Immunodiagnosis of Celiac Disease
Among Children With Chronic Diarrhea in
Gaza Strip

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Submitted in Partial Fulfillment of Requirements for The Degree of
Master of Biological Sciences/Medical Technology
Faculty of Science.

1429/2008
وقل اعملوا فسيروا اعمالكم ورسوله والمؤمنون صداق الله العظيم
Declaration

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Celiac disease (CD) is a permanent intolerance to gluten that results in damage to the mucosa of the small intestine. This damage consists of mucosal inflammation and loss of absorptive surface area and is manifested by a broad spectrum of symptoms and nutritional deficiencies. The immune mediated damage to small bowel mucosa triggered by an immune response to the gliadin fraction of gluten, a component of wheat, barely and oats. CD can present at any age after the introduction of gluten into the diet. During Infant and childhood CD can present with failure to thrive, vomiting, diarrhea, constipation, rickets, occipital seizures, and short stature. In adults, CD may present with iron-deficiency anemia, diarrhea, infertility, nausea, vomiting, abdominal pain, weight loss, and pathologic fractures. CD is associated with specific MHC class II alleles that map to the HLA-DQ locus, The HLA-DQ2, DQ8 alleles associated with increased susceptibility to CD.

Since there are no previous studies performed to evaluate the CD in our area, therefore this study was performed to determine the occurrence of CD among children suffering from chronic diarrhea in Gaza City, to investigate the role of ASMA in false negative EMAs results, to propose TTG as an alternative solution for masking problem and To highlight the CD in Gaza Strip for the first time.

The results of this study by using EMAs test showed an occurrence of CD (3.25%), but when they were analyzed by using (TTG IgG, IgA) the occurrence was (12.2%). Our results also showed that the occurrence of ASMA was (28.5%), which may mask the EMAs antibodies and hence giving false negative results of EMAs, the four positive subjects for EMAs showed positive results when they were tested for (TTG IgG, IgA), these results mean that the two tests have the same sensitivity, and finally our finding showed that the total IgA deficiency represent (33.3%) from all (TTG IgG, IgA) positive subjects, these results showed false negative results for (TTG IgA), so the class IgG of EMAs and TTG must be done to CD patients with total IgA deficiency.

It is recommended to follow the protocol in figure 6.1 for laboratory diagnosis of CD, to Introduce the genetic analysis of CD to identify persons with increased risk of having the disease, and to adopt HPE as a golden standard test for clinical assessment of the patients with CD and to confirm the disease diagnosis.
Keywords:

Celiac disease (CD), tissue transglutaminase antibodies (TTG), antiendomysial Antibodies(EMAs), HLA-DQ locus, ASMA, Jejunal biopsy, Gaza Strip, Gluten.
التشخيص المناعي لمرض حساسية القمح بين الأطفال الذين يعانون من الإسهال المتكرر

في قطاع غزة

ينتج مرض حساسية القمح عن عدم تحمل الأمعاء لمادة الجلوتين، مما ينتج عنه أضرار جسيمة للغشاء المبطن للأمعاء الدقيقة. هذه الأضرار تكون على شكل التهاب للغشاء المخاطي المبطن للأمعاء الدقيقة ونقش كبير في مساحة وحدات الاستماع (الخلمات). يتم تشخيص هذا المرض عن طريق الأعراض الكثيرة والمختلفة التي تصيب المرضى نتيجة لسوء استماع الغذاء المحضوم. رد الفعل المناعي ضد الغشاء المبطن للأمعاء الدقيقة يحدث نتيجة لتفاعل المناعي لمادة الجلوتين (GLADIN) وهي جزء من مادة الجلوتين الموجودة في القمح والشعير والجاودار.

مرض حساسية القمح يمكن أن يحدث في أي عمر بعد أن يتناول مادة الجلوتين، وظهور في الأطفال الرضع بصورة ضعيفة في النمو، قيء، إسهال، إمساك، كمثاج، تشتاجات وقصر في القامة، ولكن الأعراض في سن البلوغ تكون أكثر دم بسبب نقص الحديد، إسهال، عدم غليان، قيء وألم في البطن ونقص في الوزن وكسور بالعظام، أما الأمهات اللاتي لم يعالجن من هذا المرض يكون الطفل المولود ناقص الوزن ويزداد احتمالية الإجهاض لهذه النساء.

وبهذا أنه لا يوجد دراسات سابقة أجربت لتقديم هذا المرض في منطقتنا فقد قمت بالإجراء هذه الدراسة بهدف تحديد نسبة حدوث المرض بين الأطفال الذين يعانون من حالات الإسهال المتكرر في مدينة غزة، وأيضاً لتحقيق من مدي نتائج الASMA تأثير الأجسام المضادة مختبرات IgG, IgA على نتائج الASMA. لتشخيص المرض وأخيراً ألاقات الضوء على مرض حساسية القمح للمراة الأولى في قطاع غزة.

أظهرت الدراسة أن نسبة العامة لمريض حساسية القمح 3.25% باستخدام فحص TTG IgG, IgA. ارتفعت النسبة إلى 12.2%، وكانت جميع الحالات الإيجابية لفحص الـ EMAs إلى جانب الإيجابيات، منها 33.3% من جميع الحالات الإيجابية لفحص TTG IgA والـ Total IgA. ونسبة 33.3% من جميع الحالات الإيجابية لفحص IgA، وهذه الحالات أظهرت نتيجة سلبية لفحص IgG أو IgA، لذلك يجب عمل فحص TTG IgA لمرضى حساسية القمح الذين لديهم نقص في Total IgA.
وبناءً على نتائج هذه الدراسة فإننا نوصي باتباع الآلية المقترحة في الشكل (6.1) للتشخيص المناعي لمرض حساسية القمح، ولإضافة نوصي بعمل التحاليل الجينية الخاصة بهذا المرض لمعرفة وتشخيص الأفراد الذين لديهم عوامل الخطر الإصابة به، وأخيراً ليكون الفحص السكبي هو الخيار الأول لتأكيد التشخيص لمرض حساسية القمح.
Dedication

To the person who taught me patience, strife, and pushed me towards success in life and give me all care and happiness.

To My Father's soul

To My Mother

To My Brothers

To My Beloved Wife

To My Sons,
Mohamed, Eias, Mahmood

To My Daughter

Haya
Acknowledgment

First of all, I thank God, the beneficent and most merciful.

I would like to acknowledge all of the people who have assisted me with my work presented in this thesis. My deepest gratitude goes to my supervisor, Prof. Mohammad Eid Shubair, who has given me his utmost support, guidance in all steps through this project.

I would also like to thank Dr. Randa Al-khodary who have assisted me with my work presented in this thesis and for helping me in the practical part of research and for her permission for me to make my work in central lab.

I would like to acknowledge all staff at the department of the biological science, particularly, Dr. Abood kichaoy, Dr. Fadel A Sharif, Dr. Basem Ayish, Dr. Abdalha Abd, and Dr zeyad Dawoody.

I would like to extend my thanks to all staff at the central laboratory in Al rimal clinic for their general support, orientation, and very good comments.

I owe my thanks to the staff at the immunology department (Reem Abu Shomar, suzan Al Hassanat, ohood sarsoor) for all their helpful and support in the lab work.

In addition, I express my thanks to the staff at Ard Al Ensan association mainly Dr. Adnan Alwihady and Dr yunes Abu Noor for giving me much a time, experience meticulous valuable support and advice.

I would also like to thank my friends and colleagues mainly Mr Ehab ismaeel, Shehda barhoom and fuad el samak, for all of their support and guidance and encouraging; good luck to all.
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<td>AGAs</td>
<td>Antigliadin antibodies</td>
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<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ARA</td>
<td>IgA anti-reticulin antibodies</td>
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<tr>
<td>ASMA</td>
<td>Anti smooth muscle antibodies</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CD</td>
<td>Celiac disease</td>
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<td>EMAs</td>
<td>Antiendomysial antibodies</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>HPE</td>
<td>Histopathological examination</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IELs</td>
<td>Intra epithelial lymphocytes</td>
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<td>IFA</td>
<td>Immunofluorescence assay</td>
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<td>IFN-γ</td>
<td>Interferon γ</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility antigens</td>
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<tr>
<td>NIH</td>
<td>National Institute of Health (USA)</td>
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<td>PBS.</td>
<td>Phosphate Buffered saline</td>
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<td>RID</td>
<td>Radial immunodifusion</td>
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<td>Th1</td>
<td>T helper 1</td>
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<td>TTG</td>
<td>Tissue transglutaminase enzyme</td>
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<td>FAB</td>
<td>Fragment of antigen binding</td>
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Chapter One

Introduction
1.1. Introduction
Celiac Disease (CD) is a permanent intolerance to gluten that results in damage to the mucosa of the small intestine. This damage consists of mucosal inflammation and loss of absorptive surface area and is manifested by a broad spectrum of symptoms and nutritional deficiencies (1).

The mucosal damage is primarily cellular, untreated celiac disease is also associated with a humoral immune response directed against the reticulin and endomysium of connective tissue, “endomysial antibodies” (EMAs), and against various peptides derived predominantly from wheat, “antigliadin antibodies” (AGAs). EMAs have been proposed as the most reliable serologic marker for celiac disease (2).

Antibodies to the enzyme tissue transglutaminase (TTG) are found in an overwhelming majority of cases, and cross-react with gluten. Tissue transglutaminase modifies gluten peptides into a form that may stimulate the immune system more effectively. The use of Tissue Transglutaminase Antibody (TTG) screening may soon replace the use of the small-bowel biopsy to diagnose celiac disease in children (3).

Celiac disease shows incomplete penetrance, as the gene alleles associated with the disease appear in most patients, but are neither present in all cases nor sufficient by themselves to cause the disease. Over 95% of Celiac patients have an isoform of DQ2, major histocompatibility antigens (MHC,class II antigens) and DQ8, which are inherited in families. The reason these alleles produce an increased risk of celiac disease is that the receptors formed by these genes bind to gliadin peptides more tightly than other forms of the antigen-presenting receptor. Therefore, these forms of the receptor are more likely to activate T lymphocytes and initiate the autoimmune process (4).

The inflammatory process, mediated by T cells, leads to disruption of the structure and function of the small bowel's mucous lining, and causes malabsorption as it impairs the body's ability to absorb nutrients, minerals and fat-soluble vitamins (A, D, E and K) from food. Lactose intolerance may be present due to the decreased bowel surface and reduced production of lactase, but typically resolves once the condition is treated.
Alternative causes of this tissue damage have been proposed and involve release of interleukin 15 and activation of the innate immune system by a shorter gluten peptide. This would trigger killing of enterocytes by lymphocytes in the epithelium. The villous atrophy seen on biopsy may also be due to unrelated causes, such as tropical sprue, giardiasis and radiation enteritis. While positive serology and typical biopsy are highly suggestive of Celiac disease, lack of response to diet may require these alternative diagnoses to be considered (5).

Celiac disease affects people differently. Symptoms may occur in the digestive system like, chronic diarrhea, abdominal bloating and pain, or in other parts of the body like anemia, bone or joint pain, osteoporosis, muscle cramps, seizures, infertility, sores inside mouth, failure to thrive, itchy skin rash called dermatitis herpetiformis.

The prevalence of clinically diagnosed disease (symptoms prompting diagnostic testing) is 0.05–0.27% in various studies. People of African, Japanese and Chinese descent are rarely diagnosed; this reflects a much lower prevalence of the genetic risk factors. Population studies also indicate that a large proportion of Celiac patients is undiagnosed; this is due to many clinicians being unfamiliar with the condition (6).

A large multicentre study in the U.S. found a prevalence of 0.75% in not-at-risk groups, rising to 1.8% in symptomatic patients, 2.6% in second-degree relatives of a patient with Celiac disease and 4.5% in first-degree relatives. This profile is similar to the prevalence in Europe (7).

Treatment for celiac disease is a strict gluten-free diet. This includes elimination of the storage proteins (prolamines) of wheat, barley, rye and oats. It also includes eliminating gluten in over-the-counter medications by carefully checking the labels for ingredients such as hydrolyzed plant and vegetable proteins, modified food starch, cereal solids, and the more obvious oat hull fibers, wheat flour and glutens. Home food preparation requires a strong commitment to exclude glutens (8).
1.2. General Objectives

1. To estimate the occurrence of the celiac disease (CD) among diarrheic children in Gaza strip.

1.3. Specific objectives

2. To investigate the role of anti smooth muscle antibodies (ASMA) in false negative EMAs results.
3. To Propose TTG as an alternative solution for masking problem.

1.3. Significance

v There is a large fraction of diarrheic children who are misdiagnosed and thus don’t respond to treatment as the drugs are directed toward other disease.

v CD is currently diagnosed by EMAs in the Ministry of Health which is associated with high percentage of false negative results when compared to biopsy.

v The evaluation of CD occurrence in Gaza strip for the first time will help the decision makers to apply the suitable diagnosis and management strategies of this disease.

v To highlight the CD in Gaza Strip for the first time
1.4. Research problem

In Palestine the screening test of CD is based on one test which is (EMAs). EMAs is specific and sensitive test, its usage for diagnosis of CD might be accompanied by high percentage of false negative results. This might result from the high frequency of a positive anti smooth muscle antibodies (ASMA) that might mask EMAs when present. Moreover IgA deficiency in children may result in false negative as well. According to my experience in the field, I noticed that there is a high frequency of (ASMA) which may mask EMAs and gives false negative results of CD. According to the researcher knowledge there is no information about this disease in our area, This study aims at bringing into focus the celiac disease in Gaza Strip and propose a standard protocol to be implemented to achieve an accurate and dependable laboratory results.
Chapter Two

Literature Review
2.1 Background

Celiac disease (CD) is a gluten enteropathy occurring in both children and adults. CD also referred to as celiac sprue, gluten-sensitive enteropathy, non-tropical sprue, in addition to a host of other names. The condition is characterized by a sensitivity to gluten that results in inflammation and atrophy of the mucosa of the small intestine. Clinical manifestations include malabsorption with symptoms of diarrhea, steatorrhea, and nutritional and vitamin deficiencies. Secondary immunologic illnesses, such as atopic dermatitis, dermatitis herpetiformis, alopecia and aphthous ulcers, may be the primary presentation (9).

Normally, ingested food does not elicit a local or systemic immune response. Ingestion of protein down-regulates the intestinal immune response to that protein. This phenomenon is known as oral tolerance. In patients with celiac disease, the immune system is abnormally activated by gluten, specifically the gliadin portion of wheat protein, and prolamines (insoluble proteins) in rye, barley and oats. It is thought to result from the activation of both a cell-mediated (T-cell) and humoral (B-cell) immune response upon exposure to the glutens (prolamins and glutenins) of wheat, barley, rye, and oats, in a genetically susceptible person. Thus, celiac disease is a genetic, immunologically mediated, small intestine enteropathy in which mucosal villi are destroyed by cellular and humoral-mediated immunologic reactions to gliadin protein. The loss of functioning villi limits the ability of the small intestine to absorb nutrients, thus adversely affecting all systems of the body. The immunologic response to gluten may also occur secondarily in other bodily tissues, an example being dermatitis herpetiformis (8).

2.2 History

Celiac disease may have an ancient history dating back to the 1st and 2nd centuries AD. The first clear description was given by Samuel Gee in 1888. He suggested that dietary treatment might be of benefit. The doctoral thesis of Wim Dicke of 1950 established that exclusion of wheat, rye and oats from the diet led to dramatic improvement. Dicke's colleagues, Weijers and Van de
Kamer, showed that measurement of stool fat reflected the clinical condition. Histological abnormalities of the lining of the small intestine were demonstrated beyond doubt by Paulley in 1954 and techniques of per-oral biopsy described by Royer in 1955 and Shiner in 1956 afforded reliable diagnosis. A relationship with dermatitis herpetiformis was suggested by Samman in 1955 and established by Shuster and Marks in 1965 and 1968. The Celiac Society (now Celiac UK) was founded in 1968 and similar societies now exist across the world. They provide an extremely valuable service. Present problems include definition of the tolerated levels of gluten, whether oats are toxic for some or all celiacs and the likelihood that the condition is relatively common and frequently without classical symptoms. Hope for the future is that more convenient methods of treatment will follow better understanding (10).

In 1996, research indicated a significant reduction in malignancies when celiac disease was treated with a gluten-free diet. Researchers continue to investigate celiac disease, honing in on the exact causes and implications for treatment (11).

2.3 Epidemiology

The researchers conducted studies about the prevalence of celiac disease, they concluded that the true prevalence of CD is difficult to estimate because of the variable presentation of the disease, particularly since many patients can have little or no symptoms. With this limitation in mind, the prevalence of the disease is highest in Celtic populations where estimates of 1:300 to 1:122 have been described. The prevalence of CD in North America has been estimated to be 1:3000, while the prevalence in at-risk groups such as first-degree relatives of CD patients was 1:22. CD can affect persons of many ethnic backgrounds, but appears to rarely affect persons of purely Chinese, Japanese, or Afro-Caribbean decent (12).

The prevalence of clinically diagnosed disease (symptoms prompting diagnostic testing) it was found to be 0.05–0.27% in various studies. However, population studies from parts of Europe, South America, Australia and the
USA (using serology and biopsy) indicate that the prevalence may be between 0.33 and 1.06% in children and 0.18–1.2% in adults (13). People of African, Japanese and Chinese descent are rarely diagnosed; this reflects a much lower prevalence of the genetic risk factors. Population studies also indicate that a large proportion of celiac patients are undiagnosed; this is due to many clinicians being unfamiliar with the condition (14).

A large multicentre study in the U.S. found a prevalence of 0.75% in not-at-risk groups, rising to 1.8% in symptomatic patients, 2.6% in second-degree relatives of a patient with celiac disease and 4.5% in first-degree relatives. This profile is similar to the prevalence in Europe (15).

In Collin study the incidence of CD in adults varied from low of 1.27 in Denmark and 3.08 in England to high of 17.2 cases per 100,000 in Finland, the incidence of CD in child age 0-5 year varied from 2.15 to 51 cases per 100,000 (16).

The incidence was found to be one case in every 1,000 live birth ,with a range from one in 250 to 4000.In United state the prevalence was found to be one in 250 (17).

2.4 Pathogenesis

The pathogenesis of CD is as yet not fully elucidated. Susceptibility to CD, and its activation and perpetuation, involve a combination of environmental and genetic factors, and immunological mechanisms. (18).

Normally, ingested food does not elicit a local or systemic immune response. Ingestion of protein down-regulates the intestinal immune response to that protein. This phenomenon is known as oral tolerance (19).
2.4.1 The role of dietary proteins in disease pathogenesis.

CD is activated by proteins in the dietary cereal grains wheat, rye, and barley. Although the disease-activating proteins in these grains are widely termed “gluten” (18).

The term "gluten" is, in a sense, a generic term for the storage proteins that are found in grains. In reality, each type of protein, gliadin in wheat, secalin in rye, hordein in barley, avenin in oats, zein in corn and oryzenin in rice - is slightly different from the others. The "gluten" in wheat, rye, barley, and in a much lower amount, oats, contains particular amino acid sequences that are harmful to persons with celiac disease. The damaging proteins are particularly rich in proline and glutamine (especially the amino acid sequences which are in the following orders: Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro). As peptides, some cannot be broken down any further. In people with celiac disease, this stimulates T-cells to produce antibodies. The antibodies, in turn, attack the villi in the small intestine, reducing their ability to absorb nutrients. It is important to note that these sequences are not found in the proteins of corn and rice (20).

(Figure 2.1) Taxonomy of some dietary grains (21)
Wheat, barley, and rye, which contain the CD-activating proteins gluten, hordein, and secalin, respectively, are derived from the Triticeae tribe of the grass (Gramineae) family. In contrast, oats, which contain few CD-activating proteins, are more distantly related, as are rice, maize, sorghum, millet, Job’s tears, and teff. (21)

2.4.2 The role of Genetic factors: MHC class II HLA-DQ alleles in disease pathogenesis.

In a study reported about the genetic etiology of celiac disease, he concluded that the pathogenesis of CD is firmly rooted in host genetic factors. This was first evident from clinical observations of multiple cases of CD within families, and the high (approximately 70%–75%) rate of concordance for CD among monozygotic twins (22). It is known that CD is associated with specific MHC class II alleles that map to the human leukocyte antigen (HLA-DQ) locus. Moreover, the presence of specific HLA-DQ alleles is necessary, although not sufficient, for the phenotypic expression of CD in virtually all affected individuals, irrespective of geographic location (21). The vast majority of celiac patients have one of two types of HLA DQ, a gene that is part of the MHC class II antigen-presenting receptor (also called the human leukocyte antigen) system and distinguishes cells between self and non-self for the purposes of the immune system. There are 7 HLA DQ variants (DQ2 and D4-9). Two of these variants—DQ2 and DQ8—are associated with Celiac disease. Every person inherits two copies, one from each parent. The gene is located on the short arm of the sixth chromosome, and as a result of the linkage this locus has been labeled CELIAC1 (23). This HLA-DQ2 is present in at least 90%–95% of patients with CD, although a very small number of CD patients have been reported in whom only 1 of these DQ2 alleles is present (that is, HLA-DQB1*0202 or, rarely, HLA-DQA1*05) The HLA-DQ8 heterodimer found in the remaining 5%–10% of patients with CD is formed by the β chain and α chain encoded by HLA-DQB1*0302 and HLA-DQA1*03, respectively (21).

The association of HLA and pathogenesis was studied and found that the HLA-DQ2 and HLA-DQ8 heterodimers on antigen presenting cells (APCs) can
bind and subsequently present “gluten” peptides to populations of lymphocytes (CD4+ T cells) in the lamina propria of the small intestine (24).

Because the peptide-binding groove of HLA-DQ2 and HLA-DQ8 favors the binding of peptides with negatively charged residues at key anchor positions. Such negatively charged amino acids are largely absent from native “gluten” peptides generated in the human intestinal tract. However, this puzzle was solved after the discovery that the target antigen of an autoantibody present in many patients with CD was a calcium-dependent tissue transglutaminase (TTG) (25). Tissue TTG, which is released in the intestinal mucosa during tissue injury, has a role in tissue repair and cross-links proteins by forming isopeptide bonds between glutamine and lysine residues. However, tissue TTG also has a high avidity for “gluten” peptides and, under certain conditions (for example, low pH) and in the absence of lysine residues, can deamidate glutamine, which converts neutral glutamine to negatively charged glutamic acid. Further studies indicated that tissue TTG, has specificity for only selected glutamine residues in the glutamine and proline-rich “gluten” peptides, which depends on the amino acids neighboring the target glutamine residue. Some, but not all, of the deamidated “gluten” peptides, by virtue of having negatively charged glutamic acid residues, manifest an increased binding affinity for the disease-relevant HLA-DQ2 or HLA-DQ8 molecules (26). Once bound to HLA-DQ2 and HLA-DQ8, the “gluten”-peptide–HLA-DQ complexes can activate T cells in the mucosa of the small intestine that recognize these complexes. The production of IFN-γ is a signature of “gluten” peptide–specific HLA-DQ2– and HLA-DQ8–restricted T cells that are isolated from the mucosa of the small intestine of CD patients, and it is considered to have a key role in the downstream initiation of mucosal damage (27).

2.4.3 A model of CD immunopathogenesis

Researchers have proposed a model to conceptualize the role of the adaptive T cell response in CD pathogenesis (Figure 2.2) divides pathogenesis into 3 phases: luminal and early mucosal events; activation of pathogenic CD4+ T cells; and events leading to tissue damage. In the first phase, an individual ingests “gluten.” “Gluten” is digested to peptides, but because of the lack of
prolyl endopeptidases among the gastric, pancreatic, and brush border enzymes, residual, relatively large “gluten” peptides that are rich in proline and glutamine remain in after initial digestion. For 99% of individuals, including most of those who carry the CD susceptibility alleles that encode HLA-DQ2 and HLA-DQ8, this does not present a problem, at least in terms of developing CD. However, in those HLA-DQ2– or HLA-DQ8–positive individuals with increased susceptibility to CD, because of additional genetic and/or immunological factors and/or an adverse set of environmental events (for example, concurrent infection with an enteric virus), “gluten” peptides set in motion a series of immunological events that culminate in the immunopathology of CD (21).

Partially digested “gluten” peptides gain access to antigen presenting cells (APCs) in the subepithelial region of the small intestine, and the pathway(s) involved here is not yet determined but might include paracellular passage through a damaged epithelial cell layer, transepithelial passage that can cross the epithelial cell layer. It is probable that the uptake of “gluten” peptides into a microenvironmental milieu in the small-intestinal mucosa suited for disease development is facilitated by a transient infection or other cause of inflammation in the small intestine. Viral infections would seem to be prime culprits to set the stage for a mucosal T cell response to “gluten” peptides. Having set the conditions for developing a T helper (Th1) cell response, in the second phase, “gluten” peptides bound to HLA-DQ2 or HLA-DQ8 encounter “gluten”-specific T cells that become committed to Th1 cytokine production. In the third phase, the release of interferon γ (IFN-γ) and other cytokines, which perpetuate the ongoing response and alter key mucosal functions including intestinal permeability, can also result in the activation and release of enzymes that can damage the mucosa. This results in a loss of villous structure and crypt hypertrophy (21).
(Figure 2.2) A model of CD immunopathogenesis

2.5 Pathology

At endoscopy, the appearance of the duodenal mucosa will vary depending on the severity of the CD. Latent CD typically demonstrates no gross abnormality and demonstrates normal semicircular folds. With overt disease, the folds may show a scalloped, or notched, appearance; in severe disease, the folds may be decreased in size and number or be completely absent (28).

As the clinical and endoscopic spectrum of CD has broadened, the range of histologic findings compatible with the diagnosis of serologically positive CD has also increased:

- Increased intraepithelial lymphocytes (IEL; Fig. 2.3). This is the first and most sensitive marker of the effects of gluten on the small-bowel mucosa; thus, it is the major histological feature of CD. An increase is defined as >40 lymphocytes per 100 surface or upper-crypt
enterocytes. The vast majority are CD2+, CD3+; 70 to 90% are CD8+ T-cells. In latent forms of CD, an increase in IELs is often the only abnormality; villous architecture and lamina propria cellularity are within the normal range (29).

- Increased cellularity in the lamina propria. In CD with villous/crypt abnormalities, plasma cells, lymphocytes, and eosinophils are increased in number, particularly in the upper half of the mucosa. The number of eosinophils may be striking; however, their increase is paralleled by the increase in mononuclear inflammatory cells, a finding that is against a diagnosis of allergic enteritis. Neutrophils are also part of the inflammatory response and may be numerous in the lamina propria (30)

- Enterocyte damage (Fig. 2.4 A). In latent or minimal-deviation CD, the enterocytes are unremarkable. With severe injury, however, the apical cytoplasm is typically vacuolated; the cells themselves are shorter than normal and easily dislodged from the underlying basement membrane (31)

- Villous atrophy/crypt hyperplasia (Fig. 2.4 B). These changes represent severe damage and can only be assessed in well-oriented sections. As a rule, villous-crypt ratios can be assessed if four or more crypts in parallel, nontangential array adjacent to one another can be identified in the biopsy specimen. Taking deeper sections through the tissue block may uncover such areas. Also, it should be recalled that villi overlying and adjacent to lymphoid nodules/follicles are normally blunted or absent, and such areas should not be chosen for analysis (32).
2.5.1 Marsh stages

- Marsh stage 0: normal mucosa
- Marsh stage 1: increased number of intra-epithelial lymphocytes, usually exceeding 20 per 100 enterocytes.
- Marsh stage 2: proliferation of the crypts of Lieberkuhn.
- Marsh stage 3: partial or complete villous atrophy
- Marsh stage 4: hypoplasia of the small bowel architecture (33)

(Fig 2.3) Marsh Type I lesion. The villous is unremarkable except for a modest increase in intraepithelial lymphocytes, particularly at the villous tip (33)

(Fig 2.4) Marsh Type III lesion. A, at low power, there is virtually complete villous blunting associated with crypt hyperplasia. B, at higher power, the increased number of surface intraepithelial lymphocytes and the damage to enterocytes are evident (33).
2.6 Clinical Presentation

Infancy
During the first year of life, an infant may manifest celiac disease with intermittent vomiting, diarrhea, growth delay and failure to thrive. The incidence of this early classic presentation in infants has decreased. However, to prevent significant growth problems in infants, confirmation of celiac disease is important (34).

Childhood
Children with celiac disease may present with failure to thrive, vomiting, anorexia, short stature, anemia, hepatitis, epilepsy and other extragastrointestinal conditions. With age, these presentations become more subtle. The most frequent of their symptoms were abdominal pain, aphthous stomatitis and atopic dermatitis. Angular cheilitis and recurrent aphthous ulcers are frequent in children and adults with celiac disease (Figure 2.5 & 2.6). These clinical findings should prompt the physician to consider the diagnosis of celiac disease (35).

(Figure 2.5) Atopic dermatitis on the arm (35)
Young adults

The initial presentation of celiac disease in patients in their 20s and 30s may be dermatitis herpetiformis (figure 2.7). This condition usually appears as clear or blood-tinged vesicles symmetrically distributed over the extensor areas of the elbows, knees, buttocks, shoulders and scalp. Intense pruritus and/or burning sensations in the area occur hours before the onset of the vesicle. Dermatitis herpetiformis flares after consumption of foods containing high amount of gluten.

Small intestine biopsies from patients with dermatitis herpetiformis reveal features identical to those found in patients with celiac disease. In a study of the occurrence of malignancies and the survival of 305 patients with dermatitis herpetiformis from 1970 to 1992. It was indicated that the incidence of non-Hodgkin's lymphoma is significantly increased in patients with dermatitis herpetiformis. The results also confirmed no increase in mortality in patients with dermatitis herpetiformis who are treated with a gluten-free diet (36).
(Fig 2.7) Dermatitis herpetiformis. Symmetric vesiculation, crusts and erosions are distributed over the extensor areas of the elbows, knees, buttocks, shoulders and scalp, with a tendency to grouping of individual lesions (36).

**Adults**

Malabsorption. The varied signs and symptoms of malabsorption may be caused by celiac disease or many other diseases. Mild malabsorption may be asymptomatic. With its gradual onset, the classic manifestations of flatulence and bulky, greasy and foul-smelling stools may not be recognized by the patient as signs of celiac disease. Malabsorption should be suspected in any patient with weight loss and diarrhea, and the signs and symptoms of specific vitamin or nutritional deficiencies. The latter include visual disturbances, neuropathy, anemia, osteopenic bone disease, tetany, hemorrhagic diathesis or infertility (37).

_Anemia._ Anemia is a frequent presentation of celiac disease. In one study, 200 consecutive patients presenting to a hematology clinic were screened for antigliadin and antiendomysial antibodies. Patients with both positive titers underwent intestinal biopsy, and in 10 (5 percent), results were positive for celiac disease. The prevalence increased to 8.5 percent if the patients with macrocytic anemia and the patients with bleeding who responded to iron therapy were excluded. The authors of this study recommend including celiac screening in the diagnostic algorithm of patients with anemia(38).

Osteopenia. Osteopenia may be the initial finding in patients with celiac disease.
Seizures. There have been numerous reports of children and adults with seizures associated with celiac disease. Studies have provided some insight into this condition (39).

2.6.1 Celiac disease iceberg

The celiac iceberg is the model used to illustrate that Celiac disease varies with respect to clinical presentation and severity of symptoms. (figure 2.8)

**Clinical Celiac disease** at the top of the iceberg depicts the relatively small proportion of the celiac population with typical symptoms, e.g. weight loss, failure to thrive and diarrhea.

**Silent Celiac disease** incorporates those people who have celiac disease yet display no typical overt symptoms. Diagnosis usually occurs as a result of screening, e.g. family history or type 1 diabetes. Some in this category have mild degrees of anemia or osteopenia. Those with clinical and silent disease have a typical celiac small bowel lesion.

**Latent celiac disease** describes people who may at times have a normal mucosa on a normal diet, yet at other times show villous atrophy which is responsive to gluten withdrawal.

**Healthy Individuals** at the base of the iceberg are those who include gluten as part of their diet and have normal small bowel morphology even though they may have the same genetic constitution as those with celiac disease (40).
2.7 Differential diagnosis

The combination of clinical, serologic, and histologic findings plus response to a gluten-free diet confirms the diagnosis of CD in most patients.

The following tests anti-gliadin, anti- reticulin, IgA anti-human tissue transglutimase (TTG), and IgA anti-endomysial antibodies (EMAs) are medically necessary for any of the following indications:

1. As a preliminary diagnostic test for persons with symptoms suggestive of celiac disease; or
2. To monitor response to a gluten-free diet; or
3. For screening first-degree relatives of individuals with celiac disease; or
4. To screen persons with type 1 diabetes for celiac disease (41).
According to national institute of health ( NIH) Consensus Panel Statement on celiac disease, serological testing is the first step in pursuing a diagnosis of celiac disease. The Consensus Statement recommends that the best available tests are the IgA anti-human tissue transglutaminase (TTG) and anti-endomysial IgA antibodies (EMAs) (42).

The blood tests can be divided into 2 different types of antibodies: those which are “anti-gluten”, and those that “anti-self”. The “anti-gluten” antibodies are the anti-gliadin IgG and IgA. The “anti-self” antibodies are anti-endomysial IgA and anti-tissue transglutaminase IgA (43).

2.7.1 Blood tests for CD

A number of tests, sometimes collectively referred to as the Celiac Blood Panel, will aid the physician in diagnosis. The tests may include, but are not limited to:

• Serologic Tests
  1. EMAs (Immunoglobulin A anti-endomysial antibodies)
  2. AGA (IgA anti-gliadin antibodies)
  3. AGG (IgG anti-gliadin antibodies)
  4. TTGA (IgA anti-tissue transglutaminase)
  5. ARA (IgA anti-reticulin antibodies)

• Tolerance or Measure of Digestion/Absorption Tests
  1. Lactose tolerance test.
  2. D-Xylose test (44)

2.7.1.1 Immunoglobulin A anti-endomysium antibodies (EMAs)

The endomysial connective tissue surrounding each muscle cell contains the smallest of the various types of blood vessels, the capillary as shown in Figure 2.9 (45).
Circulating anti-endomysium autoantibodies (EMAs) are directed against anamorphous supportive tissue surrounding smooth muscle cells and myofibroblasts of the gastrointestinal tract (46).

This antibody was discovered in the early 1980’s, and rapidly gained use as part of a screening “celiac panel” by commercial labs in combination with antigliadin IgG and IgA. Its major drawbacks are that it may be falsely negative in young children, in patients with IgA deficiency and a lesser degree of villous atrophy, and in the hands of an inexperienced laboratory (43).

It was reported that the serologic detection of anti-endomysium antibodies (EMAs) is used to support the diagnosis and to screen populations at risk (47).

IgA-EMAs are measured by indirect immunofluorescence, using tissue sections from either monkey esophagus or human umbilical cord as shown in (Figure 2.10) (48).

It was reported that the sensitivity of IgA-EMAs ranges from 87% to 100%, and the specificity is between 91% and 100% (Table 2.1). Although IgA-EMAs has proved to be a highly specific marker of CD, this type of autoantibodies are not always present in children younger than 2 years of age. The IgA EMAs levels correlate with the mucosal damage and eventually disappear on a gluten-free diet (49,50).
(Table 2.1). Sensitivity and specificity of anti-gliadin IgG, anti-gliadin IgA, anti-endomysial IgA, and anti-tissue transglutaminase IgA for the diagnosis of celiac disease (50).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-gliadin IgG</td>
<td>57%-85%</td>
<td>47%-75%</td>
</tr>
<tr>
<td>Anti-gliadin IgA</td>
<td>52%-90%</td>
<td>71%-90%</td>
</tr>
<tr>
<td>Anti-endomysial IgA</td>
<td>86%-100%</td>
<td>90%-100%</td>
</tr>
<tr>
<td>Anti-tissue transglutaminase IgA*</td>
<td>85%-100%</td>
<td>91%-100%</td>
</tr>
</tbody>
</table>

The anti-endomysium antibody test is based on an immunofluorescent technique and, therefore, is time-consuming to perform, generally more expensive compared with other tests, and, because the interpretation is operator-dependent, potentially more prone to errors. However, its high specificity makes this test one of the most formidable noninvasive diagnostic tools in gastrointestinal practice (51).

(Fig 2.9) The endomysial connective tissue surrounding each muscle cell contains the smallest of the various types of blood vessels, the capillary (45)
(Fig 2.10) IgA-EMAs are measured by indirect immunofluorescence, using tissue sections from either monkey esophagus or human umbilical cord (48).

The effect of immunoglobulin IgA deficiency in patients with CD on serological tests that used in the evaluation of CD patient was assessed. The result of the study revealed that the IgA deficient patients with CD yield false negative serological results when we looking for the (IgA) of (EMAs, TTG, AGA,). So the study recommended to use the IgG for serological tests (52).

Another study recommended that the total serum IgA level is checked in parallel as CD patients with IgA deficiency may be unable to produce the antibodies on which these tests depend on. This may give false negative results. In those patients, IgG antibodies against TTG and EMAs may be diagnostic (53).

Anti-smooth muscle antibodies might mask the presence of anti-endomysial antibodies so the result of EMAs will be false negative, so they show that the
EMAs response can be effectively unmasked by increasingly diluting anti-smooth muscle antibodies-positive serum (54).

2.7.1.2 Anti-gliadin antibodies

Anti-gliadin antibodies are produced in response to gliadin which is a prolamin found in the wheat. The AGA IgG and IgA recognize a small piece of the gluten protein called gliadin. These antibodies became available during the late 1970s and were the first step towards recognizing CD as an autoimmune disorder. IgA and IgG antibodies directed against gliadin (AGA) are often elevated in untreated CD patients. However, AGA is not disease specific, and some patients with active CD lack these antibodies. In contrast, patients with diseases other than CD and healthy individuals occasionally have elevated levels of IgA-AGA and IgG-AGA (55). The sensitivity of IgA-AGA has been reported to vary from 52% to 100% and the specificity ranges between 71% and 100%. The sensitivity of IgG-AGA is similar to that of IgA-AGA, whereas the specificity of IgG-AGA can be as low as 50%. These antibodies disappear rapidly during a gluten-free diet (56).

The IgG form of this test is most useful in children less than 2 years of age and in guiding diagnosis in individuals with IgA deficiency. It is also used to monitor dietary compliance. In gluten-sensitive individuals, AGA testing is a routinely used blood test for possible presence of celiac disease, allergies or idiopathic phenomena. The measurement of AGA is done with Enzyme linked immunosorbent assay (ELISA) or radioimmunoassay (57). The importance of an ELISA test is that it is rapid, inexpensive, and run by a machine. Thus the results are independent of observer variability. (58)

Antigliadin antibody (AGA) positivity disappeared in over 50% of cases, and is therefore a poor marker for celiac disease. The researchers did follow up AGA testing on 69 adults and 47 children who participated in a much larger study conducted four years ago and found that only 26 of the adults and 21 of the children still had detectable levels of AGA in their blood samples, and none of them were positive for IgA-class anti-endomysial antibodies. The researchers concluded that the appearance of AGA should be interpreted as a non-
specific “immunomodulation phenomenon” that has low specificity as a diagnostic marker for celiac disease (59).

In a study was performed to compare the usefulness of IgG and IgA antigliadin antibodies, IgA-endomysial antibodies, and intestinal permeability in diagnosing celiac disease in 102 adult patients with nonspecific abdominal symptoms. In addition, all patients underwent small bowel biopsy as a gold standard for the diagnosis of celiac disease. The results showed that Forty-nine patients were ultimately diagnosed as having celiac disease because of flat mucosa. IgA-endomysial antibodies showed a sensitivity of 100%, but only 55% specificity. IgG and IgA-antigliadin antibodies' sensitivity (73% and 82%, respectively) and specificity (74% and 83%, respectively) were much lower than EMAs (60).

2.7.1.3 Tissue transglutaminase antibodies (TTG)

Tissue transglutaminase is an enzyme present in most organs. In the gastrointestinal tract, the enzyme is found intracellularly in enterocytes and myofibroblasts. Extracellularly, TTG is located in the membranes of enteroctyes and in the subepithelial connective tissue of the small intestine. It has been demonstrated that TTG activity is increased in the mucosal lesions of patients with active CD (61).

TTG can form cross-links between glutamine containing (acceptor) and lysine containing (donor) substrates by a transamidation reaction, which is strictly Ca^{2+}-dependent. In the absence of donor substrates, or at low pH, transglutaminase-bound acceptor molecules may become deamidated. In addition to the transamidation activity, TTG may function as an intracellular GTPase. The two enzymatic functions are mutually exclusive (62,63). Intracellularly, in the GTP-bound form, TTG adapts a closed conformation that keeps the transamidation site inaccessible. Extracellularly, in the presence of Ca^{2+}, TTG adapts an open conformation, which discloses the active site (64). TTG has a strong affinity for fibronectin, and it has been demonstrated that 2:1 complexes between TTG and fibronectin are formed upon cellular release of TTG (65). The biological function of TTG has not been clarified. However,
it has been suggested that TTG is involved in apoptosis, wound healing, and cell adhesion (66). Although TTG knock out mice are born healthy, lack of TTG in these animals cause an abnormal immune response due to the impaired capacity of macrophages to engulf apoptotic cells (67). Gliadins that contain a high percentage of glutamine are favoured acceptor substrates and TTG molecules may participate in the transamidation reaction either as acceptor or donor substrates (68). It has been revealed that TTG-gliadin complexes are formed in vitro as well as in the small intestine of CD patients. Moreover, immunostimulatory peptides of gliadin can be deamidated by TTG, which increases their binding to HLA-DQ2 and DQ8 molecules (69).

Since TTG had been first described as the autoantigen of celiac disease in 1997, it has been utilized to develop innovative diagnostic tools. However, it represents an improvement over the antiendomysial antibody assay because it is inexpensive, rapid and easy to performed. (58).

The autoantigen of the EMAs has been identified as TTG (70). The amino acid sequence of TTG is generally highly conserved among species, displaying an 80% homology between guinea pig and human TTG (71). Thus, the first generation of anti-TTG assays were based on TTG from guinea pig liver preparations (68). However, these antigens contained several contaminating proteins that decreased the specificity of these assays. Subsequently, when purified native and recombinant human TTG became available, it was demonstrated that human TTG bound IgA-TTG more potently than the guinea pig counterpart (72). ELISA using recombinant human TTG for detection of IgA-TTG were demonstrated to have sensitivities and specificities higher than 90% (73). The IgA-TTG levels decrease on a gluten-free diet and reappear after the reintroduction of gluten into the diet (74).

In a study reported by Vitoria and her colleagues to assess the sensitivity, specificity, and predictive value of TTG antibodies measured by a commercially available ELISA technique, compared with those of EMAs and IgA antigliadin antibodies (AGA) for the diagnosis of celiac disease. Twenty-seven serum samples were obtained from patients with untreated celiac disease, 37 from patients who had gluten withdrawn from their diets for
varying time spans, and 34 from control subjects without celiac disease. All were younger than 14 years. Presence of TTG antibodies and AGA was determined by ELISA and of EMAs by indirect immunofluorescence.

RESULTS: Twenty-six of 27 serum samples obtained from patients at the time of diagnosis of celiac disease were AGA positive. All 27 (concordance rate 100%) were positive for EMAs and TTG antibodies. Of the 34 control subjects; 1 was for AGA and 2 for TTG-antibodies. All 34 were negative for EMAs. Sensitivity, specificity, positive predictive value, and negative predictive value within this group were, for TTG antibodies: 100%, 94%, 93%, and 100%, respectively; for EMAs: all four indexes were 100%; and for AGA: 96%, 97%, 96%, 97%. Of 37 treated 2 AGA positive, 9 were EMAs positive, and 6 were TTG antibodies positive. The concordance rate between EMAs and TTG antibodies was 100% in the group with untreated celiac disease, 94% in the control subjects, and 76% in the group with treated celiac disease. It was concluded that Immunoglobulin A antibodies to tissue transglutaminase are new, highly sensitive, and specific markers of celiac disease. They can be determined easily by an accurate, comparatively cheap technique and thereby may advantageously replace the EMAs marker traditionally used (75).

According to the National institute of health (NIH) Consensus Statement (2004), if an individual has suggestive symptoms and negative serological tests, it may be necessary to measure serum IgA to detect a selective IgA deficiency. If an IgA deficiency is identified, an IgG-TTG or IgG-EMAs test should be performed (42).

The levels of IgG-TTG and IgA-TTG were positively correlated with the grade of mucosal villous atrophy and that the IgG-TTG levels corresponded with more severe manifestations of gluten enteropathy. Whereas elevated IgG-TTG or IgA-TTG taken alone should be interpreted with caution, the combined assessment of high IgA-TTG and IgG-TTG levels predicted a severe villous atrophy. Hence, the biopsy procedure might not be needed to diagnose CD for patients with very high levels of both IgA and IgG against TTG. During a gluten-free diet, IgA-TTG mostly disappeared prior to clinical and mucosal recovery. Interestingly, the overall clinical condition in children was better
reflected by the IgG-TTG levels. For follow-up of adult IgA competent patients, assessment of IgG-TTG is also recommended, as their presence and levels might indicate a more severe disease (76).

In another study the researchers recommended using the anti-TTG as the initial test in both population screening and for individual cases suspected of having CD on the bases of symptoms or conditions associated with disease. The positive result should be tested for EMAs as a second step in the screening process and if positive the patient should undergo an intestinal biopsy for confirmation of the diagnosis (77).

The clinical utility of a newly developed kit for antibodies IgG and IgA to human recombinant TTG in a large population of CD patients was assessed. The results showed that the test is highly sensitive and specific for the detection of CD, and the results demonstrated that the test detects a substantial number of CD cases that were not recognized by IgA EMAs (78).

2.7.1.4 Anti reticulin antibodies
IgA class reticulin antibodies react with connective tissue fibers and are most easily demonstrated on rat or mouse kidney substrates and also on rat or mouse stomach. IgA class reticulin antibodies are found in 60% of celiac disease patients. IgG class reticulin antibodies are occasionally found in other disease states, especially bullous dermatoses and in some normal subjects (79). Serum reticulin antibodies tests have been in use since 1971. The antigen has routinely been detected by a standard immunofluorescence method using unfixed cryostat sections of rat kidney, liver, and stomach as antigens. Positive results obtained by a typical staining pattern in both rat kidney and liver (80).

Two patterns that can be observed in the connective tissue fibers are referred to as reticulin 1 (R1) and reticulin 2 (R2). R1 is related to Celiac Disease, the staining pattern on a liver substrate exhibits nodular fluorescence around the portal tracts, up to the limiting plate of the hepatocyte, and a fine outline of the sinusoid. R1 sera in the kidney stains all around the tubules and the
Bowman's capsule. On smooth or striated muscle tissue there is a fluorescent "honeycomb" appearance. These antibodies are not species specific.

R2 antibodies are species specific and only react with rat tissue. R2 sera stains the thin sharp-edged fibers in the liver. In the stomach (longitudinal section), they appear as streaks between the gastric glands. These fibers are not found in great quantity in the muscularis mucosae but rather they are concentrated around the vessels in all organs. In the kidney, there is no staining of the "Bowman's Capsule" or between the tubules. Only the perivascular connective tissue stains in the liver and stomach. The staining can be mistaken for smooth muscle; care should be taken to evaluate closely. The R2 fibers are very thin and much sharper than smooth muscle fibers (81).

2.7.1.5 D-Xylose test

The D-xylose absorption test measures the level of D-xylose, a type of sugar, in a blood or urine sample. This test is done to help diagnose problems that prevent the small intestine from absorbing nutrients in food.

D-xylose is normally easily absorbed by the intestines. When problems with absorption occur, D-xylose is not absorbed by the intestines, and its level in blood and urine is low.

A test for D-xylose is done to determine if malabsorption syndrome is causing symptoms, such as chronic diarrhea, weight loss, and weakness. A person with malabsorption syndrome is unable to absorb nutrients, vitamins, and minerals from the intestinal tract into the bloodstream. It is also used to find the cause of a child's failure to gain weight, especially when the child seems to be eating enough food (82). This test was done before the year of 2000 as the only test for CD in Gaza Strip, due to the difficulty in supplying the materials for this test and the availability of fluorescence microscope in central laboratory, EMAs test replaced this test until now.

Blood levels of D-xylose are highest about 2 hours after the drinking the D-xylose solution. Almost all of the D-xylose is eliminated from the body in the urine within 5 hours. If the intestines cannot absorb the D-xylose properly, the
amount of D-xylose in the blood and urine will be very low. The normal values for the test in blood are greater than 25 mg/dl (milligrams per deciliter) at one hour after ingestion. While in urine the levels are greater than 16% of ingested dose excreted within 5 hours of ingestion.

High values may be caused by a disease, such as *Hogkin's* disease or Radiation treatment.

Low values on the other hand may be caused by a disease that interferes with the intestine's ability to absorb nutrients (malabsorption syndrome), such as Crohn's disease, celiac disease, or Whipple's disease; Inflammation of the lining of the intestine or an infection with a parasite, such as giardiasis or hookworm (83).

### 2.7.1.6 Lactose tolerance test

Lactose is a disaccharide consisting of glucose and galactose. It is found in milk and other dairy products. The concentration of lactose in human milk is about 7% whereas in cow's milk it is about 5% (84).

Patients suffering from lactose intolerance are not able to cleave the alimentary disaccharide lactose. In contrast to milk protein allergy, lactose intolerance is not an atopic reaction of the immune system, but a loss of activity of the enzyme lactase. Lactose tolerant individuals can cleave lactose into its components glucose and galactose with the enzyme lactase, which is localized in the mucous membrane of the small intestine. This biochemical process leads to a measurable increase of blood glucose concentration. Lactose intolerant patients are suffering from reduced or missing lactase activity and therefore, lactose cannot be metabolized. Thus lactose is microbially fermented in the lower parts of the intestine. The non-fermented lactose leads to an increase of the osmotic activity in the intestine. Typical symptoms are meteorism (drum belly), flatulences and diarrhea (85).
2.7.1.6.1 Reasons of lactose intolerance

Genetic defects (primary lactose intolerance) or gastrointestinal diseases (secondary lactose intolerance) can cause lactose intolerance. The most severe form of primary lactose intolerance is alactasia (congenital lactase deficiency). Alactasia is a rare inherent enzyme defect (autosomal recessive), characterized by a total loss of lactase. Disorders like diarrhea, dehydration, and malnutrition can be seen in newborn infants and may lead to severe cerebral damages (86).

2.7.1.6.2 Lactose intolerance diagnosis

Two formal tests are commonly used in patients suspected of having lactose intolerance:

v **The Lactose Tolerance Test:**

The lactose tolerance test consists of administering an oral dose of approximately 1 to 1.5 g of lactose per kg of body weight and obtaining serial blood samples for measurement of blood glucose levels. The test is positive if intestinal symptoms occur and the blood glucose level increases less than 20 mg per dL above the fasting level. However, false-positive and false-negative test results occur in 20 percent of normal subjects because of the influence of variable gastric emptying and glucose metabolism (87).

v **The Hydrogen Breath Test:**

The measurement of breath hydrogen after ingestion of 25 to 50 g of lactose is more sensitive and specific than the lactose tolerance test. The breath hydrogen test has become widely available and is often used rather than the lactose tolerance test. The breath hydrogen test is based on the principle that carbohydrate in the colon is detectable in pulmonary excretion of hydrogen and other gases. A rise in breath hydrogen concentration greater than 20 ppm over baseline after lactose ingestion suggests hypolactasia (88). Very little hydrogen is normally detectable. However, undigested lactose in the colon is fermented by bacteria and
produces various gases, including hydrogen. The hydrogen is absorbed from the intestines, carried through the bloodstream to the lungs, and exhaled. In this test, the person drinks a lactose-loaded beverage and the breath is analyzed at regular intervals. Raised levels of hydrogen in the breath indicate improper digestion of lactose. Certain foods, medications, and cigarettes can affect the accuracy of the test and should be avoided before taking the test (89).
Chapter Three

Materials and Methods
3.1 Study design

The study is a descriptive one and conducted on children suffering from chronic diarrhea, they examined for the following tests:

1. EMAs (IgA).

2. TTG. (IgA, IgG).

3. TTG. (IgA).

4. Total IgA

5. ASMA.

6. Jejunal biopsy—when available—for positive cases of any parameter to confirm the diagnosis was done.

3.2 Inclusion criteria

1. Children age above six month and less than nine years.

2. Have diarrhea more than three times daily.

3. The diarrhea not caused by an infection.

4. Patients setting in Gaza Strip

A summary of the work plan is illustrated in Figure 3.1.
Figure 3.1 A summary of the work plan.
3.3 Study population

The study population comprised 123 symptomatic Palestinian children suffering from chronic diarrhea. The samples were received from Al-Rimal Clinic and Ard Al Ensan during the period from (April to June 2008) .

3.4 Sample collection

Five ml peripheral blood were collected in vacuum plain tube under quality control assurance condition for each sample. Serum was separated in clean plastic tubes and stored at -70°C until performing the assay.

3.5 Permissions and ethical consideration

Permission was obtained from the Helsinki Committee for sample collection and oral consents from were also obtained from each patient where purpose of the study was explained and drawing extra blood if needed.

3.6 Materials

3.6.1 Reagent kits

The following assays were done:

1- EMAs (IgA) qualitative test using indirect immunofluorescence technique (IFA), is purchased from Biosystem company from Espaniol.

2- TTG (IgG, IgA) quantitative test using (ELISA) technique, is commercially available kit from Orgentic company from Germany.

3- TTG (IgA) quantitative test using (ELISA) technique, it is commercially available kit from Orgentic company from Germany.

4- ASMA: qualitative test using indirect immunofluorescence technique(IFA), it is commercially available kit from Biosystem company from Espaniol.
5- Total (IgA): quantitative test using radial immunodifusion (RID) technique, it is commercially available kit from Liofilchem company from Italy.

6- Histopathological examination were executed by specialist when available in Al-Shifa hospital.

3.6.2 Instruments and disposables

ELISA reader (Statfax), Germany.
Fluorescence Microscope, Zeius, Germany.
Graduated flasks, different sizes.
Graduated Cylinders (100, 500, 1000) ml
Syringes and needles
Plasters
Vacutainer tubes
Tube covers
Cotton
Alcohol
Distilled water
Micropipettes (10, 20, 50, 100, 1000) μl
Yellow and blue tips
Timer
Centrifuge
Deep freezer (-72°C) for specimen storage
Incubator
Vortex Mixer
3.6.3 Immunological investigations

3.6.3.1 Antiendomysial Antibodies (EMAs IgA)

EMAs was performed by applying Immunofluorescence assay (IFA) which is based on the indirect method of fluorescent antibody staining. The procedure is carried out in two steps. First, the serum antibodies bind the specific antigenic substrate forming an antigen-antibody complex. In the second step, the formed complex reacted with added fluorescein-labeled anti human antibody. If serum antibodies are present the reaction yields a bright apple-green fluorescence under the UV microscope (90).

**Principle of the test**

Serum anti-endomysium antibodies (EMAs) bind to the corresponding antigens present in a section of the lower one-third of the monkey esophagus. The resulting antigen-antibody complexes are detected by means of a fluorescein labeled anti-human immunoglobulin A, and visualized with the aid of a fluorescence microscope (91).

**Composition**

A. **Slides:** Monkey esophagus sections (endomysium) in each well.

B. **Phosphate Buffered saline (PBS) (20x):** Sodium phosphate 225 mmol/L, potassium phosphate 60 mmol/L, sodium chloride 2.3 mol/L, sodium azide 0.95 g/L, pH 7.2.

C. **EMAs Positive Control:** Human serum containing anti-endomysium antibodies), sodium azide 0.95 g/L.

C. **Negative Control:** Human serum, sodium azide 0.95 g/L.

D. **IgA FITC/Evans:** Goat anti-human IgA conjugated with fluorescein isothiocyanate (FITC), Evans blue 0.01 g/L, sodium azide 0.95 g/L.

E. **Mounting Medium:** Glycerol 78%, sodium phosphate 6 mmol/L, potassium phosphate 1.6 mmol/L, sodium chloride 60 mmol/L, sodium azide 0.95 g/L.

F. **Blotting Paper.**
Reagent preparation

PBS: The Reagent B was diluted 1/20 with distilled water. It is stable for 1 week at 2-8°C. All other reagents were provided ready to use.

Samples

Serum collected by standard procedures. Serum allowed to thaw, then diluted 1/5 in PBS. For titration of positive samples, two-fold serial dilutions were made starting from 1/5 in PBS.

Procedure

1. The reagents and samples were brought to room temperature.
2. One drop (50 μL) of the diluted sample or Control was delivered on each slide well (A), making sure that it is completely covered.
3. The slide was incubated for 30 minutes at room temperature (15-30°C) in a moist chamber.
4. Sample drops were drain off by gently tapping the inclined slide. To avoid cross contamination of the sera.
5. The slide was gently rinsed with PBS.
6. The slide was thoroughly washed by immersing in a washing tray filled with PBS for 5 minutes.
7. The slides Carefully dried off by using the blotting paper provided. The cells preparation were kept a moist along the procedure.
8. One drop of the conjugate was delivered into each well. Then the slides were incubated for 30 minutes at room temperature (15-30°C) into a moist chamber.
9. Washing (step 6) and drying (step 7).
10. Several drops of mounting media reagent were delivered on the slide and covered with a cover slip avoiding the formation of air bubbles.

Reading the slides
The slides were examined using the fluorescence microscope (250-400x). For best results, the slides were read immediately and specifically in fields in the inner part of the tissue section. As recommended by the manufacturer, fluorescent intensity in the tissue edge is not representative of the slide preparation. All sera showing network-like fluorescence labeling of the thin layer surrounding the smooth muscle fibers of monkey esophagus at the recommended dilution was considered positive (Figure 2.10). When none of the above specific stainings are observed, the result were considered negative for these autoantibodies.

v Quality controls

Positive Control and Negative Control provided with kit was tested together with the patients samples, in order to verify the assay performance. Positive Control should give the above described specific staining. Negative Control should not give any specific staining.

3.6.3.2 Antismooth Muscle Antibodies (ASMA)

The procedure of ASMA was the same steps as for EMAs test except that the slides were coated by rat kidney and rat stomach.

v Reading

Sera showing staining of the muscularis mucosae (Figure 3.3), the muscle layers of the blood vessels and the interglandular fibers in the rat stomach at the recommended dilution were considered positive. When none of the above specific stainings were observed, the result was considered negative for these autoantibodies (92).
(Figure 3.2) ASMA staining reaction on rat stomach smooth muscle, 200X (93).

3.6.3.3 Anti-Tissue-Transglutaminase (TTG IgG,IgA)

Name and intended use

Anti-Tissue-Transglutaminase Screen an indirect solid phase enzyme linked immunosorbent assay (ELISA) for the simultaneous quantitative measurement of IgG and IgA class autoantibodies against tissue Transglutaminase (TTG) in human serum. The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of celiac disease and dermatitis herpetiformis.

Principle of the test

Human recombinant tissue Transglutaminase was bound to microwells. Antibodies against this antigen, if present in diluted serum, bind to the respective antigens. Washing of the micro wells removes un specific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG and IgA immunologically detect the patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes un bound conjugate. An enzyme substrate in the presence of bound conjugate
hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of color is directly proportional to the concentration of IgG and IgA antibodies present in the original sample.

v Contents of the kits
- One divisible microplate consisting of 12 modules of 8 wells each, coated with human recombinant tissue Transglutaminase. Ready to use.
- Six vials, 1.5 ml each combined Calibrators with IgG and IgA class Anti-TTG antibodies (A-F) in a serum/buffer matrix (PBS, BSA, NaN3 <0.1% (w/w)) containing: IgG 0; 5; 10; 25; 75; and 200 U/ml and IgA: 0; 5; 10; 25; 75; and 200 U/ml. Ready to use.
- Two vials, 1.5 ml each Anti-TTG Controls in a serum/buffer matrix (PBS, bovine serum albumin (BSA), NaN3 <0.1% (w/w)) positive (1) and negative (2), for the respective concentrations. Ready to use.
- one vial, 20 ml Sample buffer (Tris, NaN3 <0.1% (w/w)), yellow, concentrate (5x).
- one vial, 15 ml Enzyme conjugate solution (PBS, PROCLIN 300 <0.5% (v/v)), (light red) containing polyclonal rabbit anti-human IgG, anti-human IgA; labeled with horseradish peroxidase. Ready to use.
- one vial, 15 ml TMB substrate solution. Ready to use.
- one vial, 15 ml Stop solution (1 M hydrochloric acid). Ready to use.
- one vial, 20 ml Wash solution (PBS, NaN3 <0.1% (w/w)), concentrate (50x).

v Storage and stability
1. The kit was Stored at 2-8 °C.
2. Microplate wells were kept and sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.
4. Test reagents were not exposed to heat, sun or strong light during storage and usage.
5. sample buffer and washing buffer are stable for at least 30 days when stored at 2-8 °C.
v Sample preparation

All patient samples were diluted 1:100 with sample buffer before assay. Then 10 μl of sample was added to 990 μl of sample buffer in a polystyrene tube. Mixed well. Controls were ready to use and need not be diluted.

v PREPARATION OF REAGENTS

• Preparation of sample buffer

The contents of each vial of the sample buffer concentrate (5x) were diluted with distilled or deionized water to a final volume of 100 ml prior to use. Stored refrigerated: (stable at 2-8 °C for at least 30 days after preparation or until the expiration date printed on the label).

• Preparation of wash solution

The contents of each vial of the buffered wash solution concentrate (50x) were diluted with distilled or deionized water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2-8 °C for at least 30 days after preparation or until the expiration date printed on the label.

v Test procedure

1. A sufficient number of microplate modules were prepared to accommodate controls and prediluted patient samples.
2. One hundred μl of calibrators, controls and prediluted patient samples were delivered duplicate into the wells.
3. Incubated for 30 minutes at room temperature (20-28 °C).
4. The contents of the microwells were discarded and then washed 3 times with 300 μl of wash solution.
5. One hundred μl of enzyme conjugate were dispensed into each well.
6. Incubated for 15 minutes at room temperature.
7. The contents of the microwells were discarded and washed 3 times with 300 μl of wash solution.
8. One hundred μl of TMB substrate solution were dispensed into each well.
9. Incubated for 15 minutes at room temperature.
10. One hundred μl of stop solution were added to each well of the modules and incubated for 5 minutes at room temperature.

11. The optical density was read at 450 nm and the results were calculated. Bi-chromatic measurement with a reference at 600-690 nm is recommended by the manufacture. The developed color is stable for at least 30 minutes. optical densities were recorded during this time.

**Interpretation of results**

In a reference value study with serum samples from healthy blood donors the following ranges have been established with the Anti-Tissue-Transglutaminase Screen tests:

**Anti-Tissue-Transglutaminase Screen**

- **normal:** < 15 IU/mL
- **positive:** >15 IU/mL

**Sensitivity**

The lower detection limit for Anti-Tissue-Transglutaminase Screen has been determined at 1.0 U/ml.

**Specificity**

The solid phase is coated with human recombinant TTG. Therefore the Anti-Tissue-Transglutaminase Screen test kit recognizes only IgG and IgA class autoantibodies specific for TTG (93,94,95,96).

**3.6.3.4 Anti-Tissue-Transglutaminase IgA**

The procedure of (TTG IgA) is the same as that used in (TTG IgG,IgA) test except that the interpretation of the result.

**Interpretation of results**

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti-Tissue-Transglutaminase IgA tests:
Anti-Tissue-Transglutaminase IgA
[U/ml]
Cut-Off: 10 [IU/ml]

3.6.3.5 Total IgA

Assay principle

Radial Immunodiffusion (RID) is a well established technique based on the binding of antigen and antibody to produce a visible precipitin ring in a gel. The IgA (antigen) in the serum which drained in the well of the plate diffused radially in the agarose gel reacting with specific antibodies incorporated in agarose gel and forming immune complexes visible ring. Diameter of the ring is directly proportion to the concentration of the IgA in the sample.

Kit Contents

- One RID plates, 12 pre-cut wells per plate
- Calibrators
- Control
- Sample diluent
- Gel sectioning blades
- Instruction leaflet and results table

Test procedure

1. The plate was removed from the envelope and left for five minutes at room temperature.
2. The wells were filled with 5µL of serum.
3. After the sample diffused, the plate was closed with the lid and incubated at room temperature for 48 hours.

Interpretation of the results

After 48 hours the precipitation ring diameter was measured, The IgA concentration was read using the manufacturer tables.
v Quality control

Quality control was carried out by performing a control of accuracy and precision inoculating a control serum on the plate in examination. The precision (95 %) and the accuracy +/- (85 %) (97).

3.7 Statistical Analysis

Data will be analyzed using appropriate SPSS version "15" for cleaning data and to obtain the frequency of the data.

3.8 Histopathological examination

Histopathological examination were executed by specialist when available.
Chapter Four

Results
4.1 Study population:
The present study comprised 123 Palestinian children complaining from the following symptoms (chronic diarrhea, failure to thrive, malabsorption, short stature). Their ages were above 6 months and less than 96 months (mean age, 38.6 and median age, 36). Among the samples, 51.6% were males and 48.4% were females. The processing and testing of the samples were conducted in the immunology department in Al – Rimal clinic.

4.2 Source of samples:
Samples were obtained from two sources; Ard- El-Ensan (68%) and Al-Rimal clinic (32%), Figure 4.1

![Figure 4.1 Source of samples](image)

All results obtained by various techniques are illustrated in figure 4.1
4.3 Preliminary study:

Initially we tested 2 samples for EMAs, the results were negative and uninterpreted due to the presence of ASMA antibodies in high concentrations due to the hook effect.

The EMAs results were negative at serum dilution 1/5, when several dilution were made (1/10, 1/15, 1/20, 1/40), EMAs test was positive at dilutions of 1/15 and 1/20, the increasing serum dilution reduces the ASMA antibodies and decreases the effect of ASMA antibodies on EMAs antibodies so EMAs test will give positive result.

In another experiment were the masking between EMAs and ASMA was clearly shown when I used EMAs positive sample and ASMA positive sample, the titer of ASMA positive sample was more than 1/160. Therefore serial dilutions were made for the serum of ASMA positive (1/5, 1/10, 1/20, 1/40). I mixed the two samples, the test (EMAs) was performed by using the esophagus section. The 1/5 and 1/10 titers were negative EMAs test, but the higher titers 1/20 and 1/40 were positive for EMAs test.
Figure 4.2: A summary of the work plan is illustrated in the following algorithm.

**Footnotes:** EMAs; Antiendomysial antibodies, ASMA; Anti smooth muscle antibodies, TTG; Tissue transglutaminase enzyme, CD; Celiac disease, HPE; Histopathological examination.
4.4 The results of EMAs

All samples were tested for EMAs, only four samples showed positive results (3.25%), others were negative. These results are illustrated in figure 4.3.

Figure 4.3 Frequency of EMAs among the study population(123 patients).
4.5 The results of ASMA

All EMAs negative samples were tested for ASMA. Only 35 samples were positive (28.45%). The rest (84) were negative. These results are shown in figure 4.4

![Figure 4.4 Frequency of ASMA positive and negative](image_url)
4.6 The results of (TTG IgG,IgA)
All positive samples for ASMA (35) were tested for (TTG IgG,IgA) and the positive EMA subjects (4) were added to be tested for (TTG IgG,IgA). 15 samples were positive, others were negative. These results are shown in figure 4.5.
Among the other 84 samples that were negative for both EMAs and ASMA, only 78 samples were tested. The results showed that all are negative for TTG also.

Figure 4.5 Frequency of (TTG IgG,IgA) positive from ASMA positive samples

Figure 4.6 Comparison between; Results of TTG and EMAs among (117) samples tested for both.
4.7 The results of Total IgA

Total IgA test performed for the 15 samples which were positive for (TTG IgG, IgA). Five samples were deficient and others were within normal range. These results are illustrated in figure 4.7.

Figure 4.7 Frequency of Total IgA results
4.8 The results of (TTG IgA)

The five deficient samples were tested for (TTG IgA). Three samples of them were negative and others were positive. These results are shown in figure 4.8.

Figure 4.8 The results of TTG IgA

4.9 The results of Histopathology

Due to the difficulty of handling the histopathological examination (HPE) samples, only five patients were tested for HPE from the fifteen positive samples positive for (TTG IgG, IgA). The diagnosis of all samples was consistent with celiac disease.
4.10 Overall of CD among examined samples

Based on the previous results the overall occurrence of CD among the study population (positive for EMAs, TTG or both) was 12.2%. These results are illustrated in figure 4.9.

Figure 4.9 frequency of CD among the study population (123 patients)
CHAPTER FIVE

DISCUSSION
The present study is one of the important contributions to the child health and growth.

A number of antibodies have been described as having an association with Celiac disease. These include AGA, TTG, EMAs antibodies. IgA anti-endomysial antibodies have consistently been shown to have high sensitivity and specificity for celiac disease and they are measured by indirect immunofluorescence assay. The main technical problem with this assay is that the presence of concomitant anti-smooth muscle antibody which makes the interpretation of the slides more difficult. The presence of a high titre anti-smooth muscle antibody may make the test uninterpretable and an alternative serological test should be performed.

**EMAs results:** In the current study, the EMAs positive samples were (4) giving the occurrence of CD among 123 symptomatic patients as of 3.25%.

These results showed that the occurrence of EMAs among CD suspicious patients was lower than expected because many samples were positive for challenge and were EMAs negative test.

When comparing these results to other studies, the current study results is lower than that reported by Rabab, In Saudi Arabia where the percentage was 7.6%, among 145 patients with suspected CD depends on serological and HPE methods [98]. In another study reported by van and west in America the prevalence of CD among suspected patients was .05%, and in other studies among different populations, the prevalence was higher than this study. The difference between this study and others could be due to several factors,
study population (selection of high risk group, symptoms, number of subjects enrolled in the study. High titer of ASMA antibodies which may mask the EMAs antibodies and gives false negative results of EMAs test. Genetic variation in the study population.

**ASMA results:** according to the finding of this study the ASMA positive samples were 35 giving a percentage of 28.5%.

When comparing these results with other studies which were performed among normal population, it showed higher percentage than the current study.

In a study reported by Anderso and his colleagues the prevalence of ASMA in normal population was (2-9%), they tested 582 normal subjects of different ages (99).

Many studies were performed to evaluate the prevalence of ASMA antibodies among normal population, the results were approximately in the range of the Anderson et al study.

According to the researcher experience in the immunology department in Al-Rimal clinic there is increased occurrence of ASMA antibodies when testing the EMAs, ASMA and antinuclear antibodies.

The high occurrence of ASMA in this study as compared to others may be due to several factors: The variation in study populations in different areas, In light of other studies ASMA antibodies may be highly occurring in normal subjects in Palestine. The normal value of ASMA in our population may be
different from other population, the samples dilution 1 to 20 and above is
considered positive ASMA test, the normal dilutions of ASMA test may be
more than 1 to 20. It is expected that there is a correlation between ASMA
antibodies and CD, so the researchers must put into focus this matter. And
Our target group comprised highly suspected children for CD, while others
determined the prevalence among normal population.

**TTG IgG, IgA results**: according to the current study, (TTG IgG, IgA)
positive results were 11 (9.2%) samples out of 119 in addition to 4 (3.25%)
EMAs positive samples.

The occurrence of CD among symptomatic subjects (119) was 9.2%. The (4)
positive EMAs samples were tested for (TTG IgG, IgA), all of the positive
EMAs samples were positive for (TTG IgG, IgA). The total number of positive
subjects was increased to (15), thus the occurrence of CD among 123
symptomatic patients was increased to reach 12.2%.

When comparing our study results to other studies, our findings are more or
less than other studies.

Our study showed lower percentage when compared with that of Liorente
which revealed 15% positive results for (TTG IgG, IgA) (100).

Most studies which are concerned with the prevalence of CD among
symptomatic patients showed lower percentage than our study.

The variation could be explained by the differences in the characteristic of our
study population (genetics, different ethnics groups, etc.), the second cause
of our increased percentage may be due to consanguinity in which the DQ2, DQ8 genes pass to the siblings and increase the risk factors for CD.

When the results of EMAs antibodies were compared to (TTG IgG, IgA) results in this study, the EMAs positive subjects were 4/123 (3.25%) samples but the (TTG IgG, IgA) positive samples were 11/123 (9.2). The variations between the two results could be explained by the high occurrence of ASMA antibodies which may mask the EMAs antibodies, so EMAs test may show false negative as reported by lasagna et al., (1999) [54].

The EMAs response can be effectively unmasked by increasing the dilutions of ASMA positive serum, but the increasing of serum dilution is not always possible, when the EMAs antibodies are present at low concentration, the increasing of serum dilution will decrease the EMAs antibodies and may give false negative results.

The preliminary study which the researcher made showed that the high titer of ASMA gives false negative EMAs test, the reason behind this matter may be due to masking of ASMA antibodies to EMAs antibodies as described above.

It is supposed that the masking is not due to binding of ASMA antibodies to EMAs antibodies or to the epitops of the endomysial tissue because the fragments of antigen binding (FAB) of the two antibodies are different.

The following explanation may reveal the issue of the false negative EMAs results in the presence of ASMA antibodies in high titer:
1. **Fluorescence masking:**

A strong positive results of ASMA means a strong Fluorescence green color in a broad area occupied by the muscle fibers when compared to the endomysial tissue which is a thin layer surrounding the muscle fibers, so the high intensity of ASMA test prevents EMAs color to be interpreted.

2. **The high titer of ASMA antibodies** may prevent the EMAs antibodies to reach the binding sites of endomysial tissue, when diluting the ASMA antibodies by titration, the EMAs antibodies will reach the endomysial tissue and gives positive results.

In various studies researchers recommended using TTG test as the initial test in both normal population screening and for suspected individuals of having CD.

**The results of total IgA:** All samples which were positive for (TTG IgG, IgA) were subsequently examined for total IgA. Ten samples showed normal results while the remaining five samples were deficient in total IgA (33.3%). Our study showed an increase in the percentage of total IgA deficient subjects when compared to other studies. In a study was reported by *Kumar et al., (2002)* the total IgA deficient subjects were 10 to 15 times more in patients with celiac disease when compared to healthy subjects. IgA deficiency is one of the most frequent immunodeficiencies. The prevalence was estimated to be one in 500 to one in 700 healthy persons, The incidence of IgA deficiency in patients with CD is somewhere between 2 and 3%, representing an increase of 10-
to 15-fold over the general population. The results of our study showed that the IgA deficient patients with CD yield false negative serological results when we are looking for the (IgA) of (EMAs, TTG, AGA). So it is recommended to test for IgG [52, 53].

The variation between our study and others may be due to the small number of subjects which were tested, so the 15 samples are not representative for the evaluation of total IgA deficiency, so we need more investigations to ensure the total IgA deficiency in CD patients. Moreover no studies were performed to evaluate the total IgA deficiency in Gaza Strip.

**The results of (TTG IgA):** The five samples which were deficient for total IgA were tested for (TTG IgA), Three samples were negative and two samples were positive.

The results of the current study have proven that in case of total IgA deficiency in patients with CD when they were tested for TTG IgA yield false negative serological results, these finding are in agreement with other studies as discussed above [52,53]. Although the results of these three samples were positive when were tested for (TTG IgG, IgA) and were negative when were tested for (TTG IgA), these results showed that the (TTG IgG) were positive. Our findings are compatible with that of other studies as discussed above. In the other two positive subjects for (TTG IgA) which were total IgA deficient, the reason behind these results is due to the accuracy of the total IgA test = +/- 15%, if I added this value to the test value, the 2 deficient subjects will be normal ones, this explanation
reveal the ambiguity from the 2 positive subjects for (TTG IgA) and total IgA deficiency.

The results of Histopathology: Due to difficulty in handling the HPE, only five samples were tested for HPE, Four of the samples were positive for (TTG IgG, IgA) and EMAs negative but the results of ASMA were positive, 1/5 subject was (TTG IgG, IgA) and EMAs positive while negative for ASMA test. The diagnosis of all samples was consistent with CD. Results are compatible with those of other studies, such as that reported by Olsson and his results who indicated that the positive (TTG IgG, IgA) is consistent with the results of HPE and is correlated with the grade of mucosal villous atrophy [75].

The five HPE subjects are neither enough nor representative to evaluate the relation between the TTG test and HPE (mucosal villous atrophy), so this need more studies to prove this matter.
Conclusions and Recommendations
6.1 Conclusions
The results of the study can be summarized as follow:

v Examination of the 123 samples by using EMAs showed an occurrence of CD 3.25%, but when were examined by using (TTG IgG, IgA) the occurrence was 12.2%.

v Our results showed that the occurrence of ASMA was 28.5% which may mask the EMAs antibodies and show false negative results.

v Positive samples for ASMA (n=35) were tested for (TTG IgG, IgA), 11 subjects of these were positive for (TTG IgG, IgA), these results prove the existence of false negative results of EMAs in the presence of ASMA.

v The four positive samples for EMAs showed positive results when they were tested for (TTG IgG, IgA), these results mean that the two tests have the same sensitivity.

v Our finding showed that the total IgA deficiency represent (33.3%) from all (TTG IgG, IgA) positive samples, these results were negative when tested for (TTG IgA), so the class IgG of EMAs and TTG must be done for CD patients with total IgA deficiency.
6.2 Recommendations

Following our results obtained in this study, I propose the following protocol was suggested to be followed for laboratory diagnosis of CD (Figure 6.1).

**Figure 6.1 protocol for laboratory diagnosis of CD.**

**Footnote**: HPE; histopathological examination, CD; celiac disease, TTG IgA; tissue transglutaminase IgA, GFD; gluten free diet, Total IgA; Immunoglobulin IgA.
The role of ASMA in (masking) the EMAs could be investigated in future research.

The prevalence of ASMA should be studied in general population to define dilution cut off for the test.

Introducing the genetic analysis of CD to identify persons with increase risk of having the disease.

Adopt HPE as a golden standard test for clinical assessment of the patients with CD and to confirm diagnosis.
References


53. korponay-szabo I,Dahlbom I,laurila k,koskinen s,woollley N,partanen J, et al. (2003) .evaluation of IgG antibodies against


### Appendix A

The study specimens results

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