamidated gliadin peptide (DGP) IgG antibodies, in the test and control samples.

The cytokine assays were carried out using sandwich enzyme linked immunosorbent assay (ELISA) technique, while the antibody assays were achieved using the indirect ELISA technique.

2.2.1 Histological processing of duodenal biopsy samples:

The biopsy tissue samples were processed according to standard hematoxylin-eosin protocol for histological slide preparation. The protocol proceeded from dewaxing using xylene to hydration passage through several sections of alcohol and then rinsed throughly with water, then hematoxylin which is a

nuclear stain is added which is succeeded by bluing with a weak alkaline solution – a step that converts hematoxylin stain to blue colour. The section is then rinsed to ensure proper staining of nuclei after which differentiation using a weak alcohol proceeds to remove non-specific background staining and improve contrast. Eosin stain, which stains non-nuclear components is then applied after which the slide passes through several gradients of alcohol to remove water and several baths of xylene to clear the tissue, making it transparent as the slide is covered and mounted for reading(Slaoui and Fiette, 2011). The reading, interpretation and grading of the slides was executed by a qualified histologist.

2.2.2 Determination the serum levels of IL-18 and sIL-2R:

The BioTech Human IL-18 & sIL-2R ELISA kits from Bioassay Technology Laboratory (China) were used to quantitatively measure the levels of IL-18 & sIL-2R respectively in serum of the test and control subjects by strict adherence to protocol provided by the manufacturer. The details of the kits components as well as the reagents and solutions used are presented in (Appendix B and C).

2.2.2.1 Assay principle of IL-18 test kit:

The BioTech Human IL-18 ELISA kit utilizes the sandwich ELISA technique. The plate comes pre-coated with human IL-18 antibody. Therefore, the IL-18 present in the sample when added, binds to the antibodies coated on the wells. The addition of the biotinylated human IL-18 antibody ensures binding to IL-18 – bound IL-18 antibody complex. Streptavidin-HRP is then added to the wells which then binds to the biotinylated complex. After incubation the unbound Streptavidin-HRP is washed away during a washing step. Substrate solutions (A & B) are then added and color develops in proportion to the

amount of human IL-18 in the sample. The reaction is terminated by addition of acidic stop solution and OD is measured at 450 nm.

2.2.2.2 Assay principle of sIL-2R test kit :

The BioTech Human IL-2SR ELISA kit also utilize the sandwich ELISA technique. The plate comes pre-coated with human sIL-2R antibody. Therefore, the sIL-2R present in the sample when added, binds to the antibodies coated on the wells. The addition of the biotinylated human sIL-2R antibody ensures binding to sIL-2R – bound sIL-2R antibody complex. Streptavidin-HRP is then added to the well which then binds to the biotinylated complex. After incubation the unbound Streptavidin-HRP is washed away during a washing step. Substrate solutions (A & B) are then added and color develops in proportion to the amount of human sIL-2R in the sample. The reaction is terminated by the addition of acidic stop solution and OD is measured at 450 nm.

2.2.2.3 Assay procedure of both IL-18 & sIL-2R kits :

The assay procedures, the components of both IL-18 & sIL-2R kits are the same and all reagents were ready to use with the exception of the wash buffer solutions and standard solutions which were in form of concentrates. However standard solution concentration for IL-18 is 128 ng/L while that for sIL-2R is 1280 ng/L.

The Wash Buffer of each kit was prepared by mixing gently, the 20 mL Wash Buffer (25x) concentrate to dissolve crystals and diluting with 480 mL distilled water, yielding 500 mL of Wash Buffer (1x). The buffer's pH was checked and maintained at pH of 7.2 before storage in the refrigerator.

For preparation of Standard solutions, 120 µL of the Standard concentrate (128 ng/L and 1280 ng/L) were reconstituted with 120 µL of Standard diluent to generate 240 µL of 64 ng/L and 640 ng/L standard stock solutions for IL-18

& sIL-2R respectively. The solutions were allowed to sit for 15 minutes after which they were briefly agitated prior to preparing of serial dilutions for standard curve.

Each standard stock solution was serially diluted at ratio 1: 2 to yield serial concentrations in 7 labeled tubes as presented in Table 2.1 & Table 2.2 for IL-18 & sIL-2R sequentially .

Table 2.1: IL-18 ELISA serial dilutions for standard curve

Tube label	S6	S5	S4	S3	S2	S1	S0*
Concentration (ng/L)	128	64	32	16	8	4	0

*The standard diluent was used for concentration 0 ng/L.

Table 2.2: sIL-2R ELISA serial dilutions for standard curve

Tube label	S6	S5	S4	S3	S2	S1	S0*
Concentration (ng/L)	1280	640	320	160	80	40	0

*The standard diluent was used for concentration 0 ng/L.

Since the assay procedure is the same for both kits, so it was mentioned once.

The standards, test and control samples were sequentially arranged in a plastic rack in accordance to their respective labels in separate racks.

The plastic microtiter plate (96 wells) was unsealed from the pouch and placed on the working bench. The standards (50 µL) were dispensed into wells labeled S0 to S7 while 40 µL of undiluted samples were appropriately dispensed into the other wells. Ten microlitres (10 µL) of biotinylated human IL-18 antibody (or biotinylated human sIL-2R antibody regarding sIL-2R assay procedure) was added to the wells containing the samples but not the standards, after which 50 µL of streptavidin HRP was added to sample and standards wells but not the blanks. The plate was shaken gently and incubated at 37^{o}C for 60 minutes.

During the incubation period, the Wash Buffer prepared earlier was collected and was used in priming the ELISA automated washer – here, a 5-wash cycle and 30 seconds soak programme was chosen. After incubation, the sealer was removed, its content was aspirated and the washing cycle was initiated. During each cycle, 350 µL of Wash Buffer was added to the wells. Complete removal of any remaining buffer was ensured by inverting the plate and blotting against clean paper towels, after which 50 µL of the Substrate Solution A and 50 µL of Substrate Solution B were added to each well. The plate was then covered and incubated in the dark for 10 minutes at 37^{o}C. After incubation the reaction was stopped by quickly adding 50 µL of Stop Solution to each well with gentle tapping to ensure thorough mixing, then the optical density (OD) of each well was read, using a microplate reader set to 450 nm wavelength.

Calculation of results: first the OD of standard S0 was deducted from the OD value of all the remaining standard and samples. A standard curve was the constructed by plotting the OD of each standard on the x-axis against the concentration on the y-axis and drawing the curve of best fit through the points on the graph. The concentrations of IL-18 and sIL-2R in the test and control samples were determined by interpolation of the standard curve. Since the samples were not diluted, there was no need to multiply the concentrations from the standard curve by dilution factor.

2.2.3 Measurement of the serum levels of anti-deamidated gliadin peptides (DGP) IgG and anti-tissue transglutaminase (tTG) IgA antibodies using indirect ELISA technique:

The EuroImmune Human anti-DGP IgG & anti tTG IgA kits from EuroImmune Medizinische Labordiagnostika (Lubeck, Germany) were used to

quantitatively measure the levels of antibodies against deamidated gliadin peptides and tissue transglutaminase enzyme respectively in serum of the test and control subjects by strict adherence to protocol provided by the manufacturer. The details of the kits components as well as the reagents and solutions used are presented in Appendix D and E.

2.2.3.1 Assay principle of Anti-DGP IgG test kit :

This kit provides a semi-quantitative or quantitative in vitro assay for human antibodies of the IgG class against deamidated gliadin (GAF-3X) in serum or plasma. The test kit contains microtiter strips, each with 8 break-off reagent wells coated with GAF-3X (antigens). In the first reaction step, diluted patient samples are incubated in the wells, and in case of positive samples, specific IgG antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labeled anti-human IgG (enzyme conjugate) catalyzing a color reaction.

Calculation of results: the amount of anti-DGP IgG antibodies in the test and control samples can be evaluated by "point-to-point" plotting of the extinction values(OD) measured for the 3 calibrators against the corresponding units (linear/linear) for calculation of the standard curve. For quantitative interpretation of results, the EuroImmun upper limit of normal range which is 25 relative unit (RU)/mL was used. The interpretation range as recommended by Euroimmun is; “Positive for ≥ 25 RU/mL and “negative “for < 25 RU/mL.

2.2.3.2 Assay principle of Anti-tTG IgA test kit :

This kit provides a semi-quantitative or quantitative in vitro assay for human antibodies of the IgA class against tissue transglutaminase in serum or plasma. The test kit contains microtiter strips, each with 8 break-off reagent wells

coated with human tissue transglutaminase(antigens). In the first reaction step, diluted patient samples are incubated in the wells, and in case of positive samples, specific IgA antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labeled anti-human IgA (enzyme conjugate) catalyzing a color reaction.

Calculation of results: the amount of anti-tTG antibodies in the test and control samples can be evaluated by "point-to-point" plotting of the extinction values(OD) measured for the 3 calibrators against the corresponding units (linear/linear) for calculation of the standard curve. For quantitative interpretation of results, the EuroImmun upper limit of normal range which is 20 relative unit (RU)/mL was used. The interpretation range as recommended by EuroImmun is; “Positive for ≥ 20 RU/mL and “negative “for < 20 RU/mL.

2.2.3.3 Assay procedure for measuring serum titer of Anti-DGP IgG & Anti-tTG IgA antibodies:

As the method of measurement is the same for both kits, therefore it was mentioned once to overcome repeating.

With the exception of the Wash Buffer, all other reagents; calibrators, enzymes, substrates and solutions came in the kit, were ready to use. The Wash Buffer 1x solution was prepared by adding 450 mL of distilled water to 50 mL of the Wash Buffer 10x concentrate.

The samples were diluted in the Sample Buffer by adding 5 µL of the sample to 1 mL of Sample Buffer and vortexed gently. One hundred microlitres (100 µL) each of the calibrators, positive control, negative control and diluted samples were dispensed into appropriately labeled wells of a microwells plate and the plate was incubated for 30 minutes at room temperature. The plate was

then washed using the initially prepared Wash Buffer 1x in an automatic plate washer. After 3 cycles of washing, the content of the plate was disposed of completely by tapping it on absorbent paper with the openings facing downwards after which 100 µL of the Enzyme Conjugate (peroxidase-labelled anti-human antibody) was added into each well. The plate was then incubated at room temperature for 30 minutes. After incubation, 3 cycles of washing were executed, then 100 µL of chromogen/ Substrate Solution was added to each well and the plate was incubated at room temperature in the dark for 15 minutes . After incubation, 100 µL of Stop Solution was quickly added to each well, the plate was gently shacked to ensure homogenous distribution of the solution and the photometric measurement of the wells was carried out using a microplate reader at 450 nm wavelength as well as a reference wavelength.

2.3 Statistical analysis:

Data analysis was conducted using SPSS (version 24.0). All quantitative variables or numbers were expressed in form of mean +/- standard deviation. Independent student's t test was used to observe statistical significant association between the cytokines and serum antibodies of the test and control samples. On the other hand, qualitative data were expressed as numbers (N) and percentages, and qualitative variables were analyzed using Chi-square test. Pearson correlation analysis was used to reveal the association between two or more of the related quantitative variables.

*A p values <0.05 were considered statistically significant**.**

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

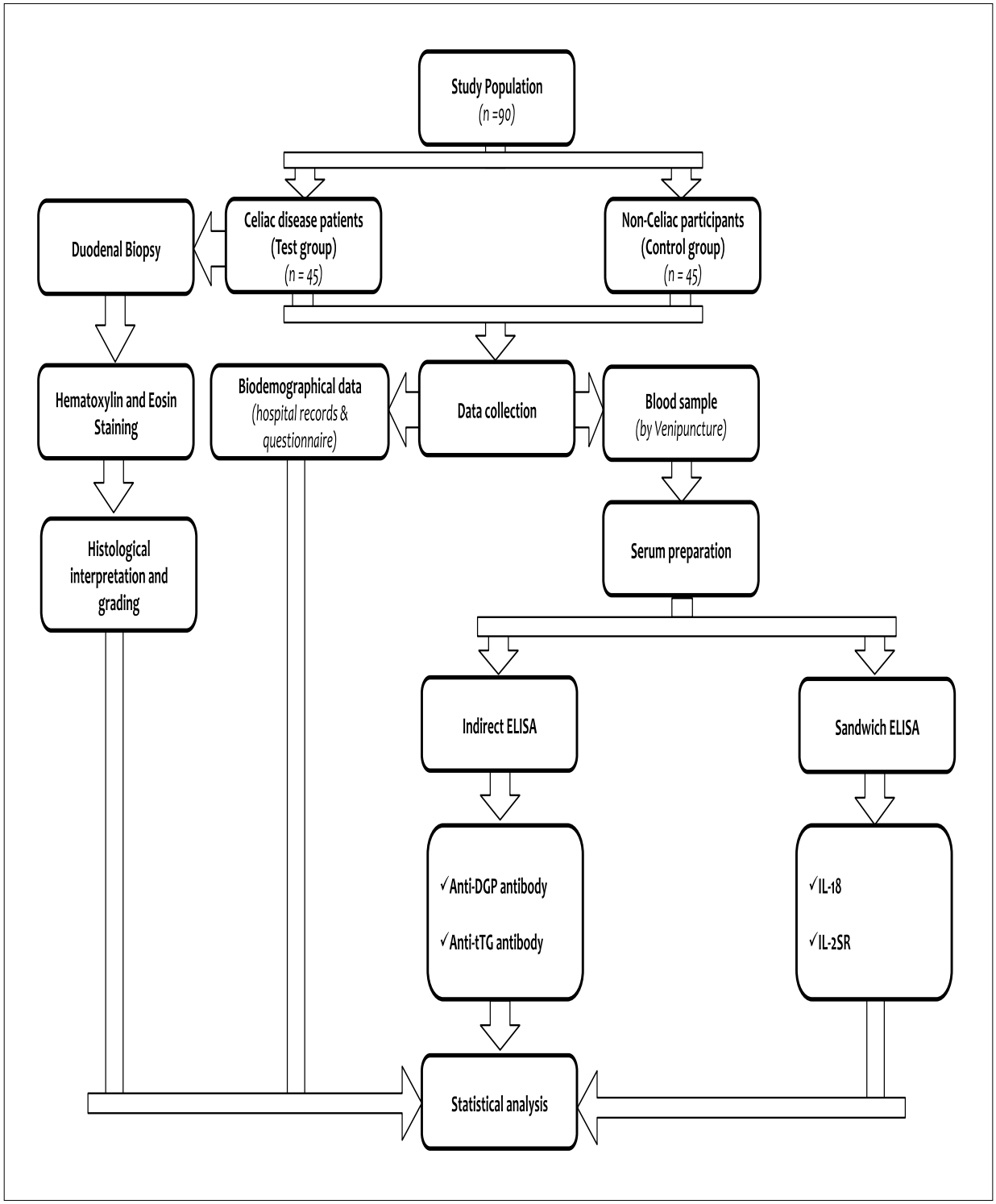

Figure 2.1: Schematic presentation showing details of the materials and methods employed in the study.

CHAPTER THREE

RESULTS

Chapter Three

The Results

3.1 Age distribution of participants:

The participant's age of the case study group ranged from 17 to 56 years with a mean± standard deviation (SD) of 33.86± 10.79 years, while that of the control group age was 30.28±10.35 years. The majority of the participants in the patient and control groups were in the age range of 20-29 years with percentages of 37.8% and 48,9% respectively. There was no significant statistical difference between the age groups of both control and patients (p=0.112) as illustrated in Table 3.1 below.

Table 3.1: The Age distribution of both patients and control groups.

Age interval (years)	**Patients N (%)**	**Mean± (SD)**	**Control N (%)**	**Mean ± (SD)**	***p* value**
10-19	2 (4.4)	33.86± 10.79	5 (11.1)	30.28± 10.35	0.112
20-29	17 (37.8)		22 (48.9)		
30-39	11 (24.4)		10 (22.2)		
40-49	9 (20)		4 (8.9)		
≥50	6 (13.3)		4 (8.9)		
Range	17-56		17-55		
Total	45 (100%)		45 (100%)		

N= number, (%)= percent , (SD)= standard deviation

3.2 The Gender of patients and control groups:

In the patient group there were 11(24.4%) male and 34(75.6%) female while in the control group, had 20(44.4%) male and 25(55.6%) female individuals. These

statistics show that there was significant difference between the patients and control groups in relation to gender (p=0.045) as shown below in the Figure 3.1.

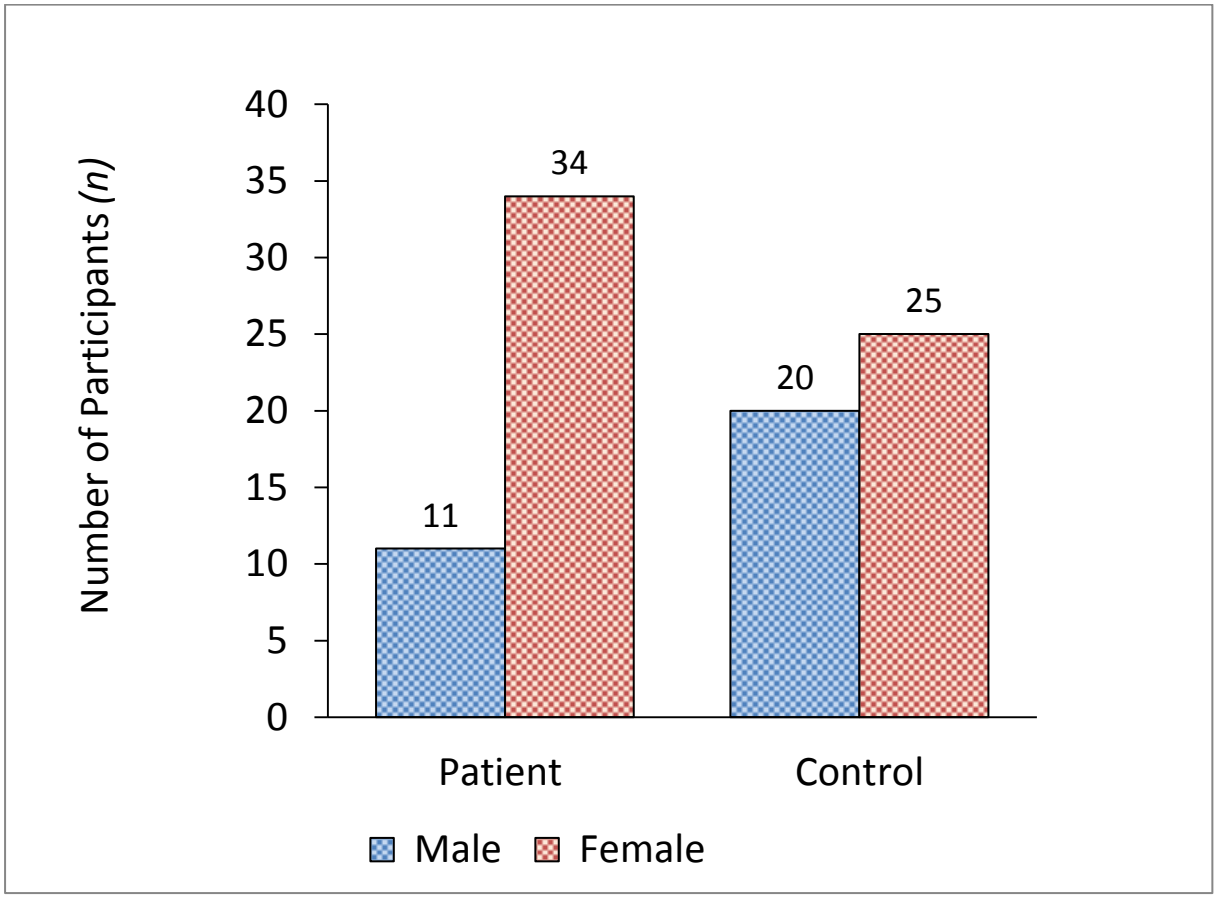

Figure 3.1: The gender distribution of the both patients and control groups.

The distribution of the participants is based on gender for the patient and control groups indicating that the number of females was significantly higher in the Patient group compared to males, while although more females were recruited in the control group relative to men, the difference in their numbers was not statistically significant.

3.3 Anemia in patients of Celiac Disease and control groups:

Regarding the anemia in the patients group, the mean value for hemoglobin was 10.68±1.71 g/dl while for the control group, the mean value was 12.52±1.65 g/dl. For ferritin, the patient group had mean value of 16.51± 15.82 ng/ml, while

for the control group, mean ferritin value was 42.63±39.41 ng/ml. With the application of t-test, the results showed significant statistical difference between the patient and control groups ($p<0.05$). Table 3.2 shows these variables.

Table 3.2: The anemia parameters for the patients and control groups.

Clinical manifestations	Patients N (%)	Mean ±SD	Control N (%)	Mean ±SD	*p* value
Anemia					
Low Hb	26 (57.8)	10.68 ±1.71	16 (35.6)	12.52 ±1.65	0.034
Normal Hb	19 (42.2)		29 (64.4)		
Total	45 (100)		45 (100)		
S. Ferritin Level					
Low	35 (77.8)	16.51 ± 15.82	17 (37.8)	42.63 ±39.41	0.0001
Normal	10 (22.2)		28 (62.2)		
Total	45 (100)		45 (100)		

N= number, (%)= percent , (SD)= standard deviation, Hb=Hemoglobin

3.4 Clinical manifestations of patients with CD in respect to the gender:

The majority of the CD patients presented with •**typical or intestinal symptoms** (73.3%) whereas ••**extraintestinal clinical features** percentage report was (26. 7%).The number and percentage of the male and female CD patients with these symptoms are illustrated in Table 3.3 below; however, the statistical records clear up that there is no significant association between them (p value more than 0.05). The results of presentation of anemia between male and female patients with CD showed predominance of anemia in female to males with statistical significant difference observed between the two gender types at ($p<0.05$). •**Typical or intestinal symptoms**: Include diarrhea,

steatorrhea, abdominal pain, abdominal bloating or distension, nausea and vomiting, poor appitite, constipation, dyspepsia, dysphagia and weight loss.

••**Atypical or extra-intestinal symptoms:** Include micronutrient deficiencies like iron deficiency anemia, short stature, growth retardation or failure to thrive, oral manifestation, menstrual disturbances, infertility, skin manifestation, osteoporosis, and lethargy…etc.

Table 3.3: The clinical manifestation of patients with CD in relation to gender.

Clinical disorder	**Male** **N (%)**	**Female** **N (%)**	**Total** **N (%)**	***p* value**
Intestinal	7 (63.7)	26 (76.5)	33 (73.3)	0.402
Extra-intestinal	4 (36.3)	8 (23.5)	12 (26.7)	
Total	11 (100)	34 (100)	45 (100)	
Anemia parameters	**Male** **N (%)**	**Female** **N (%)**	**Total** **N (%)**	***p* value**
Hemoglobin level*				0.002
Low Hb	2 (18.2)	24 (70.6)	26 (57.8)	
***Normal Hb**	9 (81.8)	10 (29.4)	19 (42.2)	
Total	11 (100)	34 (100)	45 (100)	
S. Ferritin Level**				0.032
Low	6 (54.6)	29 (85.3)	35 (77.8)	
****Normal**	5 (45.4)	5 (14.7)	10 (22.2)	
Total	11 (100)	34 (100)	45 (100)	

*normal Hb level in female ≥ 12g/dl and in male≥ 13.5g/dl
** normal s.Ferritin in female is ≥ 12ng/ml while in male ≥20 ng/ml

3.5 Serum level measurement of anti-tTG IgA in the patients and control groups:

In this study, the mean ±SD of anti tTG antibody level was 184.17±176.52 RU/mL in the patient group while in the control group was 6.45 ±2.55 RU/mL with a mean difference of 177.72 RU/mL . Patients with negative results below the 20 RU/ml constituted 28.9%(13 out of the total 45) while those with positive values(≥20 RU/ml) were 32 out of 45(71.1%).Regarding the control group ,the majority of individuals included in the study were within the negative limit;43 out of 45(95.6%). By application of T-test,there was significant difference between the patient and control groups in relation to the anti tTG measurement(p=0.001) as cleared by the Figure 3.2.

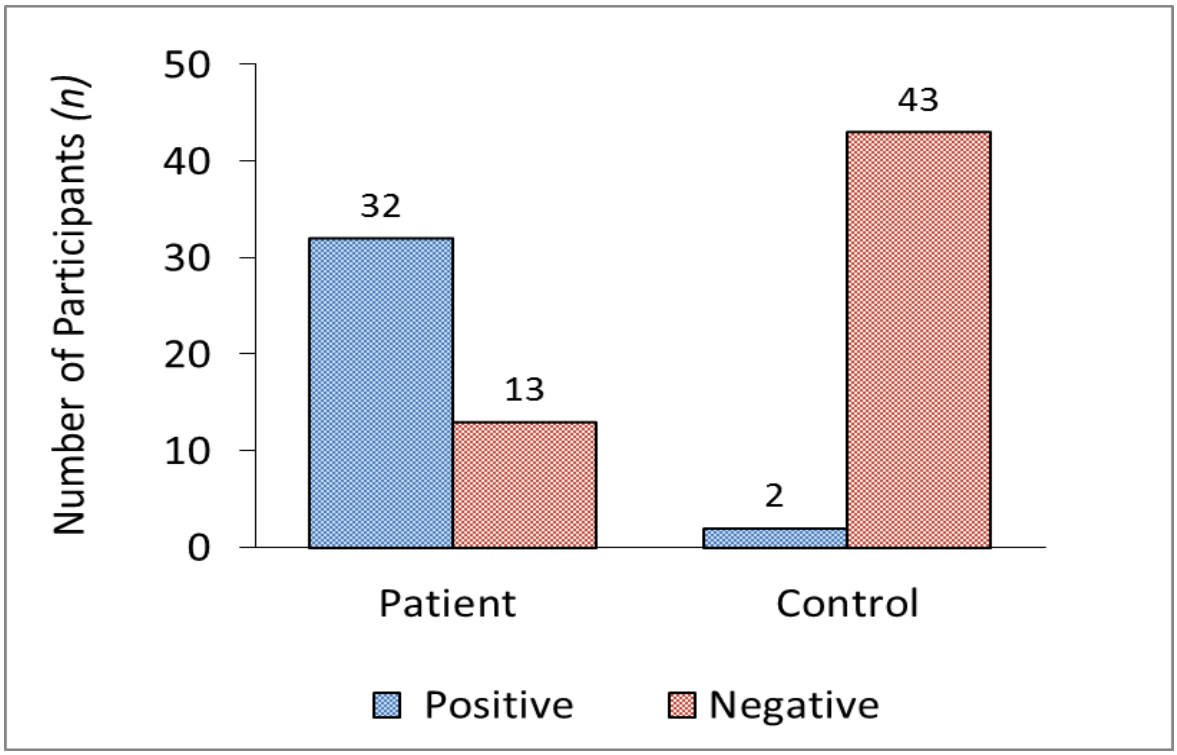

Figure 3.2: The distribution of patients and control group in relation to the serum measurements of anti-tTG antibody, p=0.001 (Positive results ■, Negative results ■).

The distribution of the participants based on serum anti-tTG antibody for the patient and control groups indicating that the number of positive values to anti-tTG was significantly higher in the patient group compared to control, while more negative values were recorded in the control group relative to the patient group, and the difference between the two groups was also statistically significant at $p<0.05$.

3.6 Serum level measurement of anti-DGP IgG in the patients and control groups:

The measurement of anti-deamidated gliadin peptide was applied in this study. The level of 25 RU/mL was chosen as the differentiation point between positive and negative results. The Table 3.4 down shows the data of the patients and control group and by application of T-test , there was significant difference between the 2 groups of study(p=0.001).

Table 3.4: Serum measurement of anti-DGP IgG in both patients and control groups.

Anti-DGP IgG	**Patients N (%)**	**Mean ±SD**	**Control N(%)**	**Mean ±SD**	*p **value**
<25 RU/mL	3 (6.7)		41 (91.1)		
≥25 RU/ml	42 (93.3)	180.77	4 (8.9)	8.49	0.001
Total	45 (100)	±158.41	45 (100)	±6.64	

*Independent-Samples t test was used.
*Anti-DGP IgG = anti-deamidated gliadin peptide of IgG type.

3.7 Serum results of soluble IL-2 Receptor(sIL-2R) in the participants:

Serum sIL-2 R was tested in both the control and patients group. The data showed that the persons in the control group have more negative (<160ng/L) scores(37 out 45),while the patients with more positive values of sIL-2R were 21 out of 45 , so there was significant statistical differences in the patient group than the control group($p<0.05$)as illustrated in the Table 3.5 .

Table 3.5: Serum sIL-2R levels in CD patients compared with healthy control group.

sIL-2 R	Patients N	(%)	Mean ±SD	Control N	(%)	Mean ±SD	*p value
< 160ng/L	24	(53.3)	198.14 ±146.21	37	(82.2)	121.05 ±130.01	0.003
≥ 160ng/L	21	(46.7)		8	(17.8)		
Total	45	(100)		45	(100)		

*t-test was applied. *sIL-2R= soluble interleukin -2 Receptor.

3.8 Detection of IL-18 serum level in the patients and control groups:

According to IL-18 kit, the 16ng/L considered as a cutoff point to differentiate between the positive & negative results. In this study, the positive Interleukin 18(IL-18) cases of patient group were 31 out 45(68.9%) while cases with negative results recorded 14 out 45(31.1%). The mean ±SD of the patients sample was 21.72 ±12.55 while of the control group was 13.87±12.17 with a mean difference equals to 7.85.In the control cases, the positive measurement of IL-18 were 6 out of 45(13.3%) cases and the negative cases constituted

86.7%(39 out of 45) .By application of the t-test , there was significant difference between the patient &control groups(p=0.001) as seen in the Table 3.6 below.

Table 3.6: Serum IL-18 levels in CD patients compared with healthy control group.

IL-18 level	Patients N (%)	Mean ±SD	Control N (%)	Mean ±SD	*p value
< 16ng/L	14 (31.1)		39 (86.7)		0.001
≥ 16ng/L	31 (68.9)	21.72 ±12.55	6 (13.3)	13.87 ±12.17	
Total	45 (100)		45 (100)		

*Independent-Samples t test was used. *IL-18= interleukin-18

3.9 Distribution of the four serological markers in the patients' group:

Table 3.7 illustrates that serum level of the anti-DGP antibody recorded the highest positive results, 93.3%(42 out of 45) patients more than the other markers, and it recorded the lowest negative results 6.7%(3 out of 45) cases. The lowest positivity was found in the sIL-2R marker 46.7% (21out of 45), this marker showed the highest negativity levels in the patient group with 53.3%(24 out of 45). The values presented in the mentioned table show significant statistical difference among the four serological markers used in the study with regards to the positivity and negativity to the markers analyzed in the CD patient groups ($p<0.05$).

Table 3.7: The association among serum results of Anti-tTG IgA , Anti-DGP IgG ,sIL-2R and IL-18 in the patients group.

Serological markers	Positive Serum level N (%)	Negative Serum level N (%)	Total N (%)	*p* value
Anti-tTG IgA	32 (71.1)	13 (28.9)	45 (100)	(0.001)
Anti-DGP IgG	42 (93.3)	3 (6.7)	45 (100)	
sIL-2 R	21 (46.7)	24 (53.3)	45 (100)	
IL-18	31 (68.9)	14 (31.1)	45 (100)	

Anti-tTG IgA=anti-tissue transglutaminase of IgA type, Anti-DGP IgG = anti-deamidated gliadin peptide of IgG type, sIL-2R=soluble interleukin -2 Receptor, IL-18=interleukin-18.

3.10 The correlation of the serum markers among them and with the anemia parameters:

The Table 3.8 explains the correlation among the serum markers and the other laboratory findings (Hb and serum ferritin).

The data shows that there is positive correlation(r=0.493) between the serum level of anti- tTG IgA and DGP IgG antibody levels with a significant value (0.001). There was weak positive correlation between the tTG IgA and the sIL-2R (r=0.112) with significant difference (p=0.046). Also there was positive correlation between tTG IgA and IL-18(r=0.469) with a significance equal to (p=0.025). While negative correlation was observed between the tTG IgA with both the Hb and serum ferritin (r=-0.554 &-0.387 respectively) with high significance (0.001 and 0.009) respectively.

The data show that there is weak positive correlation between the DGP IgG and with both sIL-2R (r= 0.377) with significance (0.011), and IL-18 (r=0.165) with significance (p=037). There was significant negative correlation between DGP

IgG and Hb(r=-0.528) with significant difference (0.001). Weak negative correlation was noticed between the DGP and s. ferritin (r=-0.301) with significance (0.044).

Considering the relationship between sIL-2R and IL-18, data recorded positive correlation (r=0.674) with significant level 0. 001.There was weak negative correlation between sIL-2R with both Hb and serum ferritin (-0.274&-0.211 respectively) with on significance. The relation of serum IL-18 with both Hb and ferritin showed negative correlation (- 0.161 and -0.109 respectively) with no significant level.

Table 3.8: The correlation of the four parameters together with the Hb and serum ferritin.

		tTG_IgA	DGP_IgG	sIL_2R	IL18
tTG_IgA	**r**		0.493**	0.112	0.469**
	Sig.		0.001	0.046	0.025
DGP_IgG	**r**	0.493**		0.377*	0.165
	Sig.	0.001		0.011	0.037
sIL_2R	**r**	0.112	0.377*		0.674**
	Sig.	0.046	0.011		0.001
IL18	**r**	0.469**	0.165	0.674**	
	Sig.	0.025	0.037	0.001	
Hb	**r**	-0.554**	-0.528**	-0.274*	-0.161
	Sig.	0.001	0.001	0.068	0.291
Ferritin	**r**	-0.387*	-0.301*	-0.211*	-0.109
	Sig.	0.009	0.044	0.163	0.477

Pearson correlation was used. Correlation is significant at p value of <0.05.
* intermediate correlation. ** strong correlation.

3.11 The distribution of the four serological markers in relation to gender of patients:

The data presented in the Table 3.9 were planned to show the possible correlation of the positive serum levels of the 4 markers used in this study with the gender of the patients .Due to the higher number of the female in the sample of the patients group, the data look as if the female group were higher in the recorded cases but the statistical analysis shows that there is no significant difference among the four markers enrolled in this study in relation to the gender of the patients($p>0.05$).

Table 3.9: The positive results of Anti-TG IgA, Anti-DGP IgG, sIL-2R and IL-18 in relation to the gender of the patients.

Serum level	Male		Total		Female		Total		*p* value
	N	(%)	N	(%)	N	(%)	N	(%)	
Anti-tTG IgA	6	(54.5)	11	(100)	26	(76.5)	34	(100)	0.422
Anti-DGP IgG	9	(81.8)	11	(100)	33	(97.1)	34	(100)	
sIL-2 R	5	(45.5)	11	(100)	16	(47.1)	34	(100)	
IL-18	10	(90.9)	11	(100)	21	(61.8)	34	(100)	

Level of significant(*p* value) at <0.05 was used. Anti-tTG IgA=anti-tissue transglutaminase of IgA type, Anti-DGP IgG = anti-deamidated gliadin peptide of IgG type, sIL-2R=soluble interleukin -2 Receptor, IL-18=interleukin-18.

3.12 Patients' serum values of IL-18 and sIL-2R in relation to both positive DGP-IgG and tTG-IgA or discrepant DGP-IgG and tTG-IgA:

Patients with positivity of both antibodies (DGP-IgG and tTG-IgA) showed the more percentages of elevated interleukins (sIL-2R and& IL-18) more than the percentages that are associated with conflicting antibodies. While patients with negative values of sIL-2R clarified the same percentages for discrepant and&

elevated both antibodies. On the other hand, patients who have negative results of IL-18 revealed nearly approximated percentages for positive and discrepant antibodies, and these differences are statistically significant ($p<0.05$) as presented in Table 3.10.

Table 3.10: Patients' serum values of IL-18 and sIL-2R in relation to both positive DGP and tTG or discrepant DGP and tTG.

		Positive Both tTG-IgA and DGP-IgG N (%)	Discrepant values* of tTG and DGP N (%)	Total N (%)	P Value
sIL-2R	Positive N (%)	17 (81%)	4 (19%)	21 (100%)	0.031
	Negative N (%)	12 (50%)	12 (50%)	24 (100%)	
		29	16		
IL-18	Positive N (%)	23 (74.2%)	8 (25.8%)	31 (100%)	0.042
	Negative N (%)	6 (42.9%)	8 (57.1%)	14 (100%)	
	Total	29	16		

* Discrepant values were either (+ve DGP-IgG and -ve tTG-IgA) or (-ve DGP- IgG and + ve tTG-IgA).

3.13 Histological outcome and MARSH grading of the patients:

The grading of the results of histological analysis of duodenal biopsy conducted on the patients based on MARSH grading is presented in the figures below. Figure 3.3 shows increased intraepithelial lymphocyte which represents

MARSH I grade, Figure 3.4 shows crypt hyperplasia and increased IEL, representing MARSH II classification and Figure 3.5 shows subtotal villous atrophy which represents MARSH III grade.

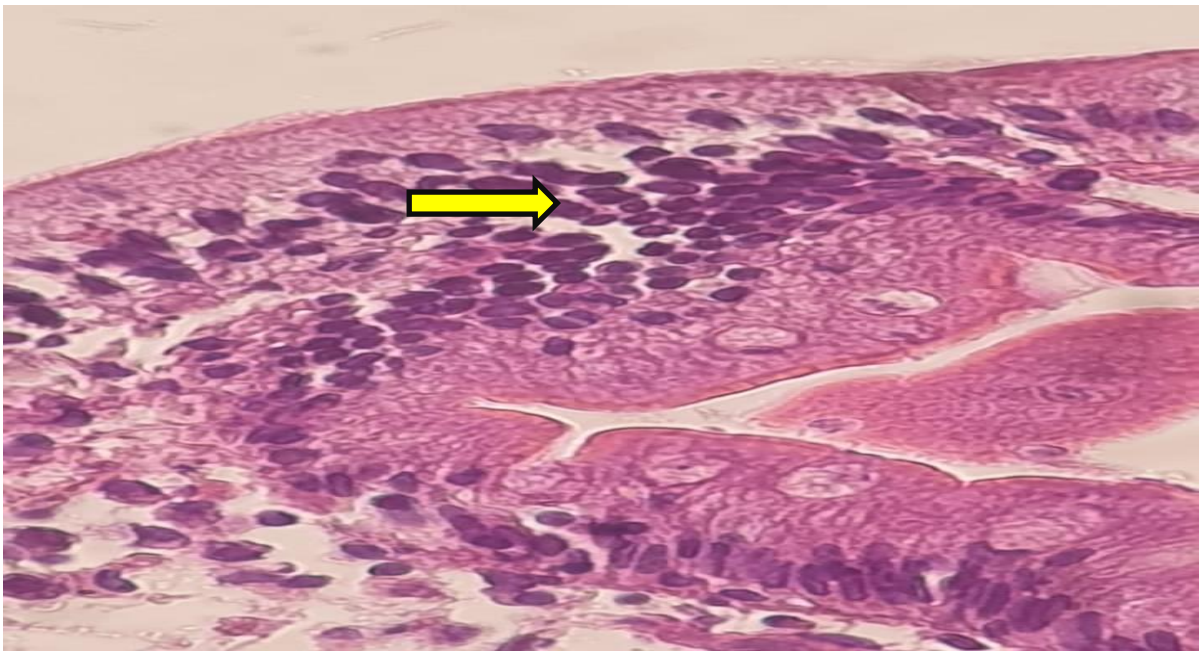

Figure 3.3: An increased intraepithelial lymphocyte (yellow arrow) in celiac duodenal tissue representing MARSH grade I (x40).

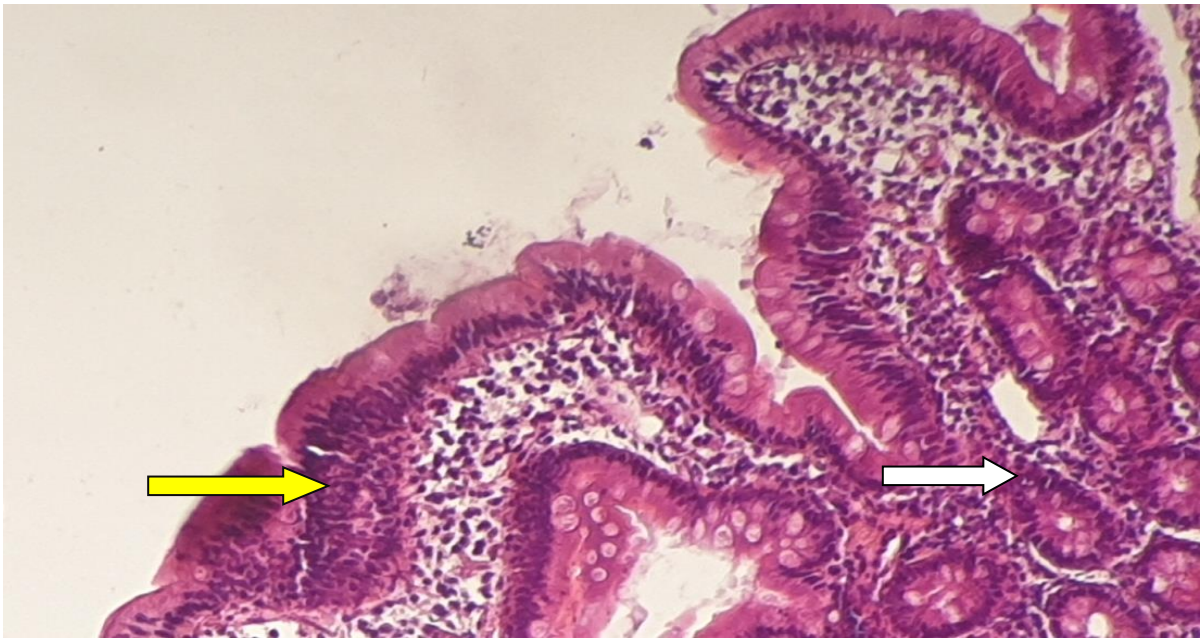

Figure 3.4: The Duodenal tissue in Celiac disease (MARSH grade II) showing crypt hyperplasia (white arrow) and increased IEL (yellow arrow) (x 10).

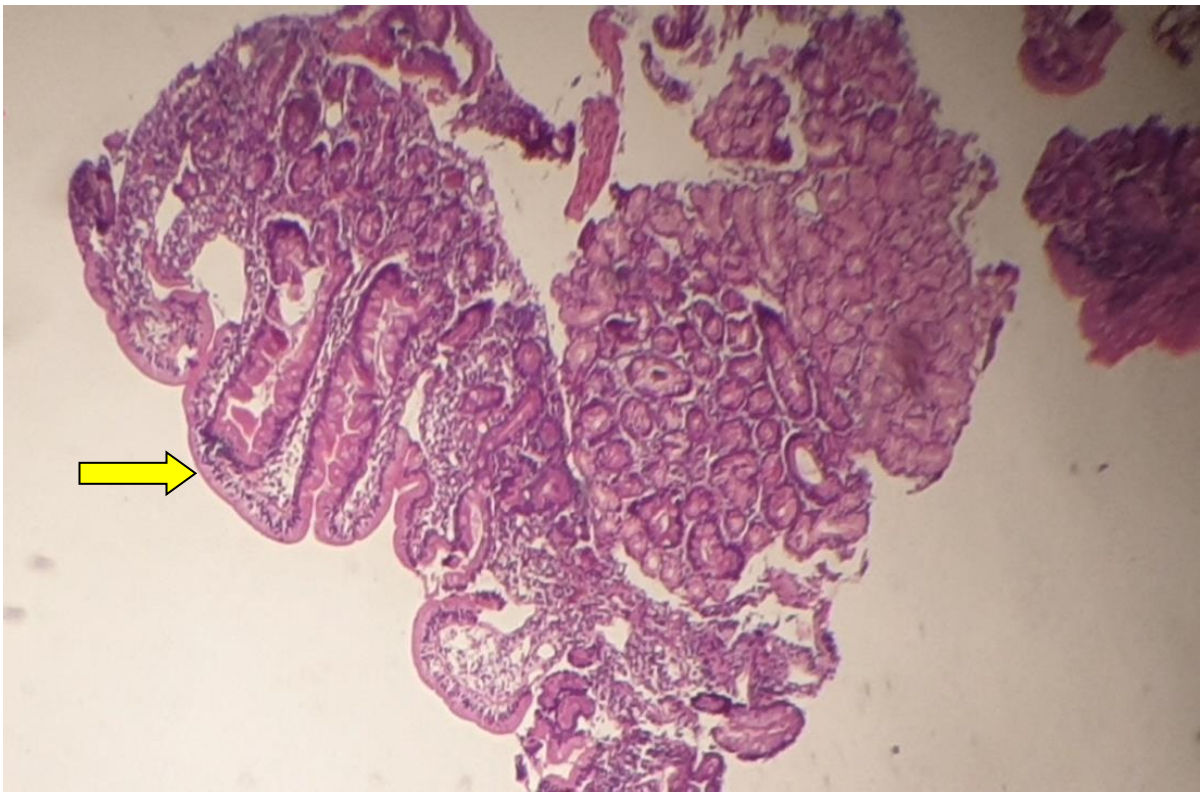

Figure 3.5: The MARSH (Grade III) Duodenal tissue in Celiac disease showing subtotal villous atrophy (yellow arrow) (X 40).

3.14 MARSH histological grades in relation to patients' gender:

Table 3.11 illustrates the distribution of the different MARSH grades of the duodenal histology in regards to the patient's gender. The females had the higher number of patients in relation to the different MARSH grades, being the highest lies within the grade I (44.1%) (15 out of 34 cases) and lowest record was within the grade II (26.5%) (9 out of 34 total female cases). Regarding male patients, the highest recorded cases were within the grade I (63.6%) (7 out of 11 cases) while rest of cases were distributed equally within Grades II and III (i.e. 2 cases for each grade). There was no significant difference between the different histological grades in relation to the gender of patients($p>0.05$).

Table 3.11: The patients MARSH Histological grades in relation to the gender.

Gender	MARSH grade I N (%)	MARSH grade II N (%)	MARSH grade III N (%)	Total N (%)	*p* Value
Male	7 (63.6)	2 (18.2)	2 (18.2)	11 (100)	0.528
Female	15 (44.1)	9 (26.5)	10 (29.4)	34 (100)	
Total	22	11	12	45	

*Chi-square with level of significance at $P<0.05$ was applied.

MARSH=Mucosal Algorithm Rules for Scoring Histology

3.15 Patients' MARSH histological grades in association with the serological markers results:

The combination of the histological diagnosis with the serological tests will clarify the severity level of CD. Each serological marker was studied independently in relation to the grades of the MARSH system. The distribution of the data reveals that there is significant difference in positive and negative limits of the (anti-tTG IgA, sIL-2R and IL-18) with the variable MARSH grading system($p<0.05$).

This relationship was not achieved in the correlation of the different duodenal histological grades with the highest and lowest limits for the anti-DGP IgG antibodies, so there is no statistical significance ($p >0.05$) as seen in the Table 3.12 .

Table 3.12: Patients' MARSH histological grades in relation to the four parameters results.

ELISA-detected parameter	MARSH grade I N (%)	MARSH grade II N (%)	MARSH grade III N (%)	*p* value
Anti t-TG IgA				
< 20 IU/ML	11 (50 %)	1 (9.1%)	1 (8.3%)	0.009*
≥ 20 IU/ML	11 (50%)	10 (90.9%)	11 (91.7%)	
TOTAL	22 (100%)	11 (100%)	12 (100%)	
Anti- DGP IgG				
< 25 IU/ML	1 (4.5%)	1 (9.1%)	1 (8.3%)	0.854
≥ 25 IU/ML	21 (95.5%)	10 (90.9%)	11 (91.7%)	
TOTAL	22 (100%)	11 (100%)	12 (100%)	
sIL-2R				
< 160ng/L	5 (22.7%)	9 (81.8%)	10 (83.3%)	0.001*
≥ 160ng/L	17 (77.3%)	2 (18.2%)	2 (16.7%)	
TOTAL	22 (100%)	11 (100%)	12 (100%)	
IL-18				
<16ng/L	3 (13.6%)	4 (36.3%)	7 (58.3%)	0.024*
≥ 16ng/L	19 (86.4%)	7 (63.7%)	5 (41.7%)	
Total	22 (100%)	11 (100%)	12 (100%)	

*P-value was calculated as less than 0.05 level.

Anti-tTG IgA=anti-tissue transglutaminase of IgA type, Anti-DGP IgG = anti-deamidated gliadin peptide of IgG type, sIL-2R=soluble interleukin -2 Receptor, IL-18=interleukin-18.

CHAPTER FOUR

DISCUSSION

Chapter Four

Discussion

Celiac disease, also known as gluten sensitive enteropathy, is an autoimmune disorder affecting persons who have genetic susceptibility, the immunopathogenesis happens in the small intestine(both in the epithelium and lamina propria)(Green and Jabri, 2006). According to the elevated incidence of CD, there are scarce Iraqi researches elaborated around the proinflammatory cytokines coordinating CD immunepathogenesis, genetic susceptibility, and the distribution of the disease in various races of Iraqi people. This study elaborated on the function of sIL-2R and IL-18 as biomarkers for assessing the severity of CD in correlation with circulating anti-tTG IgA and anti-DGP IgG matched for Socio-demographic characteristics (gender and age).

4.1 Age distribution of the participants

The age of participants elected for the study regarding CD patients and controls was from 17 years old and above i.e. adults & elderly, with the range from 17-56 years, the mean age for the CD patients is 33.86± 10.79 years, while that of control group is 30.28±10.35 years, where highest number of both groups; patients and controls were presented between 20-29 years old (adults) with percentages of 37.8% and 48,9% respectively. After comparison among cases and controls there was no statistical significance (p value=0.112). These age groups compared with many studies emphasizing that CD can be diagnosed at any age with most incidence at early childhood or in the third decade of age (Śmigielski and Gmitrowicz, 2014). However, children were not included in this study a circumstance that would not affect the findings of this study as Al-Saadi and his team reported that CD clinical presentation can occur at any time of life (Al-Saadi and Abid, 2009). Early realization about CD and the development of

the investigations may elevate CD diagnosis in adulthood (Al-Saadi and Abid, 2009).

4.2 The Gender of patients and control groups

The data of the study presented in Figure 3.1 showed that there are more females both in the patients and the controls groups, in patients the percentage of the affected female was 75.6% while the male was 24.4% with statistical difference regarding the gender observed (p=0.045).This fact was shown in many researches which declared that the CD is predominant more in the women (Dixit et al., 2014)like most other autoimmune diseases(Quintero et al., 2012), Hameed and his research colleagues also reported that CD is more in women than in men (Hameed et al., 2016) while Green and his team illustrated that female to male ratio was 2.9:1 (Green et al., 2001), but mostly with no difference between the two genders in relation to the age onset or the mode of the clinical manifestation of the CD disease(Bai et al., 2005, Caio et al., 2019a).

4.3 Anemia in patients of Celiac Disease and control groups

Anemia is a common presentation in CD and can be a preliminary symptom of the patients (Mahadev et al., 2018)**.**The observations in this study illustrated that most of patients had anemia(57.8%) with a variable degrees of lowered Hb level, while in the control group the anemia which could be of different causes, comprised 35.6% with a significant difference between the two groups(p=0.034).These results are compatible with the most published researches which studied the anemia in CD (Laurikka et al., 2018).It is well known that CD is an autoimmune disease with a subsequent destruction of the intestinal villi that leads to decreased the mucosal surface with a secondary diminished absorption of minerals including the iron(Stefanelli et al., 2020).

Measurement of serum ferritin ,one of the tools to assess the iron deficiency anemia was applied in this study and results obtained show that 77.8% of patients group had low serum ferritin in comparison with the control group(37.8%) with a significant difference (p=0.001) (Table 3.2).This outcome has been previously illustrated in many articles (Wierdsma et al., 2013, Mahadev et al., 2018, Scricciolo et al., 2020) .

In Iraq, several articles focused on the association between the CD and both iron deficiency anemia and the low serum ferritin (Majeed, 2021).

4.4 Clinical manifestations of patients with CD in respect to the gender

Concerning the association between gender and clinical manifestations, the numbers clarify that both male and female patient present intestinal (like abdominal pain, bloating and nausea) manifestation and extra-intestinal (like short stature and osteoporosis) manifestations with no significant difference between the two genders (p=0.402) as seen in Table 3.3.This outcome was previously reported by other researchers (Laurikka et al., 2018).Dian Bai *et al* showed that there are no difference regarding the age of onset or the mode of presentation between male and female(Bai et al., 2005). Other studies concluded that the clinical signs are more recorded in female, partly due the higher incidence of CD in female (Rubio-Tapia et al., 2016).

The numbers in the Table 3.3 show that the females of the patient group had lower level of Hb and serum ferritin than the males in the same group with significant difference observed between the two groups(p values for HB &serum ferritin were 0.002 & 0.032 respectively). These data are in concordance with articles done by other authors (Ciacci et al., 1995). The female predominance of celiac disease and the menstrual loss of blood and other

stressful conditions may explain the higher women incidence of iron deficiency anemia than male(Abu-Zeid et al., 2014, Stefanelli et al., 2020) .

4.5 Serum level measurement of anti-tTG IgA in the patients and control groups

In this study the level of anti-tTG IgA was analyzed since the measurement of this antibody is recognized as powerful diagnostic tool of CD (Germenis et al., 2005).The data showed that the patients who had positive values were 32 out of 45 (71.1%) and those that had negative results were 13 out of the total 45 most of whom were at borderline levels. In the control group, the negative results were 95.6% and the positive results were 2 out of 45(0.04%), with a lower mean than the patient group that showed significant difference (p=0.001).These results are compatible with those published by other authors (Klapp et al., 2013).The positive values in the control group may occur as reported in some articles (Lewis and Scott, 2006). The sensitivity of the anti tTG-IgA for untreated CD is about 93.0 % and the specificity is also 96.5%(Lewis and Scott, 2010, Lebwohl et al., 2018),while in another older study the tTG sensitivity was 92% of untreated patients(Troncone et al., 1999).

The lower positivity of the anti tTG-IgA (71.1%) in this study might be due to IgA deficiency as previously suggested by Absah et al (Absah et al., 2017) or seronegative villous atrophy CD patients (Aziz et al., 2017). Also CD patients whom suffered from malnourishment and failure to get weight may show negative score of anti tTG-IgA (Aldaghi et al., 2016).

Anti-tTG test of IgG type was not used in this study because the precision of an isolated positive values of IgG to tTG (with negative values of IgA to tTG) in diagnosing CD is unknown and may not detect CD in the presence negative scores of anti tTG-IgA (Absah et al., 2017).

As stated by the WGO diagnostic protocol, tTG are an acceptable representative of the EMA antibodies, especially in countries with limited resources ("The EMA test needs expert observers, and detecting tTG antibodies using ELISA tests should therefore be recommended in settings with low expertise")(Bai et al., 2013, Ludvigsson et al., 2014).The WGO advise the assessment of the available local resources to develop a diagnostic protocol.

4.6 Serum level measurement of anti-DGP IgG in the patients and control groups

The usage of anti-DGP IgG in celiac disease detection is of more sensitivity and specificity of the naïve gliadin antibodies and it was seen to be a superior in the detection rate than some other serological tests of CD (Vermeersch et al., 2010).In this study the positive level of anti DGP in the patient group 93.3% (42 out the total 45) while in the control group ,the positive cases were just 4 out of 45 with clear significant difference observed between the two groups ($p< 0.05$). Even in the 4 positive control cases, their scores were so close to the cutoff points (borderline level), These results are compatible with several recent researches executed globally (Kelly et al., 2015).Few articles concluded that anti-DGP can occur in healthy control or other disorders like gluten ataxia(Rashtak et al., 2011).

In this study anti DGP-IgA test was not conducted as a result of lower sensitivity by using anti DGP-IgA (87.8%) than anti tTG-IgA (93%) with no significant differences between the two tests (Lewis and Scott, 2010) , furthermore anti DGP-IgG was carried out to detect CD in patients with negative anti tTG-IgA(Tonutti and Bizzaro, 2014) on the other hand the anti DGP-IgG has sensitivity of 98% and specificity range(90.3%-100%)(Schyum and Rumessen, 2013), L.Chaisemartin *et al* insisted on that anti-DGP was a sensitive predictive marker for detecting and following CD patient and it's widely

expressed in the serum CD patients, especially those with villous atrophy.(de Chaisemartin et al., 2015). .

Detection of the serum levels of the antibodies (anti tTG-IgA and anti DGP-IgG) in this study was achieved by utilizing quantitative calculation of the data giving more precision for the diagnosis in contrast to some laboratories that yield semi-quantitative results producing uncertain assessment (Tucci et al., 2014).

4.7 IL-18 and sIL-2R as predictive markers for CD immunopathogenesis

In this study, the serum positive results of sIL-2R in the patients group indicated in 21 out of 45 (46.7%) in comparison with 8 out of 45(17.8%) in the control group with a significant difference between the 2 groups(p=0.003) as seen in Table 3.5.These serum measurements were similar to other articles enrolled in CD and clarified that serum level of sIL-2R was higher in CD cases (especially in the untreated persons) than the control group (Kapoor et al., 2013).

Table 3.6 focused on the serum measurement of IL-18 in control and patient groups which showed that IL-18 positive results in CD patients were 68.9% while the positive control cases were just 6 out of 45 (13.3%) with a significant difference between the two groups (p=< 0.05). The pathogenic role of the IL-18 in the CD has been documented and it's found that it induces the γ-INF and has effect on the epithelial apoptosis and induces the inflammation associated with intake of gluten(Garrote et al., 2008).It was seen that serum IL-18 was higher in CD patients more than the control and it was not highly enhanced in other inflammatory bowel diseases(Lettesjö et al., 2005, Sattler et al., 2015).

4.8 Distribution of the four serological markers in the patients group

The results illustrated that all markers analyzed in the study showed higher

positive serum levels in the patient groups, except for the sIL-2R which showed semi equal results between the negative and positive levels in the patient group (24 negative vs 21 positive measurements), in spite of that even the negative values were so close to the cutoff point which equals to160 ng/L. This results were in concordance with that published in 2019.(Vorobjova et al., 2019).

Table 3.7 shows that 68.9%(31 out of 45) of patients had positive results regarding IL-18 and 31.1%(14 out of 45) revealed negative levels. These percentages were similar to other articles studied the level of this proinflammatory cytokine in the CD which was seen to be more specific for CD than other inflammatory diseases (Lettesjö et al., 2005, Leach et al., 2007).

The association of the serum measurements of Anti-tTG IgA , Anti-DGP IgG ,sIL-2R and IL-18 in the patient group as seen in Table 3.7,demonstrate a significant difference of p value= 0.001which is compatible with other studies (Vorobjova et al., 2019).

4.9 The correlation of the serum markers among them and with the anemia parameters

In the Table 3.8, t-test was applied to show the correlation and the significance among the four serum markers enrolled in this study and with the anemia clinical parameters (Hb concentration and serum ferritin). There was a positive correlation among the tTG IgA and anti-DGP IgG, sIL-2R and IL-18(0.493,0.112 and 0.469) and a significance equals to 0.001,0.046 and 0.025 respectively).The correlation between tTG IgA and DGP IgG (the old and new tests of CD) was emphasized by E.Marietta *et al* who record r=0.8(Marietta et al., 2009).Also the correlation of anti-tTG IgA with the sIL-2R was consistent with that reported by an article published in 2019(Vorobjova et al., 2019).The IL-18 cytokines which was seen to be increased both in the serum and peripheral blood of patients with active CD, was observed to be correlated with other CD

serum markers especially the anti-tTG(Lettesjö et al., 2005).Tissue transglutaminase autoantibodies play an essential role in initiation the inflammatory cascades after ingestion the dietary gluten ,leading eventually to duodenal villi destruction with a subsequent decreased mineral, including the iron with the consequent development of iron deficiency anemia(Freeman, 2015).In this study, there was significant negative correlation of anti-tTG with both the Hb and the serum ferritin, which means that increased serum tissue TG is associated with reduction in the blood factors and increasing the level of anemia in CD. These data are comparable with that obtained by A. Gangi et al (Ganji et al., 2014). Interestingly, the results published by D.Vini *et al* showed that there were no correlation between the anti-tTG and the development of anemia (Deora et al., 2017), which is different from the findings of this study . Negative correlation was also observed among the anemia markers (Hb) and anti-DGP, sIL-2R and IL-18 and serum ferritin with those three markers. As mentioned above that all the three serum measured markers (DGP,sIL-2R&IL-18) are associated with the inflammatory process which leads to villous atrophy and subsequent malabsorption of minerals, especially the iron and consequently the anemia (iron deficiency anemia) which is considered as the most common type in CD(Harper et al., 2007).Moreover anemia can be caused by folate or vitamin B12 deficiency in CD (Martín-Masot et al., 2019).

4.10 The distribution of the four serological markers in relation to the gender of patients

Concerning the relationship of the serum markers (tTG, DGP, sIL-2R & IL-18) with gender of patient group, Table 3.9 shows that the positive results of the serum markers in female are more than male, possibly due to that the female patients constitute the greater portion of the sample. In spite of that, there was no significant difference between both genders (p=0.422).This confirms that these markers are independent factors in both male and female patients of CD. An

article published in 2018,declared that there is no significant difference in the serum level of tTG between male and female patients with CD (Jansson-Knodell et al., 2018).The same manner regarding the level of anti-DGP IgG in both male and female celiac patients ,there was no significant difference in the positive records in both genders ($p>0.05$). Similar findings has been reported by other authors (Jansson-Knodell et al., 2018).

The sIL-2R, a lymphocyte activation marker was recorded as positive in 45.5% of the male and 47.1% of the female with no significant difference between the 2 genders. This findings is consistent with another article published in 2013 (Kapoor et al., 2013).

In relation to the distribution of the serum IL-18 in the patients group, the data show that 90.9% of male and 61.8% of female patients have positivity with no significant difference between the two genders($p>0.05$).These data coincide with a research published by other authors (Leach et al., 2007).

4.11 Patients' serum values of IL-18 and sIL-2R in relation to both positive DGP-IgG and tTG-IgA or discrepant DGP-IgG and tTG-IgA

The Table 3.10 shows that 81% of CD patients with positive sIL-2R have also positive both tTG IgA and DGP IgG and only 19% of those patients will have positivity for either the tTG or DGP, while patients with negative sIL-2R recorded 50%(12 out of 24) get positivity for both tTG and DGP and 50% recorded positivity for one of these auto-antibodies with a significance between both groups(positive and negative sIL-2R patient) was ($p=0.031$).This refers to that CD is an inflammatory process with multiple involvement of several inflammatory mediators with the subsequent arising of the histological and clinical disorders coordinated with the celiac sprue. The usage of more than one test is beneficial in diagnosing and following up CD patients since for example

there are cases who showed seronegative results, the combined usage of IgG-DGP and IgA-tTG will increase the sensitivity of diagnosis up to 100% and specificity to 97%(Anaya et al., 2013, Iervasi et al., 2020).

Regarding the association of the patients with triple positivity IL-18, tTG and DGP recorded 23 cases (74.2%), while only 8(25.8%) patients had positivity for IL-18 and for either tTG-IgA or DGP-IgG. Patients with negative IL-18 with double positivity for tTG and DGP scored only 6 out of 14(42.9%), and 8 of such patients (57.1%) had positivity for either the tTG or DGP. There was significant difference between the patients with positive and negative results of IL-18 in relation to double or single positivity of tTG and DGP($p=0.042$).Several studies have been conducted with the aim of elucidating the effect of serum IL-18 together with tTG IgA in untreated CD and they found positive correlation and increased number of both markers(Lettesjö et al., 2005). Presence of celiac cases with selective IgA deficiency is a detectable problem which may interfere with the diagnosis of CD if it depends only on IgA tests(Chow et al., 2012) and so multiple tests were used in this study to increase the diagnosis level.

4.12 MARSH histological grades in relation to patients' gender

The histological examination of upper small intestine biopsy is still considered as the gold standard method of diagnosis of celiac disease in most cases. Correlation of the histological examination with the clinical and serological information are essential to reach the final diagnosis(Kalhan et al., 2011).The MARSH system was introduced as a grading system for the histological changes associated with CD and still used till now(Peña, 2015).In this study, both male and female patients showed that most of them have MARSH grade I ,while there was predominance of the female for the other grades(II &III),probably due to the higher number of the female patients group in this study, as illustrated in Table 3.11. In spite of these data

differences, there was no significant differences (p>0.05) between male and female in relation to the histological changes(MARSH grading) of CD. This matter concordant with other studies (Kalhan et al., 2011).

4.13 Patients' MARSH histological grades in association with the serological markers results

As mentioned above the accuracy of the diagnosis of CD is increased by combining the intestinal biopsy interpretation and the serological testing for the patients of CD. Table 3.12 explains the association between the four serological markers enrolled, with the MARSH grades of the biopsies examined in this study. It was noticed that there was significant differences(p=0.009) between the patients with positive and negative values of anti-tTG IgA regarding the histological markers and it seems that the elevation of this autoantibody is associated with progressive intestinal damage interpreted by the MARSH grading. This finding is in agreement with several studies that showed the association of anti tTG with the histological grading of CD (Hawamdeh et al., 2016, Jora et al., 2017). Regarding the results of anti-DGP IgG, there was clear differences between the positive and negative results of this serum marker in the patients group rendering this antibody as a good diagnostic tool but when applied with the intestinal histological examination, the results showed no significance in regarding the MARSH grades(p=0.854),this might be due to all cases enrolled in this study were newly diagnosed and anti-DGP is the first antibody detected at the beginning of CD inflammatory process(Lammi et al., 2015). This matter is coincided with other authors who proposed that there is no significant association between the anti-DGP antibody and the MARSH grades (Hoerter et al., 2017). Conversely, there were authors who focused on the high association of the anti DGP with the MARSH grading of the CD (de Chaisemartin et al., 2015). The positive values of sIL-2R in concerned with MARSH grades were(17 in grade I, 2 in grade II and 2 in grade III) with a

significant difference (p=0.001). Other articles documented the positive association of sIL-2R with the degree of small intestinal mucosal damage (Vorobjova et al., 2019).The essential role of the proinflammatory mediator, IL-18 was explored and it showed great influence on γ-INF production which has a central role in the inflammatory scenario of CD (Lettesjö et al., 2005, Masaebi et al., 2020).In this study,IL-18 positive results were distributed within the grades of MARSH as the following;19 cases in grade I,7 cases in grade II and 5 in grade III with significant difference from the negative cases (p =0.024).Such association of IL-18 with the MARSH grading was cleared up in other articles (Heydari et al., 2018, Masaebi et al., 2020).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

Chapter Five

5.1 Conclusions

Depending on the results obtained from this study, several conclusions can be briefly listed below:

1- Two inflammatory mediators (cytokines) were selected in this study; sIL-2R and IL-18 which participate in the immunopathogenesis of CD. Both of them showed remarkable rise of their serum levels in patients with CD including both gender in comparison to the control group. So the inflammatory process associated with CD can be monitored by detecting the serum level of these cytokines, thereby physicians can predict the severity of CD, clinical outcome and the Gluten free diet compliance.

2-The usage of anti-DGP IgG has beneficial role for diagnosing CD in patients with total IgA deficiency in whom serum level of anti-tTG IgA are negative.

3- The data of this research show that markers were applied in this study (anti-tTG IgA, anti-DGP IgG, sIL-2R and IL-18) give positive inter correlation among them, and so focusing about the multifactorial inflammatory process of the CD. These four serum markers showed negative correlation with both the Hb and serum ferritin level, making them good predictors of the anemic status of the patients with CD.

4-Anti-tTG IgA and anti-DGP IgG were applied in this study and both showed great degree of serum level rise in the patients with CD more than the control group indicating that the antibody profile of CD is still the best serological tool for diagnosis and differentiation from other clinical conditions with similar manifestations.

5- The biopsy examination applied in this study shows that there was no significant difference of the histopathological changes between male and female patients referring that the intestinal mucosal injury due to the active CD occurs in both genders .

6-The study data conducted that there was a significant association of the positive results of anti-tTG IgA, sIL-2R and IL-18 with different MARSH grading.The only exception was the anti-DGP IgG level which was scored positivity in patients with the MARSH grading but with no significant difference among the 3 grades, because all patients were newly diagnosed for CD in addition to that the increase of anti-DGP IgG level preceding the rise of other antibody levels and the intestinal mucosal changes, a truth proved by other articles.

7-In this study females were more affected with CD than males like most other AID, a finding that comes with many studies.

8-Low leveled hemoglobin and low serum ferritin were seen in most of patients affected with CD as a remarkable manifestation different from the general population, a fact proved by numerous studies.

9-Being CD have different presentations ,the combining study of different serum markers in diagnosing & monitoring CD will be more informative, boost the sensitivity of the diagnosis and will reduce the number of missed diagnosed cases.

5.2 Recommendations

Some propositions are advised to be estimated for the detection of numerous factors included in the inflammatory process in CD patients, these are:

1- Planning for a national screening program for celiac disease covering variable age groups is recommended to discover the unknown cases, since CD is an iceberg-like condition with the hidden cases are more than the discovered and its incidence is increasing.

2- Patients attending health centers with chronic anemia and low serum ferritin are mandatory to be examined for serological and histological investigations to rule out CD.

3- Since CD has a genetic basis; so genetic counseling is preferred to prove the diagnosis in the suspected cases, in addition to that first degree relatives for a patient with CD should be advised for Celiac screening with HLA typing.

4- For a definite diagnosis of CD, combination of clinical, serological and histological findings is recommended.

5- Since the histological changes associated with CD can mimic other conditions, so adding other techniques like giemsa stain to exclude infectious causes of villous atrophy and immunohistochemical stain to point out the duodenal IEL.

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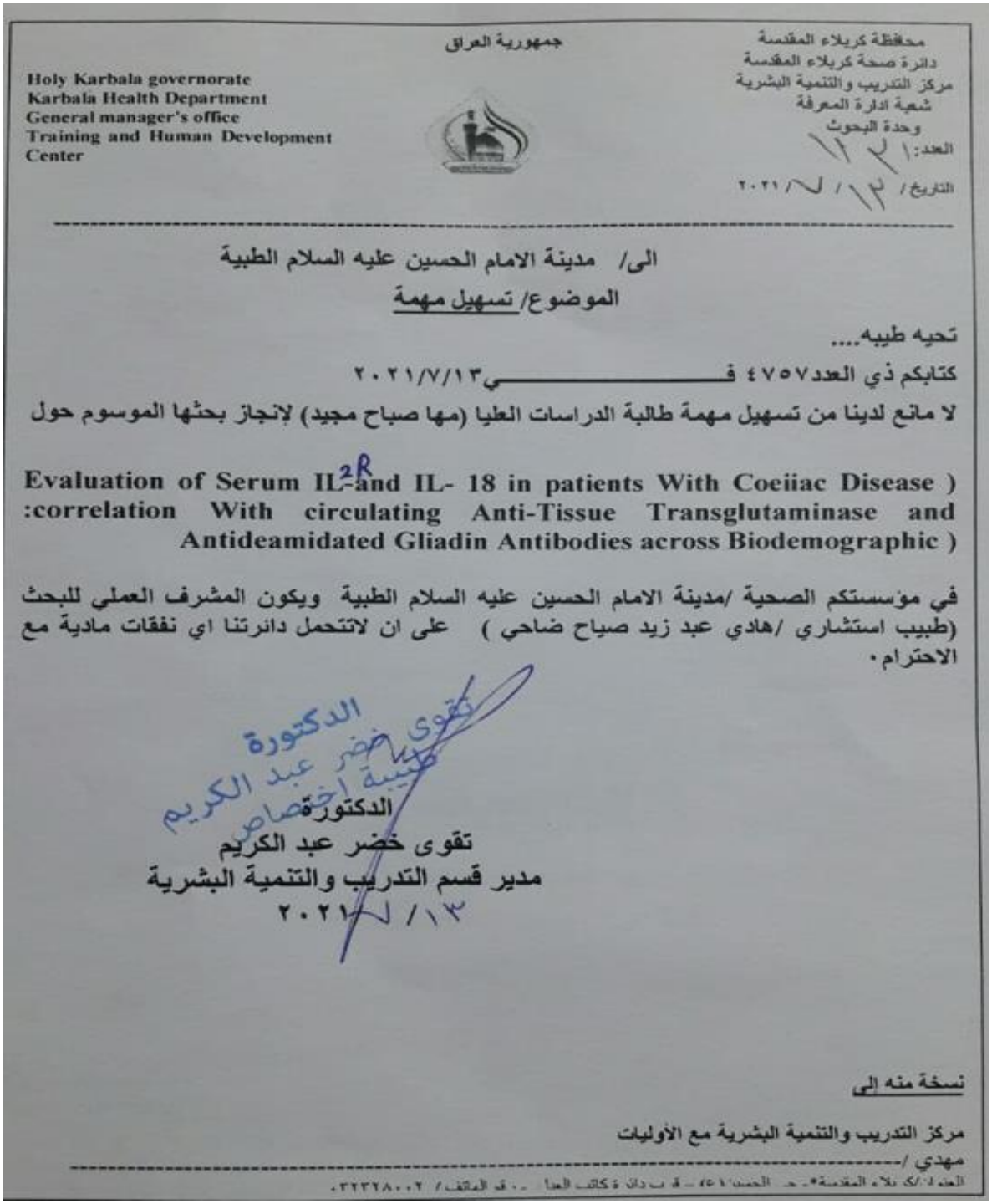
APPENDICES

APPENDICES

Appendix (A): Ethical Approval (Karbala health directorate/research unit)



جمهورية العراق

محافظة كربلاء المقدسة
دائرة صحة كربلاء المقدسة
مركز التدريب والتنمية البشرية
شعبة ادارة المعرفة
وحدة البحوث
العدد: ١٢٧
التاريخ / ١٣ / ٧ / ٢٠٢١

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

الى/ مدينة الامام الحسين عليه السلام الطبية

الموضوع/ تسهيل مهمة

تحيه طيبه....

كتابكم ذي العدد٤٧٥٧ في ٢٠٢١/٧/١٣

لا مانع لدينا من تسهيل مهمة طالبة الدراسات العليا (مها صباح مجيد) لانجاز بحثها الموسوم حول

Evaluation of Serum IL-2R and IL- 18 in patients With Coeiiac Disease) :correlation With circulating Anti-Tissue Transglutaminase and Antideamidated Gliadin Antibodies across Biodemographic)

في مؤسستكم الصحية /مدينة الامام الحسين عليه السلام الطبية ويكون المشرف العملي للبحث (طبيب استشاري /هادي عبد زيد صياح ضاحي) على ان لاتتحمل دائرتنا اي نفقات مادية مع الاحترام.

الدكتورة
تقوى خضر عبد الكريم
مديرة قسم التدريب والتنمية البشرية
٢٠٢١/٧/١٣

نسخة منه إلى

مركز التدريب والتنمية البشرية مع الأوليات
مهدي /

Appendix (B): The reagents & solutions of IL-18 ELISA kit (BIOTECH User Manual).

Reagents	**Quantity**
Standard solution(128ng/L)	0.5ml×1 (1 vial)
Pre-coated ELISA Plate	12*8 well strips×1 (96 wells)
Standard Diluent	3ml × 1vial
Streptavidin-HRP	6ml × 1vial
Stop Solution	6ml × 1vial
Substrate Solution A	6ml × 1vial
Substrate Solution B	6ml × 1vial
Wash Buffer Concentrate(25x)	20ml × 1vial
Biotinylated human IL-18 Antibody	1ml × 1vial
Plate Sealer	2 pics
User Instruction	1

Appendix (C): The reagents & solutions of sIL-2R ELISA kit (BIOTECH User Manual).

Reagents	**Quantity**
Standard solution(1280ng/L)	0.5ml×1 (1 vial)
Pre-coated ELISA Plate	12*8 well strips×1 (96 wells)
Standard Diluent	3ml × 1vial
Streptavidin-HRP	6ml × 1vial
Stop Solution	6ml × 1vial
Substrate Solution A	6ml × 1vial
Substrate Solution B	6ml × 1vial
Wash Buffer Concentrate(25x)	20ml × 1vial
Biotinylated human sIL-2R Antibody	1ml × 1vial
Plate Sealer	2 pics
User Instruction	1

Appendix (D): The components of anti-DGP IgG antibody ELISA kit (EUROIMMUN).

Reagents	Colour	Quantity
Microplate wells; coated with antigens	-----	12 x 8 well strips (96wells)
Calibrator 1	Dark red	2ml x1vial
Calibrator 2	red	2ml x1vial
Calibrator 3	Light red	2ml x1vial
Positive control	blue	2ml x1vial
Negative control	green	2ml x1vial
Enzyme conjugate; peroxidase-labelled anti –human IgG	green	12 ml x 1vial
Sample buffer	Light blue	100ml x 1vial
Wash buffer 10x concentrate	colourless	100ml x 1vial
Chromogen/substrate solution(TMB/H2O2)	colourless	12ml x 1vial
Stop solution(sulpheric acid)	colourless	12ml x 1vial
Test instruction	-----	1 booklet

Appendix (E): The components of anti-tTG IgA antibody ELISA kit (EUROIMMUN).

Reagents	Colour	Quantity
Microplate wells; coated with antigens	-----	12 x 8 well strips (96wells)
Calibrator 1	Dark red	2ml x1vial
Calibrator 2	red	2ml x1vial
Calibrator 3	Light red	2ml x1vial
Positive control	blue	2ml x1vial
Negative control	green	2ml x1vial
Enzyme conjugate; peroxidase-labelled anti –human IgA	Orange	12 ml x 1vial
Sample buffer	Light blue	100ml x 1vial
Wash buffer 10x concentrate	colourless	100ml x 1vial
Chromogen/substrate solution(TMB/H2O2)	colourless	12ml x 1vial
Stop solution(sulpheric acid H2SO4)	colourless	12ml x 1vial
Test instruction	-----	1 booklet

الخلاصة:

مرض السيلياك (الزلاقي) هو مرض مزمن من أمراض المناعة الذاتية يصيب بشكل رئيسي الجهاز الهضمي بدرجات متفاوتة من الشدة ونوع الأعراض الظاهرة. يسببه تناول الأطعمة المحتوية على الغلوتين ، والموجودة بشكل أساسي في القمح والشعير والجاودار في الأشخاص المهيئين وراثيًا من جميع الأعمار. منذ عقود ، تم التركيز على كيفية حدوث مرض السيلياك ومحاولة الربط مع اضطرابات المناعة الذاتية الأخرى في الانسان وهناك العديد من العوامل التي تحفز ظهور المرض بما في ذلك الاضطرابات المناعية والبيئية والوراثية. الهدف من هذه الدراسة هو إجراء تقييم نسبي لمستقبلات إنترلوكين ٢ في الدم (sIL-2R) وإنترلوكين ١٨ (IL-18) مع الاجسام المضادة لانزيم ترانسجلوتاميناز الأنسجة (مضاد للـ tTG) والأجسام المضادة لببتيد الغليادين المتحلل (مضاد DGP) عبر العوامل الديموغرافية الحيوية في المرضى الذين يعانون من مرض السيلياك. حيث ان إنزيم ترانسجلوتاميناز الأنسجة وببتيد الغليادين المتحلل يشاركان في سلسلة من العمليات المناعية المرتبطة بمرض السيلياك، ويعتبر sIL-2R مع IL-18 من العلامات التنبؤية للعملية الالتهابية للأمعاء الدقيقة لدى الأشخاص العراقيين البالغين المصابين بمرض السيلياك وذلك لارتفاع مستوياتهما في الدم عند هؤلاء المرضى . ولقد تم استخدام الفحص النسيجي لخزعات نسيج الاثني عشر والذي كان يعتبر حجر الزاوية في تشخيص مرض السيلياك لعدة عقود في هذه الدراسة. الطريقة: أجريت دراسة لاختبار ٤٥ من مرضى السيلياك من الفئة العمرية البالغة من كلا الجنسين لقياس مستوى مصل العلامات الأربعة باستخدام تقنية ELISA ثم مقارنتها بـ ٤٥ من الشخاص السليمين الملائمين للعمر والجنس. وتم فحص الخزعات المستحصل عليها بالمنظار من أنسجة الاثني عشر للمرضى المصابين بمرض السيلياك عن طريق تطبيق بروتوكول الهيماتوكسيلين والايوزين ومن ثم تصنيفها حسب نظام MARSH. النتائج: لوحظ ارتفاع تركيزات مضاد لـ tTG و DGP و IL-18 و sIL-2R في الدم عند غالبية مرضى السيلياك ، والتي ربما تُعزى إلى استهلاك الغلوتين في النظام الغذائي لمعظم المرشحين. وتوضح هذه الدراسة فروق ذات دلالة إحصائية في مستويات الدم للواسمات الأربعة المذكورة أعلاه بين مرضى السيلياك مقارنة بمجموعة الاشخاص السليمين ($p<0.05$). وكانت هناك ارتباطات معنوية بين مستوى الواسمات المضادة لـ tTG و IL-18 و sIL-2R مع درجات نسيجية مختلفة ($p <0.05$). ان العملية الالتهابية

المرتبطة بمرض السيلياك يمكن مراقبتها عن طريق الكشف عن مستوى الدم من السيتوكينات المشمولة في هذه الدراسة وبالتالي يمكن للأطباء التنبؤ بالنتيجة السريرية ، وتدعو هذه الدراسة أيضًا إلى التحقيق في الأشخاص الذين يعانون من فقر الدم المقاوم أو الاعراض غير المعوية والتي ربما تكون اعراض مرض السيلياك الغير مكتشف عند هؤلاء المرضى باستخدام علامات الالتهاب المصلية المتمثلة بالسيتوكينات المذكورة (IL-18 و sIL-2R) وهذا قد يقلل من الحاجة إلى تطبيق تنظير الجهاز الهضمي العلوي.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الطب- فرع الأحياء المجهرية

علاقة مستويات الانترلوكينات (sIL-2R & IL-18) في مصل الدم مع الاجسام الذاتية المضادة لدى المصابين بمرض السيلياك في محافظة كربلاء

رسالة مقدمة الى

كلية الطب/ جامعة كربلاء وهي جزء من متطلبات نيل درجة الماجستير في المناعة السريرية / فرع الاحياء المجهرية

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٢٠٢١ ميلادية

١٤٤٣ هجري