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FACOLTÀ DI MEDICINA E CHIRURGIA

Ph.D. Thesis

Identification of a new biomarker for celiac disease and other gluten-related disorders

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For all who are not able to digest wheat

Dedicated to the loving memory of Prof. **Elio Tonutti**....

Safe in the arms of Jesus

....Gone but Never Forgotten

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Introduction

Cereal crops (Wheat, barley, rye, oat, corn) are the foremost portion of human food. Among those, wheat is the most prevalent cereal grain and worldwide staple food. Due to the visco-elastic property of wheat flour dough and due to the major storage protein i.e. 'gluten', it is used extensively in the food industry primarily to prepare bread and pasta.[1] Gluten is a ubiquitous material presents in almost every diet in apparent or its concealed form. Apart from food industry gluten is used widely in cosmetics, bakeries, toiletries, beverages drugs and numerous industries.[2–4]

Consumption of gluten in the form of wheat and closely related cereals gives birth to several wheat related disorders collectively termed as **"Gluten-Related disorders (GRD)"** that include **Celiac disease (CD), Dermatitis herpetiformis (DH), Gluten Ataxia (GA), Wheat Allergy (WA), and Non-Celiac Gluten Sensitivity (NCGS).**[5] GRD gradually emerged as an epidemiologically relevant phenomenon with a global prevalence of around 5%.[5,6,7]

For GRD, the only treatment available so far is the complete avoidance of gluten and adherence to a strict gluten-free diet (GFD). Despite the well-recognized efficacy of the GFD, complete adherence to GFD is challenging to achieve, numerous studies have reported up to 50% incomplete compliance in celiac patients.[8,9] It has been calculated that gluten-free products with <20 mg/kg (or parts per million = ppm) of gluten level are safe over a wide range of daily consumption.[10] This maximum tolerable amount of gluten contamination (<20 ppm) is recommended by Codex Alimentarius, US Food and Drug Administration (FDA) and European Food Safety Authorities (EFSA).[11–13]

Among different GRD, a considerable amount of work has been done towards CD. Its pathophysiology has been extensively investigated. Some useful sero-markers have

documented that help making a diagnose of CD. For other GRD so far, there is no specific and reliable biomarker discovered. Available serological tests work well for screening and diagnosis purposes. After the diagnosis of disease, for the follow-up purpose, serological markers are not very reliable as autoantibody titers do not match with the histological findings or with the symptoms of CD.[14,15] In patients following a GFD, elevated antibodies levels take about 6-24 months to reduce in blood.[16] This is a matter of concern that the requirement of adherence to GFD vary in each GRD, while a strict and lifelong GFD is the only option for CD, NCGS requires repeated gluten challenges. A strict adherence to the GFD is certainly necessary to improve the symptoms, despite this, there are no clear guidelines to monitor the adherence to the diet moreover, there are no validated biomarkers to assess the compliance. [17]

This Ph.D. work has principally focused on the finding and validation of suitable non-invasive biomarkers for diagnosis as well for the assessment of the adherence to GFD. To fulfill this goal, I with my research team in the supervision of **Professor Carlo Catassi** have extensively investigated the efficacy of different potential blood-dependent non-invasive biomarkers for the diagnosis (**Rapid HLA DQ typing method, EMA Biopsy**) as well as for the adherence to GFD [**Alkylresorcinol (AR), GlutenImmunogenic Peptides (GIP urine test), Intestinal Fatty Acid Binding Protein (i-FABP)]**. Additionally, the efficacy of blood-independent biomarkers has also been investigated, as in the quantitative analysis of gluten in food products through different antibodies that specifically designed to investigate the gluten content in food products (e.g. R5 and G-12 ab). I have discussed the details of each biomarker in the following sections of this thesis.

Awards and Recognitions

XXIV CONGRESSO NAZIONALE SIGENP
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ESPGHAN 49th ANNUAL MEETING
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which has been held in Athens, Greece, 25 – 28 May 2016.

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CERTIFICATE OF CONFIRMATION
for
Anil K. Verma
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Università Politecnica delle Marche, Ancona, Italy

This certificate confirms that Anil K.Verma completed a research fellowship at:
The Mucosal Immunology Biology and Research Center
Massachusetts General Hospital, Boston, MA
October 23, 2016 to April 11, 2017

This collaborative research was focused on the role of gut microbiome on the onset of celiac disease in genetically-predisposed infants. The project built a multi-omic modeling of disease risk based on the clinical dataset and biospecimens that are collected on an ongoing observational birth cohort study.

Respectfully submitted by:

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Roma, 7 ottobre 2017

Il Presidente della Società Italiana di Gastroenterologia, Epatologia e Nutrizione Pediatrica

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Gentilissimo Dr. Verma,

abbiamo il piacere di comunicarle che la commissione SIGENP preposta per il Premio SIGENP 2016 per giovani ricercatori ha approvato il finanziamento della sua progetto di ricerca, da svolgersi presso il Mucosal Immunology and Biology Research Center del Massachusetts General Hospital (USA) per un periodo di 6 mesi, a partire dal 1 luglio 2016, da completare entro il 31 dicembre 2017, per un importo di euro 5000.
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Con i nostri migliori auguri

Cordiali saluti

Il segretario SIGENP
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We are pleased to confirm that

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Nutrients, Foods, Safety

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Basel, 29 October 2018

MDPI

Awards

- First award for oral presentation in Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP) Congress Rome, Italy. 2017
- "Young Investigator Travel Grant", World Congress of Pediatric Gastroenterology, Hepatology and Nutrition (WCPGHAN), Canada. 2016
- "Young Researcher Award", Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP) award, Italy. 2016
- Full Ph.D. fellowship, Università Politecnica delle Marche, for Ph.D. in Biomedical Sciences, Italy, 2015

Professional Membership

- Italian Society of pediatric gastroenterology, hepatology, and Nutrition (SIGENP)
- International Society for the study of Celiac Disease (ISSCD)

Reviewer

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- Journal of Gastroenterology and Hepatology Open (JGH Open)
- Annals of pediatrics

External Reviewer

- Nutrients
- Food
- Safety
- Sustainability

Editorial board member

- Journal of public health and nutrition
- Nutrition and public health
- International Journal of Nutrition and Food Sciences

Featured Publications:

S. No.	Title	Authorship	status	Year	Journal	Impact factor
1.	Gluten contamination in naturally or labeled gluten-free products marketed in Italy **[Project 3]**	First & corresponding	Published	2017	Nutrients	4.2
2.	Comparison of diagnostic performance of the IgA anti-tTG test vs IgA anti-native gliadin antibodies test in detection of celiac disease in the general population **[Project 8]**	First & corresponding	Published	2018	CGH	7.9
3.	Validation of a novel single-drop rapid HLA-DQ2/-DQ8 typing method to identify subjects susceptible to celiac disease **[Project 5]**	First	Published	2018	JGH Open	Newly launched journal
4.	Contribution of oral hygiene and cosmetics on contamination of gluten-free diet: do celiac customers need to worry about? **[Project 4]**	First & corresponding	Published	2019	JPGN	2.8
5.	Celiac disease in the year 2020: still increasing, largely undetected **[Project 7]**	Fourth	Under review	2019	Gastroenterology	20.9

Status of the research studies (projects) performed during Ph.D. duration (2015-2018)

Project no.	Project title	Status	Duration	Publication status
1.	Biomarkers for the adherence to Gluten	Ongoing	2018	-
2.	Per day gluten exposure determination in celiac disease patients on gluten-free diet	Ongoing	2018	-
3.	Gluten contamination in naturally or labeled gluten-free products marketed in Italy	Completed	2016-2017	Published
4.	Contribution of oral hygiene and cosmetics on contamination of gluten-free diet	Completed	2018	Published
5.	Validation of a novel single-drop rapid HLA-DQ2/-DQ8 typing method to identify subjects susceptible to celiac disease	Completed	2017-2018	Published
6.	Verification of HLA-DQ2 and HLA-DQ8 allele distribution in native south Indian population	Ongoing	2018	-
7.	Re-exploring the iceberg of celiac disease in children	Completed	2015-2017	Under review
8.	The increasing prevalence of celiac disease: what is the role of an improved diagnostic accuracy?	Completed	2017	Published
9.	Establishment of IgA EMA biopsy method	Completed	2018	-
10.	Non-immunological biomarkers for assessment of villous abnormalities in patients with celiac disease	Completed	2015-2018	Under review
11.	Gluten sensitivity in patients with cerebellar ataxia: A prospective cohort study	Completed	2015-2018	Under review

Abbreviations:

AGA	Anti Gliadin antibodies
Anti tTG ab	anti Tissue transglutaminase antibodies
AOAC	Association of Official Analytical Chemists
AR	Alkylresorcinol
ATI	Amylase-Trypsin inhibitors
AU	arbitrary units
BAT	Basophil activation test
BP	Base pair
CD	Celiac disease
DGP	De-amidated gluten peptide
DH	Dermatitis herpetiformis
EFSA	European Food Safety Authorities
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMA	Endomysial antibodies
ESPGHAN	European Society of Pediatric Gastroenterology Hepatology
EU	European Union
FA	Food allergy
FDA	Food and Drug Administration
FDR	First degree relatives
GA	Gluten Ataxia
GFD	Gluten-free diet
GIP	Gluten immunogenic peptides
GRD	Gluten-related disorders
GS	Gluten Sensitivity

HLA	Human Leukocytic Antigen
HMW-GS	High Molecular Weight Glutenin Subunits
HPLC	Hi-performance liquid chromatography
HSCT	Hematopoietic stem cell transplantation
IEL	Intraepithelial lymphocytes
i-FABP	Intestinal Fatty Acid Binding proteins
IFN	Interferon
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IUIS	International Union of Immunogenic Societies
LMW-GS	Low Molecular Weight Glutenin Subunits
MA	Molecular-based allergy
mAb	Monoclonal antibody
Mg	Milligram
ml	Mililiter
MW	Molecular weight
NCE	Non-celiac enteropathies
NCGS	Non-celiac gluten sensitivity
NPV	Negative predictive value
pH	Power of hydrogen
ppm	Parts per million
PPV	Positive predictive value
PWG	Poramin working group
RT	Real-time
sIgE	specific Immunoglobulin E

SPT	Skin prick test
TG6	tissue transglutaminase 6
TGAse	Tissue transglutaminases
Th	T helper cells
UK	United Kingdom
ULN	Upper limits of normal
USA	United State of America
WA	Wheat Allergy
WDEIA	wheat dependent exercise-induced anaphylaxis
WHO	World health organization
µl	Microliter

Chapter 1

Gluten-related disorders: An introduction

Wheat (*Triticum aestivum*) is a cereal grain which is the world's most favored staple food.[18] In 2016, global wheat production was 749 million tonnes.[19] Cultivation of wheat was started about 10,000 years ago during the Neolithic period in the fertile crescent of the middle east. The wheat kernel contains 8%–15% of protein, from which 10%–15% is albumin/globulin and 85%–90% is gluten **(**Figure 1**).**[20]

Gluten is a complex mixture of hundreds of related but distinct proteins, including some toxic proteins fractions that are mainly gliadin and glutenin.[21] Alcohol soluble gliadins are further classified in their primary structures into **α-, γ- and ώ-gliadins,** poorly alcohol soluble glutenin proteins can be divided into **high molecular weight glutenin subunits (HMW-GS), low molecular weight glutenin subunits (LMW-GS) [Figure 1].**[22] The gliadins have high proline and glutamine content, known as prolamins, characterized by high levels of glutamine (38%) and proline residues (20%). humans inherently lack endopeptidases (an enzyme) that cleave bonds between proline and glutamines. The incomplete digestion of gliadin by digestive tract enzymes leads to the generation of many bigger peptides that eventually causes dietary diseases.[20] In the last decades, multiple disorders have been reported due to the ingestion of gluten. There is a general agreement for the term **"Gluten-Related Disorders"** that is an umbrella-term to be used collectively for all such disorders with 5% worldwide prevalence. [5,23,24] GRD is the umbrella-term to be used for describing all conditions related to ingestion of gluten, the protein found in all forms of wheat, rye, and barley that include **CD, DH, GA,**

WA, and NCGS.[5] Each disorder has discussed in separate sections. Classification of GRD is shown in **Figure 2.**

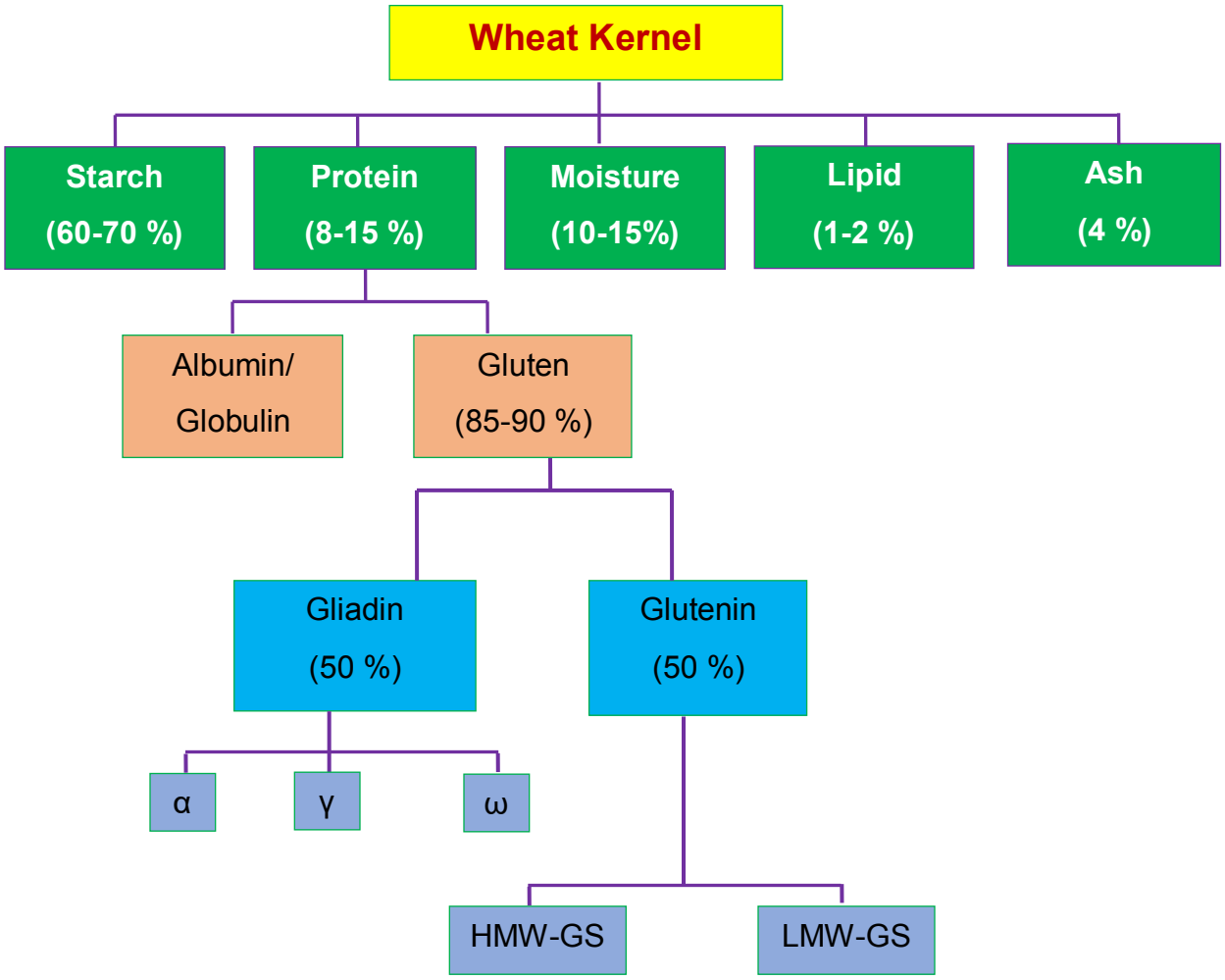

Figure 1: Breakdown of wheat components.

HMW-GS: High Molecular weight glutenin subunit; LMW-GS: Low molecular weight glutenin subunit

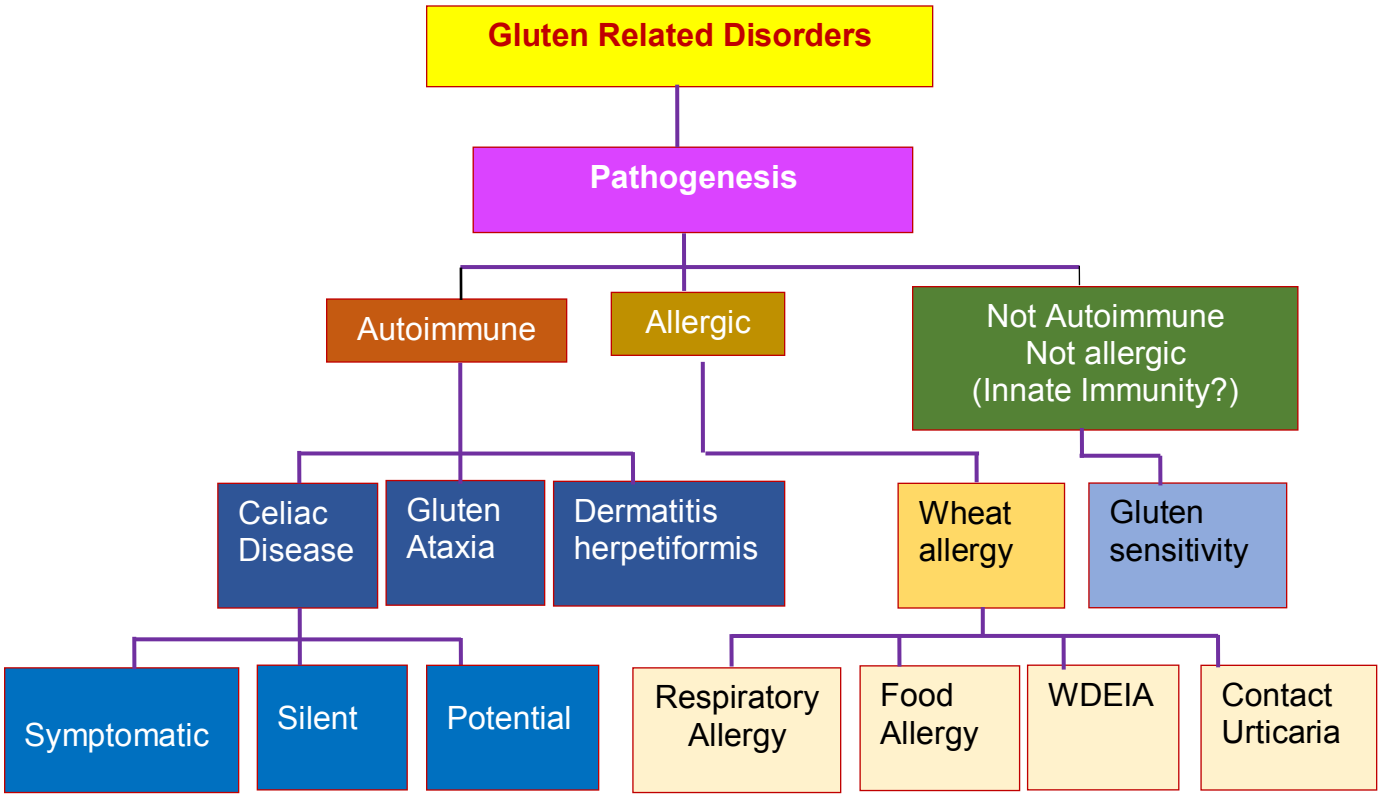

Figure 2: Proposed new nomenclature and classification of gluten-related disorders.

Celiac Disease

Celiac disease (CD) is an autoimmune condition characterized by permanent intolerance to dietary gluten, a protein complex found in wheat, rye, and barley, occurring in genetically predisposed individuals i.e individuals display HLA-DQ2 and/or HLA-DQ8 alleles. At least 1% of the general population all over the world is affected by CD. The hallmarks of the active CD are the presence of serum autoantibodies (e.g. lgA anti-transglutaminase; anti tTG ab and anti-endomysial antibodies; EMA) and a small intestinal enteropathy characterized (in typical cases) by villous atrophy, crypt hypertrophy and increased number of intraepithelial lymphocytes (IELs). Treatment of CD is based on the lifelong exclusion of gluten-containing food from the diet.GFD determines the gradual disappearance of symptoms and serum autoantibodies, and the normalization of the intestinal histological architecture. CD is explained further in **chapter 2.**

Dermatitis herpetiformis

The term dermatitis herpetiformis (DH), was introduced by Louis Adolphus During in 1884, is a chronic skin inflammatory condition due to the indolence of gluten that results in skin rashes, blisters filled with watery fluid. Elbows and upper forearms are the most affected body parts (almost 90% DH patients), other common sites are knees, shoulders, sacrum, face, scalp, neck and trunk. About 1 in every 10,000 in the UK and USA and slightly higher in Europe, 4-6 in every 10,000 in Finland and Sweden are affected with DH.[25]

DH is often reported from Asia and Africa. The age of onset of DH is about 15-40 years, DH is more common in males than in females. About 5 % of first degree relatives of DH patients develop DH. Approximately 90%-95% of DH patients express HLA-DQ2/-DQ8 haplotypes.[25,26] DH is a manifestation of CD, the exact causal mechanism is unknown to date. It is not understood very well, why only some patients with CD develop DH. In DH, IgA is present in skin and inflammatory cells, EMA and anti tTG ab occur in serum. Epidermal transglutaminase-3 (TG3) is an effective serological test for DH. Suspected individuals are advised to undergo for a skin biopsy to check the IgA uninvolved skin and could be considered for duodenal biopsy test, about 60-75% patients with DH show villous abnormalities, patients with normal endoscopy also show a minor change in the mucosa e.g. increased IELs. Diagnosis of DH is mostly dependent on serology and skin biopsy, a duodenal biopsy is not strictly advised to DH patients.[27] Once the diagnosis of DH is confirmed, a gluten elimination from the diet is recommended to the patients even they show a normal duodenal mucosa. A lifelong strict GFD heals the complications and it also helps reduce other associated intestinal conditions.[28]

Gluten Ataxia

Ataxia is a general term that defines the symptoms and signs resulting from cerebral dysfunction. Major symptoms of incoordination of limbs, gain instability slurred speech.[29] **Gluten ataxia (GA)** is an extra-intestinal, autoimmune disorder caused by the ingestion of gluten in genetically susceptible individuals and characterized by the damage to the cerebellum resulting in ataxia. GA is probably one of the commonest cause of idiopathic sporadic ataxia.[29,30]

So far, the exact prevalence of GA is not known. However, in the last two decades, multiple key studies have found a high prevalence of native anti-gliadin antibody (AGA) in GA patients. [31–33]

Elimination of gluten from the diet has shown a satisfactory improvement in GA symptoms.[29] About 95% of patients with GA have Human Leukocytic Antigen (HLA) -DQ2 or -DQ8 positive. However, 10%-15% of AGA prevalence has been reported in the general population. AGA test also remained a first line serological test for CD several years before. This evidence suggest that AGA is, however, not a specific serological test for GA. Nevertheless, in the last decades, significant deposition of transglutaminase antibodies has been found around the brain vessels (mainly in the cerebellum) of GA patients. Antibodies against tissue transglutaminase 6 (tTG6), a primarily brain-expressed transglutaminase, have been found in patients with GA.[34,35] At present, TG6 ab test in association with the AGA test is used as a serological test. However, in an interesting study, Hadjivassiliou and co-workers found that 73% of patients with idiopathic sporadic ataxia positive for AGA, were also positive for TG6 antibodies.[35] This observation suggested a correlation between AGA and TG6 positivity but does not clarify whether TG6 antibodies are more sensitive or specific for GA than AGA.[35,36]

For making a diagnosis for GA serological tests including AGA (IgG and IgA), anti-tTG2 antibodies and, if available, IgG and IgA anti-tTG6 antibodies are considered as a screening test. Considering the level of these abs, patients should undergo a duodenal biopsy. However, irrespective to the presence of an enteropathy, patients positive for any of these antibodies with no alternative cause for their ataxia should be offered a strict GFD with regular follow-up to ensure that the antibodies are eliminated, which

usually takes 6-12 months. Stabilization or even improvement of the ataxia after one year would be a strong indicator that the patient suffers from GA.[32,36]

Wheat Allergy

Wheat allergy (WA) is a specific wheat dependent allergic condition where ingested wheat causes an IgE mediated immediate allergy reaction in both adult and children. This involves urticaria, angioedema, bronchial obstruction, nausea, and abdominal pain. Depending on the route of the allergen and immunologic mechanism WA could be classified in multiple types e.g., occupational asthma (backer's asthma) and rhinitis, food allergy (FA) affecting the skin, the gastrointestinal tract or respiratory tract, wheat dependent exercise-induced anaphylaxis (WDEIA) and contact urticarial. According to the US Food and Drug Administration (FDA), the overall prevalence of WA (self-reported as well as clinically diagnosed) is 0.4%.[37] WA shows a greater prevalence in children than adults.[37,38] However, in adults most common condition is WDEIA where ingested food and physical exercise together display the most common symptoms e.g., diarrhea and bloating. Among multiples WA conditions, backer's asthma and rhinitis are the most ancient and well-recognized allergic response due to the inhalation of wheat flour.[39,40] About 10%-15% of Baker's and pastry factory workers are affected by this condition.[41] Several allergenic proteins have been recognized for WA. Recently, 21 well-classified wheat allergens listed in the updated database of WHO/IUIS allergen nomenclature.[42] Skin prick test (SPT), in-vitro specific Immunoglobulin E (sIgE) assay and functional tests (a bronchial challenge test in baker's asthma and a double-blind placebo-controlled food challenge or an open oral food challenge in FA) are considered as the first-line tests for the diagnosis of WA i.e. SPT and in-vitro sIgE assay. However,

these tests have lower sensitivity and their positive predictive value (PPV) is limited to 75%.[39] Reagents, used in commercially available SPE kits are not highly purified they are only a mixture of water and salt soluble wheat that lacks allergens from insoluble gliadin fraction and required a significant improvement in the kit performance.[39,40] On the other hand, sIgE assay is about 70-75% more sensitive that SPE test but is about 60% less specific that SPE test due to the cross-reactivity with pollens[43].

Molecular-based allergy (MA) diagnostics could be a promising method for the diagnosis of WA. In recent time, wheat flour extracts (omega-5 gliadin) based ImmunoCAP™ assay, alpha-amylase/trypsin inhibitors (ATIs) based ISAC™ assay is reasonably satisfactory markers.[40] However, with approximately 20% missed cases, sIgE to omega-5 gliadin assay is highly reliable and now widely used to identify the patients with WDEIA[44] Recently introduced flow cytometry-assisted basophil activation test (BAT) seems to be promising in vitro functional test for the diagnosis of immediate-type allergy. In clinical practice, the use of BAT is increasing. However, it is an expensive test to be used as in routine.[45,46]

Gluten sensitivity (GS)/Non Celiac Gluten Sensitivity (NCGS)

Gluten sensitivity (GS) or more simply **Non-Celiac Gluten Sensitivity (NCGS)** was introduced during the 1980s but strong attention was given in the last two decades when a large number of individuals erupted with NCGS.[47] So far, insufficient knowledge is poised about the specific diagnostic criteria, biomarker, treatment and management of NCGS. However, to date, four international scientific expert meetings have been called to make a consensus about NCGS. A panel of experts discussed the definition, diagnostic algorithm, advances, and current trends on NCGS. First, two expert meetings were organized in London, 2011 and Munich, 2012 respectively.[5,39] The third expert panel meeting was held in Salerno (Italy) 2014 to make criteria of how the diagnosis of NCGS should be confirmed, this is called **"The Salerno Expert's Criteria"**.[48] Latest meeting (4th) has been held in Bolzano (Italy) in 2018. According to Salerno criteria, "Non-Celiac Gluten Sensitivity is a non-autoimmune, non-allergic, intestinal and extra-intestinal symptoms due to the ingestion of gluten-containing food in subjects that are not affected by either CD or WA".[48]

Classical symptoms of NCGS include abdominal pain, bloating, bowel habit abnormalities (diarrhea/constipation) and systemic manifestations such as foggy mind, headache, fatigue, joint, and muscle pain. In NCGS, celiac-specific antibodies may remain absent, the villous structure remains normal with a variable status of HLA-DQ alleles (50% positivity) and variable status of native (first-generation) anti-gliadin antibody (AGA).[5]

Till date, the definite prevalence of NCGS is unknown but it is frequently reported. However, a study done in the USA estimate a high prevalence of NCGS i.e. 6%.[39] Until now, there is no specific biomarker designated for the NCGS, most specific biomarkers for CD (IgA anti tTG ab and EMA) do not provide a reliable result. Nevertheless, the IgG class of anti-native gliadin antibody (IgG AGA) test shows a somewhat satisfactory result and a recommended sero-test for suspected individuals.[31,5] The recommended for NCGS is the complete exclusion of gluten for the diet (i.e. GFD) and a gluten challenge. However, before advising a GFD, a suspected individual should undergo complete clinical and laboratory evaluation while on a normal diet (before GFD) to exclude the possibility of having CD or WA. After 6 weeks of normal diet (gluten-containing diet) the suspected individual is kept on 6 weeks of complete GFD. After this period, seven days of gluten challenge is given to the patient followed by a one-week washout period of strict GFD and by the crossover to the second one-week challenge. During all this duration patient's condition is monitored. Depending on the examination final diagnosis is made.[5]

Although so far, there is no certain biomarker for the diagnosis for NCGS, three gluten-related disorders (WA, CD, and NCGS) can be discriminated based on their combined clinical, biological, genetic and histological data following the algorithm given by Sapone et al, 2012 (**Figure 3).**[39]

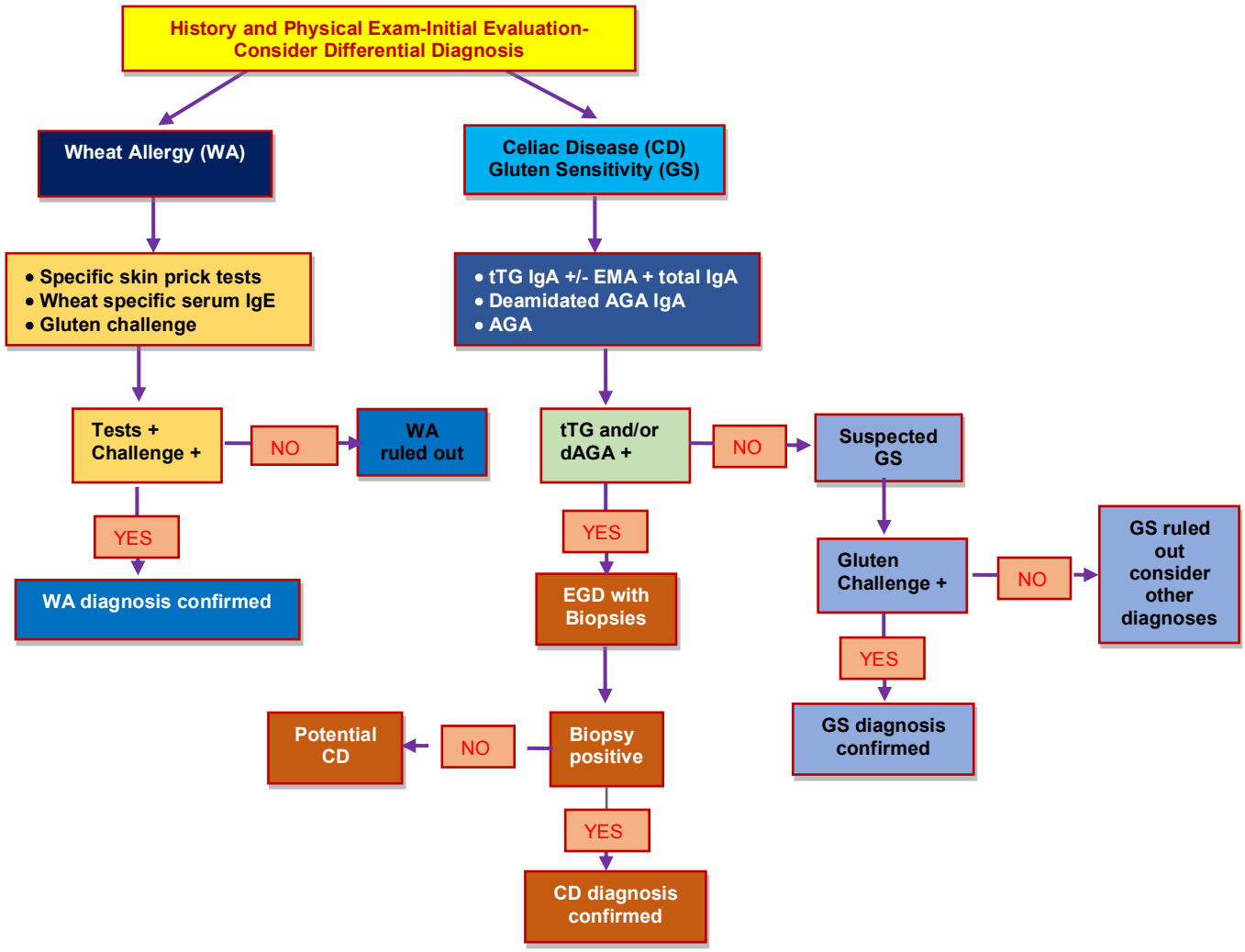

Figure 3: Proposed algorithm for the differential diagnosis of gluten-related disorders, including celiac disease, gluten sensitivity, and wheat allergy

Chapter 2

Celiac disease

Introduction

Among the wide range of adverse reactions caused by the gluten, CD is the longest-studied systemic, autoimmune disorder with the best-known pathology.

CD is a heritable, inflammatory condition of the small intestine, takes place due to ingestion of immune-dominant dietary peptide (gliadin in wheat, hordein in barley and secalin in rye) in the predisposed individuals who possess specific Human Leukocytic antigen (HLA) -DQ2 and/or HLA-DQ8 alleles. Ingestion of these peptides eventually causes villous atrophy, crypt hyperplasia, malabsorption, and extra-intestinal manifestations. Lifelong Gluten-free diet (GFD) is currently the only accepted therapy for CD that leads to normalization of the histological lesion.

Epidemiology

CD once thought to be an uncommon disease but now At least 1% of the world population is affected with CD.[49–52] While a large number of subjects are expected to have CD, the majority of them still remain undiagnosed.[53] In the year 1996, our group conducted one large CD screening study in Italian school children of 6-15 years of age reported prevalence of silent CD as 0.5%.[54] Pioneer CD population screening studies based on IgA native AGA as the first level test (conducted between 1992 and 2003) reported a prevalence of CD ranging from 0.25 to 1.13%, with an overall pooled prevalence rate of 0.5% **(Table 1)**. More recently, screening studies based on IgA anti-

tTG have described a prevalence rate ranging from 0.2 to 2.4%, with a pooled prevalence rate of 0.9% **(Table 1)**. These data suggest that CD prevalence has increased in recent years, a hypothesis that is also supported by longitudinal studies performed in North America and Europe.[55,56] Initially, it was exclusively thought as the disease of western countries but now it is considered to be relatively common throughout the world.[51,57–59] In the United States, the CD is believed to affect 0.5%-1.0% of the general population.[60] In Northern African populations (Morocco, Algeria, Tunisia, Libya, and Egypt) the prevalence of CD was 0.28%-5.6% in the general population.[61–63] In Australia and New Zealand, the overall prevalence is 1:82 (1.2%).[64] There is a large variation in prevalence of CD between countries, from the lowest record incidence of 0.2% in Germany to as high as 2-3% in Finland and Sweden.[51]

In Asian countries which shows both serological and biopsy prevalence of CD 1.6% and 0.5% respectively.[65] Among Asian countries, in India CD has been explored respectfully. Some elegant CD screening studies have been conducted in past years showed overall biopsy-proven CD prevalence in India is more or less 1% (I was among the authors in two of three studies).[65–67] Some possible reasons for increasing CD prevalence could be, due to advance diagnosis or increased awareness. Instead of the availability of several highly sensitive and specific non-invasive serological markers (e.g. anti tTG ab), intestinal biopsy is still considered as the gold standard for diagnosis of CD.[68]

Table 1: Summary of CD prevalence studies based on AGA and tTG as a first level screening tool in different countries.

Study	Place	Year	Population	Sero-marker	Prevalence: number (%)
Mazzetti et al[69]	Italy	1992	Children	AGA	12/3033 (0.39)
Catassi et al[57]	Italy	1994	children	AGA	11/3351 (0.32)
Catassi et al[54]	Italy	1996	Healthy children	AGA	82/17201 (0.47)
Grodzinsky et al[70]	Sweeden	1996	Blood donor	AGA	7/1866 (0.37)
Uibo et al[71]	Estonia	1996	Children	AGA	33/2895 (1.13)
Pittschieler et al [72]	Italy	1996	Healthy adult	AGA	29/4616 (0.62)
Montesanti et al [73]	Italy	1996	Children	AGA	4/1585 (0.25)
Ivarsson et al[74]	Sweden	1999	Adult	AGA	10/1894 (0.52)
Hovdenak et [75]	Norway	1999	Blood donor	AGA	8/2094 (0.38)
Rostami et al[76]	Netherland	1999	Blood Donor	AGA	3/1000 (0.3)
Riestras et al[77]	Spain	2000	Adult	AGA	3/1170 (0.25)
Volta et al[78]	Italy	2001	Adult/children	AGA	17/3483 (0.48)
Weile et al[79]	Denmark	2001	Adult	AGA	9/3439 (0.26)
Fasano et al [49]	USA	2003	Children	AGA	4/1281 (0.3)
				Total	**232/48897 (0.5%)**
Maki et al[50]	Finland	2003	Adolescent	tTG	37/3654 (1%)
Tommasini et al[80]	Italy	2004	Children	tTG	30/3188 (0.9)
Castano L[81]	Spain	2004	Children	tTG	7/830 (0.8%)

Menardo et al[82]	Italy	2006	Adult	tTG	10/1002 (1%)
Akbari et al [83]	Iran	2006	Adult	tTG	27/2799 (1%)
Vilppula et al[84]	Finland	2008	Adult	tTG	60/2815 (2.1%)
Mustalahti et al[51]	Finland	2010	Adult	tTG	113/4846 (2.4%)
	Germany				8/3038 (0.2%)
	Italy				32/4781(0.7%)
Marine et al[85]	Spain	2011	Children	tTG	11/780 (1.4%)
			Adult		10/3450 (0.3%)
Dalgic et al[86]	Turkey	2011	Children	tTG	95/20190 (0.5%)
Alarida et al [87]	Libya	2011	Children	tTG	19/2920 (0.7%)
				Total	**459/54273 (0.9%)**

Clinical features

CD is a lifelong disease (once a celiac, always a celiac) where individuals may present GI symptoms, extraintestinal symptoms, no sign or symptoms at all and nutritional deficiencies as well. The clinical manifestations of CD are classical (signs and symptoms of malabsorption such as diarrhea, steatorrhea, weight loss & growth failure) or non-classical and symptomatic (with evident GI and/or extra-intestinal symptoms) or asymptomatic.[88,89] There are several classical and non-classical symptoms of CD summarized in **Table 2.**

Table 2: Clinical features of celiac disease

Intestinal manifestation	
GI	Diarrhea
	Abdominal pain
	Bloating
	Constipation
Growth	Weight loss
	Short Stature
	Delayed puberty
Nutritional deficiency	Anemia
	Vitamin D deficiency
Extra-intestinal manifestation	
	Arthritis
	Dermatitis Herpetiformis
	Osteoporosis / Osteopenia
	Elevations in transaminases
	Infertility
	Dental enamel
Neurologic	Ataxia
	Epilepsy
Psychiatric	Anxiety
	Depression
Associated conditions	
	Type 1 diabetes
	Autoimmune thyroid disease
	IgA deficiency
	IgA neuropathy

Classification of CD

Significant improvement in the diagnostic methods in CD has changed the concept of understanding the presentation of CD. It is not mandatory that an individual suffering from CD will always preset the classical symptoms of CD (diarrhea, growth failure).[90] A

large number of adult CD individuals present atypical symptoms of CD. Based on the presentation, CD has been classified in the multiple forms that are described in **Table 3**

Table 3: Classification of celiac disease

Types	Features
Classical	Associated with features of intestinal malabsorption, gluten-induced villous atrophy and other classic histological features, the presence of GI symptoms (diarrhea, steatorrhea, abdominal distension, iron-deficiency anemia, and weight loss or growth failure). Children with classical CD present with chronic diarrhea, vomiting, abdominal distension and failure to thrive, muscle wasting and poor appetite and show signs of emotional distress and lethargy.
Non-classical	Associated with the atypical or extraintestinal manifestations, such as unexplained iron deficiency anemia, short stature, osteoporosis, arthritis, delayed puberty, infertility, peripheral neuropathy, hypertransaminasemia, dermatitis herpetiformis and dental enamel defects at the time of diagnosis.
Atypical	Patients generally have little or no GI symptoms but come to medication because of other reasons such as iron deficiency, osteoporosis, short stature, or infertility, generally, have fully developed gluten-induced villous atrophy. Since these patients are “asymptomatic” from the GI perspective, a large number remain undiagnosed.
Asymptomatic	Associated with no symptoms or commonly associated with CD, even on detailed questioning and presence of villous abnormalities, these patients are diagnosed only through the testing of populations enrolled in screening program or through family studies
Potential	It is characterized by the presence of CD-specific autoantibodies in the blood of patients without histological abnormalities in small intestinal biopsies or may develop intestinal damage later. These

	patients are at increased risk of developing CD as indicated by positive CD serology.
Silent	It is characterized by asymptomatic patients having gluten-induced villous atrophy. They are discovered after serologic screening or perhaps during endoscopy and biopsy for another reason. These patients are clinically silent or associated with atypical features of CD such as iron deficiency or osteoporosis.
Latent	It is associated with the previous diagnosis of CD that responded to a GFD and retains normal mucosal histology or manifests only an increase in IELs. It also represents patients with normal intestinal mucosa, on a gluten-containing diet who will subsequently develop CD.
Refractory	It represents patients with true CD (i.e. not a misdiagnosis) who do not or no longer respond to a GFD. Some of these patients develop complications such as ulcerative jejunoileitis or enteropathy-associated T-cell lymphoma.

Pathogenesis

The principal toxic component of wheat gluten belong to a family of closely related proline and glutamine-rich proteins called gliadins.[91] In-vitro and in-vivo studies (rats and human) have confirmed that CD is triggered by the presence of a 33-mer peptides (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) that develops due to the partial digestion of gliadins because the gliadin remain stable toward breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases.[15,39,40] This peptide was identified as the primary initiator of the inflammatory response to gluten in celiac sprue patients.[91] When an individual ingests gliadin in the form of wheat, is digested into

smaller peptides but because of the lack of the proly-endopeptidase among gastric, pancreatic and brush border enzyme relatively large gluten peptide that is rich in proline and glutamine remain after initial digestion.[92] High proline content renders these proteins resistant to complete proteolytic digestion in the human intestine. Proline-rich fragments of gluten that are resistant to processing by luminal enzymes survive digestion and transported across the mucosal epithelium as polypeptides **(Figure 4)**. Tissue transglutaminase-2 (tTG), a calcium-dependent autoantigen is a ubiquitous enzyme which is released in the intestinal mucosa during tissue injury, has a role in tissue repair and cross-linking proteins by forming iso-peptides bond between glutaminase and lysine residues.[93] tTG also has a high avidity from gluten peptides and under certain conditions (e.g. low pH) and in the absence of lysine residue, can deamidate glutamine, which converts neutral glutamine to negatively charged glutamic acid. These negatively charged glutamic acid residues manifest an increased binding affinity for the disease-relevant HLA-DQ2/-DQ8 molecules. Once bound to HLA-DQ2/8 molecule, the gluten peptides HLA-DQ complex can activate CD4 T-helper 1 (Th1) cells in the mucosa of the small intestine that recognize these complex.[94] The gluten-reactive CD4 T-cells produce interferon (IFN-γ) on activation. IFN-γ is also produced by T-cells in the epithelium. Interleukin (IL-15), produced by either mononuclear cells in the lamina propria or by enterocytes, stimulates T-cells to migrate to the epithelium and facilitate the killing of enterocytes by upregulated expression of mic by enterocytes and NKG2D by intraepithelial T-cells. IL-15 production is stimulated by gluten.[95,96] Gluten can also induce production of the intestinal peptide zonulin, which acts on tight junctions and increases epithelial permeability **Figure 4**.

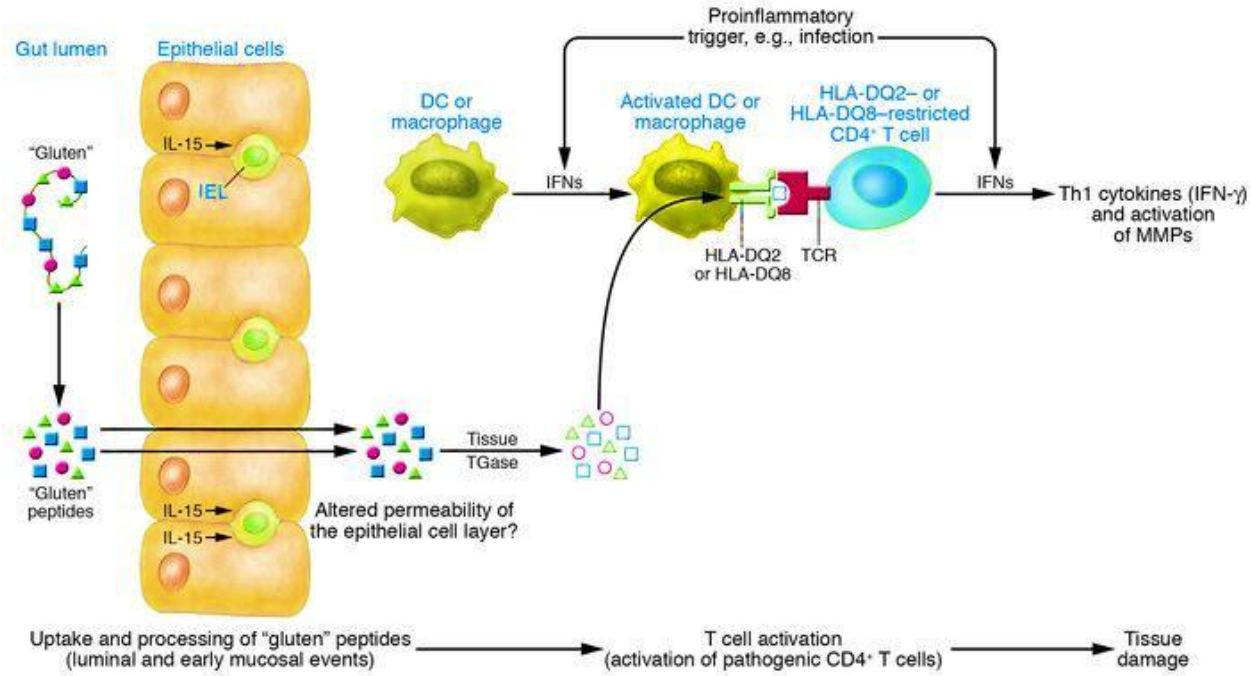

Figure 4: Pathogenesis of Celiac disease

Diagnosis and management of celiac disease

The diagnosis of CD is established by the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria (designed especially for pediatric subjects) is based on the combination of clinical manifestations, positive CD-specific serological tests and histopathological evaluation of duodenal biopsies. Following different tests are used for the CD diagnosis.

Serology: Serological tests are considered as the first line investigation for the screening of CD. Several antibodies such as anti tTG ab, EMA and DGP have been used to evaluate the patients susceptible to CD.[42] All these antibodies are based on immunoglobulin A (IgA) and G (IgG). IgA deficiency is common in CD, therefore IgG-

based tests are used for screening of IgA deficient patients to avoid the possibility of false negative results. Of all the serological tests, due to its high sensitivity and specificity, IgA anti-tTG ab is widely used sero marker for the screening of CD. The specificity of anti-tTG ab is >90% and the sensitivity is in the range of 90-96%.[97] Nowadays simple non-invasive serological markers have a major role in the diagnostic algorithm, particularly in children, due to their high sensitivity and specificity.[98]

HLA-DQ typing: HLA-DQ genes (HLA-DQ2/-DQ8) play a primary role in the development of CD. Clinically, HLA-DQ genotyping does not provide a final diagnosis of the CD but indicates the necessary predisposition required for the development of CD.[99,100] Due to its high negative predictive value (NPV), the absence of HLA-DQ predisposing alleles makes the diagnosis of the CD unlikely. Different combinations of HLA-DQ CD predisposing alleles also determines the level of risk of CD development. For instance, individuals presenting a double dose of the HLA-DQ B1*02 variant remain at high risk of developing CD in comparison to those expressing a single dose of the DQB1*02 allele.[99,100] A single HLA determination in such subjects may set them free from future surveillance and unnecessary follow-up in the clinic (repetitive serology, duodenal endoscopy/biopsy).[101] However, HLA typing is not sufficient for the diagnosis of CD because of its modest sensitivity (HLA-DQ2, 70%-99.8%; HLA-DQ8, 1.6%-38%) and specificity (HLA-DQ2, 69%-77%; HLA-DQ8, 77%-85%). HLA-DQ genotyping may be useful in the diagnosis of CD in those individuals who are having negative serology, but histological findings are suggestive of CD.[102,103]

Endoscopy: Endoscopic features such as scalloping of mucosal folds, loss of circular folds, flattening of mucosal folds, the mosaic pattern in mucosa and nodularity are

suggestive of villous atrophy in CD. However, these endoscopic features are neither sensitive nor specific enough for the diagnosis of CD as these endoscopic changes have been reported in other small bowel disorders such as tropical sprue, Crohn's disease, HIV enteropathy etc. Hence, endoscopy merely provides a means to obtain biopsies for histopathological evaluation.[104]

Histopathological analysis: Although the diagnosis of CD can be suspected on clinical or laboratory grounds, or because of serological tests. Histology of the proximal duodenum is still the gold standard for the diagnosis.[68] The histopathology of small intestinal biopsy is characterized by typical architectural abnormalities that include partial to total villous atrophy, crypt lengthening with an increase in the crypt to villi ratio, structural abnormalities in epithelial cells and increase in IELs. The histopathological changes of CD are classified using modified Marsh (mMarsh) classification (**Table 4)**.[105]

Table 4: mMarsh Classification of histological findings in CD (Oberhuber)

Marsh Type	IEL / 100 enterocytes – duodenum	Crypt hyperplasia	Villi
0	<30	Normal	Normal
1	>30	Normal	Normal
2	>30	Increased	Normal
3a	>30	Increased	Mild atrophy
3b	>30	Increased	Marked atrophy
3c	>30	Increased	Complete atrophy

According to the latest ESPGHAN criteria 2012.[106] The following guideline should be used while making the diagnosis of child/adolescent with symptoms of CD **(Figure 5)** and for asymptomatic individuals **(Figure 6)**

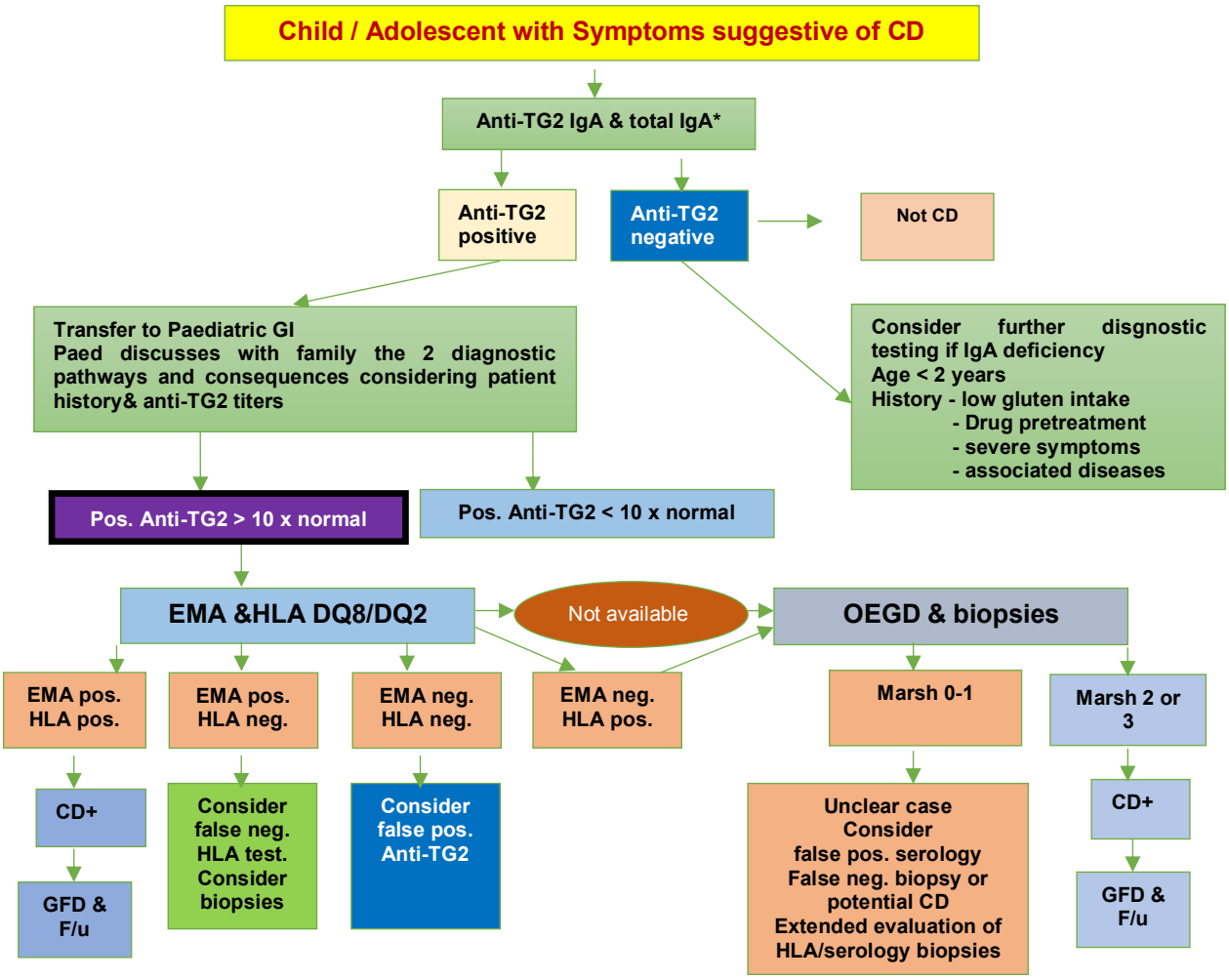

Figure 5: Symptomatic patient. CD=celiac disease; EMA=endomysial antibodies; F/u=follow-up; GFD=gluten-free diet; GI=gastroenterologist; HLA=human leukocyte antigen; IgA=immunoglobulinA; IgG=immunoglobulinG; OEGD=oesophagogastroduodenoscopy; TG2=tranglutaminase 2

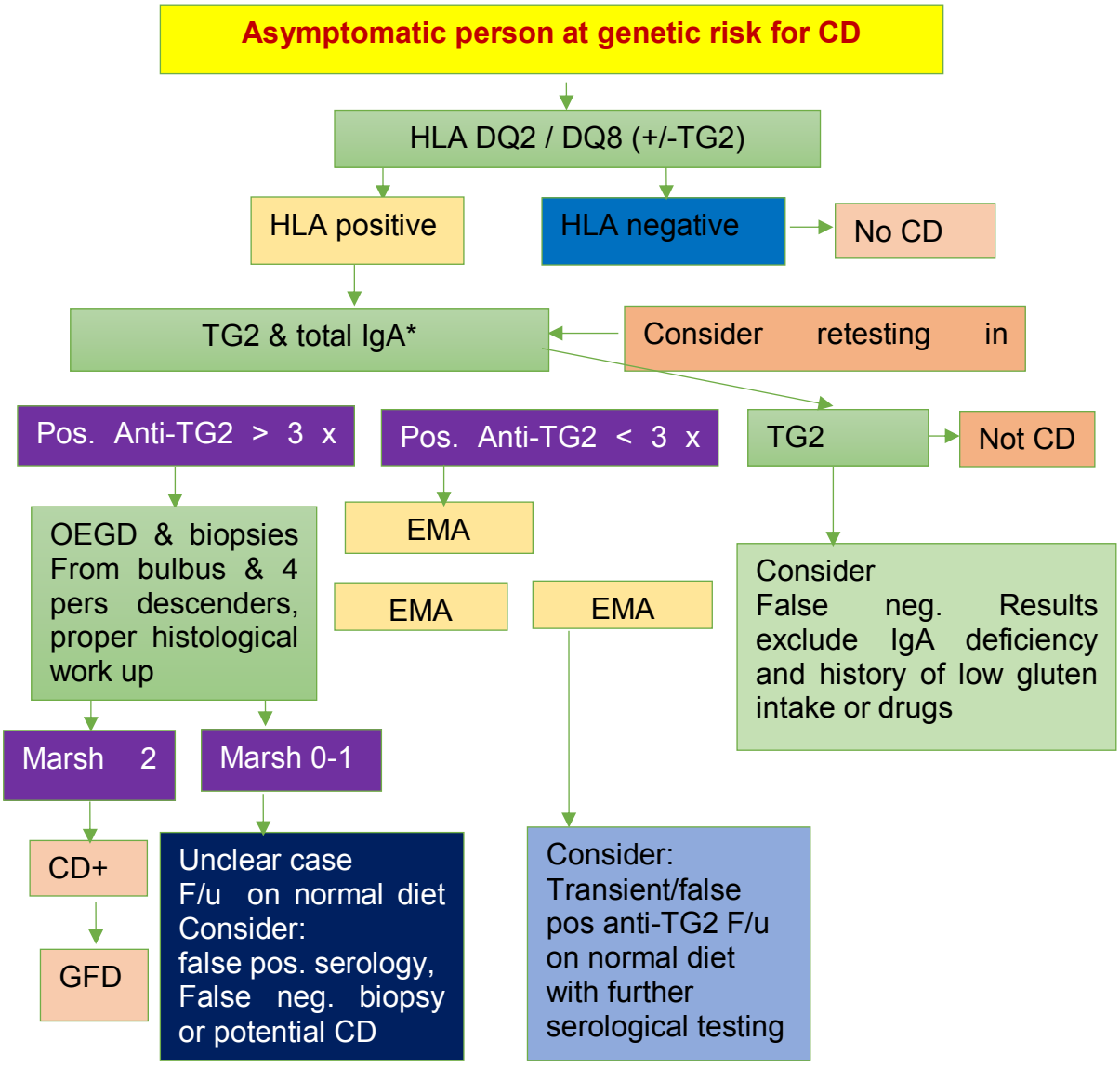

Figure 6: Diagnosis algorithm for asymptomatic patient

The latest ESPGHAN guideline precisely recommends avoiding the histological assessment in children and adolescents with signs or symptoms suggestive of CD with high anti tTG ab titers with levels >10 times Upper Limits of Normal (ULN), supported by a positive EMA test also with positive HLA-DQ2 and/or HLA-DQ8 alleles.[106]

Treatment

Currently, the only effective treatment for CD is a strict lifelong GFD which results in clinical, serological and histological improvement.[28] Unfortunately, the complete avoidance of gluten intake is extremely difficult due to hidden gluten contamination. CD patients are highly sensitive to the toxic effect of gluten. Even protracted ingestion of traces of gluten (10–50 mg daily) may damage the integrity of the small intestinal mucosa. By combining toxicity data with the observed food intake, it has been calculated that gluten-free products with <20 mg/kg (or parts per million = ppm) of gluten contamination are safe over a wide range of daily consumption. Codex Alimentarius regulation, also endorses that gluten-free food should contain < 20 ppm of gluten in total.[11] Effective therapy for CD is strict adherence to GFD which often restricts patient's social activities; affects the quality of life and causes nutritional deficiencies. Therefore, there is a need for alternative or adjunctive treatments of CD. Several treatment strategies have been under investigation. Some of these are enzyme therapy, inhibition of intestinal permeability, tTG inhibitors, HLA-DQ blockers and cytokine therapy.[107,108]

Chapter 3

History of CD diagnostic Biomarkers

Introduction of wheat in the human diet arose the possibility of development of syndromes including CD. Evidence of CD were present from 1st and 2nd centuries AD but the major breakthrough was contributed by Dr. Wim Dicke in 1950, in his Ph.D. thesis, showed that exclusion of wheat from the diet on children led to dramatic improvement in the symptoms of CD.[109] In the late 19th century, abnormalities of the lining of the small intestine in autopsy were described. Finally in 1954 abnormal mucosal lining was demonstrated by Paulley.[110] Early study of HLA (preliminary HLA B) suggested a relationship with CD considered as potential a genetic biomarker for CD. During '60s, the diagnosis of CD was based on clinical symptoms, stool characteristics, and the effects of a GFD upon symptoms and the histology of intestinal lesions. Circulating antibodies to gluten were reported in 1958 and till 1961 with other food components.[111,112] This finding was not highly specific for CD and was discoverable even in non-celiac individuals. In 1971, antibodies to reticulin was reported but due to its compromised specificity it was not accepted as a specific test for the CD.[113] During, the 1970s and the beginning of the 1990s, the discovery of autoantibodies such as reticulin and anti-endomysial antibodies in the serum of patients with CD laid the groundwork for today's understanding of CD.[114–116] As the understanding had been developed towards the pathogenesis of CD with the revelation of technology, several biomarkers were developed to diagnose CD. Most crucial biomarkers that were developed for this purpose are described below and detail of some leading biomarkers for the CD is given in **Table 5**.

1. **Native Anti-Gliadin Antibody (AGA):** In 1958, *Berger* described alcohol soluble native antigliadin antibody (AGA).[117] During 70's clinical use of AGA was started. IgA class of AGA was the first serological marker to be used for CD diagnosis and screening.[118] Although the test detected antibodies to gliadin both IgG and IgA class but the AGA test had shown a better accuracy through IgA class. During that time, AGA method was the only marker present for the diagnosis of CD.[119] However, these antibodies clearly lacked specificity. IgA AGA appeared to offer fair to good performance in children, with a sensitivity and specificity > 80% in most of them. In fact, the old ESPGHAN guidelines, in addition to the serological tests, required three biopsies for CD diagnosis.[120] Due to its less specificity AGA test was not accepted as a sole sero-test. However, during '90s some leading CD epidemiology studies were conducted AGA as the primary diagnostic marker.[52,55,65,66,69] Even though the guidelines have discouraged to use, however, this test had inappropriately, never been abandoned. It has been observed that IgG class AGA, but also IgA, are present in patients with autism.[121–125] Moreover, it has recently been observed that about half of patients suffering from NCGS do have high-IgG AGA and this remains, to date, the only laboratory marker to diagnose NCGS.[39]
2. **Anti Endomysial Antibody Test (EMA):** Discovery of anti-endomysium antibody test (EMA) has started a new era in the diagnosis of CD with remarkable specificity and sensitivity over 90%. IgA class of EMA test provided support of specificity with AGA, so, for several years, tests for AGA plus EMA were the laboratory approach suggested and most widely used. The endomysium is the perivascular connective tissue which lines smooth muscle bundles and takes up the silver stain. The target

antigen in endomysium is tissue transglutaminase-2.[126] Tissue transglutaminase is a ubiquitous calcium-dependent enzyme that crosslinks proteins. When it reacts with gliadin, neoepitopes are formed. It is thought that the immunological response to these neoepitopes may initiate the mucosal damage in CD. The commercially available tests for EMA detect IgA class auto-antibody directed against the endomysium in monkey esophagus by indirect immunofluorescence.[115] More recent work using human umbilical cord tissue as a substrate has shown improved sensitivity and correlation with villous atrophy and has overcome the ethical issue of using samples from endangered species.[127,128] The technique of indirect immunofluorescence for IgA EMA is both subjective and more labor intensive than the ELISA tests which are used for IgA and IgG AGA. However, it has been consistently demonstrated that EMA has superior sensitivity and specificity than assays for AGA and anti-reticulin antibodies. The IgA EMA test is as easy as an ELISA test. Nevertheless, it requires experienced pathology to read the EMA slide so, the prediction of EMA is observation dependent. To be 100% sure diagnosis with EMA test, a small bowel biopsy is necessary.[129] Nevertheless, First generation IgA AGA together with the combination of EMA, was a popular choice for the clinicians. During ’80-’90s, several CD population screening studies were performed in different countries using the native IgA-AGA test as the first line serological marker. [52,57,69,71]

3. **Anti-Tissue Transglutaminase antibody (anti tTG ab):** Following the discovery that calcium-dependent tTG is the major auto-antigen responsible for EMA positivity in the late ’90s by the group of Dieterich who identified the tTG or Type 2 transglutaminase (TG2) in the enzyme.[126] A simple ELISA anti-tTG test was

developed and this gradually replaced the AGA testing in first-line CD screening, due to higher sensitivity and specificity. Earlier, the commercial immunoassay kits were based on guinea pig or human extractive enzyme, based on this method in pediatric patients, the sensitivity of these tests was reported between 89% and 96% with a specificity higher than 92%.[130–133] The sensitivity in adults of the IgA anti-tTG assay, using human recombinant tTG, ranges between 95% to 100% and the specificity between 97% to 100%.[134,135] Later the commercial ELISA kits were developed with recombinant human enzymes. Subsequently showed a higher diagnostic accuracy and became almost a standard.[132,134] The sensitivity of IgA anti-tTG assays, using human recombinant tTG, in adults ranges between 95% to 100% and the specificity between 97% to 100%[134,135] with the highly reliable performance anti tTG ab was the most reliable test for CD so far. With the discovery of anti tTG ab further discovery of diagnostic tools for CD probably got a halt. This enzyme plays a significant biological role, catalyzing the bond between glutamine and lysine in different proteins. It is important in the processes of tissue repair and it is also involved in the removal of cell debris after cell death and apoptosis.[136] When excessive gluten penetrates the mucosa, an immune response with antibody formation is triggered. This response results in mucosal damage and the subsequent release and activation of transglutaminase. Gluten, being rich in glutamine, may also be the target of the enzyme, which can bind it to other proteins including transglutaminase itself. In the “Guidelines for the diagnosis and treatment of celiac disease” produced by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition and the latest ESPGHAN criteria, the IgA anti-

transglutaminase test is recommended for CD screening, rather than the EMA test.[106,137] During the last 20 years, many screening CD studies have been performed worldwide, using the IgA anti-tTG test as the first-line test.[49,50,66,80,82,90] A high concordance rate was reported between EMA and tTG test. These studies invariably reported higher CD prevalence compared to older AGA-based studies. However, it remains unclear whether this higher frequency of CD reflected a true increase in the prevalence of the disease or is related at least in part to the higher sensitivity of the IgA anti-tTG-based diagnostic algorithm.[106,138] It has already been emphasized that IgA anti-tTG autoantibody levels may reflect the degree of mucosal damage present,[139–142] although there are reports of high levels of IgA anti-tTG in patients without CD.[143] Different groups of researchers have attempted to determine whether high-IgA anti-tTG levels can justify avoiding biopsies, especially in pediatric patients.

4. **De-amidated gliadin peptides (DGP)/Second generation AGA:** The deamidated gliadin peptides (DGP) in patients with CD bind to the circulating antibodies with higher specificity than the native peptides (AGA),[144] and in particular to residues containing the PEQ tripeptide, as the core epitope in celiac patients.[145] Later it was found that this DGP ab had a higher diagnostic accuracy than the AGA test, both in terms of sensitivity and of specificity.[146] Subsequent studies performed with commercial kits confirmed the accuracy of the data reported in early studies. Data on the sensitivity of IgA antibodies (DGP-A) in adults ranged from 83.6% to 98.3% with specificity between 90.3% to 99.1%.[147–149] The sensitivity observed for the IgG class (DGP-G) was between 84.4% and 96.7% with a specificity of 98.5% to 100%. These

early studies clearly highlighted the high specificity of the anti-DGP-G, considerably better than the classic IgG AGA. A study showed that the anti- DGP, particularly of the IgG class, maintaining a high specificity, were positive in the majority of patients negative for anti-tTG, both children and patients with selective IgA deficiency.[150] These data were confirmed in further studies.[151–153] Considering the results of published studies, it is indisputable that the anti-DGP, especially of the IgG class, have a diagnostic performance higher than AGA and add value in the diagnosis of CD. Nevertheless, there is still not widespread agreement on their use. Some authors affirm that anti-DGP should be used mainly in children under 2 years of age,[154] whereas other authors regard them as a marker of diagnostic accuracy comparable to, or slightly lower than, IgA anti-tTG.[92,150,153,155–158]

Table 5: Major diagnostic biomarker for CD their sensitivity, specificity, pros, and cons

S. No.	Name	Sensitivity (range)	Specificity (range)	Pros	Cons	Use
1.	**EMA IgA**	>90% (77.9–100)	98% (90–100)	The most specific test.	• More time consuming to perform, • More expensive. • Observer dependent	To confirm tTG IgA positive patients
2.	**Anti-tTG IgA**	>95% (67–100)	>95% (92–100)	The most sensitive test Recommended for first level screening test	Lack of standardization	Best test for the initial screening of the patients
3.	**Anti-tTG IgG**	>70% (54.7–100)	>90% (80–100)	Often positive in IgA deficient patients	The variable diagnostic accuracy of commercial kits	Useful in IgA deficient patients
4.	**Anti-DPG IgG**	>90% (80.1–98.6)	>90% (90.3–100)	Often positive in antitTG IgA negative children	Less accurate with respect to anti-tTG IgA	Recommended in children and in IgA deficient patients
5.	**Anti-DPG IgA**	>90% (80.7–98.3)	>90% (86.3–99.1)	Often positive in children	Less accurate with respect to anti-tTG IgA and anti-DPG IgG	Useful in children
6.	**Anti-actin IgA**	>50% (25.7–80)	>85% (85–100)	Highly correlated to mucosal atrophy	Lack of specificity, the IFA method is highly observer and substrate dependent	Useful to evaluate mucosal injury

5. **Microbial transglutaminase (mTg):** mTg is one of the enzymes that deamidates/transamidates proteins and is capable of cross-linking numerous molecules, thereby revolutionizing the quality of food products. In fact, it is used as a major industrial glue, connecting proteins to improve products' qualities such as gelation, solubility, foaming, viscosity, water-holding, emulsion stability, texture, shelf life, etc.[159,160] It belongs to a large family of Tgs with multifunctional related proteins, widely distributed in all living organisms. The predominant and classic function of these enzymes is as protein cross-linkers; however, as more is discovered about their biology, additional roles complicate our understanding of their function in human biology and diseases. There is a rapidly expanding literature describing dysregulation of these enzymes in multiple diseases and how this contributes to the pathogenesis of human diseases. Tissue fibrosis, apoptosis, cancer and metastasis, CD, neurodegenerative disorders such as Parkinson's disease and skin diseases are just a few examples of where Tgs have been implicated .[161]

Human tissue transglutaminase (tTg) is the autoantigen and anti-tTg antibodies are the corresponding specific serological markers in CD.[161,162] Both enzymes, the tTg and mTg de/transamidated gluten, known until now to be the main nutritional environmental factor inducing CD. Previously, it was hypothesized that mTg is a new environmental enhancer of CD based on multiple observations and scientific data, but direct proofs for immunogenicity of the enzyme or its complexes in celiac patients is lacking. [163]

mTg-mediated processes can potentially open the inter-enterocyte tight junction, allowing more immunogenic foreign molecules to induce CD.[163] In order to further investigate the role of the mTg in relation to CD induction, the two enzymes were

aligned for sequence similarity and the immune reactivity of the enzyme complexed to gliadin was investigated in CD patients sera compared to controls. The current hypothesis is that if the ingested mTg has a deleterious effect on celiac patients, it must be absorbed and get in contact and stimulate the local and/or the systemic immune system, resulting in the production of specific antibodies. mTg is immunogenic in children with CD and, by complexing to gliadin, its immunogenicity is enhanced. Anti mTg- neo-epitope IgG antibodies correlate with intestinal damage to a comparable degree as anti-tTg-neo IgA. mTg and tTg display a comparable immunopotent epitope.

mTg-neo IgG could be a new marker for CD. This marker is under its validation phase and further studies are needed to explore the pathogenic potential of anti-mTg antibodies in CD.

6. **Synthetic neoepitopes tTG-DGP complex:** Recently a synthetic neo-epitopes of the Transglutaminase-Deamidated Gliadin Complex is claimed as a possible novel biomarker for diagnosing and monitoring of CD.[164] This antibody is derived from tTG-DGP complex and to determine if immune reactivity against these epitopes can identify patients of CD with mucosal healing. So far, very less known about this marker that reveals that the average antibody-binding intensity is significantly higher in serum from patients with treated but unhealed CD mucosa than in patients with treated and healed CD mucosa. The assay identified patients with mucosa healing status with 84% sensitivity and 95% specificity. The tTG-DGP complex could be a reliable biomarker to measure the immune response to epitopes accurately identified patients with CD, as well as patients with mucosal healing. Further studies for this marker is needed.[164]

Chapter 4

The need for novel CD biomarkers for adherence to GFD

The only accepted treatment for CD and other GRD is strict adherence to a gluten-free diet. In this respect, it is noted that a diet with zero gluten intake is impossible due to its ubiquity; thus, a minimal level of gluten contamination is present in the daily diet. In fact, total daily gluten consumption that could be critical for most CD patients is of <50 mg gluten, and some patients need as little as 10 mg of daily gluten to promote the development of intestinal mucosal abnormalities.[165] Although a duodenal biopsy can get a definite diagnosis but as it is an invasive process and repeat biopsy is not feasible especially in children, therefore, there is a need for accurate, non-invasive tools for managing patients to show gluten intake and avoid the harmful aftermaths. However, requirements of adherence to GFD vary in each GRD, while a strict and lifelong GFD is the only suggestion for CD, NCGS requires repeatedly gluten challenges. The protracted ingestion of traces of gluten (10–50 mg on a daily basis) may damage the integrity of the small intestinal mucosa.[165] There is no effective biomarker available so far for the specific diagnosis of GRD. However, for CD there are some sero-markers available, most reliable and first line test is tTG.[138] Unfortunately, these serological tests are limited to the diagnosis purpose and not at all suitable to test the occasional exposure of gluten. There are no reliable biomarkers for the compliance. None of the diagnostic tests is suitable to assess the adherence to GFD. So far, there is no clear guideline for exploring the adherence to GFD along with his so far, there is no consensus for suitable measurements for evaluating the compliance and outcome.[17] However, A variety of surrogate markers are available to assess the GFD compliance

including clinical assessment of symptoms, patient self-report about the level of adherence, dietary history, an evaluation carried out by a professional nutritionist, small-bowel biopsy, or serologic screening tests. Nevertheless, the lack of standardized and accurate indicators of GFD adherence is a significant problem both in the clinic and in research. Detection of Gluten Immunogenic Peptides (GIP) monitoring GFD compliance only evaluate the consequences of GFD transgressions. Moreover, they are unable to detect occasional gluten exposure that may impede total gut mucosa recovery in the celiac patient.[14]

Clinical response could not be a single method for monitoring adherence to the GFD as a large number of celiac patients are asymptomatic or minimally symptomatic at presentation and in these cases, it would not be feasible to use clinical response as an indicator of mucosal healing and GFD compliance.[14] A controlled study examining the effects of gluten challenge found that symptoms were absent in 22% of celiac patients, despite the presence of significant villous atrophy in the small bowel biopsy.[166]

Till some extent different compliance questionnaires evaluating food frequency and self-reported GFD adherence monitor the adherence status, however, these data are visual analog score scale which consists of observation that one cannot fully rely on this data.[167] Nonetheless, no quality control or standard is available for dietetic review due to local diets and habits targeting a specific structured interview related to the quality of the diet. So, there is an unmet need for a reliable non-invasive biomarker for adherence to GFD.

Chapter 5

Development of my Ph.D.: A journey from Master's to Doctorate

The journey of my Ph.D. has started with my Ph.D. enrollment for the **XXXI cycle Ph.D. course** in **'Università Politecnica delle Marche', Ancona, Italy** in November 2015, In the supervision of one of the world's best pediatrician and Father of celiac epidemiology studies **"Professor Carlo Catassi."** (Ctrl+ click to see his detail)

Since the beginning of my Ph.D. course, I focused on finding a new biomarker for the CD and other gluten-related disorders. As I have discussed **(chapter 4)**, currently there is an unmet need of reliable **Non-invasive biomarker for diagnosis and adherence to GFD**. In this Ph.D. thesis, I have investigated the efficacy of various potential biomarker **(Table 6)**. Efficacy of each biomarker was explored through an individual dedicated project. Almost all projects have been ended so far. However, some studies are in its ongoing phase, I am still working hard to finalize them. Details of each project are discussed in later sections.

My very first exposure was a CD screening study in Italian general pediatric population **(Project 7)** where I have learned the basics of conducting a screening study. Professor Catassi and his group is the most reputed research group for conducting celiac epidemiology studies. Multiple leading celiac epidemiology studies have been conducted either by his group or by his directions from different research groups. During this CD screening study, I have read about the epidemiology of CD and its world status. Newly introduced rapid HLA-DQ typing method was used as a first-level screening tool for this study **(see detail in Project 5).** Thousands of HLA-DQ typing tests were

planned to perform during this screening study. As I was a part of the study, I had to perform the HLA-DQ test and to learn this test, during last weeks of January 2016, Prof. Catassi has organized my visit to **Biodiagene S.R.L.** (the principal manufacturing company), **Palermo, Italy** to learn rapid HLA-DQ typing test. During this time, I have performed the HLA-DQ typing test my own and learn about other variants of the HLA-DQ typing test.

This HLA DQ typing test was indeed a unique test with an affordable price. Professor Catassi with the developers of the HLA-DQ typing method wanted to spread this affordable and reliable test to other scientific groups where HLA-DQ typing do not perform due to the scarcity of fund. Especially in Asian countries where sometimes fund is limited and always an issue to perform this costly test. Later during 2017, our research team has designed one study to investigate its validity in Indian CD and non-CD subjects **(Project 5).** Later, I have been awarded **First Prize for Best Oral Presentation category** during a plenary session of **Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrics (SIGENP),** meeting Rome, 2017 and later in 2018 the study has been published in Journal of gastroenterology and hepatology Open (JGH Open).

In the next phase, we have analyzed the true HLA–DQ prevalence in a less wheat eater cohort of the pure south Indian population. In this collaborative project, blood samples have been imported from South India and HLA-DQ typing was conducted through an improved version of BioDiagene quick HLA-DQ typing method. I have visited Palermo (Italy) once again to conduct the HLA characterization in late October 2018 **(Project 6).**

To learn more about CD biomarker techniques, In the month of February 2016, I have been sent to learn **anti Endomysial antibodies test (EMA)** test in the supervision of **Dr. Elio Tonutti (late), at DPT Immunologia e Allergologia Diagnostica Responsabile, Ospedale Santa Maria della Misericordia, Udine, Italy.** On the basis of this exposure, in 2018 we have established Biopsy based EMA test in our laboratory **(Project 9)**.

I have been assigned my first sole project which was based on investigating gluten content level in naturally gluten-free and food products labeled as “gluten-free” in an Italian supermarket. From April 2016 to October 2016, I have actively worked for this study **(Project 3)**. The result of this study was discussed in the form of oral presentation in **SIGENP meeting, Milan (2016)** and some other meetings at different Universities. This work was published in the journal “**Nutrients” (Impact factor 4.2).** I was the first as well as the corresponding author of the study.

For an even deep exposure of CD and other gluten-related disorders and to know and learn advanced diagnostic techniques. Professor Catassi has encouraged me to visit the state-of-art laboratory of “**Professor Alessio Fasano” (**Professor of Pediatric Gastroenterology and Nutrition and Director of the center for celiac research and mucosal immunology and biological research center, Massachusetts, USA). For my financial support, I have applied and won the **“Young researcher award” by SIGENP.** From October 2016 to April 2017, I have been in Boston (USA) and learned advanced techniques currently being applied in CD **(See International Exposure and appendix 1).**

From June 2017 to September 2017, I did another study that was based on a concept to check the sensitivity of the Anti-Gliadin IgA (AGA IgA) 'first generation AGA test' over the well-established tissue transglutaminase IgA (anti tTG IgA ab) to evaluate the rate of **"potentially missed CD cases"** by screening studies based on IgA AGA test in order to compare prevalence rates from current studies **(Project 8)**. I have discussed the result of this study in various scientific meetings. This study had been selected to present its result in the form of '**Poster of distinction'** in the **51st ESPGHAN meeting Switzerland.** The study was published, me as a first and corresponding author, in **"Clinical Gastroenterology and Hepatology" (Impact factor 7.8).**

During February 2018, our group was interested in investigating the gluten contamination in non-food products i.e. cosmetics and oral hygiene product. I have conducted this study during February to September 2018 **(Project 4)**. I have discussed study results in different scientific meetings and this work was published in **"Journal of Pediatric Gastroenterology and Hepatology"** (Impact factor 2.9), me as fist as well as a corresponding author.

I felt honored being a part of the 4th expert meeting on Gluten-related disorders, held in Bolzano (Italy) in November 2018. I have presented some of my work during this meeting.

Project 1 is a sincere approach to find some pure biomarker for the adherence of CD and **Project 2** will allow calculating the quantity of per day gluten that a CD patient ingest unintentionally. Simultaneously, I was contributing in two of collaborative works detail of the work has been mentioned in **Project 10 and project 11.**

During this period of my Ph.D. course (2015-2018), I tried my best to work with sincerity.

I consider myself very fortunate working with Professor Carlo Catassi.

Table 6: List of potential non-invasive biomarkers evaluated during Ph.D.

[LFT; Lateral flow test, SSP-PCR; sequence-specific PCR, IF; Indirect immunofluorescence assay]

S. No	Name of biomarker	Purpose	Mode
Biomarkers to assess adherence to GFD			
1.	Alkylresorcinol	Adherence to GFD	LC-MS/MS
2.	gluten immunogenic peptides (GIP urine test)	Adherence to GFD	LFT
3.	Intestinal Fatty acid binding proteins	Adherence to GFD	ELISA
Biomarkers to detect gluten level In food (Non-blood)			
1.	R-5 antibody	Quantitative analysis of gluten in food	ELISA
2.	G-12/R-5 ELISA	Per day gluten ingestion calculation in follow up pattern's	ELISA
Genetic biomarker			
1.	Rapid HLA DQ typing method	Diagnosis of GRD	SSP-PCR
CD diagnostic biomarker			
1.	Endomysial antibody biopsy (EMA biopsy)	Diagnosis	IFA

Project 1

Biomarkers for the adherence to Gluten-Free Diet

Introduction

To find a biomarker for the adherence to GFD, the efficiency of three potential biomarkers (AR, GIP, and i-FABP) were evaluated in this research study. Detail of these biomarkers is summarized below.

1. Alkylresorcinol (AR): A biomarker of whole grain

Whole grain is a rich source of dietary fiber and several bioactive components, including minerals, vitamins, tocols, phytosterols, lignans, and cinnamic acids. Together, these are sometimes referred to as the dietary fiber complex, which has been suggested to account for the positive health effects.[168] It was suggested that non-refined foods, such as whole grains, fruits, and vegetables, which provide dietary fiber along with other constituents, have a protective effect against 'Western' diseases such as coronary heart disease and colon cancer.[169] Alkylresorcinols (AR), phenolic lipids present exclusively in the outer parts of wheat and rye grains, have been suggested as biomarkers of whole grain wheat and rye intake.[170] Alkylresorcinols (1,3-dihydroxy-alkylbenzene derivatives) are non-isoprenoid phenolic lipids present in several families of higher plants, algae, mosses, fungi, and bacteria.[170] There is a large structural diversity, which is dependent on the source. In wheat, rye and barley grains, AR forms one of the major groups of phenolic compounds.[171] The most common AR compounds in cereal grains are the 5-n-alkyl-derivatives with odd alkyl chain length commonly in the range of 17-25 carbon atoms, but different derivatives including 5-alkenyl-, 5-oxoalky-, and 5-

hydroxyalkylresorcinols are also present to a minor extent. The highest proportion of unsaturated derivatives is found in rye, which contains about 20% of AR compounds other than 5-n-alkyl-derivatives (mainly 5-alkenylresorcinol).[171] Among plants commonly used for human consumption, AR is present in high amounts in rye (Secale cereal), common wheat (Triticum aestivum), einkorn wheat (Triticum monococcum), emmer wheat (Triticum dicoccon), spelt wheat (Triticum spelta) and durum wheat (Triticum durum). [172]

Alkylresorcinols are present in the outer layers of these grains, they are used as biomarkers of whole grain intake in epidemiological and intervention studies.[173] Small amounts of alkylresorcinols are found in the refined flour of wheat,[174] and the plasma of people who eat only refined wheat still contain measurable amounts of alkylresorcinols. Because alkylresorcinols are mostly present in gluten-containing grains and show a unique fingerprint of each grain, alkylresorcinols could be markers of gluten intake in individuals on a GFD. [173]

A biomarker of gluten intake has great potential for improving understanding of the effectiveness of GFDs in preventing the symptoms of CD and in ensuring adherence to GFDs during testing of other therapies for CD. To determine whether AR could function as a biomarker of gluten exposure, we are in process of measuring plasma concentrations of alkylresorcinols in patients with treated CD, patients with untreated CD, and healthy controls through LC-MS/MS.

2. Gluten Immunogenic Peptide (GIP) Urine:

Complete avoidance of gluten from food is impossible. There is a chance for occasional ingestion of gluten in the diet. Unfortunately, all the available makers (tTG, AGA, DGP) are restricted to evaluate the consequence of gluten exposure. These biomarkers are unable to detect the occasional gluten exposure that may impede total gut mucosa recovery in the CD individuals.[14] Therefore, there is a need for accurate, non-invasive tools for managing patients to show gluten intake and avoid the harmful aftermaths.

CD is triggered by the certain GIPs are resistant to gastrointestinal digestion and can interact with the immune system of patients with CD to trigger an autoimmune response against tTG and other antigens. *Shan et al. (2002)* showed by in vitro and in vivo studies in rats and humans that a 33-mer peptide from 2-gliadin is stable toward breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases. This peptide was identified as the primary initiator of the inflammatory response to gluten in patients with CD.[91] Toward the assessment of toxicity and GIP in foods for celiac patients, G12 and A1 monoclonal antibodies (mAbs) were obtained against 33-mer peptides. The reactivity of these antibodies was correlated with the potential immunotoxicity of the proteins analyzed and they proved to be useful in studies about the enzymatic detoxification of gluten.[175,176] These antibodies displayed great sensitivity to toxic peptides (besides the 33-mer peptide) from wheat, rye, barley, and varieties of oats.[175,176] To monitoring the occasional intake of gluten by determining GIP in human samples. We have checked the efficacy of G-12 antibody-based quick GIP-urine test in the urine samples of CD follow-up

3. Intestinal Fatty Acid Binding Protein (i-FABP)

Fatty acid-binding proteins are small (14-15 kDa) cytoplasmic proteins involved in the intracellular buffering, cholesterol and phospholipid metabolism, transport of long-chain fatty acids and maintenance of lipid homeostasis.[177] FABP is first discovered in 1972 however, till date nine members have been identified from different organ where they are involved in active lipid metabolism.[178] FABPs are divided into two main groups – i) those associated with plasma membrane (FABP PM) and ii) those associated with intracellular or cytoplasmic proteins (FABP C).[177]All types of FABPs are named on the basis of the tissue in which they were identified first i.e. liver (L-), intestinal (I-), heart (H-), adipocyte (A-), epidermal (E-), ileal (Il-), brain (B-), myelin (M-) and testis (T-) FABPs **(Table 7).** The expression of FABPs in each cell type reflect its lipid-metabolizing capacity. FABP content in most of the cells is generally proportional to the rates of fatty-acid metabolism.[179] All FABPs can bind with long-chain fatty acids with differences in ligand selectivity, binding affinity and binding mechanism due to small structural differences between isoforms.[180]

From the nine types of FABPs discovered, intestinal type (i-FABP) is specifically expressed in the intestine. They are encoded by FABP2 gene present on chromosome 4.[181] However, no FABP is exclusively specific for a given tissue or cell type, and most tissues express more than one isoform of FABP. The small intestine has three distinct proteins belonging to the intracellular lipid-binding protein family: the liver-type FABP (L-FABP), the intestinal FABP (I-FABP) and the ileal lipid-binding protein (ILBP), although they are distributed in different segments.[182]L-FABP is mainly expressed in the proximal part, whereas i-FABP is restricted to the distal portion of the small intestine. i-FABP is

expressed throughout the intestine, but most predominantly in the jejunum, and is in greater abundance in villous cells than in crypt cells. It is tough to identify the individual contributions of these three FABPs to lipid absorption perhaps more work is needed to detect exact contribution. It has been reported that polymorphism in i-FABP, an alanine to threonine substitution at codon 54 (Thr54), was associated with insulin resistance and decreased lipid oxidation in Pima Indians, a population with an extremely high prevalence of obesity and type 2 diabetes.[183]

Table 7: Types and characteristics of Human FABPs

S. No.	Gene	Common name	Localization	Chromosomal location	No. of amino acids
1	FABP1	Liver FABP (L)	Liver, intestine, pancreas, kidney, lung, stomach	2q11	127
2	FABP2	Intestinal FABP (I)	Intestine, liver	4q28-q31	132
3	FABP3	Heart FABP (H)	Cardiac & skeletal muscle, brain, kidney, lung, stomach, Testis, placenta, ovary	1p33-p31	133
4	FABP4	Adipocyte FABP (A)	Adipocytes, macrophages,dendritic cells	8q21	132
5	FABP5	Epidermal FABP (E)	Skin, tongue, adipocyte, macrophage, dendritic cells, mammary gland, brain, stomach, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen, placenta	8q21,13	135
6	FABP6	Ileal FABP (Il)	Ileum, ovary, adrenal gland, stomach	5q23-q35	128
7	FABP7	Brain FABP (B)	Brain, central nervous system (CNS), glial cell, retina, mammary gland	6q22-q23	132
8	FABP8	Myelin FABP (M)	Peripheral nervous system, Schwann cells	8q21.3-q22.1	132
9	FABP9	Testis FABP (T)	Testis, salivary gland,mammary gland	8q21,13	132

Methodology:

Sample selection and processing

For the conduct of the study total of 170 whole blood and urine samples proposed to be collected from the **pediatric gastroenterology division, Salesi hospital Ancona (Italy)** as well as **adult gastroenterology division, Torette hospital Ancona (Italy)**. From the total 170 samples, 90 samples proposed to be collected from the CD subjects already on GFD (45 samples of 0-2 months from the start of GFD and 45 samples of 3 months from the start of GFD), 30 samples to be collected from the CD new diagnosis patients and 50 samples from the healthy volunteers. Blood samples were processed for the respective tests as given below.

1. **Alkylresorcinol:** AR analysis is being done by LC-MS/MS technique, for this 3 ml of whole blood was collected into EDTA vial and the tube was centrifuged at 3000rpm x 10 minutes to separate the plasma. The plasma samples were stored at -80°C labeled with patients details until the use.
2. **GIP-urine test:** GIP urine test is being done by IVYCHECKJ GlutenDetect urine test kits, (Biomedal SRL, Spain). From each patient 5 mL urine sample was collected, the GIP test was performed on the same day and the rest urine sample was stored at -80°C.
3. **i-FABP:** i-FABP analysis is being by i-FABP ELISA by the commercial ELISA kits procured by Hycult Biotech, The Netherlands. For this 3 ml of whole blood was collected into heparin vial and the tube was centrifuged at 3000rpm x 10 minutes to

separate the heparinized plasma. The plasma samples were stored at -80°C labeled with patients details until the use.

Laboratory instrumentation:

1. Quantification of Plasma Alkylresorcinol through LC-MS/MS

Determination of AR is planned according to the protocol developed by Ross et al[184] where heptane was used as a solvent. This work is recently started and we are standardizing the LC-MS/MS. In the coming months, we will have some result to show.

2. Gluten Immunogenic peptide-GIP-urine test

Qualitative test

The resistance of gluten peptides to gastrointestinal digestion, particularly the immunogenic 33-mer peptide ensures that a significant portion of the ingested gluten peptides is excreted via faeces and urine. Thus, the recovery of measurable amounts of GIP from urine samples indicates recent gluten consumption and therefore constitutes an accurate and very useful method for short and long-term GFD monitoring.

To measure the occasional gluten exposure efficacy of newly developed GlutenDetect Urine Test was investigated. GlutenDetect Urine Test is a quick immunochromatographic test that allows the detection of GIPs from gastrointestinal degradation of ingested gluten in urine.

Test procedure:

Using a sterile syringe, 2 ml fresh urine sample, collected from the patient, was mixed with a conditioning solution that prepared it for the detection step **(Figure 7.1-8.2)**. This solution was shacked well for 5-10 seconds **(Figure 7.3)**. Four drops of this solution were poured on the GIP urine kit (cassette) at sample zone (S) and the cassette was incubated on room temperature for 15 minutes **(Figure 7..4)**. The detection step was based on the reaction of the 33-mer-like immunogenic peptides of gluten present in the sample with the colored conjugates. The peptide-conjugate complexes spread through the strip by capillarity.

After the incubation, the result was interpreted by the naked eye, on the basis of color change. In the presence of GIP, the result was positive and a RED line appeared in the Test Zone (T) of the cassette. The absence of the RED line indicated a negative result **(Figure 7.5).** Whether or not GIP is present in the sample, a second colored conjugate moved through the stick up to the Control Zone (C) of the cassette, where antibodies have been immobilized, resulting in a visible GREEN (control line). This result confirmed that the test had functioned properly. If the Green line did not appear, regardless of the Redline, the result of the test was considered invalid. Limit of detection (LoD) of this procedure is 2.2ng/ml of urine. The sensitivity of the test is up to 95% for the detection of 0.5 g of gluten.

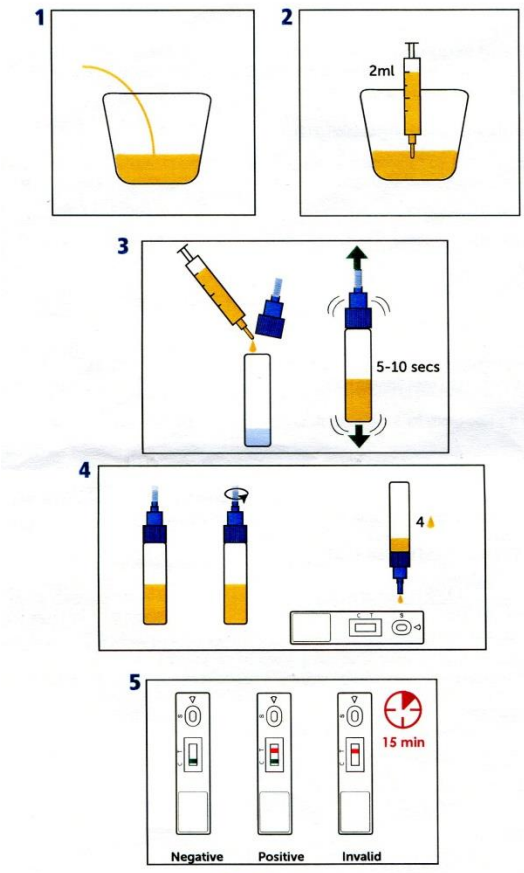

Figure 1. Test Procedure

5

Figure 7: GlutenDetect test procedure

Quantitative test

The Blomedal IVYCHECKJ GlutenDetect urine test was indeed a qualitative test. This test was perfect to know if there was any current gluten exposure in a patient. But the same was not useful to know the quantity of the gluten ingested. This qualitative test was not fulfilling our target precisely. To achieve our goal, we replaced the IVYCHECKJ GlutenDetect urine test (qualitative test) to its advanced and upgraded version i.e. Biomedal iVYCHECK GIP Urine test that is a quick and quantitative test to detect GIP in the urine sample.

Test procedure:

In a sterile urine collection cup, the patient's fresh urine was collected. From the total urine, 70µl of urine was mixed with 30 µl of conditioning solution with the use of micropipettes. This 100 µl of solution is enough for one test. Homogenize the mixture by pipetting up and down or by gently shaking the vial for 5-10 seconds **(Figure 8.1)**. The iVYCHECK GIP Urine cassette was open and place horizontally on a clean surface. Already prepared 100 µl of the homogenized mixture was applied to the sample zone (S) of the cassette **(Figure 8.2).** To detect the GIP in the urine sample, 15 minutes of incubation was given. The detection step is based on the reaction of the 33-mer-like immunogenic peptides of gluten present in the urine sample with the colored conjugates (monoclonal anti-gliadin 33-mer antibody/red-colored microsphere), previously loaded in a chromatography strip. The peptide-conjugate complexes spread through the strip by capillarity. When the complex reaches the test zone, the peptides interact with a second anti-gliadin 33-mer antibody immobilized on the membrane. This results in a RED line in the test zone (T) of the cassette which indicates a positive result. The absence of the

RED line indicates a negative result. Whether or not GIP is present in the sample, a second colored conjugate moves through the stick up to the control zone (C) of the cassette, where control antibodies have been immobilized, resulting in a visible GREEN line **(Figure 8.3).** This result confirms that the test has functioned properly. If the GREEN line does not appear, regardless of the RED line, the result of the test is considered invalid. After 30 minutes of incubation, the urine cassette was ready to read with the reader.

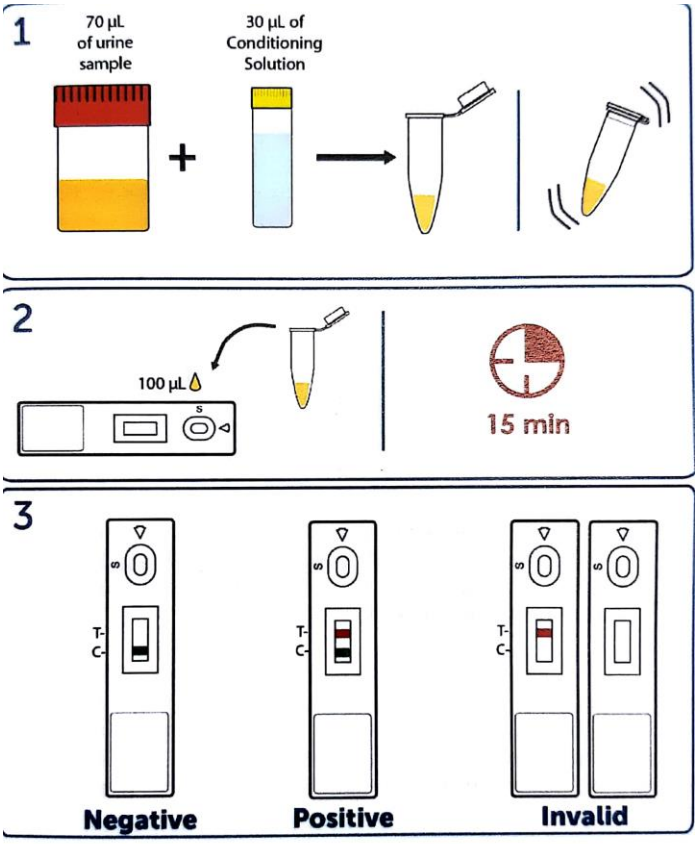

Figure 8: Test procedure for quantitave GIP urine test

Detection of GIP quantity by the iVYCHECK Reader

After 30 minutes of incubation, GIP Urine cassette was inserted in iVYCHECK Reader. The identity of the respective sample was documented in the reader and finally, the reader could read the cassette.

This technique allows two options for the reading of the results: a visual reading and a digital reading with the iVYCHECK Reader. The visual reading allows semi-quantitative results; while, iVYCHECK Reader provides a quantitative result. The iVYCHECK reader combines a highly sensitive optical detector, an integrated electronic system, and an effective data processing system.

Limit of detection (LoD) of this procedure is 2.2ng/ml of urine. The test is specific for the detection of prolamins of wheat (gliadin), rye (secalin), and barley (hordein). No cross-reactivity has been detected with naturally gluten-free food such as rice, maize quinoa, amaranth. There is no false positive reported so far.

3. Determination of i-FABP level in heparinized plasma

The plasma concentration of i-FABP was measured using Human i-FABP solid phase enzyme-linked immunosorbent assay based on sandwich principle. i-FABP ELISA test has been done according to the manufacturer's instructions.

Heparinized plasma samples were diluted in 1:1 ratio, working standards were prepared by serial dilution from the stock lyophilized standard. Standards and samples were put 100ul in each well and incubated for 1hr, ELISA wells were washed by 200ul wash buffer 3 times using a multichannel pipette. ELISA Plate was incubated with tracer and then with streptavidin solution after 1-hour incubation each time and washing were

performed with the same procedure. TMB solution was put in each well and incubated for 30 minutes at room temperature. At the final step, ELISA reaction was stopped with provided stop solution, and the ELISA plate was read in ELISA reader (thermos scientific multiscanGO) at 450nm of absorbance. Final evaluation of immunoassay was done on the basis of calibration curve drawn as per manufacturer's guidelines and the result was calculated in pg/ml. The minimum concentration that could be measured by this method is 47 pg/ml.

Results:

1. **Alkylresorcinol:** Our research team is in process of standardizing the LC-MS/MS.
2. **GIP (qualitative):** So far, 22 patients have recruited of them 18 patients already on GFD (CD follow-up patients) and 4 subjects were newly diagnosed CD patients. Out of 18 follow-up CD subjects, all 18 were found urine GIP test negative (no gluten detected in their urine sample), on the other hand, of 4 newly diagnosed CD patients all 4 were tested positive with GIP test none so far is negative from this test **(Table 8).**

Table 8: Result of qualitative GIP urine test in study subjects

Category	Number of subjects	GIP test found Positive	GIP test found Negative
CD patients on GFD	18	0	12
CD new diagnosis	4	4	0

3. **GIP (quantitative):** All 22 recruited patients samples were re-tested with quantitative GIP procedure. Out of 18 follow-up CD subjects, all 18 were found urine GIP test negative with GIP quantity <LOD i.e. 2.2ng/ml. On the other hand, of 4 newly diagnosed CD patients, all 4 were tested positive with GIP quantitative more than accepted threshold (>20 ppm) i.e. 26.85±12.1 ng/mL (mean±SD).
4. **i-FABP:** So far, 36 subjects were recruited for the study of them 13 patients were already on GFD (Follow-up CD patients), 4 were newly diagnosed cases of CD and rest 20 were healthy controls (asymptomatic healthy volunteers). Their i-FABP level is being analyzed. Follow-up CD subjects have i-FABP level 334 pg/ml, new cases of CD had 1242 pg/ml and healthy controls had i-FABP level 150 pg/ml. This result shows that New cases of CD patients have a higher level of i-FABP level in comparison to healthy subjects and patients already on GFD has their i-FABP level half the level of healthy controls **(Table 9).**

Table 9: Heparin i-FABP level in different study subject groups

S.No.	Subjects	i-FABP level (mean)
1	CD subjects on GFD (n=13/13)	334 pg/ml
2	New Diagnosis of CD (n=4/4)	1242 pg/ml
3	Healthy Controls (n=20/20)	150 pg/ml

Conclusions

The priliminary result of GIP showed that GIP qualitative as well quantitative tests are reliable to use. However, Research point of view, GIP quantitative test would be a more suitable test. Nevertheless, a large number of samples are required to verify this result.

The prilimanary result of i-FABP showed that there is a fair distinguished level of i-FABP among CD new cases, CD follow-up cases, and healthy subjects. i-FABP could be a reliable biomarker for CD patients adhered to GFD. However, in coming days more subjects will be recruited in all the groups to have a reliable robust result.

Project 2

Per day gluten exposure determination in celiac disease patients on gluten free diet

Introduction

A life-long and strict gluten-free diet is the only recommended treatment for CD. For other GRD, complete elimination of gluten from the diet for a certain period/lifelong is mandatory. Following a true GFD causes the gradual disappearance of symptoms and levels of serum autoantibodies and the normalization of the intestinal histological architecture.[10,185] However, achieving complete elimination of gluten from the diet is almost impossible.[8,9,186] As stated in an earlier section, CD patients are highly sensitive to the toxic effect of gluten. Protracted ingestion of traces of gluten (10-50 mg on a daily basis) may damage the integrity of the small intestinal mucosa, an increased number of IELs being the first marker of mucosal deterioration.[185] For a safe gluten tolerance, FDA and Codex Alimentarius established a guideline that suggests, any food that is labeled as “gluten-free” must contain <20 ppm of gluten. [11–13]

Despite knowing the adverse effects of a gluten-containing diet for CD and other GRD patients, about 50% of CD patients are failed to accomplish a recommended GFD.[8,9] The occasional and/or unintended exposure of gluten traces is enough to maintain the disease symptoms. There are numerous studies done to evaluate the compliance of CD subjects. Nevertheless, one recent study has systematically assessed the daily inadvertent gluten exposure of CD patients in a real setting.[187] However, this study was a meta-analysis and data was collected from earlier studies conducted on urine and

stool samples. Until now, there is no study has been conducted specifically on gluten-free food products available for CD patients on market. So, we have designed this study to calculate the amount of gluten that is accidentally consumed episodically and continually for those who are on GFD.

Aims of the study

•Quantification of gluten amount that is accidentally consumed by CD children on GFD

•Performance comparison of Ridascreen R-5 and Biomedal G-12/A-1 antibody-based ELISA used for the determination of gluten in food products.

Method:

Selection of food samples

For the determination of the per day gluten exposure of CD patients, a total of 45 patients who are already on GFD proposed to identify. Each patient requested to collect 10 food samples for one whole day. Considering 45 such patients, we assumed to have 450 food samples (breakfast, lunch, snacks, and dinner) in total. Each patient was provided with instructions containing the information on how to collect food samples, exclusion, and the inclusion of foodstuff. Each patient asked to fill the exact quantity of each food eaten during the day. Water, milk, fruits, vegetables, and other naturally gluten-free items were excluded for the analysis. Identified patient has been provided food collection tubes. Each food sample was given a unique code for the identification. All the food tubes collected and kept at -20°C.

Gluten quantity is being determined by the Ridascreen Gliadin sandwich R5 enzyme-linked immunosorbent assay R-7001 (R-Biopharm, Darmstadt, Germany) and GlutenTox Sandwich ELISAG12/A1 ELISA (KT-5139, Biomedal diagnostics, Spain) both, in segments. Both antibodies (R5 and G12/A1) are specially designed antibodies suitable for the specific determination of gluten in the food samples. Description and methodologies for R5 and G12/A1 antibody-based ELISAs are given in the following sections:

Ridascreen R-5 antibody-based ELISA:

The R5 monoclonal antibody (mAb) is raised against the ethanolic rye extract, specifically recognizes the prolamins from wheat (gliadins), barley (hordeins) and rye (secalins). These prolamins eventually cause mucosal damage that leads to CD. The R5 mAb-based ELISA kit is a suitable method to detect gluten in food materials.[188] The R5 mAb recognizes the epitopes (binding site) QQPFP (glutamine-glutamine-proline-phenylalanine-proline) as this epitope is in the N-terminal of the region of gluten, associated with toxicity. Sandwich ELISA based on R5 antibody recognizes the two epitopes of **QQPFP** hence, it is more specific.[189]

The R5 method is an official type I codex method for the gluten analysis, which is calibrated on the recommendation of prolamin Working Group (PWG).[190] This is the only method certified by the Association of Officials Analytical Chemists (AOAC), and is considered as the method of choice for gluten detection in food, according to the Codex Alimentarius Commission and other International Agencies and approved by (R5 ELISA) approved by the EU regulation.[11–13]

Biomedal G12/A1 antibody-based ELISA:

To recognize the toxic peptide of gliadin with high affinity two mAbs (G12 and A1) were developed against the 33-mer toxic peptide of the gliadin, that can detect the gliadin concentrations <1ng/mL.[137] Both the abs are highly sensitive and specific against the 33-mer peptide. However, the G12 mAb exhibits an affinity for the toxic peptide 8 times more than A1 mAb. G12 mAb recognized the toxic QPQLPY sequence while A1 mAb recognizes the QLPYPQP sequence of the toxic peptide of gliadin. In Biomedal ELISA based on G12 and A1 mAbs, G12 mAb (coated on the ELISA wells) is used as the detection ab and A1 (conjugate solution) is used as the capture ab. These abs are claimed to have a wider specificity for the prolamins that are more toxic for CD patients. Hence, is it very highly suitable for the detection of gliadin from the foodstuff? The G12 mAb proved to be efficient in measuring prolamin concentrations in both native and partially hydrolyzed cereals.[176]

The G12 based ELISA method had a limit of detection of 0.6 ppm gluten, 1/3 of the concentration obtained by other methods described to date. Similarly, a rapid test for the detection of gluten in solid food, drinks, and on surfaces using G12 mAb lateral flow devices (LFD) or dipsticks[191,192] as well as a competitive ELISA method was also developed for the detection of toxic gluten peptides in hydrolyzed foods[191,193] More interesting, G12 immunodepletion experiments with hydrolyzed gliadin from beers showed that this mAb recognize those with the highest immune-activity for celiac patients.[194]

All food products were subjected to gluten content determination by the Ridascreen Gliadin sandwich R5 enzyme-linked immunosorbent assay R-7001 (R-Biopharm,

Darmstadt, Germany) and by GlutenTox Sandwich ELISAG12/A1 ELISA (KT-5139, Biomedal diagnostics, Spain) at the Celiac Disease Research Laboratory of the Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy. All food samples were given a unique laboratory code for R5 and G12 ELISA and their details (breakfast, lunch, dinner, snacks) were recorded on an Excel sheet. During each run of ELISA, manufacturer's guidelines were strictly followed. Briefly, the steps of both the ELISAs procedure are as follows.

Determination of gluten content in food samples by Ridascreen R5 ELISA

Extraction and preparation of samples: Five grams of each sample was homogenized and crushed in a laboratory blender (solid food products). Each time after the crushing of a sample, parts of the blender was removed and washed with alkaline-enzyme detergent and rinse with 70% ethanol and dried before processing of another sample. Homogenized samples were stored in sterile tubes. Ridascreen R-7006 cocktail solution, containing detergents and a reducing agent, was used for the extraction of samples. 0.25 g of processed solid samples and 0.25 ml of liquid samples were measured in separate pre-labeled falcon tubes. In tannin and polyphenol containing products additionally, 0.25g of skimmed milk powder was added. After this preparation, 2.5 ml of cocktail solution was added in each tube under a chemical hood and tubes were vortexed and kept in a water bath at 50°C for 40 minutes. After the incubation, tubes could maintain room temperature and 7.5 ml of freshly prepared 80% ethanol was mixed in each tube and kept on a shaker for 1 hour. Samples were then transferred into 1.5 ml of Eppendorf tubes and centrifuged at least 2500 g for 10 minutes, the supernatant was separated and collected into another 1.5 ml Eppendorf tube and stored

at room temperature. To avoid any possible cross-contamination samples were crushed in different rooms and at a different time interval.

Gluten quantification: extracted food samples were diluted at 1:12.5 in provided sample dilution buffer, standard and samples were added in duplicate into pre-defined ELISA wells and enzyme conjugate was added to each well followed by a wash of ELISA plate by washing buffer and kept for incubation for 30 minutes at room temperature. Substrate and chromogen were added, and the reaction was stopped by provided stop solution and reading was obtained at the absorbance of 450 nm. Samples that showed an absorption value above the highest standard value were further diluted to get the absorption value within the range. The lower limit of the quantification was 2.5 ppm (mg/kg) of gliadin corresponding to 5 ppm (mg/kg) of gluten. Results were calculated by the suggested method and then entered in the Excel sheet.

Food products containing gluten level <20 ppm were considered as gluten-free while products with gluten level between 20 and 100 ppm were classified as products with low gluten contamination and products with >100 ppm of gluten were considered significantly contaminated. All products with a gluten level >20 ppm were re-extracted and analyzed second time.

Determination of gluten content in food samples by Biomedal G12/A1 ELISA

Sample extraction: Samples were homogenized in case of solid and semi-solid food samples 0.5 g of sample was measured and 5 mL of extraction buffer was added, tubes were closed properly, and a vigorous vortex was provided. In the case of non-heat treated samples sample was incubated at room temperature for 40 minutes with mild

agitation. In case of heat processed samples were kept in a water bath at 50°C for 40 minutes and mixed periodically during the incubation. Samples were then centrifuged at 2500 g for 10 minutes and transferred the supernatant in a new clean tube.

In the case of liquid samples, 0.5mL sample was measured and 4.5mL of extraction buffer was added. Samples were shed for 1-2 minutes using vortex.

Gluten quantification: Extracted food samples were diluted with provided sample dilution buffer as per given dilution table. Standard and samples were added in duplicate into pre-defined ELISA wells. In each well, 100µL of standard, positive control, negative control, and each sample was loaded and the ELISA plate was incubated for 60 minutes at room temperature. A wash was provided to the ELISA plate with 300 µL diluted wash buffer and 100 µL of ab (GlutenTox G12-HRP conjugated ab) was added in each well and another 60 minutes of incubation was provided at room temperature. After another wash, ELISA wells were treated with 100 µL of substrate solution and the plate was incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 µL stop solution. The result was calculated by plotting a standard curve (gliadin concentration y-axis and absorbance values x-axis). The reading was obtained at the absorbance of 450 nm. Samples that showed an absorption value above the highest gliadin quantification was calculated as per manufacturer's recommendations.

Food products containing gluten level <20 ppm were considered as gluten-free while products with gluten level between 20 and 100 ppm were classified as products with low gluten contamination and products with >100 ppm of gluten were considered significantly contaminated. All products with a gluten level >20 ppm were re-extracted and analyzed second time.

Daily gluten exposure quantification

The content of gluten in each food will be calculated multiplying the concentration of gluten found (1 ppm= 0.001 mg/grams) by the quantity (grams) of the specific food eaten by the subject in that day. The overall exposure to gluten /day will result from the sum of the amounts of gluten in each food (mg/day).

Quality control:

Each time absorption value of ELISA standards was assured with the quality assurance certificate provided with the ELISA kit. Random coded results were sent to the principal company for expert comments and suggestion. At different time intervals, all the group members gathered and discussed the procedure and further action.

Result: This is an ongoing project. So far, 24 patients agreed to participate in the study and have provided their per day food samples (breakfast, lunch, snacks, and dinner). In total 154 (approximately) different food items were collected. Out of 154 so far 78 food samples were investigated for their gluten content level though Ridascreen R5 as well as Biomedal G12/A1 ELISA. None of the samples have quantified more than acceptable level of gluten (20ppm) by G12 ELISA however, two samples out of 78 detected gluten level >20ppm through R5 ELISA.

Conclusion: This is just a preliminary result. Yet, about 21 patients to be recruited in the study and their one-day food sample to be investigated. In the recent future, we will be able to provide a robust result and conclusions only after final results

Project 3

Gluten contamination in naturally or labeled gluten-free products marketed in Italy

The hallmarks of the active CD are the presence of serum autoantibodies (e.g., IgA tTG and EMA) and a small intestinal enteropathy characterized, in typical cases, by villous atrophy, crypt hypertrophy and increased number of intraepithelial lymphocytes (IELs). GFD, that is the only accepted treatment of CD so far, determines the gradual disappearance of symptoms and serum autoantibodies, and the normalization of the intestinal histological architecture.[195]

Despite the availability of a wide range of natural (by origin) and industrially-prepared gluten-free food, complete avoidance of gluten from the diet is difficult to maintain. Gluten is indeed a “pervasive” nutrient that may contaminate otherwise gluten-free items along the production chain, from the field to the milling and manufacture steps.[196] Furthermore, wheat flour or purified gluten are largely added by the food industry to naturally gluten-free food, due to its technological properties, particularly the high visco-elasticity. Protracted intake of items contaminated with gluten traces may cause persistent intestinal damage and symptoms in treated CD patients.[197]

The scarcity of published data on the possible gluten contamination of nominally gluten-free products is a matter of concern. This is the reason why we decided to undertake this study. We have measured gluten in a large sample of gluten-free products that are currently on the market in Italy, using the only method (R5 ELISA) approved by the EU regulation.

A sampling plan was developed to analyze gluten-free products, including substitutes of wheat-based food, and other starch-rich food, e.g., legumes, that are extensively used in day-to-day meal preparation by individuals following a gluten-free diet. Selected products included different brands of (a) gluten-free flour, pasta, snacks, cookies, muesli, breakfast cereals, bread, and pizza; (b) rice, oats, buckwheat, quinoa, amaranth, mixed cereals, lentils, and chickpeas. Between April and October 2016, a total of 200 commercially available food products of common use were purchased in randomly chosen supermarkets in Ancona, Italy.

Food products were carefully identified and categorized into two broad categories, i.e., naturally (by origin) gluten-free products (Group 1) and labeled “gluten-free” products (Group 2). Group 1 was further divided into 1a, reporting no information of gluten content (defined herein as “products with unknown gluten content”), and 1b, reporting “may contain traces of gluten” on the label. Group 2, i.e., certified gluten-free products, were categorized as 2a, including products fulfilling the EU regulation for gluten-free products (UE 828/2014) plus the quality certification released by the Italian Celiac Society (identified by the “Crossed Ear” symbol on the package) or 2b, including gluten-free products fulfilling the EU regulation for gluten-free products only (**Figure 9**).

All food products were subjected to gluten content determination by the Ridascreen Gliadin sandwich R5 enzyme-linked immunosorbent assay R-7001 (R-Biopharm, Darmstadt, Germany) at the Celiac Disease Research Laboratory of the Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy. During each run of ELISA, manufacturer’s guidelines were strictly followed. Briefly, the steps of the ELISA procedure were as follows.

Of overall, 200 food products were analyzed: 107 in Group 1 (group 1a, n = 71; group 1b, n = 36) and 93 in Group B (group 2a, n = 45; group 2b, n = 48). Overall 173 (86.5%) products were detected with gluten level lower than 10 ppm, nine (4.5%) products contained between 10 and 20 ppm of gluten, and 18 (9%) products were detected with gluten level above the maximum tolerable of 20 ppm (15 products containing less and three products more than 100 ppm of gluten) **(Table 10)**.

In products belonging to group 1, 16 items (8%) were contaminated with more than 20 ppm of gluten, 12 (6%) from sub-group 1a (gluten content unknown) and four (2%) products from sub-group 1b ("may contain gluten") products. In group 2 (products labeled as gluten-free), only two (1%) products were found to have gluten level higher than 20 ppm. These products belonged to subgroup 2b, whereas no "Crossed-Ear" product was found to contain gluten at 20 or more ppm **(Figure 9)**. By comparing the staple ingredients, we found a significantly higher proportion of contaminated products in oats, buckwheat and lentils as compared to chickpeas, corn, mixed seeds, quinoa, and chocolate. By comparing the food categories, the lunch/dinner products were significantly more contaminated as compared to snacks.

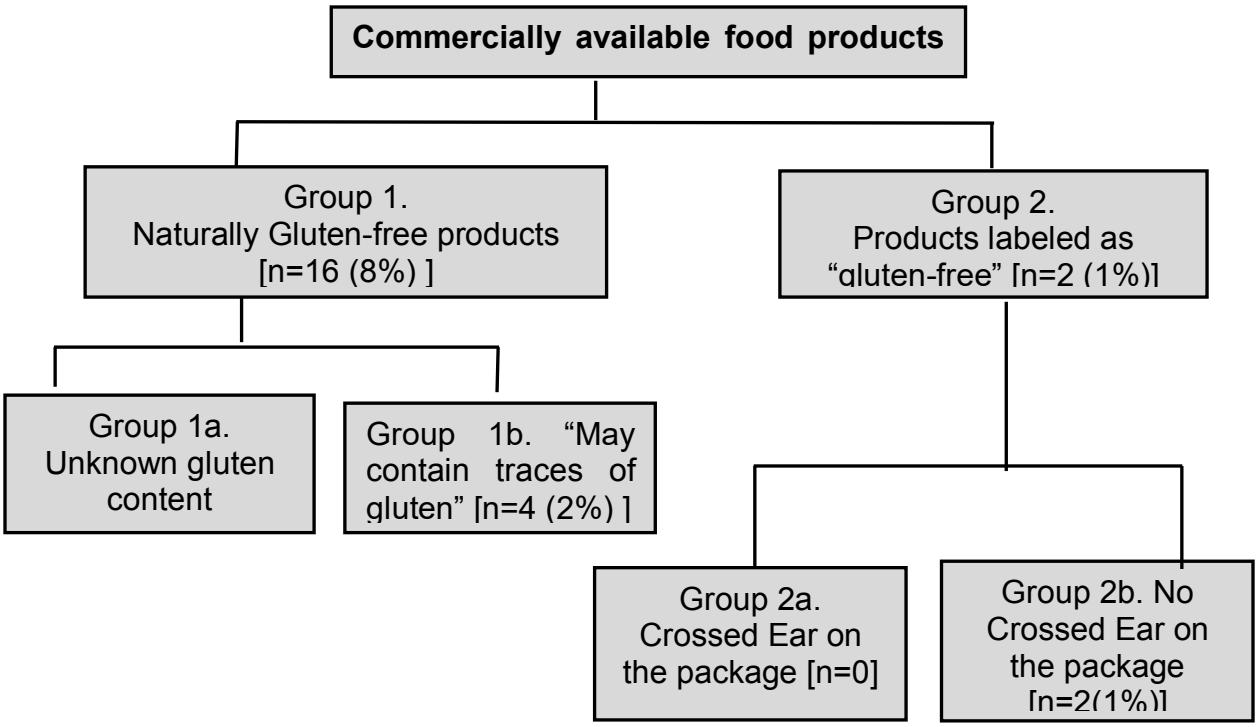

Figure 9: Number of food products containing gluten > 20 ppm in different groups

Overall 53 products belonging to six different food categories (lentils, chickpeas, beans, oats, buckwheat, and quinoa) were considered for the cost analysis. The PI was not significantly correlated to the content of gluten ($r = -0.009$; $p = 0.51$). However, a significantly different distribution of price categories was found according to the level of gluten contamination, a higher proportion of low price foods were found in products with levels of gluten > 20 ppm ($p < 0.01$).

Table 10: Level of gluten contamination in the 200 examined products

Gluten content (ppm)	Number of products	Median (range) (ppm)	Mean±SD (ppm)
<10	173	< 5 (<0.5-9.3)	n.a.
10-20	9	13.9 (10.4-17.1)	14.1±2.2
>20	18	31.7 (20.4-126.2)	49.2±35.9

n.a.= not applicable [due to the (< 5) values

This study concludes that gluten contamination in either naturally or commercial gluten-free products marketed in Italy is nowadays uncommon and usually mild on a quantitative basis. Crossed Ear and higher cost gluten-free products are in general safer than other products. Caution is however needed to interpret these findings, due to the intrinsic limitations of the analytical method for determining gluten traces in food matrixes. A program of systematic sampling of gluten-free food is needed to promptly disclose at-risk products, to ensure the safety of available products and ultimately improve the long-term wellbeing of individuals affected with CD or other gluten-related disorders.

nutrients

MDPI

Article

Gluten Contamination in Naturally or Labeled Gluten-Free Products Marketed in Italy

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Abstract: Background: A strict and lifelong gluten-free diet is the only treatment of celiac disease. Gluten contamination has been frequently reported in nominally gluten-free products. The aim of this study was to test the level of gluten contamination in gluten-free products currently available in the Italian market. Method: A total of 200 commercially available gluten-free products (including both naturally and certified gluten-free products) were randomly collected from different Italian supermarkets. The gluten content was determined by the R5 ELISA Kit approved by EU regulations. Results: Gluten level was lower than 10 part per million (ppm) in 173 products (86.5%), between 10 and 20 ppm in 9 (4.5%), and higher than 20 ppm in 18 (9%), respectively. In contaminated foodstuff (gluten > 20 ppm) the amount of gluten was almost exclusively in the range of a very low gluten content. Contaminated products most commonly belonged to oats-, buckwheat-, and lentils-based items. Certified and higher cost gluten-free products were less commonly contaminated by gluten. Conclusion: Gluten contamination in either naturally or labeled gluten-free products marketed in Italy is nowadays uncommon and usually mild on a quantitative basis. A program of systematic sampling of gluten-free food is needed to promptly disclose at-risk products.

Keywords: celiac disease; gluten-free products; naturally gluten-free; R5 ELISA; oats; buckwheat; lentils

1. Introduction

Celiac disease (CD) is an autoimmune condition characterized by permanent intolerance to dietary gluten, a protein complex found in wheat, rye and barley, occurring in genetically predisposed individuals [1]. The hallmarks of active CD are the presence of serum autoantibodies (e.g., IgA antitransglutaminase and antiendomysial antibodies) and a small intestinal enteropathy characterized, in typical cases, by villous atrophy, crypt hypertrophy and increased number of intraepithelial lymphocytes (IELs). Treatment of CD is based on the lifelong exclusion of gluten-containing food from the diet. The gluten-free diet (GFD) determines the gradual disappearance of symptoms and serum autoantibodies, and the normalization of the intestinal histological architecture [2].

Unfortunately, CD patients are highly sensitive to the toxic effect of gluten. It has been shown that the protracted ingestion of traces of gluten (10–50 mg on a daily basis) may damage the integrity of the small intestinal mucosa, an increased number of IELs being the first marker of mucosal deterioration [3]. By combining these toxicity data with the observed food intake, it has been calculated that gluten-free

products with less than 20 mg/kg (or parts per million = ppm) of gluten contamination are safe over a wide range of daily consumption [4]. The 20 ppm threshold for gluten-free food has been endorsed by the Codex Alimentarius [5] and other agencies, e.g., the US Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) [6].

Despite the availability of a wide range of natural (by origin) and industrially-prepared gluten-free food, complete avoidance of gluten from the diet is difficult to maintain. Gluten is indeed a "pervasive" nutrient that may contaminate otherwise gluten-free items along the production chain, from the field to the milling, stockage and manufacture steps [7]. Furthermore wheat flour or purified gluten are largely added by the food industry to naturally gluten-free food, due to its technological properties, particularly the high visco-elasticity. Protracted intake of items contaminated with gluten traces may cause persistent intestinal damage and symptoms in treated CD patients [8].

The scarcity of published data on the possible gluten contamination of nominally gluten-free products is a matter of concern. This is the reason why we decided to undertake the current study, by measuring gluten in a large sample of gluten-free products that are currently on the market in Italy, using the only method (R5 ELISA) approved by the EU regulation. We present here the final results of these analyses on 200 commercially available gluten-free products.

2. Materials and Methods

2.1. Collection of Food Products

A sampling plan was developed to analyze gluten-free products, including substitutes of wheat-based food, and other starch-rich food, e.g., legumes, that are extensively used in day-to-day meal preparation by individuals following a gluten-free diet. Selected products included different brands of (a) gluten-free flour, pasta, snacks, cookies, muesli, breakfast cereals, bread, and pizza; (b) rice, oats, buckwheat, quinoa, amaranth, mixed cereals, lentils, and chickpeas. Between April and October 2016, a total of 200 commercially available food products of common use were purchased in randomly chosen supermarkets in Ancona, Italy.

Food products were carefully identified and categorized into two broad categories, i.e., naturally (by origin) gluten-free products (Group 1) and labeled "gluten-free" products (Group 2). Group 1 was further divided into 1a, reporting no information of gluten content (defined herein as **"**products with unknown gluten content"**),** and 1b, reporting "may contain traces of gluten" on the label. Group 2, i.e., certified gluten-free products, were categorized as 2a, including products fulfilling the EU regulation for gluten-free products (UE 828/2014) plus the quality certification released by the Italian Celiac Society (identified by the "Crossed Ear" symbol on the package) or 2b, including gluten-free products fulfilling the EU regulation for gluten-free products only (Figure 1).

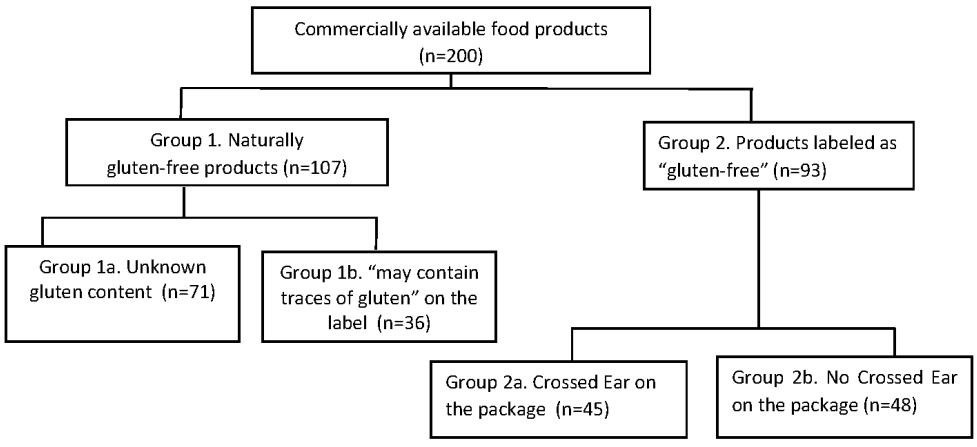

Figure 1. Types of food products analyzed in this study.

2.2. Determination of Gluten Content

All food products were subjected to gluten content determination by the Ridascreen Gliadin sandwich R5 enzyme-linked immunosorbent assay R-7001 (R-Biopharm, Darmstadt, Germany) at the Celiac Disease Research Laboratory of the Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy. During each run of ELISA, manufacturer's guidelines were strictly followed. Briefly, the steps of the ELISA procedure were as follows.

2.2.1. Extraction and Preparation of Samples

All samples were given a unique laboratory code and their details (including brand, cost, ingredient, food type, etc.) were recorded on an Excel sheet. Five grams of each sample were homogenized and crushed in a laboratory blender (solid food products). Each time after the crushing of a particular sample, parts of the blender were removed and washed with alkaline-enzyme detergent and rinse with 70% ethanol and dried before processing of another sample. Homogenized samples were stored in sterile tubes. Ridascreen R-7006 cocktail solution, containing detergents and reducing agent, was used for the extraction of samples. One-quarter gram of processed solid samples and 0.25 mL of liquid samples were measured in separate pre-labeled falcon tubes. In tannin and polyphenol containing products additionally 0.25 g of skimmed milk powder was added. After this preparation, 2.5 mL of cocktail solution was added in each tube under a chemical hood and tubes were vortexed and kept in water bath at 50 °C for 40 min. After the incubation, tubes were allowed to maintain room temperature and 7.5 mL of freshly prepared 80% ethanol was mixed in each tube and kept on a shaker for 1 h. Samples were then transferred into 1.5 mL of Eppendorf tubes and centrifuged at least 2500 g for 10 min, supernatant was separated and collected into another 1.5 mL Eppendorf tube and stored at room temperature. To avoid any possible cross contamination, samples were crushed in different rooms and at different time intervals.

2.2.2. Gluten Quantification

Extracted food samples were diluted at 1:12.5 in provided sample dilution, standard and samples were added in duplicate into pre-defined ELISA wells and enzyme conjugate was added to each well followed by wash of ELISA plate by washing buffer and kept for incubation for 30 min at room temperature. Substrate and chromogen were added and the reaction was stopped by provided stop solution and reading was obtained at the absorbance of 450 nm. Samples that showed an absorption value above the highest standard value were further diluted to get the absorption value within the range. The lower limit of the quantification was 2.5 ppm (mg/kg) of gliadin, corresponding to 5 ppm (mg/kg) of gluten. Results were calculated by the suggested method and then entered in the Excel sheet.

Food products containing gluten level lower than 20 ppm were considered as gluten-free while products with gluten level between 20 and 100 ppm were classified as products with low gluten contamination and products with more than 100 ppm of gluten were considered significantly contaminated. All products with a gluten level higher than 20 ppm were re-extracted and analyzed second time.

2.3. Quality Control

Each time absorption value of ELISA standards was assured with the quality assurance certificate provided with the ELISA kit. The result of each run was discussed with research group members and random results were sent to the principal company for expert comments and suggestion. At different time intervals, all the group members gathered and discussed the procedure and further action.

2.4. Cost Analysis: Correlation between the Cost of the Product and Gluten Contamination

If at least 5 products with similar ingredients from different brands were available, the mean price was calculated. Then, for each product the price index (PI) was calculated as the product price divided

by the mean price of the category. The PI was then categorized in 3 groups (price categories): PI < 0.75 (products with a low price), PI: 0.75–1.25 (products with an average price), PI > 1.25 (products with a high price).

2.5. Statistics

Data are presented as proportions, means and S.D., medians and range, as appropriate. The Kruskal-Wallis one-way analysis of variance was used to determine if there was statistically significant difference of gluten contamination between the four groups of products (1a, 1b, 2a, and 2b), and, if significant, post hoc test was used for multiple comparisons. Comparison between proportions of contaminated (>20 ppm) and not contaminated (<20 ppm) samples within each group was calculated by the Fisher's test. Spearman's test was used to correlate quantitative variables (prices and gluten levels). Results were found significant when $p < 0.05$. The statistical analysis was performed using the Software Program Stata System (SPSS) v17.0 (Chicago, IL, USA).

3. Results

Detection of Gluten Contamination

Overall, 200 food products were analyzed: 107 in Group 1 (group 1a, $n = 71$; group 1b, $n = 36$) and 93 in Group B (group 2a, $n = 45$; group 2b, $n = 48$). Overall 173 (86.5%) products were detected with gluten level lower than 10 ppm, nine (4.5%) products contained between 10 and 20 ppm of gluten, and 18 (9%) products were detected with gluten level above the maximum tolerable of 20 ppm (15 products containing less and three products more than 100 ppm of gluten) (Table 1).

The proportion of contaminated products (gluten > 20 ppm) according to the staple ingredient and to the food category is reported in Tables 2 and 3, respectively. In products belonging to group 1, 16 items (8%) were contaminated with more than 20 ppm of gluten, 12 (6%) from sub-group 1a (gluten content unknown) and four (2%) products from sub-group 1b ("may contain gluten") products. In group 2 (products labeled as gluten free), only two (1%) products were found to have gluten level higher than 20 ppm. These products belonged to subgroup 2b, whereas no "Crossed-Ear" product was found to contain gluten at 20 or more ppm (Figure 2). Overall, we found a significant different proportion of contamination between the four groups of products (Kruskal-Wallis, $p < 0.01$). By multiple comparison, a significant higher proportion of contaminated products was found in group 1a as compared to group 2a (16% vs. 0, $p < 0.01$) (Table 2). No significant difference was found in the proportion of contaminated products between groups 1a and 1b and between groups 2a and 2b, respectively (Table 2, Figure 3). By comparing the staple ingredients, we found a significant higher proportion of contaminated products in oats, buckwheat and lentils as compared to chickpeas, corn, mixed seeds, quinoa, and chocolate. By comparing the food categories, the lunch/dinner products were significantly more contaminated as compared to snacks.

Overall 53 products belonging to six different food categories (lentils, chickpeas, beans, oats, buckwheat and quinoa) were considered for the cost analysis. The PI was not significantly correlated to the content of gluten ($r = -0.009$; $p = 0.51$). However, a significantly different distribution of price categories was found according to the level of gluten contamination. As shown in Figure 4, a higher proportion of low price foods were found in products with levels of gluten > 20 ppm ($p < 0.01$).

Table 1. Level of gluten contamination in the 200 examined products.

Gluten Content (ppm)	Number of Products	Median (Range) (ppm)	Mean ± SD (ppm)
<10	173	<5 (<0.5–9.3)	n.a.
10–20	9	13.9 (10.4–17.1)	14.1 ± 2.2
>20	18	31.7 (20.4–126.2)	49.2 ± 35.9

n.a. = not applicable (due to the (<5) values).

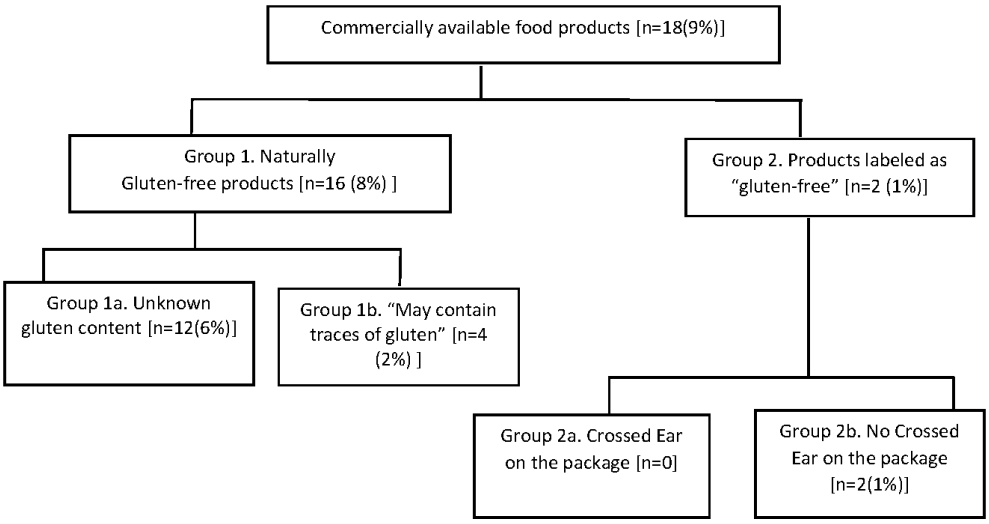

Figure 2. Number of food products containing gluten >20 ppm in different groups.

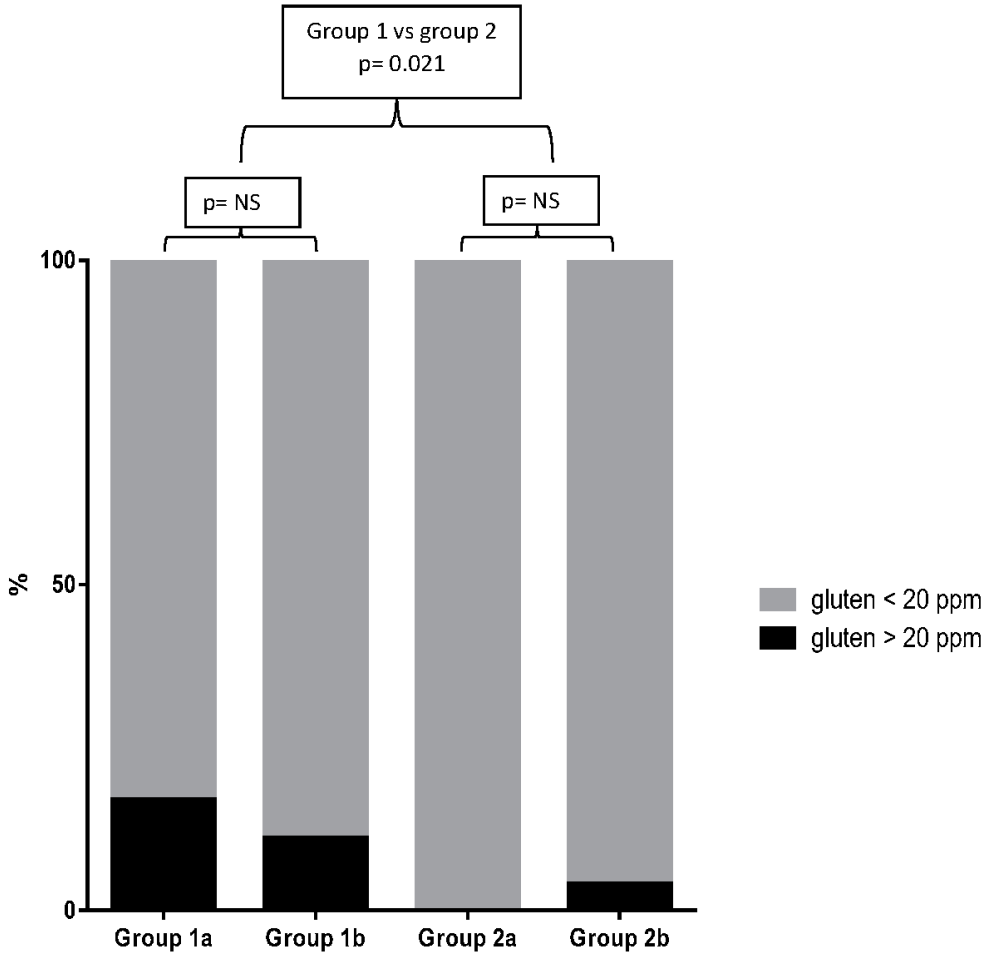

Figure 3. Percentage of contaminated products in each food group.

Table 2. Proportion of items containing >20 ppm of gluten by staple ingredient (contaminated/tested products).

Item	Overall *	Group 1		Group 2		*p*	*p*
		Naturally Gluten Free Products		Products Labeled as "Gluten Free"			
		Group 1a	Group 1b	Group 2a	Group 2b	Kruskal-Wallis	Multiple Comparisons
Amaranth	0/2	0/1	0/1	-	-	1.000	
Buckwheat	5/12	3/5	1/3	0/1	1/3	0.695	
Chickpeas	0/6	0/4	0/2	-	-	1.000	
Chocolate	0/9	0/1	0/3	-	0/5	1.000	
Coconut	1/3	1/2	-	-	0/1	0.480	
Corn	0/40	0/8	0/5	0/23	0/4	1.000	
Dry fruit	0/2	0/2	-	-	-	-	
Fruit Candy	0/4	0/1	-	-	0/3	1.000	
Fruit Jam	0/4	0/2	-	-	0/2	1.000	
Kidney Bean	0/7	0/5	0/2	-	-	1.000	
Lentil	4/17	2/6	2/11	-	-	0.495	
Mixed Cereal	1/25	0/2	0/2	0/10	1/11	0.736	
Mixed Seeds	0/12	0/8	0/1	0/1	0/2	1.000	
Oats	4/5	4/5	-	-	-	-	
Others	1/14	0/4	1/3	-	0/7	0.160	
Peanuts	1/4	1/4	-	-	-	-	
Quinoa	0/10	0/5	0/1	0/1	0/3	1.000	
Rice	1/24	1/6	0/2	0/9	0/7	0.392	
Total	**18/200**	**12/71**	**4/36**	**0/45**	**2/48**	**0.010**	**1a vs. 2a p = 0.012**

* Kruskal-Wallis $p < 0.001$; Multiple comparisons: $p < 0.001$: mixed seeds vs. oats, quinoa vs. oats, chocolate vs. oats, corn vs. oats; $p = 0.001$: chickpeas vs. oats; $p = 0.002$: corn vs. buckwheat.

Table 3. Proportion of items containing >20 ppm of gluten by food category (contaminated/tested products).

Food Category	Overall *	Group 1		Group 2		*p*	*p*
		Naturally Gluten Free Products		Products Labeled as "Gluten Free"			
		Group 1a	Group 1b	Group 2a	Group 2b	Kruskal-Wallis	Multiple Comparisons
Breakfast	0/11	0/4	0/1	0/4	0/2	1.000	
Lunch/dinner	15/88	10/45	4/25	0/12	1/6	0.348	
Snacks	2/95	2/22	0/10	0/28	0/35	0.082	
Bread	0/3	-	-	0/1	0/2	1.000	
Pizza	1/3	-	-	-	1/3	-	
Total	18/200	12/71	4/36	0/45	2/48	0.010	1a vs. 2a p = 0.012

* Kruskal-Wallis $p = 0.004$; Multiple comparisons: Lunch/dinner versus snacks: $p = 0.006$.

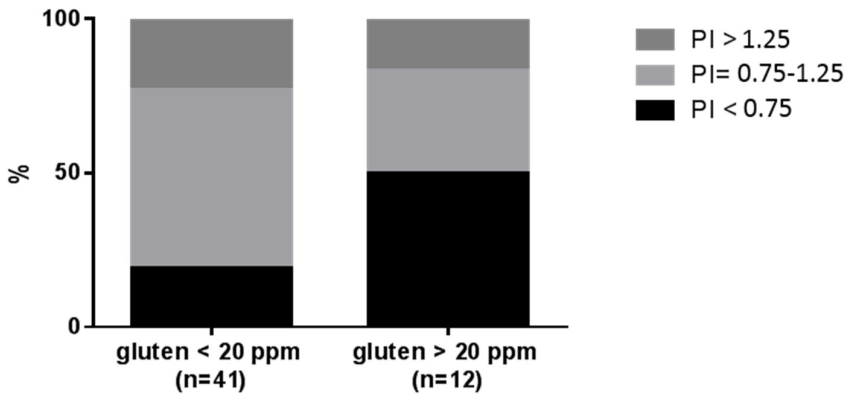

Figure 4. Distribution of price indexes (PIs) according to the level of gluten contamination.

4. Discussion

Our large survey shows that gluten contamination is low in gluten-free food marketed in Italy, both in terms of the percentage of contaminated products (9%), and the amount of gluten in contaminated products (almost exclusively in the range of the low gluten content 20–100 ppm). However, naturally (by origin) gluten-free products are at significantly higher risk of contamination as compared to products certified as gluten-free; indeed, we found that 16% of naturally gluten-free products with unknown gluten content are contaminated with respect to none of the certified gluten-free products with the crossed-ear symbol. Of note, among the certified gluten-free products without crossed-ear symbol we found that two out of 48 (4%) were contaminated with respect to none of the products with crossed-ear symbol; although this difference is not significant, it may suggest that the more stringent controls performed on "Crossed Ear" gluten-free products guarantee less risk of gluten contamination.

Our findings are more encouraging than previous studies in some American countries, e.g., contamination was found in 20.5% of gluten-free products marketed in the USA [7] and 21.5% in Brazil [9] respectively, and are in line with previous data from Canada [10] and Europe [4]. Compared to the past, the picture has clearly improved, most likely due to the worldwide implementation of the 20 ppm maximum threshold of gluten contamination established by the Codex Alimentarius in the year 2008 [5]. Based on these data and the dietary habits of the Italian population, the safety threshold of 10–50 mg of daily gluten would hardly be exceeded even by CD patients consuming very large quantities of gluten-free items (provided that no other contaminated food is eaten at the same time).

Quantification of gluten in food is difficult, for several reasons. Firstly gluten is not a single protein but a mix of different protein components (microheterogeneity) generally classified as gliadins, glutenins, globulins and albumins [11]. Measuring all these different fractions is clearly unpractical. Gliadins are the major component on a quantitative basis, and it is generally assumed that the ratio between gliadins (the fraction that is measured with the R5 test) and overall gluten is 1:2 (50%) [12,13]. Other analytical problems include the difficulty in (a) specifically quantifying all the different celiac-toxic peptides contained in gluten; (b) extracting gluten from the different food matrixes; and (c) measuring hydrolysed gluten peptides (e.g., in fermented food such as beer). Several tools have been developed for gluten quantification in food, such as the R5, the G12 and the α-20 antibody-based ELISA kits [14,15]. In the present study we used the R5 method, an ELISA test based on specially designed monoclonal antibodies raised against a pentapeptide from rye. It detects prolamins of wheat (gliadins), rye (secalins) and barley (hordeins), i.e., all the cereals that are toxic for celiac patients, in both raw (flours) and processed food products [16]. It is the only method certified by the Association of Officials Analytical Chemists (AOAC), and is considered as the method of choice for gluten detection in food, according to the Codex Alimentarius Commission and other International Agencies [5,6]. Most recent studies on gluten contamination have been performed using the same R5 test, a finding that allows comparisons among the results of different surveys [4,7,9,10,17].

As reported in previous studies [4,7,9,10,17], we found that products at significant higher risk of contamination of gluten are oats (four out of five examined items), buckwheat (5/12) and lentils-based (4/17) products. Several studies have shown that medium-high amounts of gluten-uncontaminated oats can be safely ingested by patients with CD [18,19]. Official recommendations acknowledge the safety of products containing purified oats, and several national associations for CD allow inclusion of oats in the diet of people with CD [19]. Unfortunately, the commercial oat supply is often contaminated with wheat. In Canada 88% of 133 oat samples were contaminated above 20 ppm [10]. There are possibilities for cross-contamination in the field, in the transport of the grain, in the storage of the grain, and in the milling and packaging facilities [7,10]. This is a deplorable situation since oats is rich in soluble dietary fiber, vitamins and minerals, and may unquestionably improve the nutritional value and increase the palatability of the GFD, while expanding food choices and ultimately improving the life quality of people with CD. Buckwheat is a gluten-free pseudocereal that belongs to the Polygonaceae family. Buckwheat grain is a highly nutritional food component that has been shown to provide a wide range of beneficial effects. Health benefits attributed to Buckwheat include plasma

cholesterol level reduction, neuroprotection, anticancer, anti-inflammatory, antidiabetic effects, and improvement of hypertension. In addition, buckwheat has been reported to possess prebiotic and antioxidant activities [20]. The possible gluten contamination of buckwheat has been correlated with the high content of fiber [21]. The frequent gluten contamination of lentils was somewhat unexpected, since this food is a legume and not a cereal, and its production chain is far different from wheat. Lentils are an edible pulse that is part of the human diet since the Neolitic age, being one of the first crops domesticated in the Near East. Lentils are a rich source of numerous nutrients, including protein, starch, folate, thiamin, pantothenic acid, vitamin B6, phosphorus, iron and zinc [22]. The origin of gluten contamination of lentils remains unclear. Many patients or caregivers check lentils seed by seed, and have reported that rare wheat seeds can be found mixed with lentils, most likely due to contamination occurring in the field. The practice of inspecting and washing lentils before cooking should be recommended when the package does not report any gluten-free labeling.

It is important to underscore that oats, buckwheat and lentils are nutritious dietary components that may increase the variety of carbohydrate- and fiber-rich food in the gluten-free diet. For this reason, we hope that the food industry will pay more attention in ensuring and certifying a gluten-free food chain for these important ingredients.

Finally, in the present study we also aimed to evaluate if the gluten contamination is, to some extent, related to the cost of the product. It is worth noting that we found that a higher proportion of low price foods were contaminated with respect to higher price foods ($p < 0.01$), suggesting that the lower the price the lower the quality of control on the gluten contamination.

Despite the GFD, many treated CD patients frequently show incomplete resolution of the histological intestinal damage at the follow-up intestinal biopsy, suggesting ongoing gluten ingestion [8]. Since our data and other surveys [4] found that gluten contamination of wheat substitutes does not represent a big issue in recent years, this persistent enteropathy is probably related to different sources of contamination, such as voluntary dietary transgressions, particularly in adolescents, or contaminated meals consumed outside home. Consumption of food prepared away from home plays an increasingly large role in the diet. In the US in 1970, 25.9 percent of all food spending was on food away from home; by 2012, that share rose to its highest level of 43.1 percent (data of the US Department of Agriculture, 2016; www.ers.usda.gov). In restaurants, pizzerias and cafeterias the chance of getting gluten-contamined GF food is higher than home, due to inadequate personnel training, careless use of tools/workbench and so forth. An active policy of training and education on the requirements for the GFD should be addressed to employees at food services.

5. Conclusions

Gluten contamination in either naturally or commercial gluten-free products marketed in Italy is nowadays uncommon and usually mild on a quantitative basis. Crossed Ear and higher cost gluten-free products are in general safer than other products. Caution is however needed to interpret these findings, due to the intrinsic limitations of the analytical method for determining gluten traces in food matrixes. A program of systematic sampling of gluten-free food is needed to promptly disclose at-risk products, to ensure the safety of available products and ultimately improve the long-term wellbeing of individuals affected with CD or other gluten-related disorders.

Author Contributions: Anil K. Verma designed and performed the laboratory tests; Anil K. Verma, Simona Gatti, Giada Del Baldo; Tiziana Galeazzi and Roberta Annibali, acquired the data; Anil K. Verma and Carlo Catassi wrote and drafted the manuscript; Simona Gatti and Tiziana Galeazzi conceived and designed the experiments; Simona Gatti, Tiziana Galeazzi, Chiara Monachesi, Lucia Padella, Elena Lionetti and Carlo Catassi gave technical and material support; Tiziana Galeazzi supervised the laboratory experiments; Simona Gatti, Tiziana Galeazzi, Elena Lionetti and Carlo Catassi critically revised the manuscript; Elena Lionetti and Carlo Catassi analyzed and interpreted the data. Carlo Catassi designed the overall study concept and gave administrative and financial support and supervised the study. All authors revised and approved the final version.

Conflicts of Interest: Carlo Catassi has received consultancy funds from Schär. Other authors declare no conflict of interest.

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Project 4

Contribution of oral hygiene and cosmetics on contamination of gluten-free diet

Introduction

Despite the well-recognized efficacy of the GFD, complete adherence to the diet is very difficult to achieve, with several studies reporting incomplete compliance in up to 50% of celiac patients.[8,9] The protracted ingestion of traces of gluten (10–50 mg on a daily basis) may damage the integrity of the small intestinal mucosa.[165] In a recent study, the average inadvertent exposure to gluten by CD individuals on a GFD was estimated to be ~150-400 mg/d, with a high proportion of CD patients, routinely ingesting more than 200 mg of gluten per day.[187]

The maximum tolerable amount of gluten contamination (<20 ppm) is recognized by Codex Alimentarius and also recommended by the US Food and Drug Administration (FDA) and European Food Safety Authorities (EFSA).[11–13]

Gluten is not only used in human foods. Personal care products or toiletries (toothpaste, mouthwashes, soap, shampoo, etc.) and cosmetics (lipsticks, lip balms, lip gloss, lip balms, face powder, body lotions, etc.) may contain gluten in different forms (thicker and preservatives) or gluten-derived ingredients.[2–4] Although such products should not be ingested, some amounts of these products can inadvertently be swallowed, particularly in the form of lipsticks or lip balms and toothpaste, thus leading to the possible ingestion of gluten traces (in the case of contamination) and eventually contributing to persistent intestinal damage in CD patients. To the best of our knowledge, there is a lack of

studies specifically assessing the gluten content of oral hygiene products and cosmetics, and therefore the safety of these products for patients affected with CD is unknown.

In the present study, we evaluated the gluten content of several oral hygiene products and cosmetics, widely available in the Italian market, by using the only laboratory method (R5 ELISA) for quantification of gluten that is approved by the EU regulation.

Overall 66 oral hygiene products and cosmetics were purchased from popular Italian supermarkets and pharmacies of Ancona (Italy) between February and March 2018. For the selection of products, priority was given to products from the largest producers and most popular among the public. Products were categorized as follows: 1) toothpastes; 2) dental tablets; 3) mouthwashes; 4) lip balms, and 5) lipsticks. Each group was further divided into two subgroups: a) labeled gluten-free; b) unknown gluten content**.**

All 66 products (oral hygiene and cosmetics) were analyzed for their gluten content by Ridascreen Gliadin sandwich R5 ELISA R-7001 (R-Biopharma, Darmstadt, Germany) at our Celiac Disease Research Laboratory, Department of Pediatrics, Marche Polytechnic University, Ancona, Italy. The R5 ELISA was performed as previously described in P**roject 2.**

Figure 10 shows the characteristics of all selected products. No information about gluten content was provided in any product except for 5 lip balms claimed as gluten-free without any certification.

Overall, out of the 66 selected products, 62 (94%) showed gluten level lower than 20 ppm (i.e., within the accepted level of gluten content for gluten-free products), while 4

(6%) products were detected with gluten level higher than 20 ppm. In detail, among products of group 1 (toothpaste), 3 (4.5%) were found with gluten level >20 ppm (20.7 ppm, 31.4 ppm, and 35 ppm, respectively); products belonging to group 2, 3 and 4 (dental tablets, mouthwashes, and lip balms) presented a gluten level <20 ppm, while among products of group 5 (Lipstick), 1 (1.5%) was found with a gluten level higher than the tolerated level (27.4 ppm).

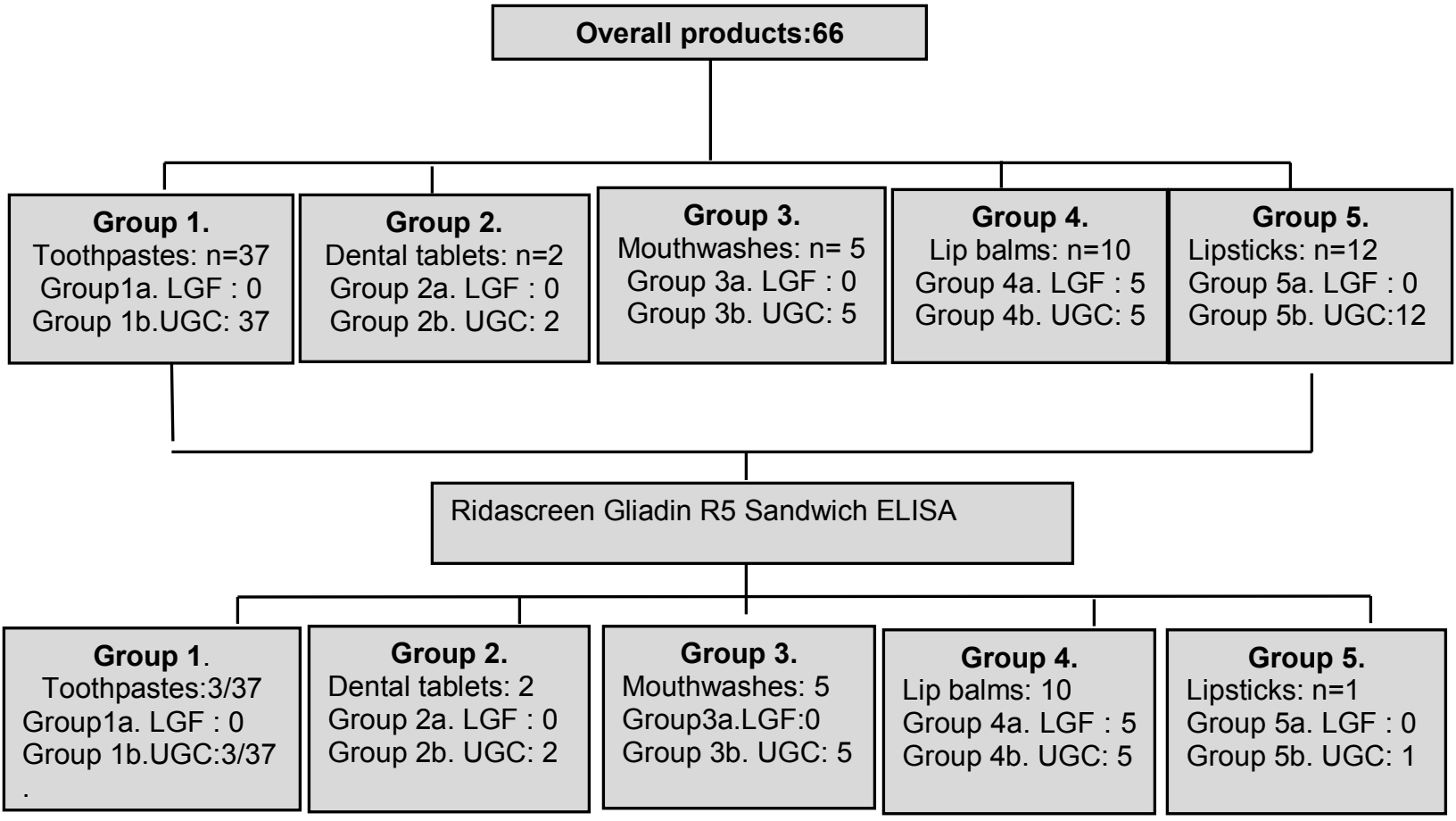

Figure 10: Type of products analyzed and their gluten content

LGF: labeled as gluten-free; UGC: unknown gluten content

The present study showed that currently, gluten contamination is not an issue in a wide array of cosmetic and oral hygiene products that are on the market in Italy.

Nevertheless, since all these products, particularly dentifrices and lipsticks, may contain gluten traces and may be ingested by patients on need to follow a strict GFD, it would be desirable that producing companies will report the information “gluten-free” or “may contain traces of gluten” on the package, therefore allowing a safe and informed choice by the celiac customers.

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Contribution of Oral Hygiene and Cosmetics on Contamination of Gluten-free Diet—Do Celiac Customers Need to Worry About?

AQ2 *Anil K. Verma, †Elena Lionetti, †Simona Gatti, †Elisa Franceschini, †Giulia Naspi Catassi, and †‡Carlo Catassi

ABSTRACT

Objectives: The only available treatment for celiac disease (CD) is the gluten-free diet. It is unclear whether the presence of gluten in oral hygiene products and cosmetics that are applied on the mouth is a reason of concern for CD patients. The aim of this study was to test the level of gluten contamination in oral hygiene and cosmetic products available in the Italian market.

Methods: A total of 66 products (toothpastes = 37; dental tablets = 2; mouthwashes = 5; lip-balms = 10; lipsticks = 12) labelled gluten-free or with unknown gluten content were randomly collected from different supermarkets and pharmacies. The gluten quantification was determined by the R5 ELISA method approved by EU regulations.

Results: Out of 66 oral hygiene and cosmetics, 62 products (94%) were found to be gluten-free (gluten level <20 ppm), while 4 (6%) (toothpastes = 3; lipsticks = 1) showed a gluten level >20 ppm (toothpastes: 20.7, 31.4, and 35 ppm; lipstick: 27.4 ppm). None of the selected products had ingredient derived from wheat, barley, or rye.

Conclusions: Gluten contamination is currently not an issue in a wide array of cosmetic and oral hygiene products that are commonly on the market.

Key Words: celiac disease, cosmetics, lipsticks, oral hygiene products, toothpaste

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What Is Known

- A complete adherence to gluten-free diet is difficult to achieve for celiac patients.
- Oral hygiene and cosmetic that are applied on the mouth may contain gluten, and, by swallowing, may contribute to a persistent intestinal damage.
- No studies specifically assessed the gluten content of these products.

What Is New

- Gluten contamination is very low in oral hygiene and cosmetics applied on the mouth.
- The amount of gluten that could be ingested by teeth hygiene or by applying lipsticks/lip-balms is almost negligible.
- Based on our findings, gluten contamination in oral hygiene and cosmetic applied on the mouth is not an issue for celiac patients.

Celiac disease (CD) is a systemic immune-mediated disorder caused by the ingestion of gluten in genetically susceptible persons. It is one of the most common lifelong disorders, affecting approximately 1% of the population in Europe and North America (1,2). The only available treatment for CD is the gluten-free diet (GFD), which consists of the dietary exclusion of grains containing gluten (ie, wheat, rye, barley, triticale, semolina or durum wheat, spelt, and kamut) (3). Despite the well-recognized efficacy of the GFD, a complete adherence to the diet is very difficult to achieve, with several studies reporting an incomplete compliance in up to 50% of celiac patients (3,4). The protracted ingestion of traces of gluten (10–50 mg on a daily basis) may damage the integrity of the small intestinal mucosa (5). In a recent study, the average inadvertent exposure to gluten by CD individuals on a GFD was estimated to be ~150 to 400, with a high proportion of CD patients, routinely ingesting more than 200 mg of gluten per day (6).

By combining the toxicity threshold data with the observed food intake, it has been calculated that gluten-free products with <20 mg/kg (or parts per million = ppm) of gluten contamination are safe over a wide range of daily consumption (7). This maximum tolerable amount of gluten contamination (<20 ppm) is recognized by Codex Alimentarius and also recommended by the US Food and Drug Administration (FDA) and European Food Safety Authorities (EFSA) (8–10).

Gluten is not only used in human foods. Personal care products or toiletries (toothpaste, mouthwashes, soap, shampoo, etc) and cosmetics (lipsticks, lip balms, lip gloss, lip balms, face

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AQ5 Carlo Catassi and Elena Lionetti are scientific consultants to Dr Schär's. The remaining authors declare no conflict of interest.

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powder, body lotions, etc) may contain gluten in different forms (thicker and preservatives) or gluten-derived ingredients (11–13). Although such products should not be ingested, some amounts of these products can inadvertently be swallowed, particularly in the form of lipsticks or lip balms and toothpastes, thus leading to the possible ingestion of gluten traces (in the case of contamination) and eventually contributing to a persistent intestinal damage in CD patients. To the best of our knowledge, there is a lack of studies specifically assessing the gluten content of oral hygiene products and cosmetics, and therefore the safety of these products for patients affected with CD is unknown.

In the present study, we evaluated the gluten content of several oral hygiene products and cosmetics, widely available in the Italian market, by using the only laboratory method (R5 ELISA) for quantification of gluten that is approved by the EU regulation.

METHODS

Collection of Products

Overall 66 oral hygiene products and cosmetics were purchased from popular Italian supermarkets (Coop Italia, Si supermarket, tigre Amico, Tigota, and KIKO Milano) and pharmacies of Ancona (Italy) by the same author (A.K.V.), between February and March 2018. For the selection of products, priority was given to products from the largest producers and most popular among public. Product details were documented in an excel sheet reporting the type of product, gluten content information, weight, ingredients, and so on (see Table, Supplemental digital content 1, *http://links.lww.com/MPG/B474*). Each product was given a unique code, and kept sealed until further investigations. Products were categorized as follows: toothpastes, dental tablets, mouthwashes, lip balms, and lipsticks. Each group was further divided into 2 subgroups: labelled gluten-free and unknown gluten content.

Determination of Gluten Content

All 66 products (oral hygiene and cosmetics) were analyzed for their gluten content by Ridascreen Gliadin sandwich R5 ELISA R-7001 (R-Biopharma, Darmstadt, Germany) at our Celiac Disease Research Laboratory, Department of Pediatrics, Marche Polytechnic University, Ancona, Italy.

Extraction of Gluten From Products

The R5 ELISA was performed as previously described (14). Eighteen products were analyzed per ELISA by the same operator (A.K.V.); each day of gluten extraction, a laboratory personal (E.F.) who was not actively involved in the R5 ELISA processing, randomly selected the products to be analyzed among those available and gave a momentary ELISA code to keep R5 ELISA operator unaware of the identity of the selected products. Five grams of toothpaste, dental tablets, lip balms, and lipsticks were measured and homogenized by a pre-washed (70% ethanol) and dried plastic spatula, 0.25 g sample from homogenized stock was measured in pre-labelled sterile tubes. On the other side, 0.25 mL of each mouthwash was measured and kept in the separate pre-labelled sterile tubes. Before and after each sample processing, laboratory balance and platform were wiped with 70% ethanol. For each sample, new spatulas were used and discarded before starting a new sample processing. In each tube 2.5 mL of Ridascreen cocktail solution (R-7006) containing detergents and β-mercaptoethanol as a reducing agent was added under a chemical hood, tubes were re-caped and sealed with paraffin films, well shaken and kept in water bath at 50°C for 40 minutes. Tubes were allowed to cool down after this incubation and each tube was treated with 7.5 mL of 80% ethanol. After 1 hour of continuous shake on a laboratory shaker, tubes were centrifuged at 2500*g* for 10 minutes, the supernatant was separated into fresh and sterile1.5 mL Eppendorf tubes and diluted in the ration of 1:12.5 ratio with provided sample dilution. Diluted samples were stored at room temperature until the ELISA was done.

Determination of Gluten Quantity

Pre-identified ELISA wells were selected for the test and samples and standards were added to the designated wells in duplicates and incubated for 30 minutes. Wells were washed three times with washing buffer before adding conjugate, another three washes were provided after conjugate and before substrate and chromogen treatment. At the final step, ELISA reaction was stopped and ELISA plate was read in ELISA reader (thermos scientific multiscanGO) at 450 nm of absorbance. Limit of quantification of Ridascreen gluten ELISA kit was as low as 2.5 ppm gliadin corresponding to 5 ppm of gluten.

Final evaluation of immunoassay was done on the basis of calibration curve drawn as per manufacturer's guidelines, gluten concentration was calculated as per Ridascreen guideline. Briefly, R5 ELISA initially calculated the gliadin concentration in μg/kg (ppb). This result was further multiplied by the recommended dilution factor of 500 obtaining value of gliadin in corresponding mg/kg (ppm). This value was then multiplied by 2 in order to obtain the final level of gluten concentration in products (gliadin usually represents 50% of the proteins present in gluten). Products quantified with gluten level >20 ppm were considered as gluten contaminated whereas products with <20 ppm of gluten were considered as gluten-free.

Quality Control

During the ELISA process recommended manufacturer's guidelines were followed. Samples and standard were run in duplicate in each ELISA. Recommended standard values matched for each ELISA test. Mean quantified value of ELISA was considered as a final result. Products quantified with gluten level ≥20 ppm were re-analyzed in next ELISA run.

The study was judged exempt from ethical review from the institutional review board of the Marche Polytechnic University (Ancona, Italy), as it does not involve people, medical records, human tissues, or animals.

RESULTS

Gluten Quantification in Study Products

Figure 1 shows the characteristics of all selected products. No information about gluten content was provided in any product except for 5 lip balms claimed as gluten-free without any certification.

Major ingredients in toothpaste, dental tablets and mouthwashes were fluorides, surfactants, antibacterial agents, remineralizes, glycerols, sorbitols, hydrated dextrates, sodium starch, glycolate, alcohol, edible oils, zinc, analgesics, betadine, hydrogen peroxide, colors, and flavorings. In lip balms and lipsticks major constituents noted were different waxes, mineral oils, silica, bismuth, sodium borosilicate, titanium dioxide, color, and additives. No ingredient from selected products was derived from wheat, barley, and rye.

Overall, out of the 66 selected products, 62 (94%) showed gluten level lower than 20 ppm (ie, within the accepted level of gluten content for gluten-free products), while 4 (6%) products were

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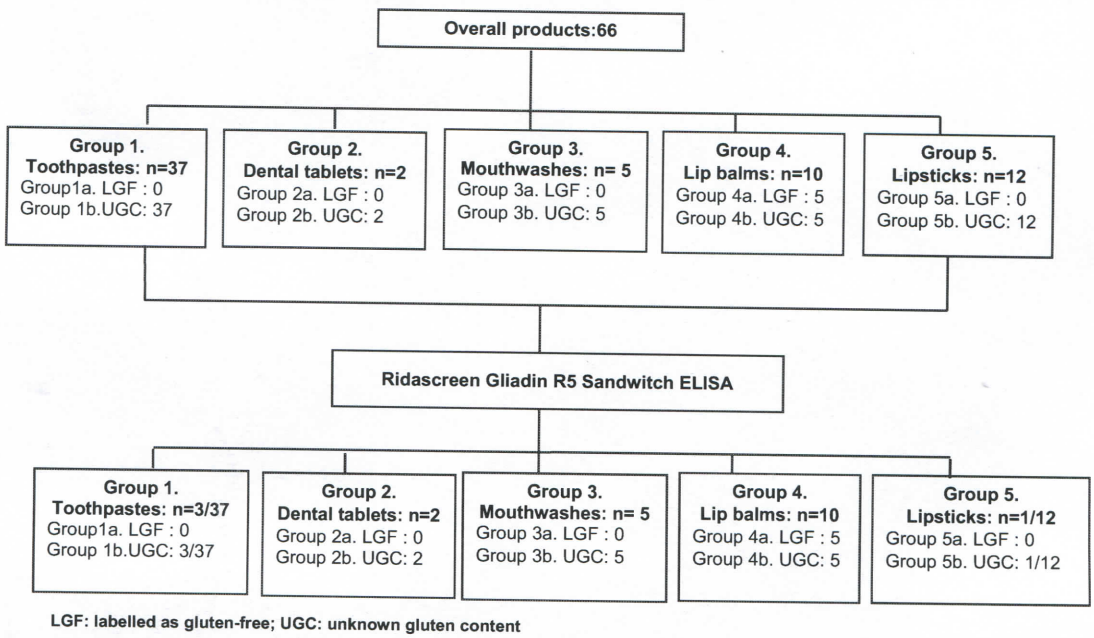

FIGURE 1. Type of products analyzed and their gluten content.

detected with gluten level higher than 20 ppm. In detail, among products of group 1 (toothpaste), 3 (4.5%) were found with gluten level >20 ppm (20.7, 31.4, and 35 ppm, respectively); products belonging to group 2, 3, and 4 (dental tablets, mouthwashes, and lip balms) presented a gluten level <20 ppm, while among products of group 5 (lipstick), 1 (1.5%) was found with a gluten level higher than the tolerated level (27.4 ppm).

DISCUSSION

Gluten might be directly added to cosmetic products or can be part of an ingredient such as wheat/barley extract, wheat germ oil, wheat germ glycerides, hydrolyzed wheat protein, and wheat starch (12). In general, gluten absorption through the skin has never been proven to be toxic for patients affected with CD, but traces of contaminating gluten might reach the oral mucosa, for instance, due to unwashed hands (11). On the other side, there is a range of oral hygiene products and cosmetics (eg, toothpastes, lip balms, and lipsticks) that are applied around or inside the mouth, allowing a higher risk of gluten ingestion. To the best of our knowledge, no data have been reported on the level of gluten contamination of commercially available toothpastes.

Only 2 previous studies evaluated gluten content in cosmetics so far (11,12). The first one was performed by using R5 ELISA on 6 products (4 lipsticks and 2 lotions) showing gluten content below quantifiable levels (11); the second one was performed on 36 cosmetic products by 3 different ELISAs, showing that gluten content varied according to the quantification method (in 6 cosmetics was 15.2 to 1453.5 ppm by one competitive ELISA, whereas 3 cosmetics contained 11.7 to 572.3 ppm gluten when quantitated with 2 sandwich ELISAs) (12).

In the present study, we primarily focused on the possible gluten contamination of products that can easily reach the mouth and eventually be ingested (ie, toothpaste, dental tablets, mouthwashes, lip-balms, and lipsticks). We analyzed a wide sample of products by using the R5 technique for gluten quantitation. To detect the specific toxic prolamin sequences of gluten there are indeed several immunoassays (ELISA Kits) based on different monoclonal antibodies (ab) such as R5, G12, and α-20 (15–17). Among all ELISA techniques, the R5 monoclonal antibody (mAb) based ELISA kit is the most suitable and accurate immunoassay to detect the gluten content. This R5 antibody mAb was developed from the pentapeptide form of rye fraction antibodies and is capable to detect the prolamins of wheat, barley, and rye. This is the only method endorsed by the Association of Official Analytical Chemists (AOAC), Codex Alimentarius Commission and other international agencies (8–10,18).

Our study found that gluten contamination was very low in oral hygiene and cosmetics products commonly available in the Italian market, both in terms of percentage of contaminated products (6%), and amount of gluten in the contaminated products (mean level 28.6 ppm, with 35 ppm as the highest gluten level). In dental tablets, mouthwashes, and lip-balms gluten level was within normal limits, (ranging from undetectable to 12.2 ppm), therefore they can be considered gluten-free. However, 3 (4.5%) toothpaste and 1 (1.5%) lipstick showed gluten quantity minimally higher than the tolerable limit (>20 ppm of gluten).

As for dentifrices, 0.25 g is the amount of paste that should be used for a single tooth cleaning, that is, a maximum of 1 g during a 24-hour period (19). Even allowing for a complete ingestion of the toothpaste, which is obviously an uncommon situation, this leads to an estimated 0.037 mg daily intake of gluten using the more contaminated toothpaste we tested in this study. Bearing in mind that the maximum tolerable daily intake of gluten by patients on treatment for CD is 10 to 50 mg (5), our data indicate that the amount of gluten that could be ingested by an accurate teeth hygiene is almost negligible. Although our findings are based on products acquired in Italy, nonetheless we believe they may be generalizable, being the majority of products analyzed in this study marketed all over the world.

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Worth noting, in our study none of the products was labelled as ''gluten-free'' and in the vast majority of them, there was no declaration of the gluten content, except in 5 lip balms (out of 10) that simply displayed a ''gluten-free'' identifier on the pack without any quantification or certification. This finding, therefore, points out the need to clarify the presence of gluten in the label of potentially ingestible body care products and cosmetics.

CONCLUSIONS

The present study showed that currently, gluten contamination is not an issue in a wide array of cosmetic and oral hygiene products that are on the market in Italy. Nevertheless, since all these products, particularly dentifrices and lipsticks, may contain gluten traces and may be ingested by patients on need to follow a strict GFD, it would be desirable that producing companies will report the information ''gluten-free'' or ''may contain traces of gluten'' on the package, therefore allowing a safe and informed choice by the celiac customer.

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Genetic biomarker for CD

Project 5

Validation of a novel single-drop rapid HLA-DQ2/-DQ8 typing method to identify subjects susceptible to celiac disease

Human Leukocyte Antigen (HLA)-DQ2 and/or -DQ8 is an essential risk-factor for CD. Presence of HLA-DQ2 and/or -DQ8 haplotypes, identified in the HLA class II histocompatibility system expressed on the surface of antigen-presenting cells (APC), mainly macrophages, dendritic cells, and B cells. Risk of CD is excluded if both genotypes are absent. [102]

The HLA-DQ2 heterodimer conferring susceptibility to CD is formed by an alpha chain encoded by HLA-DQA1*05 allele and a beta chain encoded by HLA-DQB1*02 allele (now defined as HLA-DQ 2.5).[198] These two HLA alleles (in either *cis* or *trans* position) are present in at least 90% of patients with CD.[199,200] The HLA-DQ8 heterodimer is found in 5% of CD patients and is formed by an alpha chain and beta chain encoded by HLA-DQA1*03 and HLA-DQB1*03:02, respectively.[199,201] However, HLA-DQ2 or HLA-DQ8 are expressed in almost 30-55% of the general population.[202,203] HLA-DQ2/-DQ8 typing is currently considered as an additional diagnostic test for CD, particularly useful in taking clinical decisions where there are discrepancies between celiac specific serological tests and intestinal mucosal biopsies and for the screening of 'at-risk' individuals. such as first-degree relatives (FDRs) of patients with CD, patients with type 1 diabetes, and patients with chromosomal diseases such as William's syndrome, Turner syndrome, and Down's syndrome.[204–209] Due to its high negative predictive

value, it is helpful in excluding the subjects requiring further investigations and may find application in both mass screening or case finding strategies.

In the recent European pediatric guidelines (ESPGHAN) for the diagnosis of CD, HLA typing is indicated as an adjunctive and an appropriate test to avoid the mucosal biopsies, in patients having symptoms and high titer of celiac autoantibodies (i.e. IgA anti-transglutaminase).[106]

Conventional HLA DQ typing method is very costly and tough to perfume. To make the HLA-DQ typing easy and inexpensive, we proposed a new-fangled, reliable and an unambiguous sequence-specific primer based rapid single PCR HLA-DQ typing method "Celiac Gene Screen" developed by BioDiagene S.R.L (Palermo, Italy). This quick HLA-DQ typing test isolates DNA in approximately 1 minute and total PCR (amplification of HLA-DQ alleles) run in 90 minutes. Celiac Gene Screen HLA-DQ typing kit delivers information on the presence/absence of HLA-DQ2 and/or -DQ8 alleles (yes/no) with no identification of all critical HLA alleles necessary to identify the CD risk heterodimers.

The aim of this study was to validate the accuracy of the "Celiac Gene Screen" by comparison of this new test with the conventional HLA-DQ alleles typing test (SSOP-PCR method).

During June 2017, rapid HLA-DQ typing method, "Celiac Gene Screen", was performed on the stored EDTA blood samples collected from the bio-repository of the Celiac Clinic of the Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, New Delhi, India. This study was approved by the Ethics Committee of the institution.

In the present study, we included overall 400 blood samples. For determining the diagnostic performance of the Celiac Gene Screen kit, we tested 100 blood samples in which HLA genotyping had already been characterized by the conventional SSOP-PCR with the Luminex-based One Lambda (LAB Type SSO Class II DQA1/DQB1 typing kit, CA, United States). In remaining 300 EDTA blood samples where HLA-DQ status had not been assessed previously, 141 samples were collected from patients with CD (having IgA anti tTG ab positivity and villous abnormality of modified Marsh grade 2 or more); 56 samples from first-degree relatives (FDRs) of patients with CD and 103 IgA anti-tTG negative samples (controls).

Of 100 blood samples with known status of HLA-DQ2 and -DQ8, 79 samples were HLA-DQ2 and/or-DQ8 positive and 21 samples were HLA-DQ2 and -DQ8 negative by the conventional SSOP HLA typing method. These 100 samples were re-genotyped by Celiac Gene Screen kit, all of 79 HLA-DQ2 and/or -DQ8 positive samples were positive and all the 21 HLA-DQ2 and -DQ8 negative samples typed negative by Celiac Gene Screen Kit as well. This shows an excellent concordance (100%) rate between HLA testing by SSOP HLA typing method and Celiac Gene Screen method. Among 300 samples with unknown HLA-DQstatus, 118/141 (84%) from patients with CD, 48/56 (86%) from FDRs of CD and 52/103 (50%) from controls typed positive for HLA-DQ alleles. **(Figure 11)**.

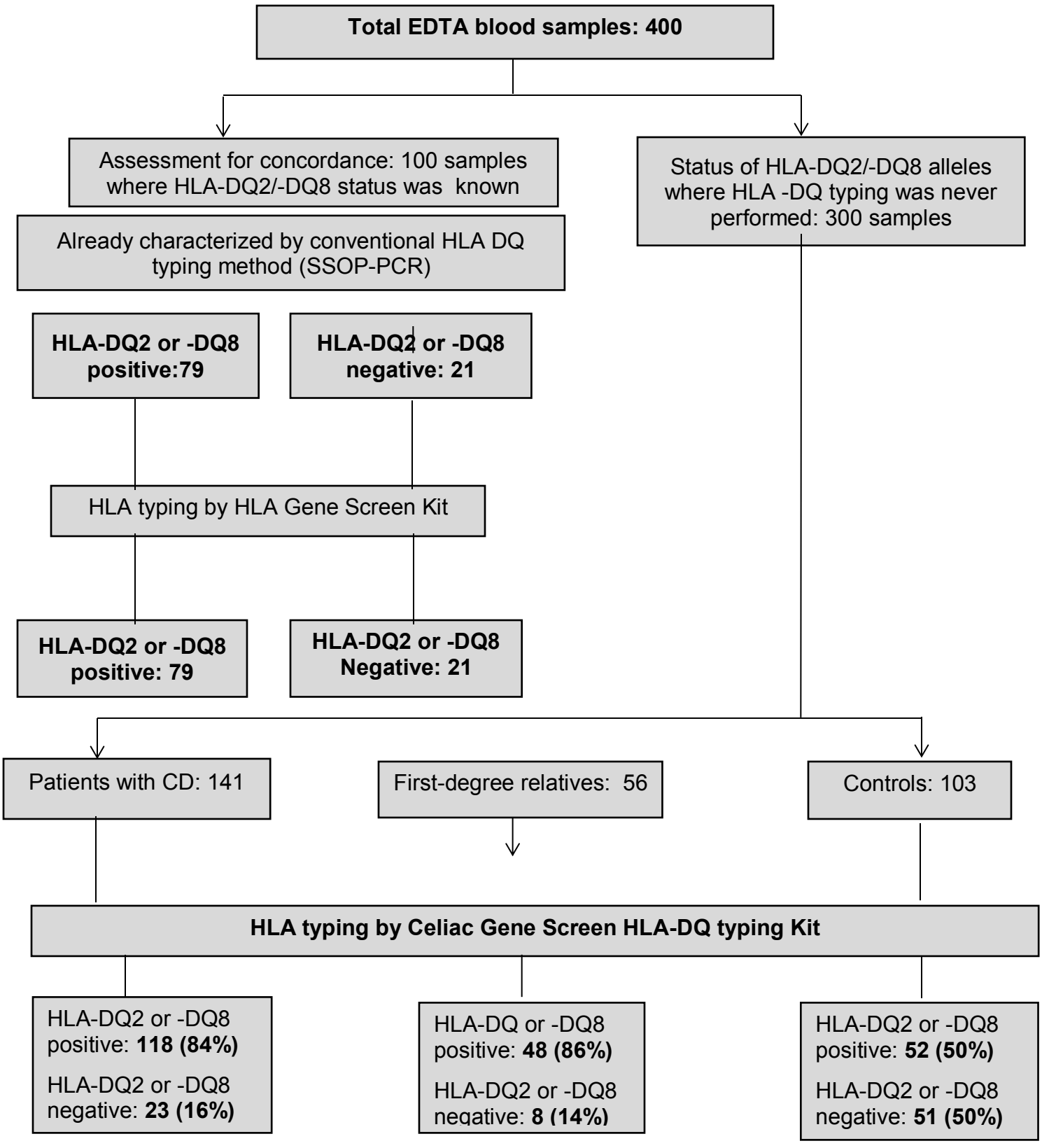

Figure 11: HLA-DQ2/-DQ8 status in the samples selected in the study

In conclusion, Celiac Gene Screen method showed an excellent concordance with the conventional HLA-DQ typing test (SSOP-PCR). This is a rapid, simpler to conduct and relatively less expansive method for specific and unambiguous detection of CD-associated HLA alleles. Celiac Gene Screen kit can be an effective tool for CD-associated HLA screening, that could be particularly useful whenever problems of budget limitations hamper the possibility to apply this important diagnostic method.

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ORIGINAL ARTICLE

Validation of a novel single-drop rapid human leukocyte antigen-DQ2/-DQ8 typing method to identify subjects susceptible to celiac disease

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Key words
alleles, celiac disease, genotyping, HLA, polymerase chain reaction, susceptibility.

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Declaration of conflict of interest: Elena Lionetti and Carlo Catassi are scientific consultants of Dr. Schär's. All other authors declare no conflict of interest.
Author contribution: Anil K Verma did the interpretation of data and statistical analysis. Anil K Verma and Alka Singh did the selection of biological material and performance of HLA-DQ typing. Carlo Catassi, Vineet Ahuja, Govind K Makharia designed the overall concept and supervised the study. Anil K Verma, Carlo Catassi, Govind K Makharia drafted the manuscript. Elena Lionetti, Simona Gatti, Tiziana Galeazzi, Chiara Monachesi, Elisa Franceschini, and Vineet Ahuja provided critical analysis of final outcome of the results and its analysis, and review of the manuscript. Carlo Catassi and Govind K Makharia provided administrative and financial support and final approval of the version to be published. All authors read and approved the final version of this manuscript.
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Abstract

Background and Aim: Human leukocyte antigen (HLA)-DQ2 and/or -DQ8 is an essential risk factor for celiac disease (CD). About 90–95% of patients with CD carry HLA-DQ2/-DQ8 alleles, and HLA-DQ typing is considered an additional diagnostic test. Conventional polymerase chain reaction (PCR)-based HLA-DQ typing methods are expensive, complex, and a time-consuming process. We assessed the efficacy of a novel HLA-DQ typing method, "Celiac Gene Screen," for the detection of CD-associated HLA haplotypes.
Methods: To assess the diagnostic performance of the Celiac Gene Screen test, 100 ethylenediaminetetraacetic acid (EDTA) blood samples, already characterized by the conventional HLA-DQ typing method, that is, PCR sequence-specific oligonucleotide probes (PCR-SSOP), a concordance between both the methods were explored. For validity, a further 300 EDTA blood samples with unknown HLA-DQ status were genotyped using the Celiac Gene Screen test, including 141 samples from CD, 56 first-degree relatives (FDRs) of CD and 103 samples from controls.
Results: Of the 100 samples with known status of HLA-DQ alleles, 79 samples were HLA-DQ2 and/or -DQ8 positive, and 21 samples were HLA-DQ2 and/or -DQ8 negative by conventional PCR. These 100 samples were re-typed using the Celiac Gene screen kit; all 79 positives were typed positive, and 21 negatives were typed negative for HLA-DQ alleles. Among 300 samples with unknown HLA-DQ status, 118 of 141 (84%) patients with CD, 48 of 56 (86%) FDRs of CD, and 52 of 103 (50%) controls typed positive for HLA-DQ alleles.
Conclusions: The Celiac Gene Screen HLA-DQ typing method showed excellent concordance with the conventional HLA-DQ typing method and could be a cost-reducing and effective method for CD-associated HLA screening.

Introduction

Celiac disease (CD) is a chronic inflammatory condition of the small intestine, initiated by the ingestion of dietary gluten peptides (contained in wheat, rye, and barley) in genetically susceptible individuals.[1] The presence of HLA-DQ2 and/or -DQ8 haplotypes, identified in the HLA class II histocompatibility system expressed on the surface of antigen-presenting cells (APC), mainly macrophages, dendritic cells, and B cells, is considered as a necessary risk factor for the development of

CD. Risk of CD is excluded if both genotypes are absent, with nearly 95% confidence.[2]

In a recent systematic review and meta-analysis, we observed that 1.37% of the global population is seropositive for CD, and 0.68% of the global population is estimated to have biopsy-confirmed CD.[3] Furthermore, various landmark studies have reported a worldwide increase in the prevalence of CD in the past few decades.[3–7] While CD was thought to be uncommon in India, in a pan-India population-based study involving more than 23 331 subjects, we observed that 0.68% of Indians have either potential CD or CD.[8] While a large number of subjects is expected to have CD, the majority still remains undiagnosed.[5]

The HLA-DQ2 heterodimer associated with the susceptibility to CD is formed by an alpha chain encoded by the HLA-DQA1*05 allele and a beta chain encoded by the HLA-DQB1*02 allele (now defined as HLA-DQ 2.5).[9] These two HLA alleles (in either *cis* or *trans* position) are present in at least 90% of patients with CD.[10,11] The HLA-DQ8 heterodimer is found in 5% of CD patients and is formed by an alpha chain and beta chain encoded by HLA-DQA1*03 and HLA-DQB1*03:02, respectively.[10,12] However, HLA-DQ2 or HLA-DQ8 are expressed in almost 30–55% of the general population.[13,14] HLA-DQ2/-DQ8 typing is currently considered as an additional diagnostic test for CD, particularly useful when taking clinical decisions where there are discrepancies between celiac-specific serological tests and intestinal mucosal biopsies and for the screening of "at-risk" individuals, such as first-degree relatives (FDRs) of patients with CD; patients with type 1 diabetes; and patients with chromosomal diseases such as William's syndrome, Turner syndrome, and Down's syndrome.[15–20] Due to its high negative predictive value, it is helpful in excluding the subjects requiring further investigations and may find application in both mass screening or case finding strategies. A combination of different serological tests (e.g. IgA anti-transglutaminase, anti-endomysial ab) with HLA DQ-2/-DQ8 genotyping can strengthen the serological test result and could minimize the burden of unnecessary duodenal biopsies.[21] In the recent European pediatric guidelines (European Society for Pediatric Gastroenterology Hepatology and Nutrition [ESPGHAN]) for the diagnosis of CD, HLA typing is indicated as an adjunctive and an appropriate test to avoid the mucosal biopsies in patients demonstrating symptoms and a high titer of celiac autoantibodies (i.e. IgA anti-transglutaminase).[22]

There are several HLA-DQ typing approaches currently available, such as polymerase chain reaction-sequence-specific primers (PCR-SSP), PCR-sequence-specific oligonucleotide probes (PCR-SSOP), real-time PCR (RT-PCR), and microarray.[2] Commercially available kits used to perform these tests are expensive and time consuming, and their methodologies are complex, requiring a well-trained technician to perform the test. Duration of analysis, costs, and availability of these tests limit their widespread use in the clinical practice. There is an unmet need for an accurate, quick, easy, and affordable method to perform HLA-DQ typing.

To make HLA-DQ typing easy and inexpensive, we proposed a new-fangled, reliable, and unambiguous sequence-specific primer-based rapid, single PCR HLA-DQ typing method, "Celiac Gene Screen," developed by BioDiagene S.R.L (Palermo, Italy). This quick HLA-DQ typing test isolates DNA in approximately 1 min and runs total PCR (amplification of HLA-DQ alleles) in 90 min. The Celiac Gene Screen HLA-DQ typing kit provides information on the presence/absence of HLA-DQ2 and/or -DQ8 alleles (yes/no), without identification of all critical HLA alleles necessary to identify the CD risk heterodimers.

The aim of this study was to validate the accuracy of the "Celiac Gene Screen" through a comparison of this new test with the conventional HLA-DQ alleles typing test (SSOP-PCR method).

Methods

During June 2017, the rapid HLA-DQ typing method, "Celiac Gene Screen," was applied to the stored EDTA blood samples collected from the biorepository of the Celiac Clinic of the Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, New Delhi, India. This study was approved by the Ethics Committee of the institution.

Selection of blood samples. In the present study, we included a total of 400 blood samples that had been collected in EDTA blood collection vials on earlier occasions and were stored in the biorepository of our institution. These samples had been collected during a community prevalence study that was conducted earlier and also from patients with CD being followed up at our center. Written and informed consent was requested from all the participants during the respective studies. To determine the diagnostic performance of the Celiac Gene Screen kit, we tested 100 blood samples in which HLA genotyping had already been characterized by the conventional SSOP-PCR with the Luminex-based One Lambda (LAB Type SSO Class II DQA1/DQB1 typing kit, CA, USA). In the remaining 300 EDTA blood samples where HLA-DQ status had not been assessed previously, 141 samples were collected from patients with CD (having IgA anti-tTG ab positivity and villous abnormality of modified Marsh grade 2 or more), 56 samples from FDRs of patients with CD and 103 IgA anti-tTG negative samples (controls).

The procedure of the Celiac Gene Screen kit. The Celiac Gene Screen kit involves three major steps, including preliminary lysis of EDTA blood sample, DNA amplification (one reaction/test), and fluorescence detection using a BioRun Reader. The total procedure time of this test to obtain the final result is less than 2 h. During the HLA-DQ typing procedure, manufacturer's guidelines were strictly followed. Major steps of the procedure are described below.

Step 1: lysis of blood samples collected in EDTA tubes. Tubes containing EDTA blood samples were allowed to thaw at room temperature and vortexed gently. For each blood sample, 200 µl of extraction buffer (provided with the Celiac Gene Screen kit) was poured in a 1.5-ml Eppendorf tube. Ten microliter of EDTA blood was mixed with the extraction buffer and incubated at room temperature for 1 min. This step facilitated quick DNA isolation. Of this lysate, 2 µl was used further for the DNA amplification.

Step 2: amplification of HLA-DQ alleles. In 0.2-ml PCR tubes provided with the Celiac Gene Screen kit, containing dried primers/probes for a housekeeping gene (internal control) and the target alleles, 18 μl of ready-to-use Taq mix (provided with the kit) and 2 μl of isolated DNA were mixed by pipetting. PCR tubes were vortexed for about 15 s and were then placed into a thermocycler. After 90 min of PCR run, tubes were ready for use in the analysis.

Step 3: detection of HLA-DQ alleles. PCR tubes were placed in the BioRun Reader. In-built software analyzed all the data at once and gave the interpretation in 10–20 s. The Celiac Gene Screen identifies the alleles DQB1*02 codifying for the beta chain of the DQ2 antigen and the DQB1*03:02 alleles codifying for the DQ8 beta chain. In each BioDiagene PCR tube, two fluorescence probes corresponding to primers were also coated, one that recognized the housekeeping gene and the second one that was perfectly complementary to that of the target DNA. The tubes were excited with two well-defined wavelengths. The increase in the reported fluorescent signal was directly proportional to the number of amplicons generated. Therefore, depending on the fluorescence value in the tube, the BioRun Reader reported the presence or absence of CD-associated alleles in the sample.

Statistical analysis. Quantitative variables were summarized as the mean ± SD; 95% confidence interval (CI) was calculated using the Software Program Stata System (SPSS) IBM version 25, Chicago, USA.

Results

Concordance of Celiac Gene Screen Kit with conventional HLA-DQ2 and -DQ8 typing method. Of 100 blood samples (mean age ± SD: 25.3 ± 11.1 years; 40 males) with a known status of HLA-DQ2 and -DQ8, 79 samples (mean age ± SD: 24.5 ± 10.7 years, 31 males) were HLA-DQ2 and/or -DQ8 positive, and 21 samples (mean age ± SD: 28.4 ± 12.5 years; 9 males) were HLA-DQ2 and -DQ8 negative by the conventional SSOP HLA typing method. These 100 samples were regenotyped using the Celiac Gene Screen kit, and all of the 79 HLA-DQ2 and/or -DQ8 positive samples were positive, and all the 21 HLA-DQ2 and -DQ8 negative samples were typed negative by the Celiac Gene Screen Kit as well. This shows an excellent concordance (100%) rate between HLA testing using the SSOP HLA typing method and the Celiac Gene Screen method (Fig. 1).

HLA DQ status in CD samples. Of 300 samples with unknown HLA-DQ status (mean age ± SD: 30 ± 14.7 years; 145 males), 141 blood samples (mean age ± SD: 30 ± 14 years; 68 males) were from patients with CD. Of these, 118 (84%, 95% CI: 78–90) samples (mean age ± SD: 26.2 ± 13.5 years; 57 males) showed HLA-DQ2 and/or -DQ8 positivity, and 23 (16%, 95% CI: 10–22) samples (mean age ± SD: 30.7 ± 14.1 years; 11 males) were HLA-DQ2 and -DQ8 negative (Fig. 1).

HLA DQ status in FDRs of patients with CD. Of 56 samples (mean age ± SD: 27.4 ± 13.9 years; 33 males) from FDRs, 48 (86%, 95% CI: 85–87%) samples (mean age ± SD: 28.1 ± 14.4 years; 32 males) tested positive for HLA-DQ2 and/or -DQ8. On the other hand, eight (14%, 95% CI: 5–23) samples (mean age ± SD: 23.2 ± 10.8 years; 1 male) were HLA-DQ2 and/or DQ8 negative (Fig. 1).

HLA-DQ status in control samples. In 103 samples (mean age ± SD: 38.4 ± 13.5 years; 44 males) from control subjects, 52 (50%, 95% CI: 40–60) samples (mean age 36.7 ± 13.2 years; 18 males) were HLA-DQ2 and/or -DQ8 positive, whereas the remaining 51 (50%, 95% CI: 40–60) samples (mean age ± SD: 40.3 ± 13.9 years; 26 males) were reported to be HLA-DQ2 and -DQ8 negative (Fig. 1).

Discussion

HLA-DQ genes (HLA -DQ2/-DQ8) play a primary role in the development of CD. Clinically, HLA-DQ genotyping does not provide a final diagnosis of the CD but indicates the necessary predisposition required for the development of CD.[23,24] Due to its high negative predictive value, the absence of HLA-DQ predisposing alleles makes the diagnosis of CD unlikely. Different combinations of HLA-DQ CD predisposing alleles also determine the level of risk of CD development. For instance, individuals presenting a double dose of the HLA-DQ B1*02 variant remain at high risk of developing CD in comparison to those expressing a single dose of the DQB1*02 allele.[25,26] At-risk subjects, such as FDRs of patients with CD; patients with other autoimmune diseases (type 1 diabetes); and individuals with Williams, Turner, and Down's syndrome showing HLA-DQ2 and -DQ8 negativity, particularly in Western countries, remain out of risk of developing CD on a lifelong basis.[16–20] A single HLA determination in such subjects may set them free from future surveillance and unnecessary follow-up in the clinic (repetitive serology, duodenal endoscopy/biopsy).[27]

Furthermore, a duodenal biopsy may be avoided in some subjects if one demonstrates that the patient has a genetic susceptibility to CD by showing the presence of HLA-DQ2/-DQ8 haplotype if they have a positive antiendomysial antibody (EMA) and high titer of antitissue transglutaminase antibody (IgA anti-tTG ab). However, the latest ESPGHAN guideline specifically recommends avoiding histological assessment in children and adolescents with signs or symptoms suggestive of CD with high anti-tTG ab titers with levels >10 times the upper limits of normal (ULN), supported by a positive EMA test, also with positive HLA-DQ2 and/or HLA-DQ8 alleles.[22] The ESPGHAN guideline also recommends HLA-DQ-2.5/-DQ8 typing in all type 1 diabetes mellitus patients for screening of CD and encourages the assessment of HLA-DQ2.5/-DQ8 genotyping as the first screening tool in asymptomatic individuals with a family risk of CD.[22]

Conventional HLA-DQ typing is an expensive method. Due to its high cost, HLA-DQ typing is not performed in many studies, and a considerable number of at-risk individuals do not undergo this test.[28] At our center (AIIMS) in New Delhi, HLA-DQ2/-DQ8 typing (high resolution) costs approximately 3000 Indian rupees (INR)/test (approximately US Dollar $46/test), while private laboratories in New Delhi offer this test at about

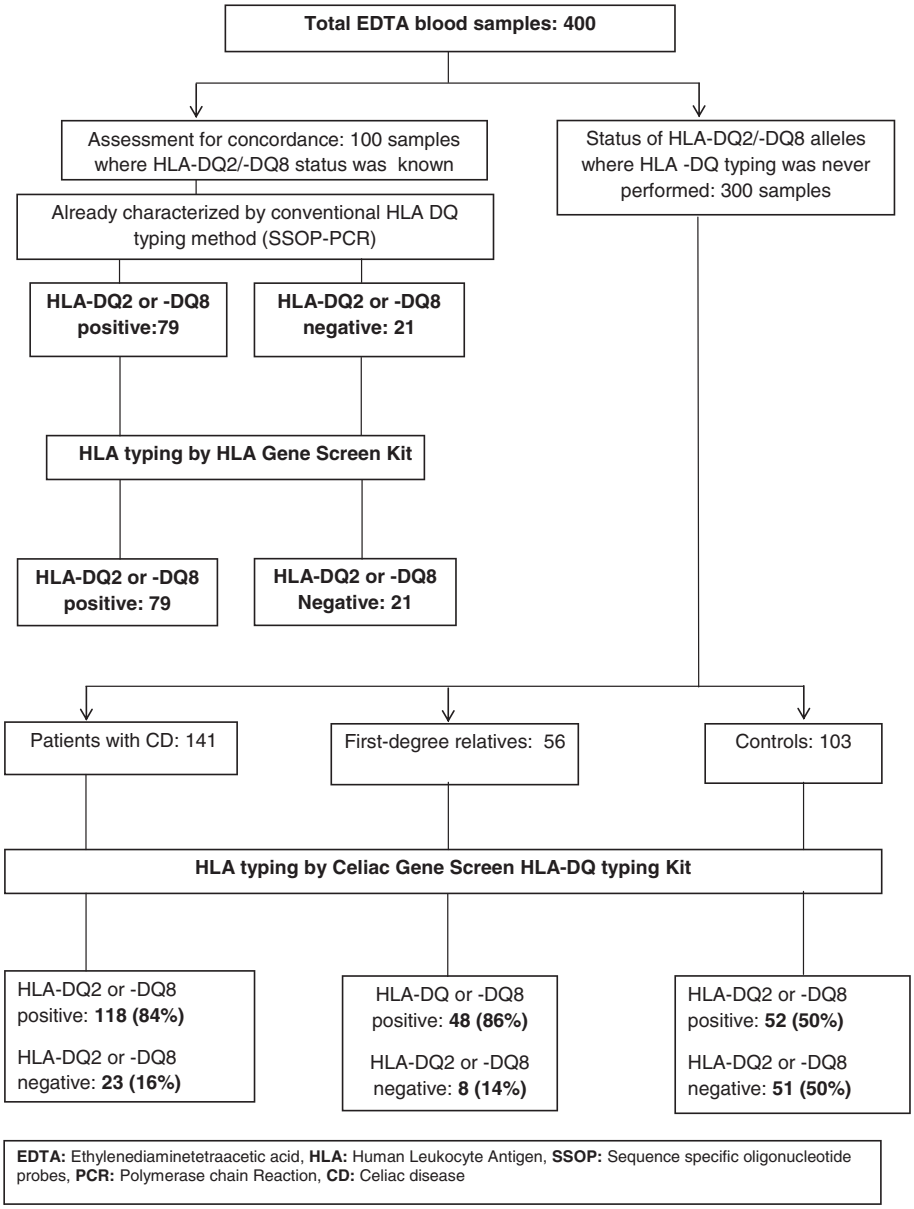

Figure 1 HLA-DQ2/-DQ8 status in the samples selected in the study. CD, celiac disease; EDTA, ethylenediaminetetraacetic acid; HLA, human leukocyte antigen; PCR, polymerase chain reaction; SSOP, sequence-specific oligonucleotide probes.

9000 INR/test (approximately USD138/test). In Italy, the cost of HLA genotyping varies from €46 to €180/test. In other parts of Europe, HLA-DQ typing costs €167–€326/test, and within the United States, in the nonprofit private medical center, HLA-DQ typing costs approximately USD 350/test.[29,30] The cost of HLA-DQ genotyping in our study was much lower (per test price by manufacturer, about €15, excluding all other laboratory costs). However, we wish to emphasize that the Celiac Gene Screen kit is a low-resolution test most suitable for CD-associated screening studies. It covers the set of critical HLA alleles necessary to identify the CD risk heterodimers (DQB1*02 and DQB1*03:02 alleles). An affordable price for HLA-DQ typing may increase the use of this test in developing countries, especially for CD screening studies, and may help physicians resolve many clinical dilemmas related to CD diagnosis.

In the present study, we compared the efficacy of a novel rapid HLA-DQ2 and/or -DQ8 typing kit ("Celiac Gene Screen Kit") with that of the conventional SSOP HLA-DQ typing method. We observed an excellent (100%) concordance rate in the reporting of two methods. We also determined the HLA-DQ status in blood samples collected from different groups (CD, FDRs of CD patients and controls) where HLA-DQ status was not known previously. In the present study, we observed that 84% of patients with CD were HLA-DQ2/-DQ8 positive compared with approximately 90–95% Caucasian CD patients in Europe and the United States.[10,11] The results of this study

match the observations in our previous population-based study, where we observed that about 76% of patients from the northern part of India detected to have CD were HLA-DQ2/-DQ8 positive.[8] There may be another HLA genotype in the rest of North Indian patients with CD. A high throughput HLA DQ allele characterization may identify the risk alleles for these 23 (16%) negative HLA but confirmed CD samples. However, these 23 CD patients who were typed negative for HLA-DQ alleles had a high titer of anti-tTG ab and had villous abnormality of Modified Marsh grade between 3a and 3c (Appendix S1, Supporting Information). One pediatric patient was exempted from duodenal endoscopy biopsy test as he or she had a significantly higher level of anti-tTG ab. In FDRs of CD patients and control subjects, the percentage of HLA-DQ2 and/or -DQ8 positivity was in line with other well-designed CD prevalence studies.[31–33]

Small sample size was a limitation of this study. A higher number of samples could have provided a more robust result. This test does not provide homo- or heterozygous status; instead, it only reveals the presence or absence of CD-associated alleles (HLA-DQ2/-DQ8). Nevertheless, this method can be useful in screening both individuals at risk of CD and the general population. The advantage of this test is to provide HLA-DQ2 and/or-DQ8 status quickly. Setting up this machinery in standard-level laboratories is feasible and quite easy. Unlike the conventional HLA-DQ typing method, this method does not involve complex steps and is not restricted to deeply trained and experienced laboratory personnel.

To make HLA-DQ typing easy, more than a few sincere efforts have been made to deliver a simpler but reliable HLA-DQ typing test. In recent times, several easy-to-perform commercial HLA-DQ typing kits have been introduced, claiming to be able to characterize celiac-associated genes (HLA-DQ genes) specifically, unambiguously, and exactly by using conventional HLA-DQ typing methods with overlapping sensitivity to the conventional ones. While these tests are easier than the conventional method, they require critical steps to be performed as DNA isolation has to be performed either manually (that takes several hours) or using a rapid kit-based method for DNA amplification (PCR run), and the amplicons have to be run on an agarose gel to read for interpretation of HLA-DQ status.[13,24,34,35] The celiac gene screen HLA-DQ typing test makes every step easier, from DNA isolation (few minutes) to PCR run (90 min), and results in interpretation (within minutes). Furthermore, to the best of our current knowledge, in comparison to conventional and other commercially available HLA-DQ typing methods we have explored, the Celiac Gene Screen HLA-DQ typing method costs less and is probably the easiest, quickest, and most economical and reliable method to identify celiac-associated alleles.[24,29,30]

In conclusion, the Celiac Gene Screen method showed excellent concordance with the conventional HLA-DQ typing test (SSOP-PCR). This is a rapid, easy-to-conduct, and relatively less expensive method for the specific and unambiguous detection of CD-associated HLA alleles. The Celiac Gene Screen kit can be an effective tool for CD-associated HLA screening, which could be particularly useful whenever problems of budget limitations hamper the possibility of applying this important diagnostic method.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Appendix S1 Clinical features of celiac disease patients, typed HLA-DQ2/-DQ8 negative using the Celiac Gene Screen method.

Project 6

Verification HLA-DQ2 and HLA-DQ8 alleles distribution in native South Indian Population

Introduction

At least 1% of the general population in Europe and North America is affected with CD.[49,50,52] However, the vast majority of the CD cases still remain undiagnosed. CD affects predisposed individuals, who carry specific genetic features identified in the HLA-II (human leukocyte antigen) histocompatibility system, HLA-DQ2, and HLA-DQ8 haplotypes, due to the ingestion of immune-dependent dietary peptide gluten found mainly in wheat barley and rye. About 90% of CD patients display the HLA-DQ2 heterodimer (formed by α chain and β chain encoded by HLA-DQA1*05 and HLA-DQB1*02 allele) and rest 10% of CD patients display HLA-DQ8 allele (formed by α chain and β chain encoded by HLA-DQA1*03 and HLA-DQB1*0302 alleles)[199]. Development of CD without expressing HLA DQ2/8 heterodimers is an extremely uncommon situation.

On the other hand, data from literature, confirmed by our group reported that 40% of the general population (Non-CD subjects) have either HLA-DQ2 or HLA-DQ8 positivity. In the past, CD was considered a rare disorder mostly affecting individuals of European origin. In 1978, Simoons et al. formulated a theory on the evolutionary history of the disease and postulated that the spreading of wheat consumption from the so-called

"Fertile Crescent" in the Middle East along the Mediterranean countries to northern Europe exerted negative selective pressure on genes predisposing to CD. The higher frequency of the HLA-B8 allele (at that time this was the only gene known to be associated with CD) and the resulting higher frequency of CD in north-western Europe were attributed to a lack of exposure to cereals until recent times.[210] In one of our previous study, we have found a significant correlation between wheat consumption and the frequency of the HLA-DQ2 and/or DQ8 worldwide.[211] We have documented that worldwide frequencies of HLA-DQ2 was 0-28% and HLA-DQ8 frequency was 1-9% that denotes that HLA-DQ2 frequency is much higher in comparison to HLA-DQ8 frequency.[211] However, recent data from Argentina and Chile estimated a higher HLA-DQ8 frequency in comparison to HLA-DQ2 allele frequency in the general population.[212–214] An unpublished work on HLA-DQ from south India has shown a high prevalence of HLA-DQ8 allele in native southern Indian population. The aim of this study was to verify the prevalence of HLA-DQ2 and HLA-DQ8 genotypes in the non-celiac native population of South India.

Methodology

For the conduct of the study, we have collaborated with the Department of Gastroenterology, Institute of Gastroenterology, Hepatobiliary Science and Transplantation, SRM Institutes for Medical Science, Vadapalani, Chennai, INDIA. A collaborative project was defined and the approved by the local ethical society (SRM Institute, INDIA).

Subjects

Recruitment of subjects was performed in the native south Indian population, originated from different south Indian states but living in the Vadapalani district of Chennai city (Tamilnadu) INDIA. Between July 2018 and August 2018, overall 211 healthy subjects (asymptomatic) with unknown HLA-DQ status, including adults (age range 19-85 years) and children (age range 10-18 years) were selected for the study. A drop of blood from middle finger was collected on protein saver cards (product code 903, GE health care Europe GmbH, Milano). The dried Blood Spot (DBS) cards were given a specific code and kept on room temperature. After the ending of the blood sampling process, the DBS cards were shipped to our laboratory, Celiac disease Research Laboratory, Ancona, Italy.

HLA-DQ typing :

HLA-DQ characterization was performed using Celiac gene screen real-time and DQ-CD Typing plus kit developed by BioDiagene SRL (Palermo, Italy). HLA-typing was done in two steps. All 211 samples were typed with Celiac gene screen real-time kit (step 1) to identify the HLA-DQ2 and/or HLA-DQ8 status (yes/no), all samples with positive HLA-DQ2/DQ-8 alleles were re-typed for complete celiac associated allele analysis and their homozygous/heterozygous status (step 2) using DQ-CD Typing plus kit. Steps of HLA-DQ typing are given below:

Processing of DBS cards

Two 3mm diameter discs were cut using the Harris Uni-Core pen, before and after every disc, the tip of the pen was washed with 5% of bleach solution and dried. One out of two cored samples (DBS disc) was transferred Into 96 well RT-PCR plate provided by the BioDiagene (to identify HLA-DQ2 and /or HLA-DQ8 status) and another disc was transferred into to 1.5 ml of sterile eppendorf tubes (to analyze alleles associated to CD). During this process cross-contamination between the discs were highly avoided.

Step 1: HLA-DQ typing for celiac associated genes (Celiac gene screen)

Celiac Gene Screen RT Kit is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains gene-specific primer and probe pairs for HLA-DQ alleles associated with CD and a housekeeping gene.

Lysis of blood sample using a dried blood spot card/DNA extraction

In 3mm DBS card disc prepared already was kept in individual 1.5 ml eppendorf tube. Two hundred microliters (µl) of lysis buffer was poured into each PCR tube and a vortex was provided and the PCR plate was incubated for 15 min at 70°C. After 15 minutes, when PCR-plate temperature maintained room temperature 180µl of 'Neutralization Solution' was added and the plate was vortexed. This lysate is ready for the final PCR reaction. Both the HLA-DQ typing kits used the same process of blood lysis.

Amplification of HLA-DQ genes using Real-time PCR (RT-PCR)

In each 0.5 ml PCR tube containing dried primers, 18µl of Taq Mix and 2 µl of DNA was added. A mixing through pipetting and vortexing was provided roughly about 10 seconds. This prepared tube was ready for amplification. The program of RT-PCT was set as per the manufacturer's guidelines.

Data Analysis / Interpretation of Results

The results were interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line. DNA HLA-DQB1*02 and HLA-DQB1*0302/0305 are detected on the FAM channel and IC on the HEX/VIC channel. Thus, genomic DNA samples positive for HLA-DQ alleles associated with CD as well as the Positive Control show amplification in both, the HEX and FAM channel. Negative samples show amplification in the HEX channel only. Fluorescent levels and corresponding amplification curves are automatically displayed in amplification plots in the real-time PCR software.

Step 2: Complete celiac associated gene typing (DQ-CD typing plus)

DQ-CD typing kit is designed to identify the HLA class II alleles associated with CD and for the direct identification of samples homozygous for DQB1*02 allele. This kit covers the alleles HLA II DQA1*0201, DQA1*03, DQA1*05, DQB1*02, DQB1*0301/04, DQB1*0302, DRB1*03, DRB1*04, DRB1*07, DRB1*11, DRB1*12. DQ-CD Typing Plus also identifies the risk of developing CD based on the targeted alleles **(Table 11)**. DQ-

CD Typing Plus foresees preliminary lysis of blood sample, DNA amplification (PCR) and agarose gel electrophoresis. For one patient sample, 8 PCR reactions (1 strip) were performed to carry out one complete gene test with the identification of HLA DQB1*02 homozygous status.

Amplification of HLA-DQ alleles

One dedicates DQ gene plus strip contains 8 tubes that contained Primer Mix (dried primers), per sample. The first tube is marked and contains primers for the DQA1*0201 allele. Considering one strip (8 tubes) were needed for one sample, the number of strips was determined, for each sample, 2 µl of DNA extract was poured into Taq Mixed tube (containing 180 µl of Taq Mix solution), This solution (Taq Mix and DNA) was mixed by vortexing. Fifteen µl of mix solution (Taq Mix and DNA) was pipette in each PCR tube for one strip per sample. The PCR-plate was vortex and kept in RT-PCR machine for the amplification with the temperature condition set by the manufacturer. After 2 hours of RT-PCR run, the tubes were ready for a read through agarose gel electrophoresis.

Gel electrophoresis

The working solution of TBE buffer was prepared by dissolving one pouch of TBE powder (provided by Biodiagen DQ-plus kit) in 1L of distilled water. Precast gel (provided by BioDiagen DQ-plus kit) was kept into electrophoresis chamber and the chamber was filled with TBE buffer. Four µl of PCR product was loaded into the gel well and the electrophoresis was performed at 100fV for 20 min. The system was stopped when bands were separated.

Interpretation of result

For each sample 8 PCR tubes were analyzed, one for each target allele. Each tube contained primers for the amplification of the internal control (visualized as a band of 796 bp) and for the target allele. Each reaction tube has two possible results:

1. Presence of two bands:

- A band with a high molecular weight (796 bp) corresponding to the control
- A band with a lower molecular weight corresponding to the presence of the target allele

2. Presence of one band:

- A band with a high molecular weight (796 bp) corresponding to the control; this result indicates that the sample tested does not carry the target allele.
- The last tube of the strip contains the primers for the identification of the HLA DQB1*02 homozygous status. The interpretation of the result obtained from this reaction tube is as follows:
    - Presence of both the internal control band (796 bp) and of the 111 bp band: a non-homozygous sample for DQB1*02 allele.
    - Presence of the internal control band (796 bp) and absence of the 111 bp band: sample homozygous for DQB1*02 allele.

Table 11:Celiac-associated alleles identified by HLA-DQ CD typing plus kit

Lane	1	2	3	4	5	6	7
Allele	**DQA1*0 201**	**DQA1* 03**	**DQA1* 05**	**DQB1* 02**	**DQB1* 0301/04**	**DQB1* 0302/0305**	**DQB1*02 homozygous status**
MW (bp)	170	183	186	205	250	119	111

Results:

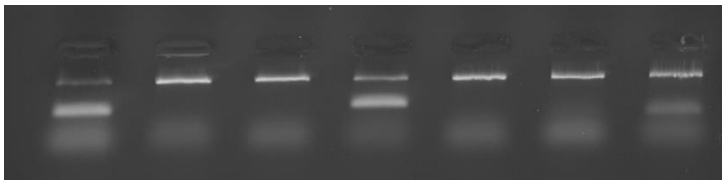
All 211 native south Indian general population blood spot samples received from adult gastroenterology division, Chennai (INDIA) were typed for CD-associated HLA-DQ alleles. Out of 211 blood spot samples, 207 samples were from a native population of Tamil Nadu state, 2 samples were formed native population of Andhra Pradesh state and rest 2 samples werefrom native population of Kerala State. Out of total 211 samples 187 samples were from healthy adults (mean age± SD: 33.5 ± 14.8 years, 66 males; 121 females) and 24 samples were from pediatric subjects (mean age± SD: 16.5 ± 2.53 years, 12 males; 12 females).

In the first step, of total 211 samples, 88 (42%) of samples were typed as HLA-DQ2 and/or -DQ8 positive that shows 42% of HLA-DQ prevalence in native south Indian general population. In the second step, all HLA-DQ2/8 positive samples (n= 88) were re-typed for complete celiac associated allele analysis through BioDiagen HLA-DQ typing plus kit. Details of celiac-associated alleles typed, and their status has been given in Error! Reference source not found.

Table 12:Celiac-associated alleles found in selected 88 HLA-DQ2/8 positive samples

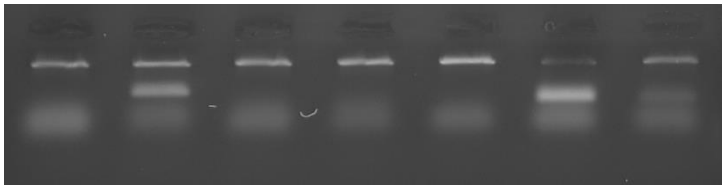
Subjects (N=88)	DQA1 *0201	DQA1 *03	DQA1 *05	DQB1 *02	DQB1 *0301/04	DQB1 *0302	DQB1*02 homozygous status	Haplotype
25	+			+			Heterozygous	DQ2/2.2/2.5
35		+				+		DQ8
5	+					+		DQ8
1		+		+			Heterozygous	DQ2.5
8		+			+			DQ8/7
14			+	+			Heterozygous	DQ2.5

Out of 88 samples, 40 samples were typed positive for HLA-DQ2 haplotype and 48 samples were typed positive with HLA-DQ8 haplotype that showed 19% prevalence of HLA-DQ2 and 23% prevalence of HLA-DQ8 in the native south Indian general population. Result of this study also shows a higher prevalence of HLA-DQ8 alleles in South Indian Native populations **(Table 12)**. Representative gel pictures have been given in **Figure 12 and Figure 13.**



Lane	1	2	3	4	5	6	12
Target Allele	**DQA1* 0201**	DQA1* 03	DQA1* 05	**DQB1* 02**	DQB1* 0301/0304	DQB1* 0302/0305	**Heterozygous Status DQB1*02**

Figure 12: DQ2.2 serological Haplotype Sample: Detected alleles: DQA1*02:01 – DQB1*02; heterozygous status for the DQB1*02 allele



Lane	1	2	3	4	5	6	12
Target Allele	DQA1* 0201	**DQA1* 03**	DQA1* 05	DQB1* 02	DQB1* 0301/0304	**DQB1* 0302/0305**	**Heterozygous Status DQB1*02**

Figure 13: DQ8 serological Haplotype Sample Detected alleles: DQA1*03 – DQB1*03:02/03:05;

Conclusions:

Results of this study indicate that unlike European countries where HLA-DQ2 prevalence is higher than HLA-DQ8 in the general population, in non-celiac south Indian native population HLA-DQ8 prevalence is higher than HLA-DQ2 prevalence. As we have already confirmed in our previous study, the possible reason could be the introduction of wheat in the diet. Most probably south Indian population had adopted wheat in their diet later. However, this study requires a large number of native south Indian as well as native north Indian sample to confirm this finding.

Future directions:

In this study, we have not included HLA-DQ typing of native North Indian blood samples. The data of HLA-DQ2 and HLA-DQ8 of north India was considered from the previous studies. We are in process of HLA-DQ characterization in native north Indian samples.

Project 7

Re-exploring the iceberg of celiac disease in children

Introduction: In our one of the landmark study, we aimed to assess the current prevalence of CD autoimmunity and overt CD in Italian school-age children by using HLA typing as the initial screening test and to redefine the clinical spectrum of the CD.

Methods. Children aged 5-11 years attending primary school in Ancona and Verona (Italy) were invited to participate in the CD screening, by determination of the HLA CD-predisposing genes (first-level), and total serum IgA plus IgA class anti-tissue transglutaminase (tTG) in children showing HLA positivity (second-level). Anti-endomysial antibody (EMA) was determined in cases showing anti-tTG positivity. The diagnosis of CD was performed according to the ESPGHAN criteria.

Results. Overall, 5,705 children were contacted and 4,570 children (80.1%) participated in the study. In the screened population, there were 23 cases of known CD (0.40%, 95% CI: 0.24-0.56%). Out of 4,570, 1,960 children showed CD-compatible HLA haplotypes (43%, 95% CI = 40.8-45.2). The prevalence of CD autoimmunity in the HLA positive subjects was 5.7% (95% CI = 4.6-6.8), and 54 children satisfied the diagnostic criteria for CD. The overall prevalence of CD in the screened sample was 1.77% (95% CI: 1.38-2.17). After adjustment for the different screening algorithm, CD frequency has more than doubled in Italian children during the last 25 years (from 0.76% to 1.77%), with 70% of cases still escaping diagnosis.

Conclusions. This study shows that the prevalence of CD in Italian children is now getting close to 2%. The reasons for the impressive prevalence raise in recent years need further investigations. Despite increasing awareness of CD clinical variability, a large proportion of cases still remains undiagnosed.

This study is now completed and under consideration for the publication in *Gastroenterology*

Project 8

The increasing prevalence of celiac disease: what is the role of an improved diagnostic accuracy?

Introduction

In recent years, population CD screening studies documented an increase in the prevalence of CD.[49,51,52,57,76] However, the contribution to this raising prevalence of an increased sensitivity of the diagnostic algorithm, based on the determination of serum IgA class anti-transglutaminase antibody (anti tTG ab) nowadays and IgA anti-native gliadin antibodies (AGA) during the '80-'90s, 6 has never been precisely estimated. The aim of this study was to compare the diagnostic performance of IgA anti-tTG with that of IgA AGA in a population CD screening study.

We investigated 138 sera samples from school children (5-10 years of age) participating in a CD screening project in Italy during the years 2015-2016. Forty-six of them showed positivity of both IgA anti-tTG and EMA (overt or potential CD) while 92 were IgA anti-tTG and EMA negative (non-CD controls).

IgA AGA was analyzed by a commercially available sandwich-type enzyme immunoassay (Eurospital, Trieste, Italy). All sera samples showing IgA AGA level > 5.5 arbitrary units (AU)/ml were considered positive.

Out of 46 untreated CD sera, 28 samples, from 10 males and 18 females, were IgA AGA-positive and 18 showed IgA AGA within normal limits. Out of the 92 control sera,

4 samples showed “false positivity” of the IgA AGA test, while the remaining 88 samples showed IgA AGA antibodies within normal limits **(Figure 14)**.

The sensitivity, specificity, positive and negative predictive values of the IgA AGA were 61%, 96%, 87.5%, and 83%, respectively. Based on IgA AGA results, the rate of missed CD cases in the screening project was 39%.

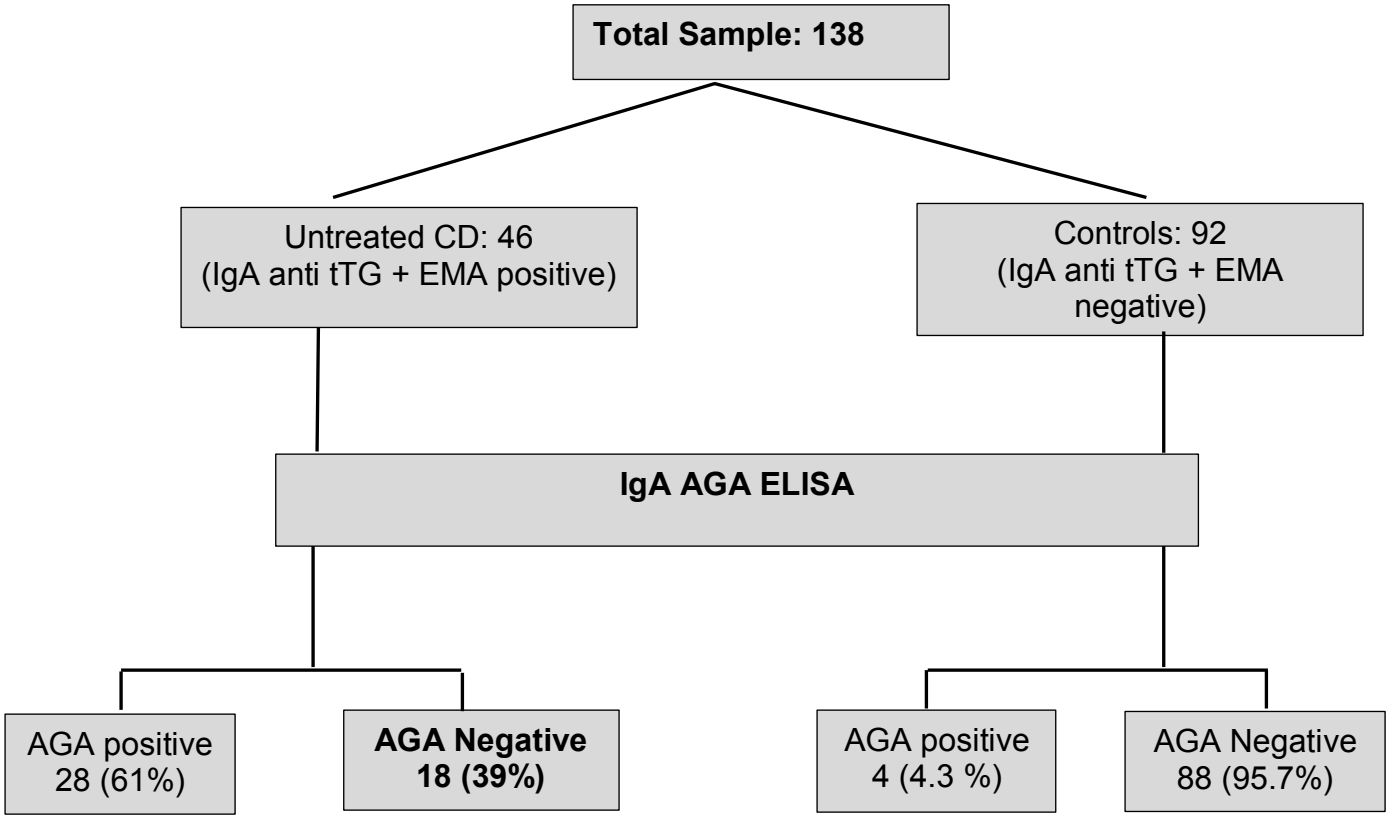


Figure 14: IgA AGA status in children with untreated CD and unaffected controls

CD: Celiac Disease; tTG: anti-transglutaminase antibodies; EMA: anti-endomysium antibodies; AGA: anti-native gliadin antibodies

This study definitively confirms the higher sensitivity of IgA anti tTG ab test in comparison to IgA AGA to detect CD autoimmunity in screening studies of the general population. These data suggest that the recently observed increase in CD prevalence is a complex phenomenon, partially related to a “true” increase explained by environmental changes (e.g. intestinal infections, the composition of intestinal microbiota, etc.), as also shown by longitudinal data in the US, and a “false” increase due to the higher accuracy of the current diagnostic algorithm.[55] Our work quantifies the contribution of this improved accuracy to the observed increase in the prevalence of CD.

Research Correspondence

Comparison of Diagnostic Performance of the IgA Anti-tTG Test vs IgA AGA Test in Detection of Celiac Disease in the General Population

Short Title: Serological screening of celiac disease

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Conflict of interest: A.K.V.,S.G.,T.G.,C.M.,E.F.,L.B.,N.S. and M.C. None to declare; **E.L. and C.C.** are scientific consultants of Dr. Schär's.

Author contributions: S.G., L.B., E.F., E.L. collected the biological samples and selected the matched controls. **A.K.V., T.G and C.M.** stored the biological samples and performed the antibody testing (IgA AGA ELISA). **N.S.** performed the antibody testing (IgA, IgA anti tTG ab, EMA); **A.K.V**. analyzed and interpreted the data and made the statistical analysis; **A.K.V., S.G., E.L. and C.C.** wrote and drafted the manuscript; **S.G., E.L., T.G., M.C., E.F. and C.C.** critically reviewed the manuscript; **C.C.** designed the overall study concept and gave administrative and financial support and supervised the study. All authors revised and approved the final version.

Introduction

Celiac disease (CD) is a systemic autoimmune disorder, characterized by a small intestine enteropathy, caused by the ingestion of gluten-containing cereals (wheat, rye and barley) in genetically predisposed individuals.[1] Almost 1% of the population in Europe and North America is affected with CD.[1-2] In recent years, population screening studies documented an increase in the prevalence of CD.[1-5] However, the contribution to this raising prevalence of an increased sensitivity of the diagnostic algorithm, based on the determination of serum IgA class anti-transglutaminase antibody (anti tTG ab) nowadays and IgA anti native gliadin antibodies (AGA) during the '80-'90s, [6] has never been precisely estimated. The aim of this study was to compare the diagnostic performance of IgA anti-tTG with that of IgA AGA in a population CD screening study.

Samples and Methods

We investigated 138 sera samples from school children (5-10 years of age) participating in a CD screening project in Italy during the years 2015-2016. Forty-six of them showed positivity of both IgA anti-tTG and EMA (overt or potential CD) while 92 were IgA anti-tTG and EMA negative (non-CD controls).

IgA AGA was analyzed by a commercially available sandwich-type enzyme immunoassay (Eurospital, Trieste, Italy). All sera samples showing IgA AGA level > 5.5 arbitrary units (AU)/ml were considered positive.

IgA anti-tTG and EMA antibodies were determined using commercially available kits, QUANTA Flash® h-tTG IgA (Inova Diagnostics, San Diego CA, USA) and Nova Lite® Monkey Esophagus (Inova Diagnostics, San Diego CA, USA), respectively. Total serum IgA

concentrations were determined using nephelometric technique and compared with cut off values for age.

Results

Out of 46 untreated CD sera, 28 samples, (61%, 95% CI: 47-75) from 10 males and 18 females (mean age ± SD: 8.4 ± 1.2 years), were IgA AGA positive and 18 (39%, 95% CI 25-53; from10 males, 8 females; mean age 8.5 ± 1.3 years) showed IgA AGA within normal limits. Out of the 92 control sera, 4 samples (4.3%, 95% CI 0.2-8.4) (all from females; mean age 9.1 ± 1.4 years) showed "false positivity" of the IgA AGA test, while the remaining 88 samples (95.7%, 95% CI 94-100, from 40 males and 48 females; mean age 8.3±1.1 years) showed IgA AGA antibodies within normal limits (Fig. 1).

The sensitivity, specificity, positive and negative predictive values of the IgA AGA were 61%, 96%, 87.5% and 83%, respectively. Based on IgA AGA results, the rate of missed CD cases in the screening project was 39% (95% CI: 25-53).

Discussion

Our study compares the diagnostic performance of the two serological tests mostly used in CD population screening, i.e. IgA native AGA (old studies) versus IgA anti tTG ab (recent studies). As for IgA AGA, a systematic review with metanalysis on the accuracy of antibody tests for CD diagnosis reported sensitivity ranging from 60.9 to 96%, significantly lower than that of IgA anti-tTG (73.9-100%).[7] However, all previous reports compared the diagnostic performance of CD antibodies in selected groups of patients, mostly subjects with symptomatic disease. This is the first study to investigate the diagnostic performance of these tests in a sample of the general population. According to our data, almost 40% of IgA tTG

positive celiac children were missed by a screening approach based on IgA AGA as the initial investigation.

Our study also found a significant proportion of "false positive" IgA AGA samples, in fact, 4.3% of the control sera samples (anti-tTG and EMA negative) showed IgA AGA positivity. Theoretically, these could be cases of non-celiac gluten sensitivity (NCGS). However, due to lack of specifically collected clinical information, we could not evaluate this hypothesis in our patients.

This study definitively confirms the higher sensitivity of IgA anti tTG ab test in comparison to IgA AGA to detect CD autoimmunity in screening studies of the general population. These data suggest that the recently observed increase in CD prevalence is a complex phenomenon, partially related to a "true" increase explained by environmental changes (e.g. intestinal infections, the composition of intestinal microbiota, etc), as also shown by longitudinal data in the US,[8] and a "false" increase due to the higher accuracy of the current diagnostic algorithm. Our work quantifies the contribution of this improved accuracy to the observed increase in the prevalence of CD.

Fig. 1: IgA AGA status in children with untreated CD and unaffected controls

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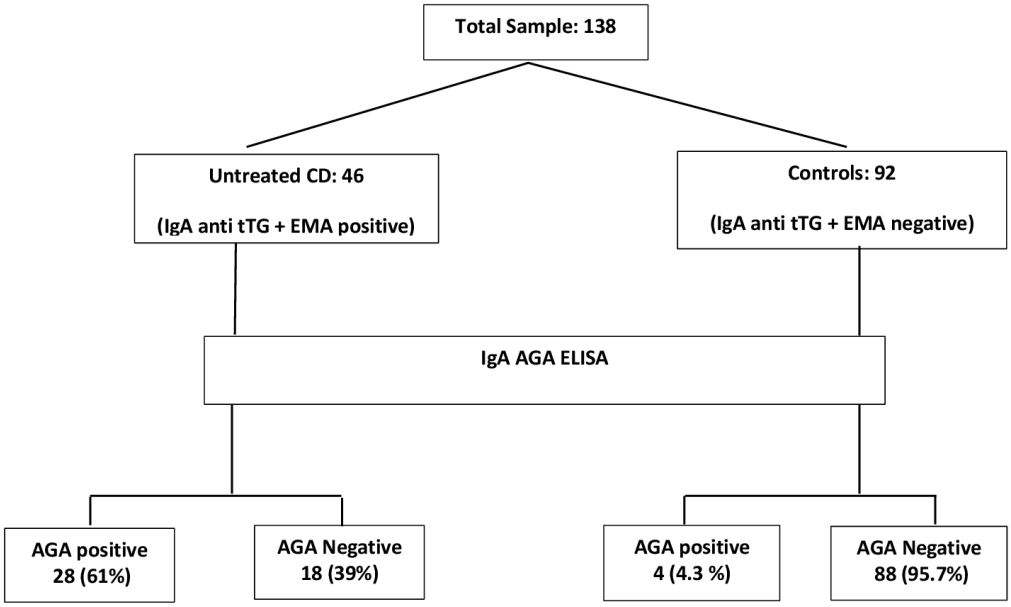

CD: Celiac Disease; tTG: anti-transglutaminase antibodies; EMA: anti-endomisium antibodies; AGA: anti-native gliadin antibodies

Project 9

Establishment of IgA EMA biopsy method

Introduction

Conventional IgA anti-endomysial antibody (EMA) method is used with blood sample which is the most specific and sensitive observer dependent diagnostic test used for the diagnosis for CD. Currently, an updated method has been introduced that allows performing the EMA test from the jejunal biopsy sample, which is even a highly specific test. We wish to establish this new method in our laboratory. For this purpose, **IgA anti-endomysium-biopsy kit (8726) were purchased from Eurospital Trieste, Italy**.

The IgA EMA biopsy method was established during July 2018 – August 2018 in our laboratory, Celiac Disease Research Laboratory, Department of Pediatrics, Marche Polytechnic University, Ancona, Italy. For the result interpretation, we have collaborated with **Dott. ssa. Barbara Cinti,** and her team, Laboratorio Analisi, Ospedale Riuniti (Torette) Ancona.

The principal of the test

The antiendomysium-biopsy kit is an indirect immunofluorescence assay on monkey esophagus sections for the detection of “in-vitro-synthesized” EMA from cultured bioptic fragments of the jejunal mucosa. The antiendomysium-biopsy kit is used for the detection of class IgA antibodies which are produced “in vitro” by human bioptic fragments. The antibodies are directed against endomysial antigens. After the addition

of a secondary fluorescein-labeled antibody, the antigen-antibody complex is visualized by means of an indirect fluorescence microscope.

Methodology:

The **Antiendomysium-biopsy kit** has provided crucial materials. Detail of the materials is given in **Table 13**. The Duodenal biopsy samples (3-3.5 mm diameter, equivalent to 40 mg) were obtained by endoscopy section, department of Pediatrics, Ospedale sales, Ancona Italy, and kept into a sterile saline buffer until the transfer into the culture medium. In case of testing, biopsy specimen was from a first biopsy (active phase), the specimen was kept into culture medium vial. If the testing specimen was the second biopsy (after a gluten-free diet, a disease in remission), the biopsy specimen was cut into two pieces having similar size and placed into two pre-labeled culture vials. In one culture vial, 50 µl of activating reagent was added. Culture vials were incubated for at least 72 hours at 37°C in an incubator.

On the day of slide preparation, EMA slide was kept on room temperature for serial minutes and under the laminar flow hood, 40 µL positive control was added on the first section of the slide. Then 40 µl of each culture medium from the previously incubated vials were added the remaining slide sections. Slides were placed in the moist chamber and incubate at room temperature at 15-30°C for 60 minutes. After this incubation, drops of the sample were removed by tilting the slide on a piece of absorbent paper, avoiding cross-contamination. By means of a squeezing bottle, slides were washed with a light flow of washing solution avoiding any cross-contamination. Slides were dipped in a container with the washing solution and incubated for 5 minutes at room temperature. After 5 minutes of washing mild shaking, washing solution was discarded and a fresh

washing solution was added in the container solution and again 5 minutes of wash was provided. After washing step slide spaces were dried using the perforated absorbent paper. Forty µL of the conjugate was added to each slide section and 30 minutes of incubation was give the slide under laminar flow hood. Again, a wash was provided exactly as first wash process. After the final wash, the mounting medium was added to each slide section and a cover glass was placed on the slide carefully avoiding any bubble on sections.

Table 13: Materials and their details provided with the Eurospital Antiendomysium-biopsy kit

S.No.	Material	Use
1.	Culture medium	For the incubation of the biopsy sample
2.	Glass Slide	Coated with monkey esophagus section
3.	Coverslip	To cover the prepared EMA slide
4.	positive control	To control the EMA process
5.	conjugate	Containing secondary fluoresces- -labeled the antibody
6.	activating reagent	To detect EMA (only for the patient who are on GFD)
7.	Mounting solution	Glycerol phosphate as a preservative
8.	Wash buffer	For washing slides

Slide interpretation by Indirect Immunofluorescence microscopy

EMA slides were examined under the fluorescence microscope using 20x - 40x magnifications. Culture medium showing interstitial positiveness of Muscolaris mucosa and Muscolaris externa was considered positive. Always use positive control as a reference. In the case of active-phase CD patients, the positive culture medium was observed due to the presence of newly synthesized antibody. Samples collected during the remission phase (CD patients already of GFD) showed antibody response only after stimulation by the activating reagent. Culture medium containing a fragment of the same origin but without activating reagent considered as no positive response. If a significant positiveness were seen without the activating reagent, it was considered as a minor diet mistake. Some representative slides of EMA result are given in **Figure 15.**

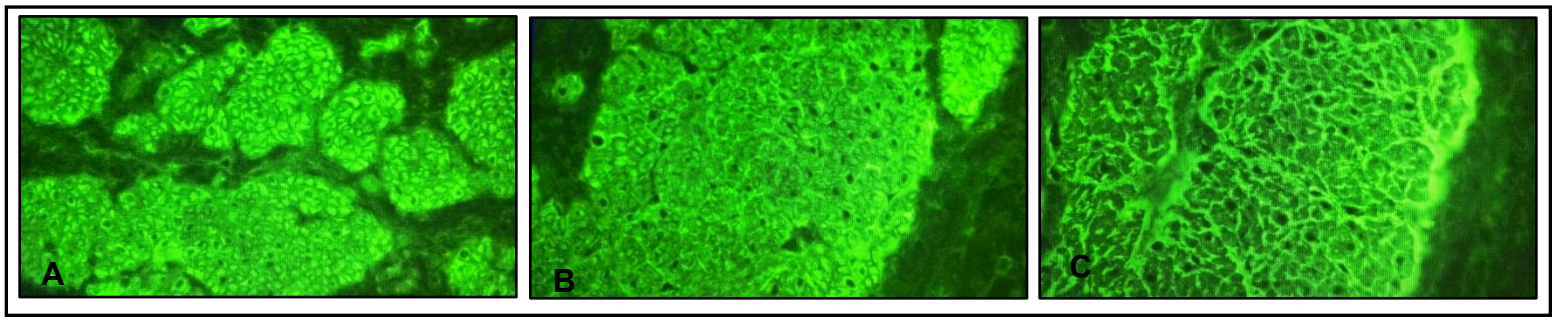

Figure 15: IgA EMA representative slides: (A) EMA negative slide; (B) EMA moderate positive slide; (C) EMA strong positive slide.

Collaborative Projects

Project 10

Non-immunological biomarkers for assessment of villous abnormalities in patients with celiac disease

Summary:

Villous atrophy is the hallmark of CD diagnosis and management that requires mucosal biopsies, that is an invasive procedure. Unfortunately, a non-invasive marker for assessing such atrophy is still not available. Therefore, I have collaborated for this study to assess few molecules which have been explored as a non-invasive marker of villous injury in patients with CD, included potential Biomarkers for this study were **Citrulline** (a synthetic marker for enteropathy), **i-FABP** (a marker for enterocytic injury) and regenerating gene **1α (Reg1α)** (a marker of enterocyte regeneration).

Levels of citrulline and i-FABP in plasma and Reg1 α in serum were estimated in treatment naïve patients with CD (n=131), non-celiac enteropathy (n=21) and controls (healthy controls, n =216 and disease controls, n=133). The expression of i-FABP and Reg1α were also checked in duodenal biopsies using immunohistochemistry and quantitative PCR. In order to validate citrulline synthesis in the intestinal mucosa, the expression of pyrolline-5-carboxylate synthase (P5CS), a rate-limiting enzyme in citrulline synthesis, was performed in duodenal biopsy by immunohistochemistry (IHC). Forty-six patients with CD and 12 patients with non-celiac enteropathies (NCE) were re-evaluated following six months of GFD and specific treatment of enteropathy, respectively. For further confirmation, the utility of above markers, a human model was selected having cycles of enterocyte injury and recovery such as patients with hematological malignancies receiving high-dose chemotherapy for HSCTs (n=70), their samples were obtained at various time points both before and after HSCT.

Levels of citrulline were estimated using high-performance liquid Chromatography (HPLC) by following standardized protocol. Estimation of i-FABP and Reg1 α was done by ELISA, as per manufacturer's protocol. Protein expression of P5CS, i-FABP, and Reg1α was done by IHC while gene expression by qPCR as per

standardized protocol. Statistical analysis was performed using Stata 11 software, StataCorp LP, Texas, USA.

Following objectives were proposed in this study

I. Establishment of normal levels of plasma citrulline, i-FABP, Reg1α and to establish if the level of plasma citrulline, i-FABP and Reg1α remain stable over 3 months in healthy volunteers.

II. To study the marker's synthetic functions of enterocytes

- To study the correlation between severity of villous abnormalities and plasma citrulline level.
- To quantify the level of proline-5-carboxylate synthase (P5CS) in the small intestinal biopsies and its correlation with plasma citrulline level

III. To study the marker of villous injury (release after an injury to enterocytes)

- Correlation between severity of villous abnormalities and serum intestinal-fatty acid binding protein (i-FABP) level
- To study the expression of the i-FABP gene in the small intestine of patients with CD and other chronic enteropathies

IV. To study the expression of Regenerating (Reg) gene (regulates expression of a gene) in the small intestinal mucosa

- Level of expression of Reg1α gene in the small intestine and its correlation with the severity of villous abnormalities
- Level of serum Reg1α protein in the serum and its correlation with the severity of villous abnormalities

V. Correlation between levels and expression of above-mentioned biomarkers in a model where there is a cycle of villous injury, villous recovery and again villous injury (patients undergoing high dose chemotherapy)

The mean concentration of citrulline and Reg1α in healthy individuals at week 0 and at week 12 were stable whereas i-FABP concentration fluctuated.

Moreover, the levels of citrulline in the plasma of patients with CD at baseline was significantly lower as compared with controls and the level get increased after 6 months of GFD in them. However, no significant difference in the concentration of plasma citrulline in patients with NCE compared with controls. However, in case of

tissue expressions, P5CS seemed to be declined in the mucosal biopsies of patients with CD compared to control which get increased after 6-months of GFD, no change in expression in patients with NCE.

i-FABP concentration was observed to be elevated significantly in patients with CD compared to control, the level declined after 6 months GFD. However, no significant change in i-FABP level between controls vs NCE. Increased and then decreased the concentration of Reg1α also followed the same pattern of i-FABP. The tissues expressions of i-FABP in the mucosal biopsies were predominantly low in patients with CD compared to control which get increased after 6-months of GFD. The same pattern was observed in tissues expression of Reg1α. However, there was no significant observation in patients with NCE compared to controls.

In a human model of enteropathy, sequential decrease and then increase in citrulline levels occurred, following a pattern of enterocyte injury and recovery, which corresponded to total leucocytes count in peripheral blood. However, we did not observe the expected cyclical pattern of i-FABP in the human model of enteropathy unlike that was seen with citrulline levels. Moreover, levels of Reg1α didn't follow any cyclical pattern, unlike citrulline.

After looking up the performance of abovementioned marker, we have calculated optimal cut-off values in order to discriminate patients with villous abnormalities from controls. The best cut off value for citrulline and i-FABP were found to be <30μM/L and ≥1100pg/ml, respectively. At the calculated cut-off values, citrulline and i-FABP were able to discriminate between normal and enteropathic mucosa with a sensitivity of 78% and 40% respectively and specificity of 95% each.

On the basis of consistent changes in the level of plasma citrulline in all above experimental groups along with changes in expression pattern of P5CS (supporting marker of citrulline), we have concluded that citrulline level estimation is the most reliable marker for predicting presence of villous abnormalities and its potential for avoiding biopsies in approximately 78% of subjects with 95% specificity.

Project 11

Gluten sensitivity in patients with cerebellar ataxia: A prospective cohort study

Cerebellar ataxia is a heterogeneous group of disorders which can be familial or sporadic. Sensitivity to gluten has been implicated in the pathogenesis of sporadic cerebellar ataxia. Since there is a paucity of data on the prevalence of gluten sensitivity in ataxia patients from Asian countries, we screened well-characterized progressive cerebellar ataxia patients for the presence of gluten sensitivity.

A cohort of 192 familial and sporadic patients with progressive cerebellar ataxia, were screened for the presence of gluten sensitivity. Four milliliters of blood was drawn from each consenting participant for estimating levels of IgA AGA, IgG AGA, IgA anti-TG2 Ab, and IgA anti-TG6 Ab to screen gluten ataxia. The detection of all markers in serum was performed using highly specific enzyme-linked immunosorbent assay (ELISA) kits. The data on their genetic testing for spinocerebellar ataxia (SCA) 1, 2, 3, 12, Friedreich's ataxia (FRDA), and brain imaging were reviewed. Individuals screened positive for either anti-tTG-Ab and/or AGA were invited for further evaluation including upper gastrointestinal endoscopic examination and duodenal biopsies. Diagnosis of CD was made on the basis of a combination of a positive anti-tTG Ab and biopsies showing villous abnormalities of Marsh grade 2 or more.

All the cases (N=192; M: F= 146:46) included in the study, manifested the features of the progressive cerebellar Ataxia. Out of 192 patients, 99 and 77 had sporadic and familial cerebellar ataxia, respectively. The genetic mutation for SCA 1, 2, 3, 12 and

FRDA was confirmed in 76 (40%) patients. Forty-two (21.8%) patients had either one or more serological test positive for gluten sensitivity; IgA-AGA (20/192; 10.4%), IgG-AGA (2/87; 2.3%), anti-TG2 Ab (1/141; 0.71%) and IgA anti-TG6 Ab (23/186; 12.3%). 20 of 32 (63%) seropositive patients had cerebellar atrophy on brain imaging. While we invited all the 42 patients detected positive for anti-tTG Ab, IgG-AGA or IgA AGA, only 10 agreed to undergo endoscopic examination and duodenal biopsies. None of these 10 biopsies showed any evidence of enteropathy. We also confirmed the age at disease onset and disease duration in patients with and without a positive test for gluten sensitivity, to see whether it affects the age or not. However, we observed there was no definite correlation between the age at the onset of the disease and presence of gluten sensitivity.

In conclusion, gluten-sensitivity related antibodies are present in Indian patients with cerebellar ataxia, both sporadic and familial, as well those with and without genetic mutations, although not as common as described from Europe. The available evidence, including results of the present study, suggests a role of gluten sensitivity in patients with cerebellar ataxia. Forty-two (21.8%) patients had either one or more serological test positive for gluten sensitivity Furthermore; gluten may not be causing gluten ataxia directly, the evidence support gluten to be a disease modifier.

International Exposure: Exposure In USA

Duration: October 2016 to April 2017

For a deep exposure of CD and other gluten-related disorders in deep and to know and learn advanced diagnostic techniques. I have been visited the state-of-art laboratory of **Professor Alessio Fasano** (Professor of Pediatric Gastroenterology and Nutrition and Director of the center for celiac research and mucosal immunology and biological research center, Massachusetts, USA) as visiting scholar.

I have been funded through **"Young researcher award"** by SIGENP organization and partially by **Università Politecnica delle Marche, Ancona, Italy.** During the six months of the visit I have learned the following techniques:

1. **Isolation of PBMC by density gradient centrifugation**
2. **Isolation of Neutrophils**
3. **Protocol cells staining for cytofluorimetric analysis**
4. **Isolation of DNA from stool samples (Power Soil DNA isolation from MOBIO)**
5. **Organoid Culture**

(See Appendix 1 for full detail)

Appendix 1

SIGENP "Young Researcher Scholarship Report" 2016-17

Six months research report conducted at the Mucosal Immunology and Biology Research Center, Massachusetts General Hospital, Boston (USA)

Duration: October 2016 to April 2017

Submitted by:

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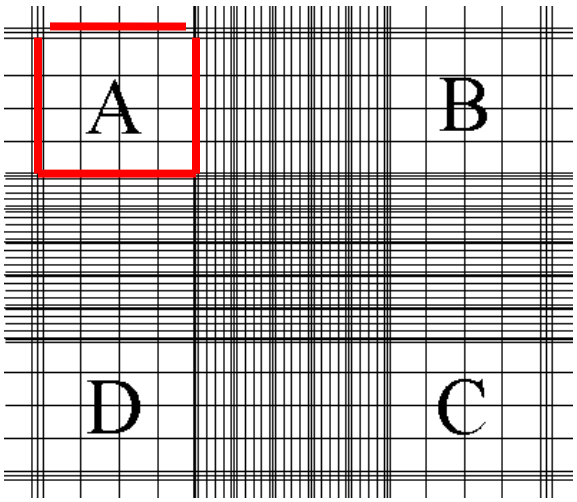
The following laboratory techniques have been learned with the guidance of **Dr. Gloria Serena.**

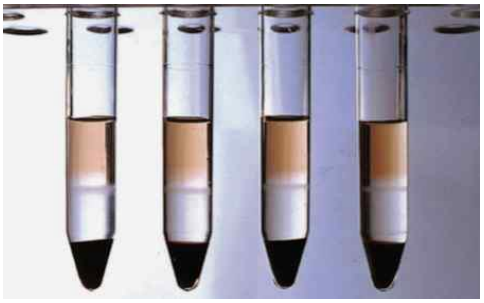
1. Isolation of PBMC by density gradient centrifugation

- Split blood in 50 ml falcon and add the same volume of PBS 1X to obtain a ratio 1:1 (for ex. If I have 25 ml of blood, I need 25 ml of PBS 1X)
- In another 50 ml falcon add Ficoll in a ratio 1:2 with blood + PBS 1X (for ex. If I have 50 ml tot of blood + PBS I need 25 ml of Ficoll)
- Add very carefully and slowly the blood + PBS 1X on the top of Ficoll (leaning horizontally the falcon with Ficoll).
- Centrifuge at 2100 rpm for 30' without brake
- Aspirate the yellowish ring containing the PBMC with a transfer pipette and transfer it in new 15 ml falcons. Add PBS 1X up to 15 ml.
- Centrifuge at 1200rpm for 10' with brake high.
- Discard supernatant (by quickly pouring it in the waste) and combine the cells from each tube together. Bring volume up to 15 ml.
- Count cells *
- Centrifuge at 1200 rpm for 10' with brake high and freeze cells

*Counting cells: 10ul of cells + 10ul of Trypan Blue in a 96 wells plate
Pipette 10 ul of cells in the hemocytometer.
Count cells in one of the square made of 16 squares. (A, B, C, D). You can count one or more and calculate the average.

FORMULA: ((N cells counted X 2)X 10^4)X N ml total





2. Protocol to freeze PBMC

- Centrifuge the cells for 10 minutes at 1200 rpm, high brake
- Discard the supernatant and resuspend the cells (between 5- 10 ^6 cells) in 500 µl of RPMI + P/S + L- Glu + 20% FBS and transfer them in cryovials. **IMPORTANT TRY TO NOT EXCEED 10* 10^6 CELLS per CRYOVIAL!!!!!**
- Add 500 µl of medium (RPMI + P/S + L-Glu + FBS 20% + 20% DMSO) already prepared and chilled on ice in each tube.
- Mix very well and chill in ice. Store in -80 over night and then store in liquid nitrogen. **IMPORTANT: CELLS SHOULDN'T STAY MORE THAN 3 MONTHS in the -80 freezer!!!!!** Store them in Liquid nitrogen as soon as possible.

3. Protocol to defreeze PBMC

- Take the vials from the liquid nitrogen tank and transfer them to ice. Warm them as soon as possible in a 37 degree bath and wait until the cells and medium are almost completely defrosted (check if they move rolling the vials)
- Transfer them to ice
- Transfer the cells to 15 ml tube (more or less 2 ml of medium with cells in each tube) and add culture medium (Complete RMPI + 10% FBS) up to 10-12 ml
- Centrifuge for 10 minutes at 1200 rpm
- Discard the supernatant and resuspend all the cells together in 2 ml of culture medium.
- Wash the tubes with other 2 ml of culture medium and transfer them to the tube with the cells.

4. Isolation of Neutrophils

- Prepare a bucket of ice
- Lysis buffer and HBSS without magnesium and calcium need to be cold
- Perform the isolation of PBMC protocol by density gradient centrifugation up to the Ficoll step
- Discard PBMC and fill ring + Serum
- Add cold lysis buffer up to 50 ml, flip gently the falcon and keep on ice for 10-20 mins until transparent
- Centrifuge 1500 rpm for 10 Min no break at 4ºC
- If the pellet is white proceed to *
- If the pellet is still red: discard the soveanatant and add cold lysis buffer up to 10 ml, flip gently the falcon and keep on ice for 5-10 mins, then proceed to *
  *
- Centrifuge at 1500 rpm for 10 min no break at 4ºC
- Discard the supernatant

Preparation of Lysis buffer

155mM NH_4CL - 1M= 53.49 g/L
155mM = 8.29 g/L or 4.14/500 ml

10 mM $KHCO_3$ - 1 M = 1g/L
10mM= 1g/L or 0.5 / 500 ml

0.1mM EDTA - Stock = 05M
2 ml EDTA /L buffer or 1 ml/ 500 ml

Prepare in deionized H_2O
Filter sterilized and stored at 4 ºC

5. Protocol cells staining for cyto-fluorimetric analysis

6.

- Prepare surface Ab master mixes in Brilliant Violet staining buffer (if not also FACS buffer works).
- Need 0.5 ul of Ab per well and a stain volume of 50 ul (ex: for 20 wells 6 ul of each Ab in 1ml of buffer).

_ # samples tot: MIX for # TOTAL VOLUME → _#__ ul/each Ab + __#_ ul of BUFFER

- Aliquot cells (we do 1*10^6 _ maximum 2*10^6) into 96 V-bottom plate (1 sample/well).
- Bring to 200 ul with FACS buffer.
- Spin 1,350 rpm brake high for 5 minutes and flick (throw away liquid but no tapping).
- Add 50 ul of Ab mix to each well.
- Incubate on ice or in the fridge fro 35-40 minutes.
- Wash by adding 150 ul FACS buffer to each well. Spin at 1350 rpm brake high for 5 minutes and flick.
- Resuspend cells in 200 ul of FIX/PERM buffer
- Wrap in aluminum foil and incubate in the fridge for 30-60 minutes.
- Prepare Ab/PERM buffer mix (0.3 ul Ab in 50 ul of PERM BUFF 1X per sample)
- Wash twice with 150 ul of PERM BUFFER 1X (1350 for 5 minutes) and flick.
- Add Ab/PERM buffer mix (50 ul/sample) and incubate in the fridge for 30-60 minutes.
- Wash with 150 ul PERM BUFFER 1X (1350 rpm for 5 minutes) and flick.
- Resuspend cells in 200 ul FACS buffer in the fridge until analysis.

SURFACE MARKERS:
CD16 PeCY5
CD303a
CD19 Alexa700
CD304 APCe780
CD11c BV421
CD123 BV605
CD8a BV711
CD56 BV786
CD4 BUV737

TF:
RORgT Alexa488
PLZF PE
FOXP3 PeCF594
GATA3 BUV395

7. Isolation of DNA from stool samples (Power Soil DNA isolation from MOBIO)

- Add 0.25 grams of stool or biosolid in Dry Bead Tube
- Add 750 µl of Bead Solution to the Dry Bead Tube. Gently vortex to mix.
- Check Solution C1. If Solution C1 has precipitated, heat solution to 60°C until dissolved before use.
- Add 60 µl of Solution C1 and invert several times or vortex briefly.
- Heat the tubes at 65°C for 10 minutes.
- Secure the bead tubes horizontally using the Vortex Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
- Centrifuge the tubes at 13,000 x g for 1 minute.
- Transfer the supernatant to a clean 2 ml Collection Tube. Expect between 400 to 500 µl of supernatant.
- Add 250 µl of Solution C2 and vortex briefly to mix. Incubate at 4°C for 5 minutes.
- Centrifuge the tubes at 13,000 x g for 1 minute.
- Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.
- Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
- Centrifuge the tubes at 13,000 x g for 1 minute.
- Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube do not transfer more than 750 µl at this step.
- Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.
- Load 650 µl of supernatant onto a Spin Filter and centrifuge at 13,000 x g for 1 minute. Discard the flow through and repeat until all the supernatant has been loaded onto the Spin Filter.
- Add 500 µl of Solution C5 and centrifuge for 1 minute at 13,000 x g.
- Discard the flow through.
- Centrifuge again for 1 minute at 13,000 x g.
- Carefully place the Spin Filter in a clean 2 ml Collection Tube. Avoid splashing any of Solution C5 onto the Spin Filter.
- Add 100 µl of Solution C6 to the center of the white filter membrane.
- Centrifuge at 13,000 x g for 1 minute and discard the Spin Filter basket.
- The DNA in the tube is now ready for any downstream application. No further steps are required.

Note: Solution C1-6 were provided with the commercial kit.

8. Amplification of 16S rRNAgene with 5 prime HOT MASTER MIX

- Prepare the template/primer mix in a 0.2ml tube by adjusting the total volume to the values given in the following table with molecular biology-grade water.
- Dispense the appropriate volume of the master mix into a PCR tube
- Add the template/primer mix to the PCR tube containing the master mix. Close tube and mix well. If necessary, centrifuge briefly to collect liquid at bottom of tube.
- Start the PCR program on a thermal cycler. The thermal cycler should be preheated (>90ºC) before placing the PCR tubes on the cycler block.

Table: PCR components for various reaction volume

Component	Reaction volume (25ul)	Final concentration
Forward primer	1	100-200 nM
Reverse primer	1	100-200nM
Template DNA	1	50 pg-200ng gDNA
Molecular biology grade water	12	
Master mix	10	1x including 2.5 mM Mg^{2+}

Table: PCR condition

PCR Cycle	Temperature	PCR product size		
		100-500 bp	500-1000 bp	1-5 kb
Initial denaturation	94ºC	2 min	2 min	2 min
Cycled template	94 ºC	20 sec	20 sec	20 sec
Cycled primer annealing	50-70 ºC	10 sec	10 sec	20 sec
Cycled primer extension	60-70 ºC	20-30 sec	40-50 sec	1 min/kb

9. Organoid Culture

Lab Supervisor: Dr. Stefania Senger, PhD

Organoid culture is emerging as a crucial culture method. It allows us to study how cells interact with each other in normal and diseased patients and can be used in drug screenings. Organoid cultures give impression of in-vitro 3D cells. Their use facilitates an understanding of biological processes of cells like renewal, mutation and damage.

Researchers all over the world are developing methodologies for establishing organoid cultures for their particular interest. Intestinal organoid culture in this segment is finding its future and certainly, in times to come, is going to open gates for new lines of research. **Dr. Stefania Senger** is working on intestinal organoid culturing, particularly for understanding intestinal pathologies including host-enteropathogen and microbiota interaction, IBD and gluten-related disorders. This method will be very advantageous for celiac disease research. She has provided me the opportunity to observe her laboratory techniques. She is in the process of developing new methodologies; her current process is not mentioned as it not yet published. Following is the published protocol for the intestinal organoid culture; this culture method has two broad sections:

Section 1: Isolation and processing of intestinal biopsies (Concise)

Duodenum Crypt isolation (based on Sato 2009)

The protocol can be scaled up based on the size of biopsy to be dissociated.

- Collect 3 to 4 duodenum biopsies (Duodenum/jejunum/ ileum or full)
- Transfer biopsies in a 14ml falcon containing cold PBS; P/S
- Incubate (5 mins)
- Remove buffer
- add Dissociation Buffer:
- Incubate buried in ICE 30 min
- Shake vigorously (~20 sec @ 120 shakes/min)
- Collect the supernatant in a fresh tube **add 1ml FBS** and set aside [Label it as Fraction 1]
- Repeat 5 to 8 5 times.
- OPTIONAL: Filter on a 70um mesh and set aside adding 10% FBS
- Centrifuge @ 800 rpm for 10 mins
- Re-suspend
- Evaluate the crypts purity/number of each fraction and transfer the crypt enriched fraction in 1.5ml vial (save the other fractions as pellet in trizol)
- Spin down at 1300 rpm (refrigerated microcentrifuge)
- Resuspend according to further application: Standard stem cell medium for culturing

Section 2: Passaging Intestinal Organoids

- On the day of passaging, remove the previously prepared complete Organoid Growth Medium from fridge or freezer and leave to warm to room temperature (15 - 25°C).
- Add 1 mL Gentle Cell Dissociation Reagent on top of the exposed dome in each well and incubate at room temperature (15 - 25°C) for one minute.
- Pre-wet a 1000 µL pipette tip with the Gentle Cell Dissociation Reagent in the well, and use it to break up the dome and organoids by pipetting up and down approximately twenty times.
- Use the same pipette tip to transfer the suspension to a 15 mL conical tube. Rinse the culture well with an additional 1 mL Gentle Cell Dissociation Reagent and add this to the 15 mL tube.
- Incubate the 15 mL tubes at room temperature (15 - 25°C) on a rocking platform at 20 rpm for 10 minutes.
- Centrifuge the tubes at 290 x g and 2 - 8°C for five minutes, then gently pour off and discard the supernatant.
- Wash the pellets by suspending in 10 mL cold (2 - 8°C) DMEM/F-12 using a pre-wetted 10 mL serological pipette, centrifuging at 200 x g at 2 - 8°C for five minutes, then gently pipetting off as much DMEM/F-12 as possible without disturbing the pellet and discarding the supernatant.
- Add 150 µL room temperature (15 - 25°C) complete Organoid Growth Medium to the pellet in each tube. Add 150 µL undiluted Matrigel Matrix to each tube and pipette up and down ten times to suspend the pellet. Avoid introducing bubbles.
- For each tube, pipette 50 µL of the medium/Matrigel suspension into the center of each of four wells of a pre-warmed 24-well plate to form domes in the center of each well.
- Place the lid on the culture plate and incubate at 37°C for 10 minutes to set the Matrigel.
- Gently add 750 µL room temperature (15 - 25°C) complete Organoid Growth Medium to each well by pipetting the medium gently down the sidewall of the well. Do not pipette the medium directly onto the domed cultures.
- Add sterile PBS to any unused wells.
- Place the lid on the culture plate and incubate at 37°C and 5% CO2.
- Exchange the culture medium three times per week by carefully aspirating the existing liquid medium, keeping the pipette tip at the edge of the well bottom. Replace with 750 µL fresh, room temperature (15 - 25°C) complete Organoid Growth Medium. Using this culture system, organoids can be passaged indefinitely.

Abstract presented in scientific conferences for the study projects

Project 3: Gluten contamination in naturally or labeled gluten-free products marketed in Italy

❖ **Abstract presented in scientific conferences:**

- **World Congress of Pediatrics Gastroenterology Hepatology and Nutrition (WGPGHAN)-2016, Canada**
  Verma AK, Catassi C, Gatti S, Galeazzi T, Monachesi C, Padella L, Baldo GD, Annibali R, Lionetti E. Detection of gluten content in the naturally gluten-free and gluten free labeled commercially available food products in Italy. JPGN 2016. Vol 63 (Suppl 2): S160

- **XXIII-Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP)-2016, Milan (Italy)**
  Verma AK, Gatti S, Galeazzi T,Monachesi C, Padella L, Baldo GD, Annibali R, Lionetti E, Catassi C. Detection of gluten content in the naturally gluten free and gluten free labelled commercially available food products in Italy. Digestive and liver diseases 2016. Vol 48 (Suppl 4): e279

❖ **Publication: Nutrients (Impact Factor 4.2)**

Verma AK, Gatti S, Galeazzi T, Monachesi C, Padella L, Baldo GD, Annibali R, Lionetti E, Catassi C. Gluten Contamination in Naturally or Labeled Gluten-Free Products Marketed in Italy. Nutrients. 2017 Feb 7;9(2). pii: E115. PubMed PMID: 28178205.

Project 4: Contribution of oral hygiene and cosmetics on contamination of gluten-free diet

❖ **Abstract presented in scientific conferences:**

- **XXV-Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP)-2018, Salerno (Italy)**
  A.K. Verma, E. Lionetti, S. Gatti, E. Franceschini, G. Naspi Catassi, C. CatassiContribution of oral hygiene and cosmetics to contamination of a gluten-free diet: do Celiac customers need to worry? Dig. and Liv. Dise 2018. Vol 50, issue 4: e398

❖ **Publication: Journal of Pediatric Gastroenterology, Hepatology and Nutrition JPGN (Impact factor: 2.8)**

Verma AK, Lionetti E, Gatti S, Franceschini E, Catassi GN, Catassi C. Contribution of Oral Hygiene and Cosmetics on Contamination of Gluten-free Diet: Do Celiac Customers Need to Worry About? J Pediatr Gastroenterol Nutr. 2019;68:26-29.

Project 5: Validation of a novel single-drop rapid HLA-DQ2/-DQ8 typing method to identify subjects susceptible to celiac disease

❖ **Abstract presented in scientific conferences**

- **51st Annual Meeting, European Society of Pediatrics Gastroenterology Hepatology and Nutrition (ESPGHAN)-2018, Geneva, Switzerland.**
  Anil Kumar Verma, Alka Singh, Simona Gatti, Elena Lionetti, Tiziana Galeazzi, Vineet Ahuja, Carlo Catassi, Govind K Makharia. Validation of a novel single-drop rapid HLA-DQ2/-DQ8 method to identify people susceptible to coeliac disease. JPGN 2018. Vol 66, Supp. 2: 237

- **XXIV-Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP)-2017, Rome**
  A.K. Verma, A. Singh, S. Gatti, E. Lionetti, T. Galeazzi, V. Ahuja, C. Catassi, G. MakhariaValidation of a novel single-drop rapid HLA-DQ2/-DQ8 method to identify people susceptible to celiac disease. Dig. and Liv. Dise 2017. Vol 49, issue 4: e273

- **17th International Celiac Disease Symposium (ICDS) -2017, New Delhi, India**
  Verma A, Singh A, Gatti S, Gatti S, Lionetti E, Lionetti E, Galeazzi T, Galeazzi T, Ahuja V, Catassi C, Makharia GK. Validation of a novel single-drop rapid HLA DQ2/DQ8 method to identify people susceptible with celiac disease. ISSCD. 2017: 034

❖ **Award and honors**

- 1st award for oral presentation in Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP) Congress Rome, Italy. 2017

- The result of this work was selected to present as **poster of distinction** during 51st Annual Meeting, European Society of Pediatrics Gastroenterology Hepatology and Nutrition (ESPGHAN)-2018, Geneva, Switzerland.

❖ **Publication: Journal of Gastroenterology and Hepatology Open (JGH Open)**

Verma AK, Singh A, Gatti S, et al. Validation of a novel single-drop rapid human leukocyte antigen-DQ2/-DQ8 typing method to identify subjects susceptible to celiac disease: A rapid human leukocyte antigen-DQ typing in celiac disease. JGH Open 2018;2:311–316.

Project 7: Re-exploring the iceberg of celiac disease in children

❖ **Abstract presented in scientific conferences**

- **XXIV-Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP)-2017, Rome**
  S. Gatti, T. Galeazzi, **A.K. Verma,** E. Franceschini, R. Annibali, G. Del Baldo, A. Palpacelli, A. Marchesini, C. Monachesi, L. Balanzoni, A. Colombari, N. Scattolo, M. Trevisan, M. Cinquetti, E. Lionetti, C. Catassi. Re-exploring the iceberg of celiac disease in children: Results of a multicenter Italian screening project, based on a rapid HLA DQ typing test. Dig. and Liv. Dise 2017. Vol 49, issue 4: e271

- **Prolamin Working Group meeting WG-2017, Minden (Germany)**
  S. Gatti, T. Galeazzi, **A.K. Verma,** E. Franceschini, R. Annibali, G. Del Baldo, A. Palpacelli, A. Marchesini, C. Monachesi, L. Balanzoni, A. Colombari, N. Scattolo, M. Trevisan, M. Cinquetti, E. Lionetti, C. Catassi. Re-exploring the iceberg of celiac disease in children: Results of a multicenter Italian screening project, based on a rapid HLA DQ typing test.

- **17th International Celiac Disease Symposium (ICDS)-2017 , New Delhi, India**
  Gatti S, Galeazzi T , **Verma A ,** Franceschini E , Palpacelli A , Del Baldo G , Annibali R ,Marchesini A , Monachesi C , Balanzoni L , Colombari A , Trevisan M , Scattolo N , Cinquetti M , Lionetti E , Catassi C. Re-exploring the iceberg of celiac disease in children: preliminary results of a multicenter Italian screening project based on a rapid HLA DQ typing test.ISSCD. 2017: 012.

- **World Congress of Pediatrics Gastroenterology Hepatology and Nutrition (WGPGHAN)-2016, Canada**
  Gatti S, Galeazzi T, **Verma AK,** Palpacelli1 A, Annibali R , Baldo GD, Franceschini E, Balanzoni L, Colombari AM, Monachesi C, Scattolo N, Cinquetti M, Giovanni MM, Lionetti E, Catassi C. Re-exploring the iceberg of celiac disease in children: preliminary results of a multicenter Italian screening project based on a rapid HLA DQ typing test. JPGN 2016. Vol 63 (Suppl 2): S413

- **XXIII-Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica (SIGENP)-2016, Milan**
  Gatti S, Galeazzi T, **Verma AK**, Franceschini E, Palpacelli1 A, Baldo GD, Annibali R , Monachesi C, Scattolo N, Cinquetti M, Lionetti E, Catassi C. Re-exploring the iceberg of celiac disease in children: preliminary results of a multicenter Italian screening project based on a rapid HLA DQ typing test. Digestive and liver diseases 2016. Vol 48 (Supl 4): e247

Project 8: The increasing prevalence of celiac disease: what is the role of an improved diagnostic accuracy?

❖ **Abstract presented in scientific conferences**

- **51st Annual Meeting, European Society of Pediatrics Gastroenterology Hepatology and Nutrition (ESPGHAN)-2018, Geneva, Switzerland.**
  Anil Kumar Verma, Simona Gatti, Tiziana Galeazzi, Chiara Monachesi, Elisa Franceschini, Linda Balanzoni, Novella Scattolo, Mauro Cinquetti, Elena Lionetti, Carlo Catassi. The increased prevalence of celiac disease: what is the contribution of an improved diagnostic accuracy? JPGN 2018. Vol 66, Supp. 2: 236

❖ Award and honors

The result of this work was selected to present as **poster of distinction** during 51st Annual Meeting, European Society of Pediatrics Gastroenterology Hepatology and Nutrition (ESPGHAN)-2018, Geneva, Switzerland.

❖ Publication: Clinical Gastroenterology and Hepatology (CGH), Impact factor: 7.9

Verma AK, Gatti S, Lionetti E, Galeazzi T, Monachesi C, Franceschini E, Balanzoni L, Scattolo N, Cinquetti M, Catassi C. Comparison of Diagnostic Performance of the IgA Anti-tTG Test vs IgA Anti-Native Gliadin Antibodies Test in Detection of Celiac Disease in the General Population. Clin Gastroenterol Hepatol. 2018. 5;30325-2.

Project 10: Non-immunological biomarkers for assessment of villous abnormalities in patients with celiac disease

❖ **Abstract presented in scientific conferences**

•51st Annual Meeting, European Society of Pediatrics Gastroenterology Hepatology and Nutrition (ESPGHAN)-2018, Geneva, Switzerland.

Alka Singh, **Anil Kumar Verma,** Prasenjit Das, Shyam Prakash, Siddhartha Datta Gupta, Lalit Kumar, Vineet Ahuja, Govind K Makharia.Non-invasive biomarkers for assessment of villous abnormalities in patients with coeliac disease and other enteropathies: An alternative to mucosal biopsies. JPGN 2018. Vol 66, Supp. 2: 8

•17th International Celiac Disease Symposium (ICDS) -2017, New Delhi, India

Singh A, **Verma A,** Das P, Jindal G, Prakash S, Nayak B, Gupta S, Kumar L, Ahuja V, Makharia GK. Non-invasive biomarkers for assessment of villous abnormalities in patients with celiac disease and other enteropathies: An alternative to mucosal biopsies. ISSCD. 2017: 006

•Digestive Disease Week (DDW)-2016, Chicago, Illinois, USA

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•Asian Pacific Digestive Week (APDW)-2016, Kobe, Japan

Kumari A, Makharia G, **Verma A,** Prakash S, Das P, Pathak M, Sreenivas V, Ahuja V, Kumar L. Assessment of the reliability of plasma citrulline and i-FABP as non-invasive markers of enteropathy: a study in a human model. JGH; 2016, Volume 31, Issue Supplementation 3, Page 1

❖ Award and honors

•1st award for oral presentation in 17th International Celiac Disease Symposium (ICDS)-2017 New Delhi, India

•Young Investigator Award (1st Prize) in Indian Society of Gastroenterology (15-18th December 2016) New Delhi, India

Project 11: Gluten sensitivity in patients with cerebellar ataxia: A prospective cohort study

❖ Abstract presented in scientific conferences

- **57th Indian Society of Gastroenterology conference (ISGCON) – 2016, New Delhi (India)**
  Inder Singh, **Anil K Verma,** Isha Singh, Alka Singh, Varun Suroliya, Urvashi Hooda, Mohammed Faruq, Vineet Ahuja, Govind K Makharia, Achal K Srivastava. Gluten sensitivity in Indian patients with progressive cerebellar ataxia. Indian J Gastroenterol (December 2017) 36(Suppl 1): A1–A105

- **17th International Celiac Disease Symposium (ICDS) -2017, New Delhi, India**
  Singh I, **Verma A,** Singh I, Singh A, Hooda U, Ahuja V, Faruq M, Srivastava A, Makharia GK.Gluten sensitivity in patients with cerebellar ataxia: A prospective cohort study. ISSCD. 2017: 029

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1. **Verma AK,** Lionetti E, Gatti S, Franceschini E, Catassi GN, Catassi C. Contribution of Oral Hygiene and Cosmetics on Contamination of Gluten-free Diet: Do Celiac Customers Need to Worry About? J Pediatr Gastroenterol Nutr. 2019;68:26-29.
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Publications under review:

1. Simona Gatti; Elena Lionetti; Linda Balanzoni; **Anil K Verma;** Tiziana Galeazzi; Rosaria Gesuita; Novella Scattolo; Mauro Cinquetti; Alessio Fasano; Carlo CatassiCeliac disease in the year 2020: still increasing, largely undetected. **Gastroenterology**
2. Inder Singh*, **Anil K Verma*,** Isha Singh, Alka Singh, Varun Suroliya, Urvashi Hooda, Mohammed Faruq, Vineet Ahuja, Govind K Makharia, Achal K Srivastava. Gluten sensitivity in Indian patients with progressive cerebellar ataxia. **Journal of Neurology**
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3. Singh A, **Verma A,** Prakash S, Das P, Pathak M, Sreenivas V, Ahuja V, Kumar L. Makharia G. Assessment of the reliability of plasma citrulline and i-FABP as non-invasive markers of enteropathy: a study in a human model. **Journal of Gastroenterology.**

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Choose…Analyse….Decide….

once decided…….deliver the work

-Prof.Carlo Catassi