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Assessment of anti-tissue transglutaminase-IgG with selected lipid panel tests among Helminths and H.pylori positive and non infected school aged children in Ziway Oromia region, South East Ethiopia

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This is to certify that the thesis prepared by Mekdes Alem, entitled: Assessment of anti-tissue transglutaminase-IgG with selected lipid panel tests among Helminths and H.pylori positive and non infected school aged children in Ziway Oromia region, South East Ethiopia and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Clinical Chemistry specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

Anti-tTGIgG -Anti tissue transglutaminase Immunoglobulin G

CD- Celiac Disease

ELISA -Enzyme Linked Immunosorbent Assay

GFD - Gluten free diet

H.Pylori- Helicobacter pylori

HLA- Human leukocyte antigen

IgA- Immunoglobulin A

IgG- Immunoglobulin G

LDL-C - Low density lipoprotein cholesterol

OD - Optical density

OR - odds ratio

TC - Total Cholesterol

TG - Triglyceride

tTG - tissue Transglutaminase enzyme

Abstract

Background: Celiac disease (CD) is an autoimmune disorder triggered by consuming a protein called gluten. It is determined by both environmental and genetic factors. Infection by Helminths and H.pylori are among the environmental factors involved that enhance anti-inflammatory response. Different auto antibodies and antibodies are used in the diagnosis of celiac disease, but Anti-tTG antibodies are highly sensitive and specific for the diagnosis of CD. IgG-based tests are useful for detecting celiac disease. Since Celiac disease is one of the areas that did not get much attention in its diagnosis; this study could give some insight on the necessity of doing more research in the area and gives a better understanding on the association between celiac diseases with that of lipid profiles, *H.pylori* and Helminths infection

Objective: The objective of this study was to assess anti-tissue transglutaminase IgG antibody with selected lipid panels among *H.pylori* and Helminths positive and non infected Children.

Methods: A cross sectional retrospective and prospective study was conducted in Ziway, South east of Addis Ababa, Ethiopia by using random sampling method. The retrospective part included taking serum samples that were collected and stored during another study and careful review of study groups sociodemographic and medical data. The samples that were kept refrigerated at -80ºC were used for analysis of anti tissue transglutaminase IgG and lipid profiles as for the prospective part of the study starting from May 2020 to June 2020. The transglutaminase IgG measurement used principle of Enzyme immunoassay for optical density measurement based on commercially available kit. While the lipid panels used kinetic assay that were measured on Cobas 6000 fully automated chemistry machine. The obtained data was entered and analyzed by using SPSS version 23 and descriptive analysis, Chi-square and logistic regression were used to see any associations and P-value <0.05 was considered as statistical significant.

Result: A total of 97 samples of children with age group between 4-14 years old were involved in this crossectional study. 19% of study participants tested positive for anti-tissue transglutaminase IgG and Females had a higher magnituse of anti-tTG IgG than males (24.5 % vs 11.4%). A significant association was found between Helminths and anti-tTGIgG [OR 3.45 (95% CI: 1.057, 11.265), P=0.033] However this study failed to show association between anti-tTGIgG and *H.pylori* as well as with lipid profiles.

Conclusion: Although the relation between *H. pylori* and Celiac diseases is controversial, our result doesn't proof *H.pylori* can shield against celiac disease so measures to protect oneself against *H.pylori* should be continued. On the other hand, even though more trials are required, there is a strong association between Helminthes infection and anti-tTGIgG where children not infected with Helminths had a 3.45 times greater risk of developing a positive anti-tTGIgG. Although a significant difference was not found in this study between lipid profiles and anti tissue transglutaminase, it is advisable to assess lipid profiles of celiac patients as shown in other studies so as to minimize the long term effect associated with lipid profile alteration, Coronary artery disease for instance. We strongly recommend large scale case control study to understand the exact relationship between celiac diseases and other co-morbidities.

Key Words: Celiac Disease, anti-tissue transglutaminase IgG, lipid panels.

1. Introduction

1.1.Background

Celiac disease (also referred to as celiac sprue, non-tropical sprue, and gluten-sensitive enteropathy) is an autoimmune disorder triggered by consuming a protein called gluten, which is found in wheat, barley and rye. It is a chronic, immunologically determined form of enteropathy affecting the small intestine in genetically predisposed children and adults [1,2].

The development of celiac disease is determined by both environmental and genetic factors. The most important genetic factor identified is the human leukocyte antigen (HLA) locus. The HLA antigen results in the activation of T lymphocytes, whose secretion products play a key role in causing mucosal lesions [3].

The tissue transglutaminase enzyme (tTG) is the target antigen of auto antibodies found in the serum of patients with CD. The most physiologically important function of tTG is to deamidate the glutamine residues of the gliadin peptides and convert them into glutamic acid; this modification makes the gliadin negatively charged, allowing it to bind with HLA-DQ2/DQ8 antigens, with the consequent exposure of the neopeptides for recognition by T cells. In people who are genetically predisposed to CD, the development of a T- and B-cell mediated immune response leads to the synthesis of anti-tTG immunoglobulin (Ig) A antibodies and proinflammatory cytokines, resulting in chronic inflammation and progressive destruction of the intestinal mucosa [4].

The active phase of CD is accompanied by elevated levels in serum of immunoglobulin A (IgA) autoantibodies against endomysium (IgA-EmA) and tissue transglutaminase (IgA-tTG) and the presence of these antibodies is frequently used as a selection criterion for jejunal biopsy. Determination of the Immunoglobulin G (IgG) class of antibodies against endomysium (IgG-EmA), and tTG (IgG-tTG) has been suggested as an alternative for the identification of IgA-deficient subjects with CD [5].

As shown in figure 1 in an active CD, gluten peptides that are left undigested enter into the intestinal mucosa and since their sequence is rich in Q-X-P motifs, they are a preferential substrate for tissue transglutaminase (Ttgase). This enzyme can deamidate neutral glutamine into

a negatively charged glutamic acid that facilitates its binding into the peptide pocket of *HLA-DQ2 (DQ8)* that is expressed by antigen presenting cells. This presentation then promotes activation of gliadin specific Th1 CD4 response in intestinal *lamina propria* which induces mucosal damage by Interferon (IFN) participation. [6]

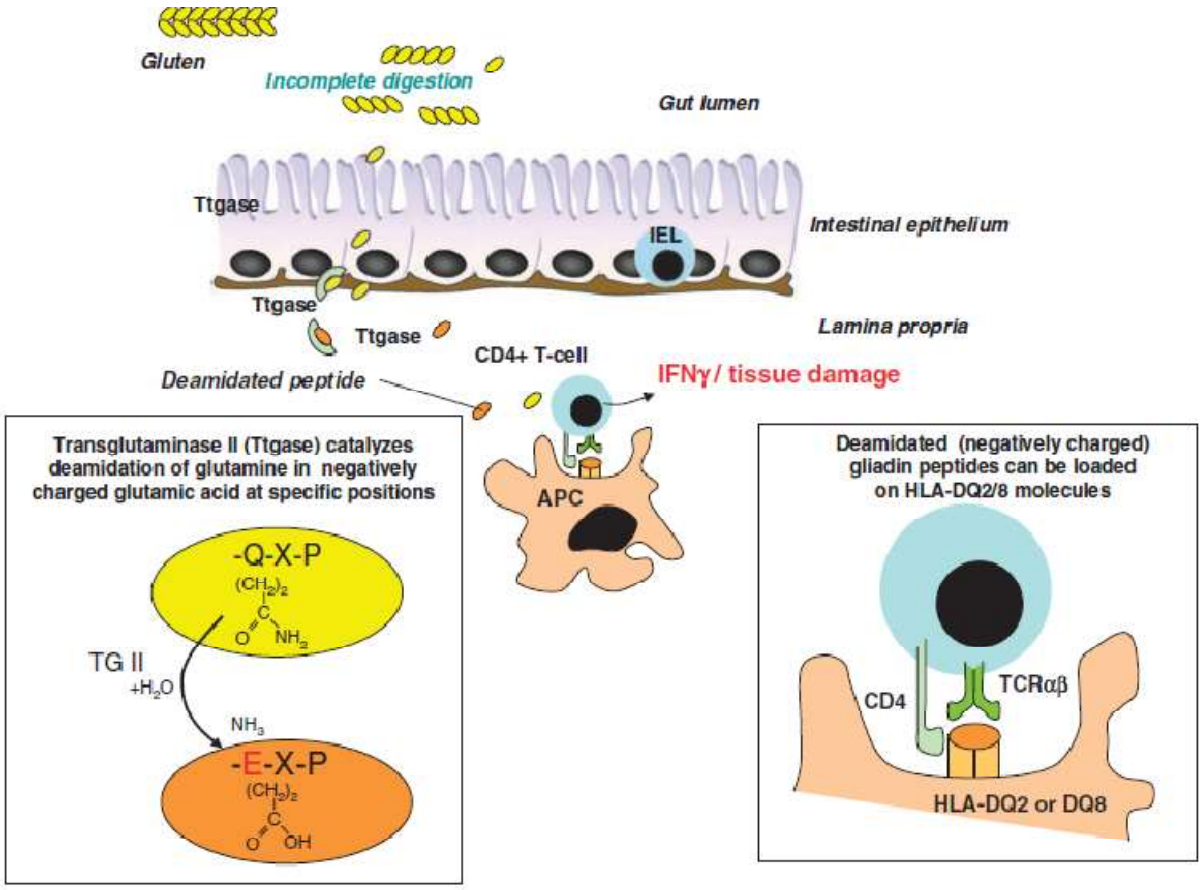

Fig 1. Possible mechanism of celiac disease pathogenesis [Taken from: Meresse B *et al*. Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. *Nature publishing group* 2008.]

Celiac disease belongs to a small group of medical disorders that doesn't have specific symptoms to point to. According to the world gastroenterology Organization, Celiac disease may be divided into two types: Classical and non-classical. In Classical celiac disease; patients have signs and symptoms of malabsorption including diarrhea, steatorrhea (pale, foul smelling, fatty stool) and weight loss or growth failure. In non-classical Celiac disease; patients may have mild gastrointestinal symptoms without clear signs of malabsorption or may have seemingly unrelated

symptoms. They may suffer from abdominal distention and pain, iron deficiency anemia, chronic fatigue, reduced bone mass and bone fracture and vitamin deficiency (folic acid and B12).

Celiac disease may also be silent or asymptomatic where patients donot complain of any symptoms but still experience villous atrophy damage to their small intestine [2].

The number of ways celiac disease can affect patients combined with lack of training in medical schools contributes to the poor diagnostic rates [7].

Since celiac disease is so hard to diagnose, people can have it for years so the long term damage to the small intestine can affect other parts of the body [8].

It has been recently hypothesized that the following environmental factors are possibly involved in the switches of the tolerance–immune response balance: the amount and the quality of ingested gluten, the type and duration of wheat dough fermentation, early infant feeding, the spectrum of intestinal microorganisms and how they change over time and intestinal infections [3].

Helicobacter pylori infection is one of the most common infections worldwide. It has been hypothesized that microbial exposure may affect the risk of CD, and chronic gastric infections that are capable of inducing duodenal ulcers and influencing the systemic immune responses may trigger autoimmunity in the small intestine [9, 10].

Parasitic Helminths evolved with the mammalian immune system, promoting their own survival by altering host immune responses. The immune response induced by these worms is accompanied by the activation of intestinal mast cells, eosinophils, goblet cells, enterocyte proliferation and intestinal contractility. Granuloma formation then occurs, isolating the eggs and larvae, and inducing tissue repair leading to a predominantly anti-inflammatory response; reduced exposure to infections in early childhood might result in an increased risk of allergic disease later in life [11]. Thus, Helminths stimulate gluten tolerance among CD patients [12]. Helminths infection was found to inhibit T-helper 1 mediated autoimmune disease and T-helper 2 mediated allergy. Recent studies have shown an inverse association between Helminths infection and inflammatory diseases[13].

Lipids are a class of compounds that are soluble in organic solvents and nearly insoluble in water. They are either compound that yield fatty acids on hydrolysis or complex compounds that combine with fatty acids to form esters [14].

Two Important lipids; Cholesterol and triglycerides are transported in the blood by lipoproteins [15]. Lipoproteins assist lipids in their transportation in the plasma from their site of synthesis to the various tissues since they are insoluble in plasma. Much attention has been focused on lipids and lipoproteins mainly because of their strong association with coronary heart disease [14]. Currently the only treatment for celiac disease is a strict lifelong gluten free diet (GFD) [16]. To improve food in consistency and palatability lipids are greatly present in GFD [17].

Untreated patients affected by celiac disease are at high risk of malnutrition including alteration in lipid metabolism mainly low serum total cholesterol and low High density lipoprotein as a consequence of malabsorption [18]. Different auto antibodies and antibodies are used in the diagnosis of celiac disease, but Anti-tTG antibodies are highly sensitive and specific for the diagnosis of CD. IgG-based tests are one of the most useful auto antibody tests for celiac disease [2].

1.2.Statement of the problem

Celiac disease is a major public health problem worldwide. Although initially it was reported from countries with predominant Caucasian populations, it now has been reported from other parts of the world. It is a global disease and the global seroprevalence and prevalence of CD are 1.4% and 0.7%, respectively. The prevalence of CD varies with sex, age, and geographic location. The prevalence of CD has increased over time from 0.6% in 1991 to 2000 to 0.8% between 2001 and 2016 [19].

In settings where diarrheal infections remain as common causes of childhood morbidity and mortality, it is not difficult to postulate that celiac disease is a cause that can easily escape attention. At the same time it is reasonable to suppose that continuing non-diagnosis may well lead to severe illness and death in affected children. Celiac disease and its under-diagnosis have led to consider how much it might contribute to the global burden of childhood morbidity and mortality [20].

Recent epidemiological studies show that the burden of celiac disease is underestimated not only in Europe, but also among the populations of Mediterranean regions such as the Middle East and North Africa. Egypt and all North African countries are significant producers of wheat and largely rely on Barley for beer-brewing. Also the diffusion of Pasta across all the Mediterranean is now the reason for an enormous increase in gluten intolerance that is truly at epidemic level. However this epidemic is not fully recognized since a sizable number of cases are neither diagnosed nor cared for [21].

In the United States, CD is believed to affect 0.5%-1.0% of the general population. The overall prevalence of CD in Western populations is close to 1% (1:100) and may be higher in Northern European countries. The Scandinavian countries, Ireland, and the United Kingdom population tended to show a higher prevalence of CD of approximately 1.0%-1.5%. CD is a relatively common cause of chronic diarrhea in Iran, Iraq and Kuwait and has been diagnosed in 2%-8% of patients in Iran, Israel and Saudi Arabia [22].

The low awareness of CD often leads to a delay in diagnosis which contributes to more than 20 associated symptoms and conditions including growth failure, infant malnutrition, gastrointestinal disease, liver conditions and mortality [21].

As far as my literature search goes, there is no published study in Ethiopia in this regard. So the purpose of this study was to diagnose celiac disease using the anti-tissue transglutaminase IgG

antibody while determining its association with selected lipid profiles in *H.pylori* and Helminths positive and non infected children.

1.3. Significance of the study

Celiac disease is one of the areas that did not get much attention in its diagnosis; so this study could give some insight on the necessity of doing more research in the area so as to have a routine diagnostic procedure to minimize its under diagnosis in our country.

It was aimed on this study to have a better understanding on the association between celiac diseases with that of lipid profiles, *H.pylori* and Helminths infection so that more studies could follow relating the impact of celiac disease with more metabolic risk factors that could cause lifelong complicated chronic diseases.

This study could also serve as reference for researchers and clinicians to pay attention to the disease.

2. Literature Review

In 2012 a cross-sectional study was performed in New York; a total of 2689patients had CD and *H.pylori* prevalence were significantly lower and it was concluded that *H.pylori* presence and CD are inversely associated [21]. Another cross-sectional study in 2017 showed of 324 children (1-18 years) diagnosed with CD, *H.pylori* was seen in 37(11.4%) of patients. The prevalence of *H.pylori* in patients without CD was significantly higher. It was summarized that celiac disease and *H.pylori* infection had inverse relationship [23].

Lebwohl and his colleagues in a cross-sectional study done in United States in 2013 found a strong, inverse association between *H. pylori* presence and CD in a total of 2,689 patients diagnosed with CD. In patients without CD, *H. pylori* prevalence was found to be 8.8% while it was only 4.4% in patients with CD. The inverse relationship remained strong for all age groups, for both males and females, and when accounting for socioeconomic status [24].

Of 31 patients who were diagnosed with CD; 15 patients were positive for *H.pylori* and 16 patients were negative. Although *H.pylori* positivity was found with lower rate in CD group; no statistical difference were found compared to patients who did not have CD in a 2016 retrospective study conducted in Turkey [25].

Two Experimental Clinical trials done in the United States in 2011 show that basal production of Interferon- (IFN-γ) and Interleukin(IL-17A) from duodenal biopsy culture was suppressed in hookworm-infected participants compared to uninfected controls. Increased levels of $CD4^{+}CD25^{+}$ cells in the circulation and mucosa were associated with active celiac disease. The study showed that this accumulation also occurs during a short-term (1 week) oral gluten challenge, and that hookworm infection suppressed the increase of circulating $CD4^{+}CD25^{+}$ cells during this challenge period. When duodenal biopsies from hookworm-infected participants were re stimulated with the immune dominant gliadin peptide QE65, robust production of IL-2, IFN-c and IL-17A was detected, even prior to gluten challenge while participants were strictly adhering to a gluten-free diet. Intriguingly, IL-5 was produced only after hookworm infection in response to QE65 [26].

In a study by Croese *et al* in Australia on 12 CD patients infected with *Necator Americanus* larvae undergoing gluten diet found Helminths stimulate gluten tolerance among CD patients minimizing villous atrophy and lower production of IgA anti tissue transglutaminase [27].

It was found in a cross-sectional study conducted by Norsa L *et al* in Israel and Italy that the most common CVD risk factors in celiac disease were high fasting triglycerides (34.8%), elevated blood pressure (29.4%), and high concentrations of calculated LDL cholesterol (24.1%) and that there was a significant increase in both total Cholesterol and HDL cholesterol on celiac patients who followed a strict gluten free diet (GFD)[16].

In a 2011 case control study by Leeds *et al* in United Kingdom found that at diagnosis of CD, patients had lower HDL cholesterol and lower total cholesterol that significantly improved after 1 year on GFD [28].

Salardi *et al* in 2016 performed a case control study in Italy on the influence of celiac disease on lipid profiles and found that before the beginning of GFD, HDL values were lower than control groups which significantly increased after GFD was started with mean values of 60.9±13.7 vs 51.3±13.6mg/dl. This study was able to show a more evident reduction in HDL-C at diagnosis of CD that was highly restored after GFD [29].

Despite wheat and its products are widely consumed in Ethiopia, and hence gluten intolerance may be under diagnosed in the country, no such studies are found in the published literatures.

3. Objectives

3.1.General Objective

To assess anti-tissue transglutaminase IgG antibody with lipid panels in *H.pylori* and Helminths positives as well as non infected Children at Ziway, South East of Ethiopia

3.2.Specific Objectives

To assess association of anti tissue transglutaminase IgG antibody and *H.pylori* infection in children at Ziway, South East of Ethiopia

To assess association of anti tissue transglutaminase IgG antibody and Helminthes infection in children at Ziway, South East of Ethiopia

To determine association of anti tissue transglutaminase IgG antibody and lipid profiles of children at Ziway, South East of Ethiopia

4. Materials and Method

4.1.Study Area

The study was conducted in Ziway, Ethiopia. Ziway is a town in Oromia National Regional State, Adami Tulu Jiddo Woreda, at a distance of 160 Km from Addis Ababa in the East Shewa zone of Ethiopia. Its Economy is based on fishing and horticulture. The 2007 National Census reported a total population of 43,660 of which 22,956 were men and 20,704 were women. Study participants whose samples were used for this study are from Sher Elementary School and Batu Elementary School [30].

4.2.Study Design and Period

This study was a cross sectional retrospective and prospective study. The retrospective part included taking serum samples that were collected and stored during another study on association of Atopy with Helminths and *H.pylori* in 2016 and careful review of study groups sociodemographic and medical data. The serum samples that were kept refrigerated at -80ºC were further analyzed for anti tissue transglutaminase IgG and lipid profiles accounting for the prospective part of the study starting from May 2020 to June 2020.

4.2.1. Source Population

All school aged Children that the sample was taken during study period.

4.2.2. Study Population

All school aged Children tested for *H.pylori* and Helminths.

4.3.Inclusion and exclusion Criteria

4.3.1. Inclusion Criteria

School aged students tested for *H.pylori* and Helminths.

4.3.2. Exclusion Criteria

Samples which were insufficient for laboratory determination of anti-tissue transglutaminase IgG and lipid profiles.

Serum samples that failed inclusion criteria of test analysis (Hemolyzed, Lipemic and Icteric)

4.4.Study Variables

4.4.1. Dependent Variable

Magnitude of anti-tissue transglutaminase IgG

4.4.2. Independent Variable

- Sex
- Age
- Lipid panels
- *H.pylori* Antigen
- Helminths

4.5.Measurement and data collection

4.5.1. Sample size determination

The sample size was determined by using the formula for cross sectional studies

$$n=\frac{(Z_{\alpha/2})^2 P(1-P)}{d^2}$$

where;

n=sample size, at 95% Confidence interval Z value (α=0.05) =1.96,

P= prevalence which is 0.5% (0.005) [19]

d=precision or margin of error, since the prevalence is below 10% we take d=1% (0.01)

$$n=\frac{(1.96)^2 0.005(1-0.005)}{0.01^2} = 191.12=191$$ [31]

The minimum sample size that could be used was 191 but because most samples got excluded as a result of being insufficient to proceed with more tests performed we used correction formula as follows

New ss=$\frac{ss}{1+\frac{ss-1}{pop}}$

Where; ss= sample size, Pop= Population

$\frac{191}{1+\frac{191-1}{200}}$= 97.9~98

4.5.2. Sampling Method

The sampling method in the original study was systematic random sampling method; the current study conveniently took all the serum samples of children that were also tested for *H.pylori* antigen and Helminths. Even though the calculated sample size was 98; one sample was excluded due to Hemolysis making the totally used sample size for further tests in this study 97.

4.5.3. Data collection Procedure

The serum samples used were already collected and stored in refrigerator at -80^{o}C and it was further analyzed for this study in the lab for anti tissue transglutaminase IgG antibody Semi-quantitatively and selected Lipid profiles (Total cholesterol, Triglyceride and Low density Lipoprotein).

4.5.4. Sample stability

Serum samples can be used as a source of data for lipid profile determination after being stored for more than 10 years if stored properly ,preferably at -80^{o}C [32].

4.5.5. Principle of Laboratory Analysis for Anti- tissue transglutaminase IgG

Auto antibodies of the diluted patient samples, positive control, and calibrators react with human tissue transglutaminase immobilized on the solid phase of a microtiter plate. Anti-huTransG guarantees the specific binding of anti-tTG IgG autoantibodies of the specimen under investigation by employing highly purified, activated recombinant human tTG for coating. Following an incubation period of 60 min at room temperature (18…25°C), unbound serum components are removed by a wash step.

The bound auto antibodies react specifically with anti-human-IgG-antibodies conjugated to horseradish peroxidase (HRP) within the incubation period of 30 min at room temperature. Excessive conjugate is separated from the solid-phase immune complexes by the following wash step.

HRP converts the colorless substrate solution of 3,3',5,5'-tetramethyl-benzidine (TMB) added into a blue product. This enzyme reaction is stopped by dispensing an acidic solution (H2SO4) into the wells after 15 min at room temperature turning the solution from blue to yellow.

The optical density (OD) of the solution at 450 nm is directly proportional to the amount of specific antibodies bound.

4.6. Operational Definitions

Celiac Disease- defined by this study as children Positive for anti-tTG IgG antibody test.

Lipid Profiles- tests including Total Cholesterol, Triglycerides and Low density Lipoprotein.

School aged Children-according to this study are age groups from 4 years upto 14 years old

(4-14 yrs)

4.7. Data Quality Assurance

All the tests performed were assessed by using a control that was run accordingly with the sample. Calibrators were used for the determination of Anti-tissue transglutaminase Ig-G and tests were assumed valid when the mean OD of the standard 1 is< 0.7 and the mean OD of the standard 4 is >1.2 according to the manufactures instruction for the ELISA determination of IgG anti-tTG (Generic Assays).Controls were also run for lipid determination and SOPs were followed.

4.8. Data analysis and Interpretation

All the data were entered and analyzed on SPSS version 23 using descriptive analysis, Chi-square and logistic regression to see any associations and P-value <0.05 was considered statistically significant. Then the results were presented in words, charts and tables.

4.9. Ethical Consideration

Ethical approval was obtained from the Department Research and Ethical Review Committee (DRERC) of the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University. The samples used for this study were from a project which had Institutional approval.

4.10. Dissemination of results

Results of this study were presented to the Department of Medical Laboratory Sciences, College of health Sciences, Addis Ababa University. Manuscript was prepared for publication on peer reviewed journals and the findings will be presented at scientific conferences.

5. Result

A total of 97 samples of children with age group between 4-14 years old were involved in this cross-sectional study. On the socio-demographic features, majority of the study participants were females (54.6%) with age group between 4-9 years (63.9%); Helminths positivity was 16.5% while *H.pylori* antigen positives accounted for 15.5%. Most of the mothers had a formal education (69.1%) and were house wives (41.2%) as shown in Table 1.

Table1. Sociodemographic and clinical features of study participants at Ziway, South East Ethiopia (n=97) from May 2020 to June 2020

Variable	***Category***	***Number***	***Percent***
Sex	Male	44	45.4
	Female	53	54.6
Age(Years)	4-9	62	63.9
	10-14	35	33.1
Maternal occupation	House wife	40	41.2
	Farming and related	2	2.1
	Trading and related	20	20.6
	Government employee	6	6.2
	Others	29	29.9
Maternal education	Non formal	30	30.9
	Formal	67	69.1
Helminths	Negative	81	83.5
	Positive	16	16.5
H.pylori antigen	Negative	82	84.5
	Positive	15	15.5

Figure 2 below summarizes the overall anti tissue transglutaminase IgG magnitude among the study participants with those tested positive for the antibody accounting for 18.5% of the total study participants.

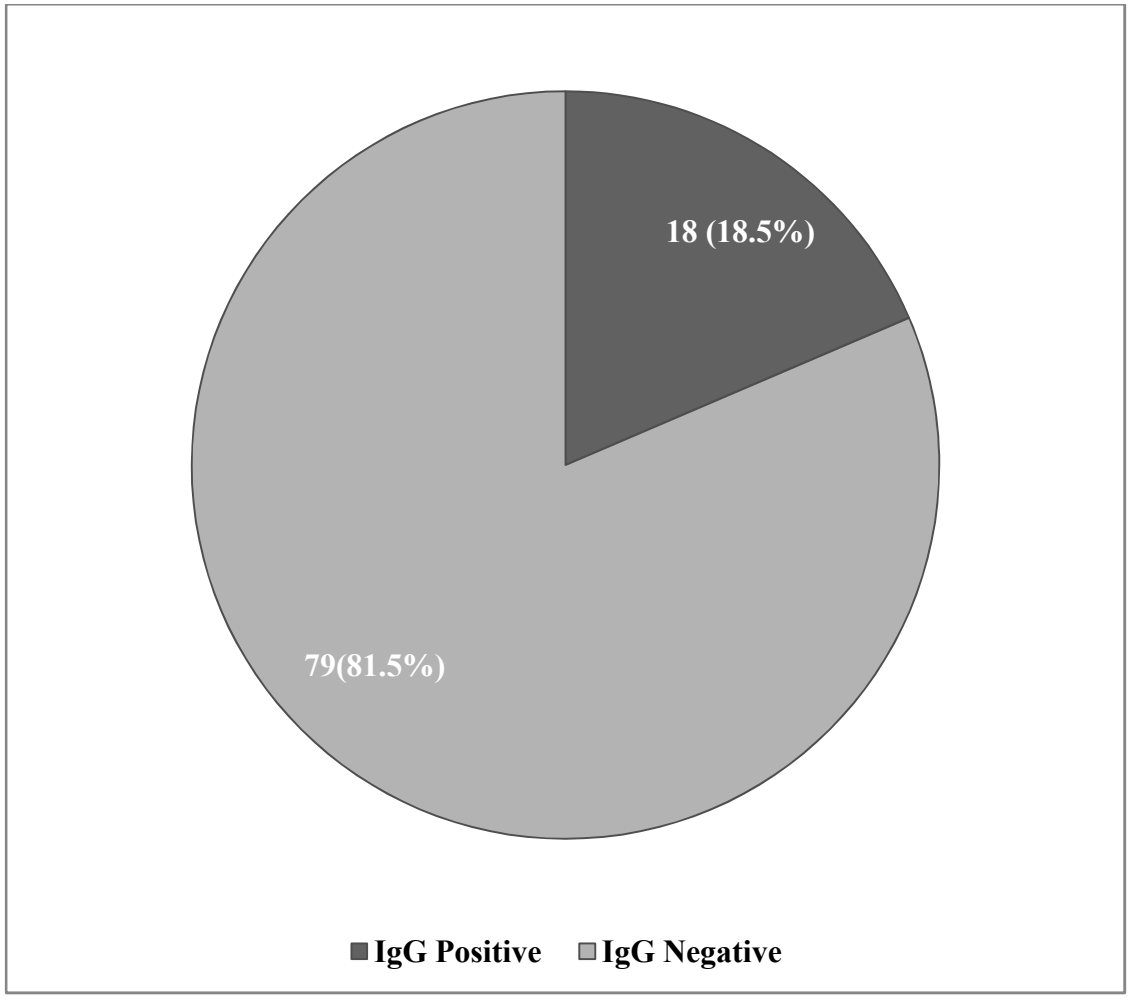

Figure 2. Magnitude of anti tissue transglutaminase IgG of study participants at Ziway, South East Ethiopia (n=97) from May 2020 to June 2020

As shown in table 2 females had a higher magnitude of anti-tTG IgG than males (24.5 % vs 11.4%) in addition age groups with 10-14 years showed a higher magnitude of anti-tTGIgG than those age groups of 4-9 years (25.7% vs 14.5%). No significant difference was seen among the different variables mentioned below in the table and anti-tTGIgG. However, significant association was found between anti-tTGIgG and helminthes [OR 3.45 (95% CI: 1.057, 11.265), P=0.033] ,where children not infected with helminthes had a 3.45 times greater risk of developing a positive anti-tTGIgG , although this association was lost when multivariate analysis was done indicating that Helminths may had been confounded with another variable so further study is suggested.

Table 2.Association between risk factors and anti-tissue transglutaminase IgG among children at Ziway, South East Ethiopia (n=97) from May 2020 to June 2020

Variables		***Anti-tissue transglutaminase IgG***				***P-value***
		Negative	***Positive***	Total	Crude OR(95%CI)	
Age	4-9	53(85.5%)	9(14.5%)	62		
	10-14	26(74.3)	9(25.7%)	35	2.038 (0.723,5.746)	0.173
Sex	Male	39(88.6%)	5(11.4%)	44		
	Female	40(75.5%)	13(24.5%)	53	2.535 (0.826,7.783)	0.097
H.pylori	Negative	68(82.9%)	14(17.1%)	82		
	Positive	11(73.3%)	4(26.7%)	15	1.8 (0.491,6.358)	0.380
Helminths	Negative	69(85.2%)	12(14.8%)	81		
	Positive	10(62.5%)	6(37.5%)	16	3.45 (1.057,11.265)	0.033
P-value <0.05 shows significant difference						

After doing independent t-test the overall mean lipid profiles of study participants showed all the results were within range for both positive and negative subjects of anti-tissue transglutaminase IgG. However there was no significant difference observed in lipid profiles and anti-tissue transglutaminase IgG status of study participants as shown in table 3.

Table 3 Mean lipid profiles versus anti-tTG IgG status among children at Ziway, South East Ethiopia (n=97) from May 2020 to June 2020

Lipid Profiles (mean±SD)	***Anti-tTGIgG***		***P-value***
	Negative	Positive	
TC	113.5±27.2	113.2±24.1	0.631
TG	125.4±49.3	130.8±40.1	0.763
LDLC	67.4±25.4	67.4±19.3	0.902

Normal range Total cholesterol(TC) 0-200mg/dl

Triglycerides(TG) 0-200mg/dl

Low density lipoprotein cholesterol (LDLC) 0-100mg/dl

P-value <0.05 shows significant association.

6. Discussion

Recently different researches performed on simple, sensitive and specific screening tests like the anti tissue transglutaminase antibody assays in the developing areas of the world, where there is a growing usage of wheat, shows that gluten intolerance had been more than the expected rate showing its under diagnosis. On the other hand, it has been hypothesized that microbial exposure like Helminthes and *H pyplori* may affect the risk of CD, Studies have shown that Helminths stimulate gluten tolerance among CD patients [10, 12]. As a result, CD is not considered as a disease of priority and hence studies are very limited in resource constrained settings. It was with this background that the current study aimed to determine anti tissue transglutaminase IgG level in children infected with Helminthes and H pylori as well as non-infected children.

Accordingly, out of 97 school aged children in this study 18(18.5%) tested positive for Antitissue transglutaminase IgG. A study performed by Cataldo *et al* (2007) on Celiac disease in developing countries among Turkish school children showed that prevalence of celiac disease with no classical symptoms among healthy individuals to be 0.8%; however, the prevalence was lower than the current study [33].That may be due to the fact that our test was only focused on anti tissue transglutaminase IgG because of resource limit to perform more tests like the antitissue transglutaminase IgA and tissue biopsy that can confirm celiac disease to show its exact prevalence.

The current study showed celiac affected females more than males (24.5 % vs 11.4%) which is against a study by Fayed *et al* that found males were highly affected by celiac disease than females (72.7% vs 27.3%) [34]. This is explained as women have a combination of two X chromosomes that leaves them genetically more susceptible to celiac disease since their immune system is more reactive than male that can easily and maybe incorrectly modify itself in fight against infection that results in excessive activation leading to autoimmune diseases such as Celiac disease [35]. Also majority of study participants in this study are female probably accounting for the higher proportion.

A study by Lebwohl *et al*, 2013 on a confirmed celiac disease, *H.pylori* was detected in only 4.4% of patients which was relatively lower than a result on this study that found of positive anti-tTGIgG study participants 26.7% tested positive for *H.pylori* although no significant association was found [24]. Additional study by Maxim *et al* found that among children diagnosed with CD as well as *H.pylori* only 5(21.7%) had a serious damage of the gut suggesting protective role of *H.pylori* against severity of celiac disease [36].

However this study failed to show any association between anti-tissue transglutaminase IgG and *H.pylori* infection. This is more related with a prospective study by Dore *et al* and a crossectional study by Basyigit *et al* 2017, where both studies found no association between celiac disease and *H.pylori* infection [10, 37]. This may be due to the fact that studies showing the direct effect of *H.pylori* on gluten or its effect on the immune response to suppress autoimmunity have not been developed yet so more trial studies are required on this area to show a clear association between the two variables.

A clinical trial by McSorley *et al* 2011, on effect of Hookworm to suppress celiac disease showed that individuals purposely infected with different Helminthes (Hookworm) showed lower levels of cytokines in response to gluten administration that supports the idea that Helminths have a protective effect against Celiac disease [26] supported with additional clinical trial by Croese *et al* that followed a purposeful infection of celiac patients with Ubiquitous *Necator americanus* that resulted in a modified host immune response to gluten by enhancing mucosal IL-1β and IL-22 and suppressing IFN-γ and IL17A levels following gluten challenge[26].Although we did not perform any clinical trial and were not able to confirm celiac disease, our result of p-value shows a significant association between Helminthes and anti-tTGIgG that should be proven with a more detailed study in the future.

Ciacci *et al* showed plasma cholesterol was inversely related to prevalence of celiac disease ($p<0.001$) mentioning all patients diagnosed with celiac disease had a plasma cholesterol level < 156mg/dl [38], supporting this most of the results on the level of total cholesterol in this study fell below 156mg/dl although no association was found between the two variables.

Our result of no association was in favor of a study by Rosenthal *et al* that found no significant difference between celiac patients and lipid profiles mainly total cholesterol, HDL-C and LDLC.

[39].This suggests that lipid profile alteration may not only be as a result of fat malabsorption but also enterocyte physiology.

Taken together, the current cross-sectional study for the first time demonstrated the magnitude of anti-tissue transglutaminase IgG antibody level and its association with H pylori and helminthes in school aged children. With the limited number of children involved it was possible to demonstrate an association with Helminthes infection where children positive for helminthes were 3.45 times less likely to be positive for the antibody. With further studies in the future this study can give a better insight on therapeutic development options for celiac disease.

7. Strength and limitation

7.1. Strength

This study is the first to be performed in children in our country so it might serve as a reference for other more researches coming ahead.

7.2. Limitation

It was not possible to perform more tests like tissue transglutaminase IgA and tissue biopsy to confirm celiac disease so it was difficult to show a clear association. The sample size is also limited to a small group of children. Also other factors like breast feeding status of children, hygiene status and the like were missing to drive a clear association between celiac and other co morbidities. However, the study still shades some light as the study demonstrated an association between helminthes and anti-tissue transglutaminase IgG levels.

8. Conclusion and recommendation

8.1. Conclusion

Although the relation between *H. pylori* and Celiac diseases is controversial, our result doesn't proof *H.pylori* can protect against celiac disease so measures to protect oneself against *H.pylori* should be continued. On the other hand, even though more trials are required, there is a strong association between Helminthes infection and anti-tTGIgG where children not infected with Helminths had a 3.45 times greater risk of developing a positive anti-tTGIgG. Although a significant difference was not found in this study between lipid profiles and anti tissue transglutaminase, it is advisable to assess lipid profiles of celiac patients as shown in other studies so as to minimize the long term effect associated with lipid profile alteration, Coronary artery disease for instance.

8.2. Recommendation

Celiac disease related studies are rare in our country so doing more research on the area in a well-designed manner is recommended since our life style is turning to more gluten use in our daily life with wheat, barley and their products being consumed more often. Also since gluten free diet can reverse long term effect of Celiac disease, as shown with different studies having an early diagnosis is advisable.

This study focused on anti-tissue transglutaminase IgG for celiac disease determination; but it is more advisable to perform tissue transglutaminase IgA and more celiac tests by following the correct algorithm to confirm celiac disease and do more association in a large scale study.

It is also strongly recommended to have large scale case control study to understand the exact relationship between celiac diseases and other co-morbidities.

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Annex I: Procedure for the determination of Anti-tissue transglutaminaseIgG by ELISA

First dilute patient sera with sample diluent (C) 1 + 100 (v/v), e.g. 10 µl serum + 1.0 ml sample diluent (C). Then follow the procedure below;

1. Bring all reagents to room temperature (18-25°C) before use. Mix gently, avoid foam.
2. Dispense **100 µl**calibrators 0 - 4 (quantitative) or **100 µl**of calibrator 1 (semi-quantitative) **100 µl**positive control **100 µl**diluted patient samples into the respective wells.
3. Seal plate, incubate **60 min** at room temperature.
4. Decant, then wash each well **three** times using **300 µl**wash solution (made of B).
5. Add **100 µl**of conjugate (D) solution to each well.
6. Seal plate, incubate **30 min** at room temperature.
7. Decant, then wash each well **three** times using **300 µl**wash solution (made of B).
8. Add **100 µl**of substrate (E) to each well.
9. Incubate **15 min** protected from light at room temperature.
10. Add **100 µl**of stop solution (F) to each well and mix gently.
11. Read the OD at **450 nm** versus 620 or 690 nm within **30 min** after adding the stop solution.

Annex II: Principles of selected lipid panel tests on cobas 6000

The Roche/Hitachi Cobas 6000 analyzer series is a fully automated, random-access, software-controlled system for immunoassay and photometric analyses intended for qualitative and quantitative in vitro determinations of a wide variety of tests. It is optimized for workloads using a combination of photometric and ion-selective electrode (ISE) determinations (c501 module), and electrochemiluminescence (ECL) technology in the immunoassay analysis module (e601 module).

Total Cholesterol

In this enzymatic method esterified cholesterol is converted to cholesterol by cholesterol esterase. The resulting cholesterol is then acted upon by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminophenazone in the presence of peroxidase to produce a colored product that is measured at 505 nm (secondary wavelength = 700 nm). The final step is known as the Trinder reaction. This method is a single reagent, endpoint reaction that is specific for cholesterol.

Triglycerides

The method is based on the enzymatic hydrolysis of serum or plasma triglyceride to glycerol and free fatty acids(FFA) by lipoprotein lipase (LPL). The glycerol is phosphorylated by adenosine triphosphate(ATP) in the presence of glycerolkinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). G-3-P is oxidized by glycerophosphate Oxidase(GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide.

A red chromogen is produced by the peroxidase (POD) catalyzed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxidase (H_2O_2), proportional to the concentration of triglyceride in the sample.

Triglycerides + H_2O $\xrightarrow{\text{LPL}}$ Glycerol+ 3FFA

Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol-3-P + ADP

Glycerol-3-P + O2 $\xrightarrow{\text{GPO}}$ DHAP+ H_2O_2

4-AA+4 Phenol $\xrightarrow{H_2O_2/\text{POD}}$ Quinoneimine + H_2O

Low density Lipoprotein Cholesterol (LDL-C)

The procedure comprises two steps.

In the first step Cholesterol in lipoproteins other than LDL in the test sample are decomposed by the simultaneous action of cholesterol esterase (CE) and cholesterol Oxidase (CO) at pH 7.0, giving as end products Cholestenone and hydrogen peroxide, the latter being decomposed to water and oxygen by catalase.

In the second step as a surfactant which specifically acts on LDL is added to the reaction product of the first step being the remaining cholesterol quantified by a Trinder's type reaction in which the aniline derivate, HDAOS, and 4-aminoantipyrine (4-AA) as a coupling reagent are condensed by the H_2O_2 in the presence of peroxidase (POD) to form a red quinoneimine dye proportional to the concentration of LDL-cholesterol present in the sample.

Annex III- Laboratory protocol for H.pylori stool Ag Rapid Test

Procedure A: Solid stool samples

1. Collect a random stool sample in a clean, dry receptacle.

2. Open the stool collection device by unscrewing the top and use the collection stick to randomly pierce the stool sample in at least five different sites. Do not scoop stool sample as this may lead to an invalid test result.

3. Ensure stool sample is only in the grooves of the collection stick. Excess stool sample may lead to an invalid test result.

4. Replace the collection stick and tighten securely to close the sample extraction tube.

5. Shake the sample extraction tube vigorously.

Procedure B: Watery stool samples

1. Collect a random stool sample in a clean, dry receptacle.

2. Open the sample extraction tube by unscrewing the top.

3. Fill the plastic dropper with the sample; dispense 2 drops (70-85 μL) into the sample extraction tube.

4. Replace the collection stick and tighten securely to close the stool collection device.

5. Shake the sample extraction tube vigorously.

Note: Specimens extracted may be stored at 2°C-8°C for up to 3 days. If longer storage is required, freezing at ≤-20°C is recommended. 74

Procedure

Step: 1 Bring the specimen and test components to room temperature if refrigerated or frozen.

Step: 2 when ready to test open the pouch at the notch and remove the test device. Place the test device on a clean, flat surface.

Step: 3 shake the sample collection tube vigorously to ensure a homogenous liquid suspension.

Step: 4, Position the stool collection device upright and twist off the dispenser cap. Holding the sample extraction tube vertically, dispense 2 drops of the solution into the sample well of the test device. Do not overload sample.

Step: 5 set up the timer.

Step: 6 Results can be read 15 minutes after adding the specimen. Positive results can be visible in a time period as short as 1 minute.

Do not read results after 20 minutes. To avoid confusion, discard the test device after interpreting the result.

Interpretation of Assay Result

1. Negative Result: If only the C line is developed, the test indicates that no detectable H.pylori antigen is present in the specimen. The result is negative.

2. Positive Result: If both C and T lines are developed, the test indicates the presence of H.pylori antigen in the specimen. The result is positive.

3. Invalid: If no C line is developed, the assay is invalid regardless of any colour development on the T line as indicated below. Repeat the assay with a new test device.

Annex IV- Laboratory protocol for Helminths determination

Formol ether concentration technique

1. Using a rod or stick, emulsify an estimated 1 g (pea-size) of faeces in about 4 ml of 10% formol water contained in a screw-cap bottle or tube.

Note: Include in the sample, faeces from the surface and several places in the specimen.

2. Add a further 3–4 ml of 10% v/v formol water, cap the bottle, and mix well by shaking.

3. Sieve the emulsified faeces, collecting the sieved suspension in a beaker.

4. Transfer the suspension to a conical (centrifuge) tube made of strong glass, copolymer, or polypropylene. Add 3–4 ml of diethyl ether or ethyl acetate.

Caution: Ether is highly flammable and ethyl acetate is flammable, therefore use well away from an open flame, e.g. flame from the burner of a gas refrigerator, Bunsen burner, or spirit lamp. Ether vapour is anaesthetic, therefore make sure the laboratory is well-ventilated.

5. Stopper* the tube and mix for 1 minute. If using a Vortex mixer, leave the tube unstoppered and mix for about 15 seconds (it is best to use a boiling tube).

Do not use a rubber bung or a cap with a rubber liner because ether attacks rubber.

6. With a tissue or piece of cloth wrapped around the top of the tube, loosen the stopper (considerable pressure will have built up inside the tube).

7. Centrifuge immediately at 750–1 000 g (approx. 3000 rpm) for 1 minute. After centrifuging, the parasites will have sedimented to the bottom of the tube and the faecal debris will have collected in a layer between the ether and formol water

8. Using a stick or the stem of a plastic bulb pipette, loosen the layer of faecal debris from the side of the tube and invert the tube to discard the ether, faecal debris, and formol water. The sediment will remain.

9. Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Tap the bottom of the tube to resuspend and mix the sediment. Transfer the sediment to a slide, and cover with a cover glass.

10. Examine the preparation microscopically using the 10x objective with the condenser iris closed sufficiently to give good contrast. Use the 40xobjective to examine small cysts and eggs. To assist in the identification of cysts, run a small drop of iodine under the cover glass. Although the motility of Strongyloides larvae will not be seen, the non-motile larvae can be easily recognized.

11. If required, count the number of each species of egg in the entire preparation. This will give the approximate number per gram of feces.

Annex V- Data collection form for lipid panel tests

ID	*Total Cholesterol (mg/dl)*	*Triglycerides(mg/dl)*	*LDLC(mg/dl)*

Annex VI: Declaration

The undersigned declares that this thesis is as a partial fulfillment of the requirements for the degree of Master of Science from Addis Ababa University and it complies with the regulations of the University and meets the accepted standards with respect to originality and quality. I also agree to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports.

Mekdes Alem

Signature: _____ Date:_____

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