Clinical, biochemical and immunogenetical characteristics of celiac disease in Albanian pediatric patients from Kosovo

Ramosaj-Morina, Atifete

Doctoral thesis / Disertacija

2019

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Atifete Ramosaj-Morina

Clinical, biochemical and immunogenetical characteristics of celiac disease in Albanian pediatric patients from Kosovo

DISSERTATION

Zagreb, 2019

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Atifete Ramosaj-Morina

Clinical, biochemical and immunogenetical characteristics of celiac disease in Albanian pediatric patients from Kosovo

DISSERTATION

Zagreb, 2019

This doctoral thesis was conducted in the Paediatric Clinic, University Clinical Center of Kosovo and Department of Transfusion Medicine and Transplantation Biology, Tissue Typing Centre, Clinical Hospital Centre, Zagreb

Mentors:

- Ass. Prof. Renata Žunec PhD - Department of Transfusion Medicine and Transplantation Biology, Tissue Typing Centre, Clinical Hospital Centre Zagreb
- Prof. Mehmedali Azemi, MD- Department of Gastroenterology, Pediatric Clinic University Clinial Center of Kosovo

Table of contents

Abbreviations

APCs - antigen-presenting cells

AHRQ - Agency for Healthcare Research and Quality

CD - celiac disease

CHC - Clinical Hospital Centre

CI - confidence interval

QOL - quality of life

DGP - deamidated gliadin peptide

EMA - anti-endomysial antibody

ELISA - enzyme-linked immunosorbent assay

ESPGHAN - European Society for Paediatric Gastroenterology, Hepatology and Nutrition

FDRs - first degree relatives

FTT - failure to thrive

GFD - gluten free diet

HF - heterodimer frequency

HLA - human leukocyte antigen

IEL - intraepithelial lymphocyte

IgA - immunoglobulin A

IgG - immunoglobulin G

IL - interleukin

LMIC - Low and/or Middle Income Countries

MHC - major histocompatibility complex

NK - natural killer

NUL - normal upper limit

OR - odds ratio

P - p value

SBB - small bowel biopsies

SD - standard deviations

ST - short stature

TG2 - tissue transglutaminase 2

T1DM - diabetes mellitus type 1

UCCK - University Clinical Centre of Kosovo

UGIE - Upper gastrointestinal endoscopy

ULN - upper limit of normal

W/L - weight for length

1. INTRODUCTION

1. 1. History of Celiac disease

A physician, named Aretaeus of Cappadocia, influenced by Greek culture and living in the first century AD, wrote about "The Coeliac Affection". In fact, he named it "koiliakos" after the Greek word "koelia" (abdomen). His description was: "If the stomach is irretentive of the food and if it passes through undigested and crude, and nothing ascends into the body, we call such persons coeliacs".

In 1888, English doctor Samuel Gee, a leading authority in paediatric diseases, took full credit for the modern description of celiac disease, when giving a lecture to medical students on the "celiac affection," the milestone description of this disorder in modern times. Gee first described celiac disease (CD) as a disorder with onset usually between 1 and 5 years of age, with diarrhea, abdominal distension and failure to thrive as the most important symptoms (1).

The disease cause was unknown, but it was noticed that patients recovered when they were put on a restricted diet. Different diets were used; in 1924 Sidney Hass recommended that bananas were the answer to the condition. He published a medical paper outlining his "banana diet" which restricted other forms of carbohydrates, thereby unknowingly cutting out many foods containing gluten. In 1941, the Dutch paediatrician Willem Karel Dicke discovered that children with CD benefited when treated with a wheat-free diet. This understanding came about due to the recovery of children during the war, when grains such as wheat, rye and barley were in short supply.

The next major breakthrough came in the mid-50s, when Margot Shiner described a new jejunal biopsy apparatus with which she successfully reached and biopsied the distal duodenum. In the mid to late 60's, it had become clear that CD could be diagnosed with the jejunal biopsy showing atrophy of the villi (2).

The anti-gliadin antibodies were detected and reported by Berger in 1964. While, seven years later Seah et al. identified, for the first time, not an anti-food protein, but an actual auto-antibody in the serum of celiac children: the anti-reticulins (3).

In late 80's, many studies declare that almost all CD patients share a particular combination of a human leucocyte antigen II (HLA class II) DQA and DQB genes; i.e. they may share a particular cis- or trans-encoded DQα/ß heterodimer (4, 5).

In Germany, Schuppan et al., were attempting to identify the target antigen of anti-endomysial antibody (EMA) that was being used so successfully as a diagnostic screening test for CD. They discovered that the target antigen of anti-endomysial antibody was an enzyme, tissue transglutaminase, also termed TG2 (6).

In the late 80's, a large multicentre Italian study demonstrated that by relying on strict clinical and laboratory criteria, a correct diagnosis of CD could be reached in 95% of cases by limiting to the one initial biopsy, and new diagnostic guidelines were published in 1990 by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (7).

After 90`s, CD was increasingly accepted as an example of an autoimmune disease, associated with a specific HLA class II- DQA and DQB genes (either DQ2 or DQ8) and the missing autoantigen was finally identified in the enzyme "tissue transglutaminase". At long last, there was universal acceptance that celiac disease is an autoimmune condition whose trigger (gluten) and autoantigen (tissue transglutaminase) are known.

1.2. Epidemiology of CD

In the last decades a high number of epidemiological data have been reported. Nowadays, celiac disease is one of the most frequent genetically based disorders in humans, among both children and adults.

CD originally thought to almost exclusively affect white Europeans, is now known to be widely distributed worldwide. Epidemiological studies conducted in areas supposedly free of CD, including Africa, the Middle East, Asia and South America, show that the disease was previously underdiagnosed. This provides evidence that CD is one of the most common genetic diseases, resulting from both environmental (gluten) and genetic factors.

For the majority of countries, the CD prevalence is unknown. It must be noted that some studies report prevalence of CD based on serology, others on celiac compatible small bowel biopsies (SBB) and a few on serology, biopsy and response to gluten challenge.

Europe is historically considered a geographical area at high frequency, with a prevalence of 1% (8), which may be higher in Northern European countries (9-11).

The Scandinavian countries, Ireland, and the United Kingdom's population tended to show a higher prevalence of CD of approximately 1.0%-1.5%. A study of SBB obtained from healthy Dutch blood donors shows that the prevalence of CD-compatible biopsies of 1:330 (12). The prevalence of CD among 3654 children (age range, 7-16 years) in Finland was at least 1:99 based on serum autoantibodies and SBB abnormalities (13).

Although it was thought that some countries, including United States, were exempt from this disease, recently it has been shown that a prevalence is similar as in Europe, namely 0.5%-1.0% of the general population (14).

Higher frequency of CD have also been shown outside Europe and the United States among populations of predominantly European extraction (e.g., New Zealand and Australia). Many studies have shown that CD is not exclusive for industrialized countries, there is an increased incidence even in North Africa, Middle East and India, with an incidence overlapping those of European countries (15-17).

Iceberg model is used to describe the epidemiology of CD (18). The typical CD cases, usually diagnosed because of suggestive complaints, make up the visible part of the celiac iceberg, expressed by the incidence of the disease in quantitative terms. In developed countries, however, for each diagnosed case of CD, an average of five cases remain undiagnosed (the submerged part of the iceberg) (19).

The crude incidence is defined as the number of new cases per 100,000 population-at-risk per year and cumulative incidence as the number of new cases per 1,000 live births.

The crude incidence of CD in adults varies from lows of 1.27 in Denmark and 3.08 in England to a high of 17.2 cases per 100,000 patient per years in Finland (20-22) where specific efforts have been undertaken to encourage screening for CD. The crude incidence of CD in children age 0 to 15 years varied from 2.15 to 51 cases per 100,000 patient-years, while the relative risk (RR) of CD was greatest for the 0 to 2-years old age group, as well as for females, and varied from 32.26 to 42.4 and from 1.9 to 3.34, respectively (23-25). The cumulative incidence at age 5, when reported, varied between 0.089 and 9 cases per 1,000 live births (26).

Regarding to gender distribution, females seem to be more frequently affected, although data on sex differences are poor (27-29). The female to male ratio in the patients age groups 0-15 years

range from 1.6-3:1. This difference was not found in screening studies, suggesting the underdiagnosing in daily practice in males (30, 31).

1.3. Definition and classification of CD

CD is an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals and characterized by the presence of a variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy. CD-specific antibodies comprise autoantibodies against TG2, endomysial antibodies (EMA), and antibodies against deamidated forms of gliadin peptides (DGP) (32).

CD is triggered by the ingestion of gluten and related prolamines that are harmful to patients with CD. The term gluten indicates a complex of water insoluble proteins (gliadins and glutenins) found in wheat. Other prolamins showing similar immunogenic properties are also found in rye (secalins), barley (hordeins) and other closely related grains (33).

Gluten is poorly digested in the human intestine with or without CD. Gluten peptides cross intact into the submucosa of the small intestine, where the human enzyme TG2, also referred to as tissue transglutaminase (tTG) deamidates gluten peptides, allows for high-affinity binding to HLA-DQ2 and HLA-DQ8 molecules, subsequently triggering an inflammatory reaction in patients with CD (34).

Several classifications of CD have been used, most important with distinction forms according to Oslo classification: classical, non-classical, symptomatic, subclinical and potential CD (35, 36).

Classical CD refers to a presentation with signs and symptoms of malabsorption: diarrhoea, steatorrhoea, and weight loss or growth failure. Children with Classic CD are often characterized by failure to thrive, diarrhoea, muscle wasting, poor appetite and abdominal distension. It is usually manifested between ages six to 24 months (37).

Non-classical seem to be one of the most common forms. These patients generally have little to no gastrointestinal (GI) symptoms, but seek medical attention because of other reasons, such as: iron deficiency, osteoporosis, short stature, or infertility. Patients with monosymptomatic disease (other than diarrhoea or steatorrhoea) usually have non-classical CD.

4

Subclinical CD is a term used to describe the disease that is below the threshold of clinical detection without signs or symptoms sufficient to trigger CD testing in routine practice.

Symptomatic CD is characterized by clinically evident GI and/or extraintestinal symptoms attributable to gluten intake. The clinical manifestations of CD vary from little to a wide spectrum of symptoms.

Potential CD is defined by the presence of CD-specific antibodies and compatible HLA, but without histological abnormalities in duodenal biopsies. The patient may or may not have symptoms and signs and may or may not develop a gluten dependent enteropathy later.

1.4. Immunopathogenesis of CD

Susceptibility to CD, its activation and perpetuation, involve a combination of environmental and genetic factors through some immunological mechanisms (38).

The role of dietary proteins - CD is triggered by the ingestion of gluten and related prolamines that are harmful to patients with CD. The term gluten indicates a complex of water insoluble proteins (gliadins and glutenins) found in wheat. Other prolamins showing similar immunogenic properties are also found in rye (secalins), barley (hordeins) and other closely related grains.

Genetic factors - Nowadays, it is known that CD is associated with specific MHC class II alleles that map to the HLA-DQ locus. Moreover, the presence of specific HLA-DQ alleles is necessary, although not sufficient, for the phenotypic expression of CD in virtually all affected individuals, irrespective of geographic location.

The HLA-DQ2 heterodimers, that confer susceptibility to CD, are formed by a β chain encoded by the allele HLA-DQB1*02 (either HLA-DQB1*02:01 or *02:02) and an α chain encoded by the allele HLA-DQA1*05. The HLA-DQ2 heterodimer is present in at least 90%–95% of patients with CD, although a very small number of CD patients in whom only one of these DQ2 alleles is present have been reported (that is, HLA-DQB1*02 or, rarely, HLA-DQA1*05) (39, 40). While, HLA-DQ8 heterodimer found in the remaining 5%–10% of patients with CD, is formed by the β chain and α chain encoded by HLA-DQB1*03:02 and HLA-DQA1*03, respectively (41).

Adaptive immunity - activation of HLA-DQ–restricted mucosal T cells and the role of tissue TGase - HLA-DQ2 and HLA-DQ8 heterodimers on antigen presenting cells (APCs) can bind and subsequently present "gluten" peptides to populations of CD4+ T cells in the lamina propria

of the small intestine (42). TG2-ase also has a high avidity for "gluten" peptides and, under certain conditions (for example, low pH) and in the absence of lysine residues, can deamidate glutamine, which converts neutral glutamine to negatively charged glutamic acid while, HLA-DQ2 and HLA-DQ8 favours the binding of peptides with negatively charged (43, 44). In a further series of events, the APCs present some of these peptides to HLA-DQ2– and HLA-DQ8–restricted populations of CD4+ T cells that become activated and release mediators that ultimately lead to tissue damage.

Role of innate immunity and intraepithelial lymphocytes (IELs) in CD - Recent studies suggest that activation of the innate immune system is important in the pathogenesis of CD. In particular, an increase in the number of IELs in the mucosa of the small intestine is a characteristic feature of CD (45,46). Following activation, IELs from patients with CD change from being typical antigen specific T cells to being natural killer (NK) - like cells able to mediate epithelial cell damage through the recognition of stress-induced molecules on intestinal epithelial cells. The cytokine interleukin 15 (IL-15) takes centre stage in this process (47).

1.5. Clinical presentation of CD

Recognition and assessment of coeliac disease can be difficult because of the variety of presenting signs and symptoms that can vary according to patients' age (48). In children, CD can present with a broad range, from absolute absence of symptoms to pronounced clinical GI or extraintestinal manifestations (32, 49) (see Table 1).

Table 1. Clinical manifestations of CD

GI manifestations	Extraintestinal manifestations
-Chronic diarrhea,	-Failure-to-thrive and stunted growth
-Chronic constipation	-Delayed puberty and/or amenorrhea
-Abdominal pain	-Anaemia and chronic fatigue
-Nausea and vomiting	-Decreased bone mineralisation (osteopenia /osteoporosis) and dental enamel defects
-Distended abdomen	-Irritability and neuropathy
-Steatorrhea	-Arthritis/ arthralgia
	-Increased levels of liver enzymes

GI manifestations usually appear in clinically diagnosed childhood CD, mostly represented by: chronic diarrhoea (in about 50% of patients), abdominal pain, chronic constipation, nausea vomiting, distended abdomen.

A shift from GI symptoms to extraintestinal symptoms seems to have occurred in children with CD. It is unclear whether this finding reflects a true clinical variation or an improved recognition of non-gastrointestinal forms of CD because of increased awareness of the disease.

Researchers have found good evidence that failure to thrive and stunted growth may be caused by CD. The risk of CD in patients with isolated stunted growth or short stature (SS) has been calculated as 10% to 40%. On the other hand, in some populations, CD is diagnosed in approximately 15% of children with iron-deficiency anaemia (50).

The following findings may also be present when CD is diagnosed: chronic fatigue, irritability, neuropathy, epilepsy, arthritis/arthralgia, osteoporosis, alopecia, amenorrhoea and/or delayed puberty, aphthous stomatitis, dermatitis herpetiformis, and abnormal liver biochemistry. The fact that CD is common and has protean manifestations means that the diagnosis is easily missed unless physicians and other health care providers include CD in the differential diagnosis of common conditions such as iron anaemia and vitamin B12 deficiency, mild chronic diarrhea, recurrent abdominal discomfort, failure to thrive as well as less common manifestations such as skin rashes, hair loss, neurological disorders, osteoporosis and osteomalacia, unexplained hypertransaminasemia or infertility.

In brief, the first and most critical step in making a diagnosis of this commonly overlooked disorder is to think of celiac disease as a diagnostic possibility (51).

1.6. Associated conditions

Since CD is an inflammatory disorder, induced by a known exogenous factor, a set of conditions can be associated with celiac disease. The term "associated conditions" refers to states that are found more frequently in affected patients. These conditions include genetic, autoimmune or neurological disorders (52).

Nowadays, good evidence is in existence for the increased prevalence of CD in patients with autoimmune diseases such as diabetes mellitus type 1 (T1DM). The prevalence of CD in T1DM

has been investigated extensively and is 3% to 12%. The Agency for Healthcare Research and Quality (AHRQ) report a paper that included 21 studies on T1DM with biopsy proven CD. For all the included studies, the minimum prevalence of CD in T1DM by serology was 1% and the maximum was 12%. By biopsy, the minimum and maximum prevalence were 1% and 11%, respectively (53).

In addition, CD occurs more frequently than expected by chance in children with Turner syndrome (54) or Down syndrome. A 10 to 20-fold increase in CD prevalence has been reported in subjects with selective IgA deficiency (55).

Moreover, in patients affected by CD it has been reported an increased prevalence (nearly, 2%-5%) of thyroid disorders (i.e., hyperthyroidism-Graves's disease or hypothyroidism-Hashimoto's thyroiditis), diagnosed either before rather than after the diagnosis of gluten-enteropathy (56). These two conditions share genetic risk factors represented by HLA-DQ2 and DQ8. HLA-DQ2 and DQ8 haplotypes have been associated with Hashimoto's thyroiditis, while HLA-DQ2 association is less clear in Graves' disease.

A potential link between CD and different neurological disorders has been reported. Although ataxia is a neurological disorder indicated in some patients with CD, the most frequent neurologic condition in celiac disease subjects is epilepsy, showing a prevalence between 1.2% and 5% (57).

The involvement of the liver is common among patients affected by CD. The prevalence of CD in Williams syndrome is higher than in the general population from 6% to 9% (58).

Low bone mass is common in subjects with newly diagnosed CD. The mechanism for this effect may be due to malabsorption of vitamin D and calcium and decreased intake of calcium due to lactose intolerance. However, low bone mass may be due not only to osteoporosis but also to osteomalacia. CD among patients with low bone mass have been performed in Europe. CD was found in 3.4 percent of adults with low bone mass but reduced bone density is common in both adults and children with CD.

Malignant diseases are more frequent in patients with long-term untreated classical CD. Small-bowel adenocarcinoma, esophageal and oropharyngeal squamous-cell carcinoma, and non-Hodgkin's lymphoma occur more often in CD patients than in healthy control individuals.

CD may be associated with delayed menarche, premature menopause, amenorrhea, recurrent abortions, and fewer children (59).

On the other hand, some individuals with CD report diminished quality of life (QOL). One group of researchers found that age at diagnosis affected perceived QOL, with those diagnosed at a younger age reporting higher QOL scores (60, 61).

1.7. Family history of CD

Many early case reports documented the occurrence of CD in siblings, identical twins, parent and child pairs, as well as more extended kindred's. At least 20 percent of index cases will have an affected family member if screening is done. Identical twins have a 75–100 percent concordance rate for the disease. Siblings are at the next highest risk at 7–20 percent concordance rate. It has been suggested that if siblings share the same HLA disease risk haplotype, their risk approaches 40 percent. These results contribute for future recommendations for CD screening frequency and follow-up duration of relatives of celiac patients (51, 62).

1.8. Diagnostic tools

1.8.1. Serum antibodies

In many studies, a number of serological markers have been repeatedly shown to be highly sensitive and specific for untreated CD.

On the basis of the target antigens, serologic tests for celiac disease can be divided into two groups (18).

1. Autoantibodies:

- Antiendomysial (EMA) and anti-tissue transglutaminase (tTG) antibody tests

IgA EMA bind to endomysium, the connective tissue around smooth muscle, producing a characteristic staining pattern that is visualized by indirect immunofluorescence. The test result is

reported simply as positive or negative, since even low titre of serum IgA endomysial antibodies are specific for CD. The antigen against which antiendomysial antibodies are directed is tTG. Anti-tTG antibodies. Enzyme-linked immunosorbent assay (ELISA) tests for IgA anti-tTG antibodies are now widely available and are easier to perform, less observer-dependent, and less costly than the immunofluorescence assay used to detect IgA endomysial antibodies.

2. Antibodies targeting the offending agent (gliadin):

- Conventional antigliadin antibodies (AGAs) (nowadays considered obsolete for diagnostic purposes)

- Antibodies against synthetic deamidated gliadin peptides (DGPs) **-** are recommended in patients <2 years old, since this may appear earlier than anti-tTG antibodies in very young children with CD.

Usually all of these antibodies are based on immunoglobulin A (IgA) class. In patients with IgA deficiency, the same type of antibodies, but of the IgG class, can be detected. Specifically, IgG-based tests are useful for detecting CD in selected IgA-deficient patients (63, 64).

The sensitivity and specificity of anti-tTG on diagnosis of CD is well documented. Assays using human recombinant tTG, improved high sensitivity of about 98 (78-99) percent and similar specificity of 98 (91-99) percent (65).

According to systematic reviews and studies in low-risk and high-risk populations, EMA-IgA is both highly sensitive and specific. Most studies demonstrate EMA sensitivities in excess of 95% (86%-100%) in children and 90% in adults with specificities above 98% (97%-100%) in both age groups (66).

Depending on different population studies, IgA anti-DGP can be nearly as sensitive as IgA anti-tTG (67).

Nevertheless, recent studies have shown that IgA anti-tTG perform significantly better and is significantly less costly than IgA anti-DGP testing. A review done in 2510 published articles, conclude that IgA-EMA and IgA-anti-TG2 tests appear highly accurate to diagnose CD (68).

AGA tests are no longer routinely recommended for diagnosing CD, because of their lower sensitivity and specificity.

1.8.2. Small bowel biopsies

CD affects the mucosa of the proximal small intestine, with damage gradually decreasing in severity towards the distal small intestine, although in severe cases the lesions can extend to more distal areas and patchy distribution, in some cases.

The definitive diagnosis of celiac disease is made by identification of characteristic histologic changes on biopsy of the duodenum during upper gastrointestinal endoscopy while the patient maintains a gluten-containing diet (69).

It has been suggested that villous lesions rarely coexist with histologically normal mucosa but others describe CD as exhibiting a patchy distribution thus implying a need for multiple biopsy specimens to secure a diagnosis. Based on this new guidelines on CD, four to six duodenal biopsies are recommended for evaluation of CD, due to the patchy nature of histopathology features.

Biopsies should be taken from the bulb (at least 1) and from the second or third portion of the duodenum (at least 4) (70).

Whole spectrum of histological signs may be present in CD, ranging from a normal villous architecture to severe villous atrophy. According to the Marsh classification and its modification, lesions include infiltrative, hyperplastic, and atrophic patterns (71, 72). Consequently, if histology shows lesions that are consistent with CD (Marsh 2–3), then the diagnosis of CD is confirmed.

The pathology report should include a description of the orientation, evaluation of villi (normal or degree of atrophy), crypts, villus/crypt ratio, and number of IELs (73). IELs in numbers >25/100 epithelial cells suggest an infiltrative lesion. Anyway, histological damage is considered characteristic, but not pathognomonic, of celiac disease, as similar lesions are seen in several other disorders.

1.8.3. Genetic tests in CD

HLA is the name for the Mayor Histocompatibility Complex (MHC) in humans; it is a genomic region located in the chromosomal position 6p21 and contains a large number of genes related to the immune response. HLA genes encode antigen presenting proteins that are expressed in most human cells and are essential for the ability of the organism to distinguish between self and foreign molecules. As previously mentioned, the HLA region is the most important susceptibility locus in CD and explains around 40% of the genetic component of the disease.

Molecular studies have revealed that the factors directly implicated are the HLA class II genes encoding both HLA-DQ2 and -DQ8 molecules (see Figure 1).

More than 95% of patients with CD share the HLA-DQ2 heterodimer, either in the cis (encoded by HLA-DR3-DQA1*05:01-DQB1*02:01) or the trans configuration (encoded by HLA-DR11-DQA1*05:05 DQB*03:01/DR7- DQA1*02:01 DQB1*02:02) and most of the remainder have the HLA-DQ8 heterodimer (encoded by DQA1*03:01-DQB1*03:02) (75, 76).

CD is a multigenetic disorder, which means that the expression of these HLA-DQ2 or HLA-DQ8 molecules is necessary, but not sufficient to cause disease, because approximately 30% to 40% of the white population holds the HLA-DQ2 haplotype and only 1% develops CD (77). The strong relation between HLA genetic factors and CD is illustrated by the effect of the HLA-DQ2 gene dose on disease development; HLA-DQ2 homozygous individuals have at least 5 times higher risk of disease development compared with HLA-DQ2 heterozygous individuals.

The sensitivity of HLA-DQ2 is high (median 91%; p25–p75 86.3%– 94.0%), and if combined with HLA-DQ8 (defined that at least one of them is positive), it is even higher (median 96.2%; p25–p75 94.6%–99.8%), making the chance to have CD for an individual who is negative for DQ2 and DQ8 very small (78). The specificity of HLA-DQ2 is low (median 74%; p25–p75 65%–80%).

The specificity of HLA-DQ8, evaluated in 9 studies, had a median of 80% (p25–p75 75%–87.5%). The specificity of the combination HLA-DQ2/DQ8 varies widely in different study populations, from 12% to 68% with a median of 54%.

As the chance of an individual negative for HLA-DQ2 or HLA-DQ8 having CD is small, the main role of HLA-DQ typing in the diagnosis of CD is its negative predictive value (96%-98%) to exclude the disease or to make it unlikely (79-81).
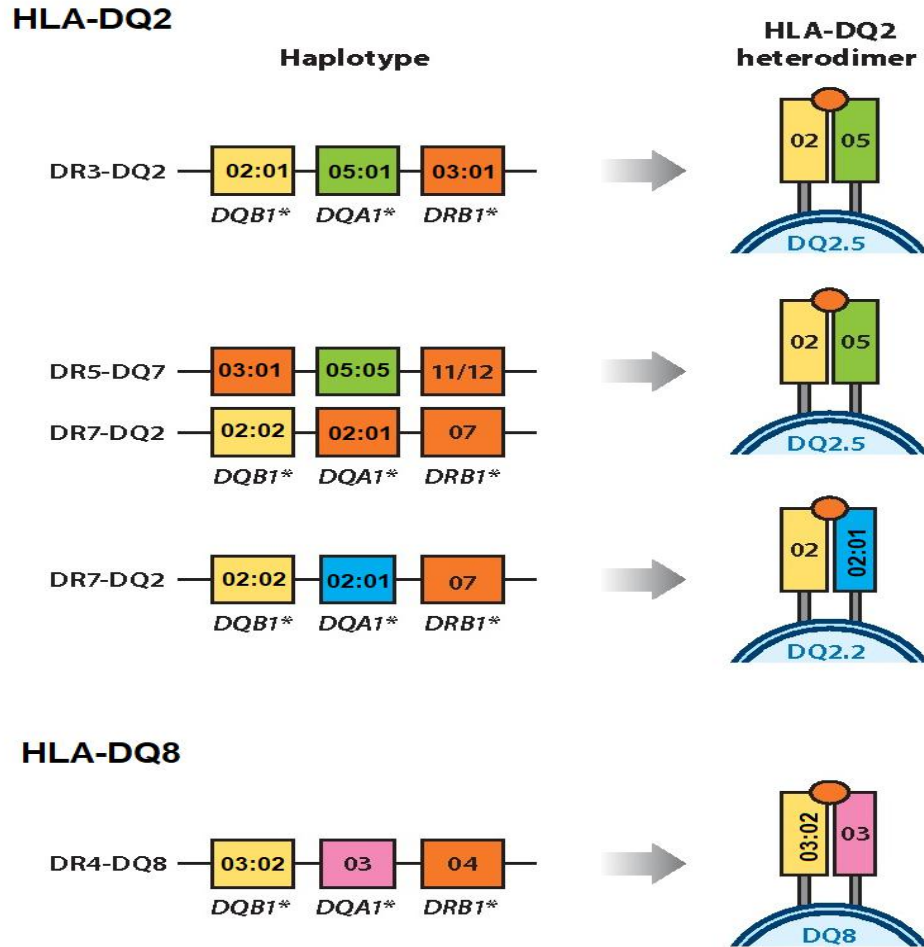
Figure 1. Association of the HLA locus with CD. HLA-DQ2 molecule is the major factor conferring risk to CD. Most celiac patients express the heterodimer HLA-DQ2.5, encoded by the alleles HLA-DQA1*05 (α chain) and HLA-DQB1*02 (β chain), that can be present in cis in the DR3-DQ2 haplotype or in trans, in the heterozygotes DR5-DQ7 and DR7-DQ2.2. The HLA-DQ2.2 heterodimer, a variant of HLA-DQ2 encoded by the alleles HLA-DQA1*02:01 and HLA-DQB1*02:02, confer a low risk to develop the disease. Most of the patients that are negative for DQ2 express HLA-DQ8, encoded by the DR4-DQ8 haplotype (74).

Literature data reports that the relative risk for celiac disease associated with genotypes (DQ2 and/or DQ8 molecules) could be different among different geographic areas.

The frequency of DQ2 and/or DQ8 heterodimer DQA1*05-DQB1*02, DQ2.5, in *cis* or *trans* configuration, and/or DQA1*03-DQB1*03:02 in the Moroccan population was found in 87% of patients with CD (82). Slightly higher frequency was reported by Megiorni et al.

(77), 91% of CD patients carried DQ2 and/or DQ8 heterodimers while among the DQ2/DQ8-negative individuals, the frequencies of cases carrying DQB1*02 (β2), DQA1*05, or neither of the two alleles were 6%, 2.1%, and 0.9%, respectively.

In a study from Slovenia in 2006, the DQ2 and DQ8 frequency was reported to be 97.6% (83), while in 2016 it was reported that frequency of DQ2 heterodimer (in *cis* and/or *trans*) in CD patients was 89.7%, while 5.88% carried DQ8 heterodimer, all together giving a DQ2 and/or DQ8 frequency in this population of 95.6 % (84).

A study from Libya performed in CD patients found out a DQ2 and/or DQ8 heterodimer prevalence of 97%, out of them 13% were DQ8 positive (85).

In a study from Serbia, 94.5% (86) of patients carried alleles that encode DQ2 protein variant and 2.7% carried alleles that encode DQ8 protein variant, comprising total of 97.2%. The results of Rostami-Nejad et al. (87) on Iranian people, 97% of the patients diagnosed with CD had HLA-DQ2 and/or DQ8 heterodimer, with 83.03% of cases carriers of an HLA-DQ2 heterodimer, either in homozygous or heterozygous state, while 13.5% were DQ8 carriers.

Anyway, there are some published studies in the literature reporting the highest incidences. In the study conducted by Sumnik et al. (88) in Czechoslovakia in 2000, the incidence of HLA-DQ2/DQ8 was 100% as well as in the study conducted by Neuhausen et al. (89) in Israel.

It is recommended that clinicians should not classify patients only as DQ2 and DQ8 positive or negative, but must also consider the presence of DQB1*02 and DQA1*05 alleles alone, regardless of their relative low risk for CD development. In study from Brazil, from a total of 100 CD children, 51 (51%) tested positive for DQ2.5 only; 22 (22%) for DQ2.5/DQ2.2; 5 (5%) for DQ2.2 only; 7 (7%) for DQ2.5/DQ8; 6 (6%) for DQ2.2/DQ8; 6 (6%) for DQ8 only; 1 (1%) was DQB1*02 positive; 1 (1%) showed low risk predisposing single allele and 1 (1%) tested negative for all of the searched alleles (90).

Additionally, the recent study (91) reports that 5.8% of patients lacking HLA-DQ2 and HLA-DQ8 variants, carried DQB1*02 allele, therefore this allele should be recognized as risk allele for disease occurrence. It may be useful to consider HLA-DQ genotype gradient risk in selecting individuals who must undergo recurrent clinical and serologic follow-ups, especially in high-risk groups.

1.9 New guidelines for CD diagnosis

According to modified European guidelines (32), in symptomatic children with anti-TG2 titres more than ten times the upper limit of normal (>10 times ULN), SBB for diagnosis of CD may be omitted. In these circumstances, it is necessary to perform further laboratory tests (EMA, HLA) to make the diagnosis of CD without biopsies. Moreover, antibody positivity should be verified by EMA from a blood sample drawn at an occasion separate from the initial test to avoid false-positive serology. If EMA testing confirms specific CD antibody positivity in this second blood sample, then the diagnosis of CD can be made and the child can be started on a gluten free diet (GFD). It is advisable to check for HLA types in patients who are diagnosed without having an intestinal biopsy to reinforce the diagnosis of CD (see Figure 2 and 3).
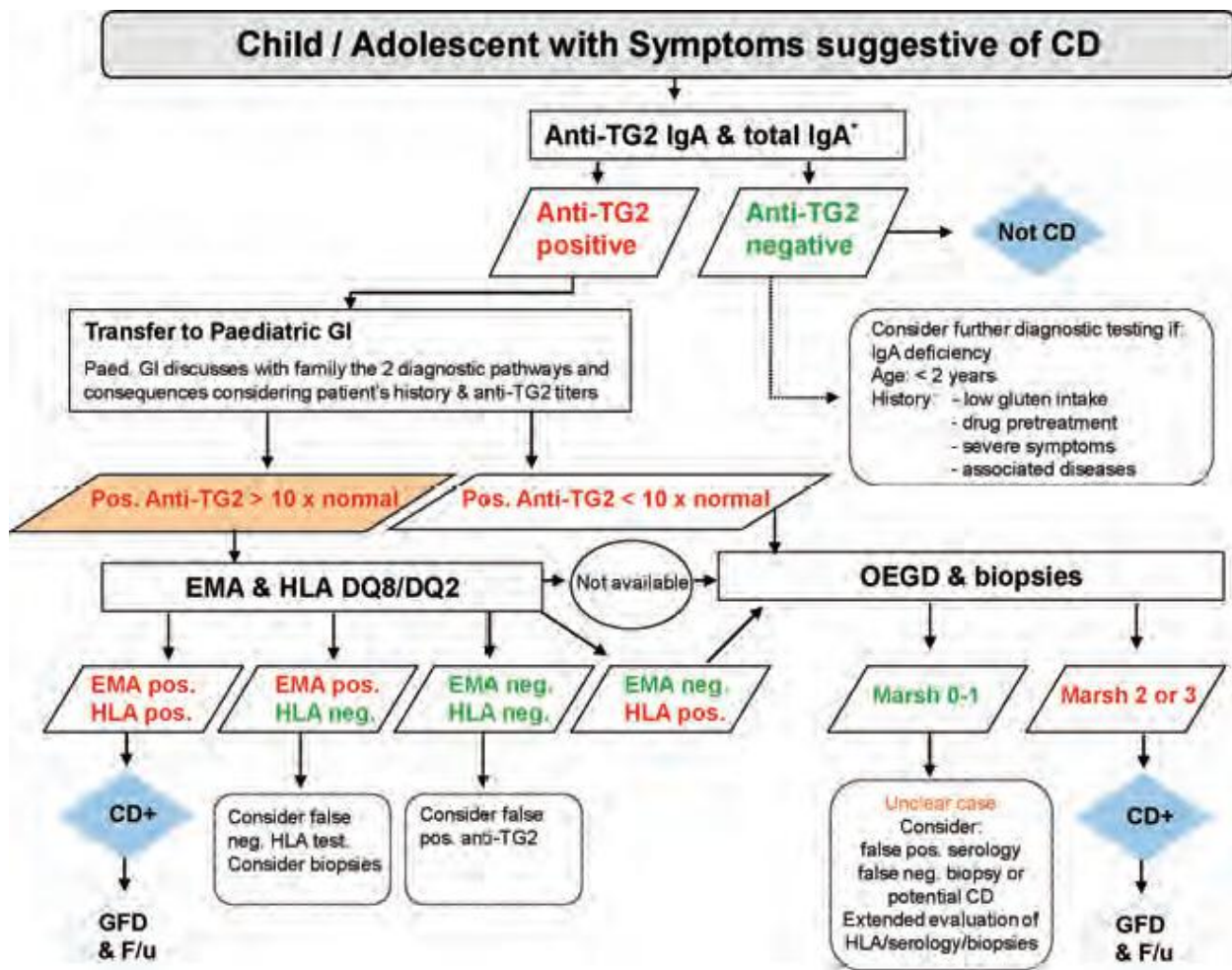
Figure 2. Symptomatic patient. CD=coeliac disease; EMA=endomysial antibodies; F/u=follow-up; GFD=gluten-free diet; GI=gastroenterologist; HLA=human leukocyte antigen; IgA=immunoglobulin A; IgG=immunoglobulin G; OEGD=oesophagogastroduodenoscopy; TG2=transglutaminase type 2.

Source: European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Guidelines for the Diagnosis of Coeliac Disease Journal of Pediatric Gastroenterology and Nutrition 54(1):136-160, January 2012.
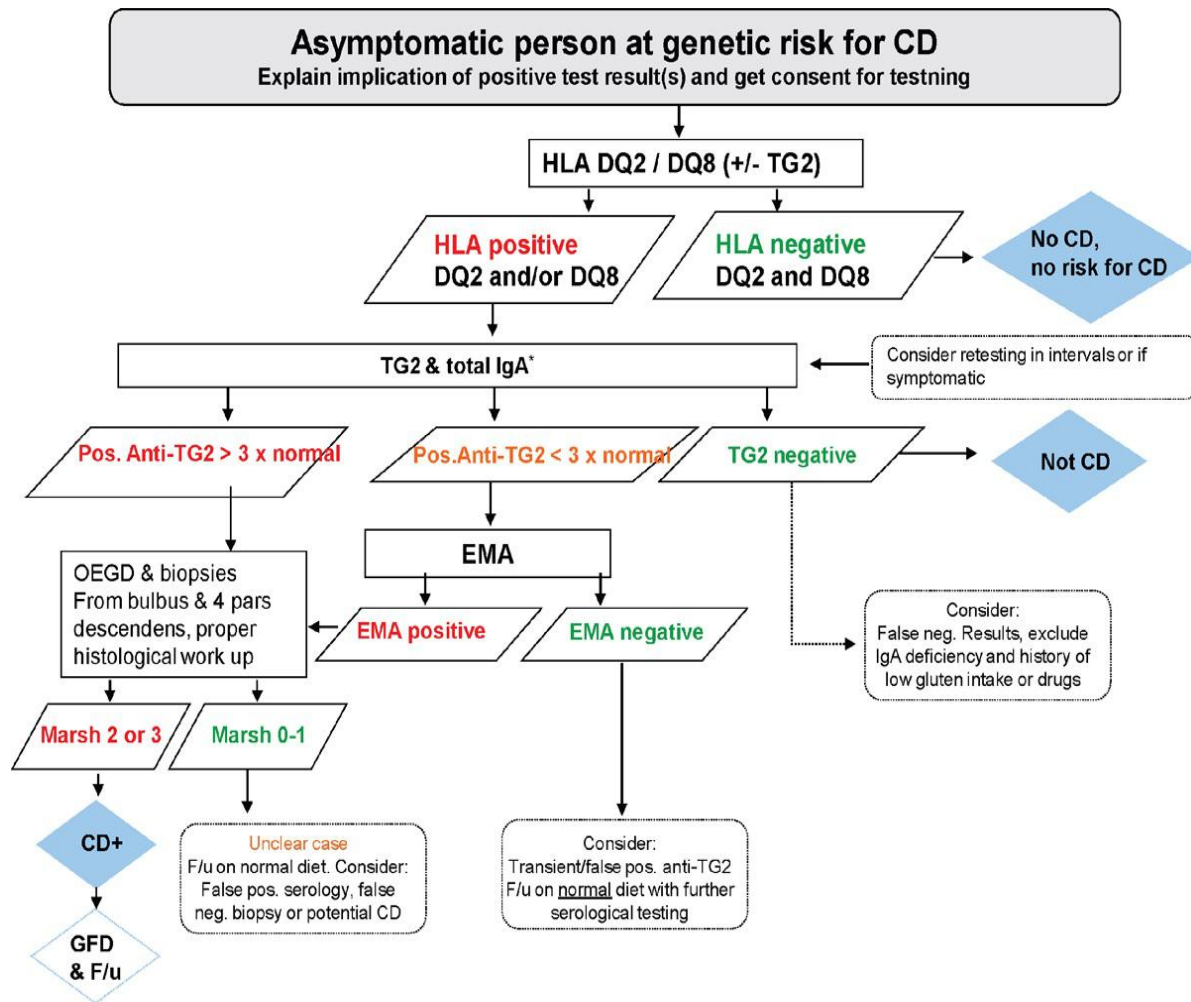
Figure 3. Asymptomatic patient. See Fig. 2 for definitions.

Source: European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Guidelines for the Diagnosis of Coeliac Disease Journal of Pediatric Gastroenterology and Nutrition 54(1):136-160, January 2012.

1.10. Treatment and prognosis of CD

The overall accepted treatment for CD is a strict gluten-free diet for life. A balanced GFD based on a combination of naturally gluten-free foods and certified processed gluten-free products, will result in symptomatic, serologic, and histological remission in most patients (92, 93). Growth and development in child return to normal with adherence to the gluten-free diet, and many disease complications in adults are avoided.

Approximately 70% of patients report an improvement in symptoms within 2 weeks after starting the gluten-free diet, on the other hand the complete histological resolution is not always achieved, or may take years (94, 95). Patients who are extremely ill may require hospital admission, repletion of fluids and electrolytes, intravenous nutrition and, occasionally, steroids. Patients with severe cases who require hospitalization are described as having a celiac crisis. Patients with newly diagnosed CD should be referred to an expert dietitian, because the GDF requires knowledge not only of hidden sources of gluten, but also of healthy gluten-free substitute grains that provide adequate fibre and nutrients. Upon diagnosis, patients should be tested for micronutrient deficiencies, including iron, folic acid, vitamin B12, and vitamin D. Patients should be encouraged to eat natural high-iron and high folate foods, especially if a deficiency in these minerals is documented. However, not all dietitians are familiar with the intricacies of a GDF, and for this reason local or national support groups provide most of the required information.

Even thought, the adherence to a GFD is considered a safe treatment for CD, some recent study reported a relationship between increased heavy metals concentrations especially arsenic, lead, mercury, and cadmium in CD patients who follow a GFD. This is suspected to be the result of drinking water and eating food, such as fish and rice-based products (96). Moreover, some other studies show that the urinary concentration of arsenic was associated significantly with consumption of rice, which is used as a major gluten-free staple (97). Heavy metals can bioaccumulate over time and cause an array of adverse health effects. Bioaccumulation of heavy metals could mainly depend on the food types ingested or their accumulation could also be due to a genetic predisposition of CD subjects to retain them. Further studies are needed to clarify the clinical significance of increased heavy metal levels in patients with CD who follow a GFD.

Following a GFD in low and/or middle-income countries (LMIC) has additional barriers. Many studies from these countries show that the non-availability of gluten-free products is the most

common barrier to adherence to a strict GFD. This is in part owing to a lack of gluten labelling on food products in a majority of LMIC. In addition, because of a lack of specific regulations, the risk of contamination by gluten is even higher in LMIC and can occur at any stage during its production and final purchase. In LMIC, families with limited resources face an additional risk of gluten contamination at home, especially if the same utensils are used for storing, cooking and handling (rolling pin, surface griddle, and oil for frying) gluten containing foods and gluten- free foods. Furthermore, in the majority of health-care centres in LMIC, there is a lack of dietitians trained in the nuances of a GFD (98, 99).

Even though the only treatment for CD is a GDF, there is interest among patients in a medical therapy to replace or help with a GFD. Recent progress made in understanding the pathogenesis of CD has opened the door for a variety of new non-dietary treatments which may be used at least as adjunctive therapy (100). However, to the date, only a limited number of experimental therapies for CD have been assessed in phase I-II randomised, controlled clinical trials. Larazotide acetate (AT-1001), which is assumed to hinder the paracellular passage of gluten through the epithelial barrier into the lamina propria by inhibiting tight junctions, has been studied in almost 100 patients to date, but an effect on hard end points, such as protection of mucosal integrity, needs to be demonstrated (101). Though fewer patients were studied, evidence for the efficacy of the endopeptidases contained in ALV003 which break down gluten to less or non-immunogenic peptide fragments is more obvious. On the other hand, other therapies like TG2 inhibition, preventing immunogenic potentiation of gluten, therapy targeted at immune cells (102, 103) blocking the binding of deamidated gluten peptides to CD-specific HLA proteins (104) or vaccination to induce tolerance to ingested gluten are less advanced but have potential for high efficacy.

Anyway, CD prognosis is excellent if the treatment begins on time and if it is consistently implemented. Most of the complications may be prevented by early detection of disease and lifelong adherence to the GFD. The patient should be regularly monitored and advised for antibodies check-up   (anti tTG) in 6 months after the start of GFD and continue once per year or less frequently in asymptomatic patients to monitor adherence to GFD.

Challenges for the next decade include reducing the burden of treatment by providing easier access to inexpensive gluten-free foods and developing non-dietary approaches to increase the efficacy of treatment.

2. HYPOTHESIS

HLA DQ2 and DQ8 heterodimers are predisposing genetic factors in Albanian paediatric celiac disease patients from Kosovo as in the majority of populations studied so far, but specific HLA haplotypes are expected, especially in DQ2 and/or DQ8 heterodimer negative patients.

3. AIM

3.1. General aim

General aim of the research is to analyze the frequency of HLA-DQ2 and DQ8 heterodimers as immunogenetic predisposing factors in the population of Albanian celiac disease children from Kosovo

3.2. Specific aims

- Analyse HLA-DRB1, DQA1 and DQB1 class II polymorphisms in healthy control group
- Analyse HLA-DRB1, DQA1 and DQB1 class II polymorphisms in patient group
- Determine HLA class II predisposing factors by comparison of two studied groups
- Compare the HLA class II gene and haplotype frequency data with other European populations.
- Determine population specific HLA class II genes and haplotypes involved in disease predisposition.

4. MATERIALS AND METHODOLOGY

4.1. Materials

Subjects

The study subject were children aged 17 months to 18 years treated for CD from 2005 to 2016 at Pediatric Clinic, University Clinical Center of Kosovo (UCCK), as a referral center for CD. Patients of non-Albanian nationality, and those older than 18 years at the time of diagnosis were excluded from the study.

Seventy-two paediatric patients were enrolled in the study. All of them were clinically suspected and had positive serology (anti-TG2) for CD. Among them, 3 were siblings of index cases, thus they were excluded from further analysis, leading to the patient group consisting of 69 unrelated children. HLA-A, B, DRB1, DQA1, DQB1 typing has been performed for all patients, while EMA testing and SBB were performed for 59 and 24 patients, respectively (see Figure 4).

For the clinical data and HLA allele frequency data analysis the entire group of 69 unrelated patients were divided into 2 groups. In the first group, consisting of 60 patients, CD diagnosis was established according to old 90's ESPGHAN guidelines for 24 patients and for 36 patients, according to 2012 criteria. Nine patients were analysed and discussed separately due to uncompleted diagnostic tools to fulfil criteria for CD diagnosis.

Separate analysis has also been carried out for three CD siblings, as well as for three healthy siblings of index cases.

Control group consisted of 124 unrelated healthy children, without a history of autoimmune diseases, gender and age matched. Out of them, 64 were females and 60 males, while mean age at the time of sample collection was 8.7 years (SD±5.8), and age ranged from 1 to 18 years.

This cross-sectional retrospective study was performed in Pediatric Clinic, University Clinical Center of Kosovo (UCCK), as a referral center for CD. HLA typing was performed in Tissue Typing Center, Department of Transfusion Medicine and Transplantation Biology, Clinical Hospital Center, Zagreb.

The study was approved by the Ethics Committee of the UCCK and CHC Zagreb.

Informed written consent was obtained from parent/guardian of all children involved in the study.
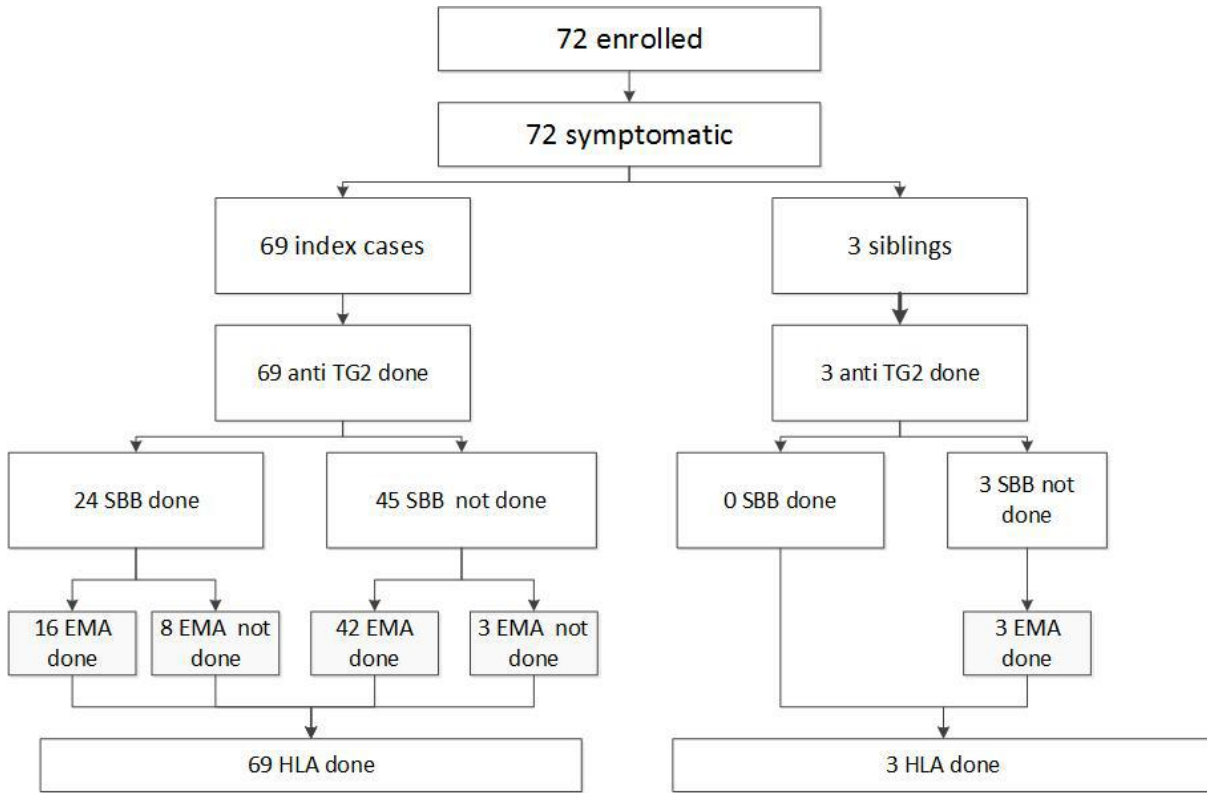
Figure 4. Algorithm for CD diagnosis conducted in all seventy-two children. Anti TG2- tissue transglutaminase 2, EMA-endomysial antibody, SBB-small bowel biopsies, HLA HD- HLA heterodimer; not done-not tested.

4.2. Methodology

General data (name, gender, age breastfeeding, age at gluten/solid foods introduction in the diet) and clinical presentation at time of diagnosis were collected through individual interviews. In the case of young children, the information was provided by parents, whereas adolescents described their symptoms directly to the physician. Patients` growth parameters were reported following the current guidelines that recommend the assessment of body weight percentiles in children according to their age and sex. We used World Health Organization standards to determine the weight for length (W/L) percentile. All information was reviewed by the investigators to validate the data and create a clear database. The following parameters were studied: age at diagnosis, the

delay in diagnosis (which is defined as the interval between the first presentation of symptoms and the definitive diagnosis of CD), sex, clinical symptoms, family history, associated diseases, and nutritional status. All cases were divided into 3 age groups: 1–5, 6–10, 11–18 years old.

Clinical presentation, manifested with: diarrhea, abdominal distention, weight loss, failure to thrive and/or anorexia, was classified as classical CD, while the presence of atypical symptoms such as abdominal pain, constipation, short stature, vomiting, anaemia, delayed puberty, was considered to be non-classical.

Serological tests were performed in accredited laboratory; Enzyme-linked immunosorbent assay by LIAISON® Analyzer (CLIA immunoassay auto-analyzer) was used for anti-TG2 testing. The manufacturer`s cut-off for positive anti-TG2-IgA antibodies was 15 U/mL, while for anti-TG2-IgG, 12 U/mL. EMAs were determined by indirect immunofluorescence using monkey esophagus tissue (Binding Site, Birmingham, UK); with a 1:5 dilution as a cut-off.

Upper gastrointestinal endoscopy with biopsies (UGIE) - was done in department of abdominal surgery and Institute of Pathology in UCCK. UGIE was done under sedation with a standard forward-viewing video-endoscope (GIF-Q145, Olympus Optical Co. Ltd., Tokyo, Japan). Biopsies were taken from both, proximal and distal part of duodenum, including one from the bulb, following the ESPGHAN recommendation of 4 to 6 specimens per procedure.

Histological examination - the histopathology report has included the description of the orientation, the presence or not of normal villi, the degree of atrophy and crypt elongation, the villus/crypt ratio, the number of intraepithelial lymphocytes (IELs). The numbers of IELs were determined with immunohistochemical staining for CD3 and CD8. The grading was done according to the revised Marsh-Oberhuber classification, as Marsh I to III (Marsh IIIa, Marsh IIIb, and Marsh IIIc). Marsh score of 2-3 was considered confirmative for CD.

HLA typing - three milliliters of blood was taken from each child included in the study. EDTA blood samples were stored frozen at -30°C until all samples were collected. Deoxyribonucleic Acid (DNA) isolation and HLA typing was performed at Tissue Typing Center, Clinical Department of Transfusion medicine and Transplantation Biology, KBC Zagreb. Genomic DNA was purified from whole blood with the MagNA Pure Compact Instrument using the corresponding commercial kit MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics GmbH, Penzberg, Germany). Final DNA concentration was 10-200 ng/µl. HLA genotyping was performed using the Lifecodes HLA-SSO typing procedure based on the

hybridization of labeled single stranded PCR product to SSO probes. Amplification of DNA was done using Biometra T professional standard thermocycler (Biometra GmbH, Göttingen, Germany). After the hybridization, the samples were processed using the Luminex200 instrument (Luminex Corporation, Austin, USA). The Luminex results were analyzed using Quicktype for Lifecodes 3.3. Software.

DQ heterodimer types were defined by the presence of one of the HLA-DQA1 and DQB1 alleles in the individual genotype. The first number in HD type stands for the serological equivalent of DQB1 allele, while the second number stands for DQA1 gene.

Following these rules HD types were denoted as follows:

DQ2.2 = DQA1*02:01-DQB1*02:02;

DQ2.3 = DQA1*03:03-DQB1*02:02;

DQ2.5 = DQA1*05:01/*05:05-DQB1*02:01/*02:02;

DQ4.3 = DQA1*03:03-DQB1*04:02;

DQ4.4 = DQA1*04:01-DQB1*04:02;

DQ5.1 = DQA1*01:01/*01:02/01:03/*01:04-DQB1*05:01/*05:02/*05:03;

DQ6.1 = DQA1*01:02/*01:03- DQB1*06:01/*06:02/*06:03/*06:04/*06:09;

DQ7.3 = DQA1*03:01/*03:02/*03:03-DQB1*03:01;

DQ7.5 = DQA1*05:05-DQB1*03:01/*03:04;

DQ8.3 = DQA1*03:01/*03:02-DQB1*03:02;

DQ9.2 = DQA1*02:01-DQB1*03:03;

DQ9.3 = DQA1*03:03-DQB1*03:03;

The presence of HLA-DQ2.5/DQ8/DQ2.2 genotypes was categorized according to the presence of CD predisposing HLA heterodimer types, as follows:

DQ2.5/DQX - HD DQ2.5 in single dose, the other heterodimer is other than DQ2.5, DQ8 and DQ2.2 (e.g. DQA1*01:01, *05:01; DQB1*02:01, *05:02)

DQ2.5*cis* - DQA1 and DQB1 alleles on the same chromosome

DQ2.5*trans* - DQA1 and DQB1 alleles on different chromosomes, one on each

DQ8/DQX - HD DQ8 in single dose, the other heterodimer is other than DQ2.5, DQ8 and DQ2.2 (e.g. DQA1*01:01, *03:01; DQB1*03:02, *05:02)

DQ2.2/DQX - HD DQ2.2 in single dose, the other heterodimer is other than DQ2.5, DQ8 and DQ2.2 (e.g. DQA1*01:01, *02:01; DQB1*02:02, *05:02)

DQ2.5/DQ2.5 - HD DQ2.5 in double dose, homozygous genotype (e.g. DQA1*05:01, *05:01; DQB1*02:01, *02:01)

DQ8/DQ8 - HD DQ8 in double dose, homozygous genotype (e.g. DQA1*03:01, *03:01; DQB1*03:02, *03:02)

DQ2.2/DQ2.2 - HD DQ2.2 in double dose, homozygous genotype (e.g. DQA1*02:01, *02:01; DQB1*02:02, *02:02)

DQ2.5/DQ8 - HD DQ2.5 and DQ8 in single dose each, heterozygous genotype (e.g. DQA1*03:01, *05:01; DQB1*02:01, *03:02)

DQ2.5/DQ2.2 - HD DQ2.5 and DQ2.2 in single dose each, heterozygous genotype (e.g. DQA1*02:01, *05:01; DQB1*02:01, *02:02)

DQ8/DQ2.2 - HD DQ8 and DQ2.2 in single dose each, heterozygous genotype (e.g. DQA1*02:01, *03:01; DQB1*02:02, *03:02)

DQX/DQX - both heterodimers other than DQ2.5, DQ8 and DQ2.2 (e.g. DQA1*01:01, *01:02; DQB1*05:02, *06:03)

4.3. Statistical analyses

Descriptive statistics were presented as means and standard deviations (SD).

HLA-A, B, DRB1, DQA1 and DQB1 allele frequencies were calculated by the GeneRate program (http://geneva.unige.ch/ahpd/). The Hardy-Weinberg equilibrium, estimates of haplotype frequencies and measures and tests of significance for linkage disequilibrium (LD) were performed using the software PyPop (Python for Population Genomics), a software for large-scale HLA population analyses, originally developed for the analysis of data for the 13th International Histocompatibility Workshop and Conference (105).

The significance of differences in allele and haplotype frequencies among patients and controls were evaluated using the chi square test, while Fisher's exact test was used if any of the values in 2x2 tables less than 5. Sensitivity, specificity, positive predictive value, and negative predictive value have been calculated using standard 2x2 contingency table. The strength of association between allele/haplotype polymorphisms and disease risk was assessed by odds ratio (OR) with the corresponding 95% CI. A p value of <0.05 was considered statistically significant. Analyses were performed using MedCalc Version 18.10.2. and SPSS software (SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY, USA).

5. RESULTS

5.1. Healthy control group

Control group consisted of 124 unrelated healthy children, without a history of autoimmune diseases, gender and age matched. Out of them 64 were females and 60 males while mean age at the time of sample collection was 8.7 years (SD±5.8), and age ranged from 1 to 18 years. All individuals have been typed for HLA-A, B, DRB1, DQA1 and DQB1 gene and allele polymorphisms.

5.1.1. HLA-A, -B and DRB1 gene frequencies

The estimated gene frequencies for the HLA-A, B and DRB1 loci studied in 124 healthy children, are shown in Table 2. Sixteen HLA-A, 24 HLA-B and 13 HLA-DRB1 different genes were detected in the control group. At HLA-A locus, the most frequent gene, A*02 was present with a frequency of 33%. Three other genes exhibited frequencies over 10% (A*24, A*03, and A*01), and together with A*02 represent 66% of all HLA-A genes detected. Three genes were not detected, HLA-A*34, A*36 and A*80.

At HLA-B locus, out of twenty-four identified, ten alleles were present at >3% contributed 77.44% of total. The most frequent one was B*51 with a frequency of 16% and together with B*18, and B*35 which had a frequency greater than 10%, represent 42% of all B genes detected. Twelve HLA-B genes were not detected, B*42, *45, *46, *48, *54, *59, *67, *73, *78, *81, *82 and *83. Out of thirteen HLA-DRB1 genes detected, four genes had a frequency higher than 10%: the most frequent one HLA-DRB1*11 (22.18%) followed by HLA-DRB1*13 (14.52%), HLA-DRB1*16 (13.71%) and HLA-DRB1*04 (11.26), representing jointly 61.67% of the total.

The less frequent genes at all three loci, present in only one individual, were A*31, A*66, B*50 and DRB1*09.

Table 2. HLA-A, -B and DRB1 gene frequencies in the control group

CONTROL GROUP (N=124)											
A*	n	GF (%)	IF (%)	B*	n	GF (%)	IF (%)	DRB1*	n	GF (%)	IF (%)
01	26	10.48	21	07	15	6.05	12	01	10	4.03	8
02	81	32.66	65	08	14	5.65	11	03	18	7.26	15
03	27	10.89	21	13	6	2.42	5	04	28	11.26	23
11	10	4.03	8	14	3	1.21	3	07	17	6.85	13
23	9	3.63	7	15	9	3.63	7	08	5	2.02	4
24	30	12.10	24	18	33	13.31	26	09	1	0.40	1
25	2	0.81	2	27	6	2.42	5	10	4	1.61	3
26	16	6.45	13	35	31	12.50	25	11	55	22.18	44
29	3	1.21	2	37	5	2.02	4	12	4	1.61	3
30	5	2.02	4	38	15	6.05	12	13	36	14.52	29
31	1	0.40	1	39	6	2.42	5	14	18	7.26	15
32	16	6.45	13	40	13	5.24	10	15	18	7.26	15
33	2	0.81	2	41	4	1.61	3	16	34	13.71	27
66	1	0.40	1	44	14	5.65	11				
68	17	6.85	13	47	3	1.21	3				
69	2	0.81	2	49	8	3.23	6				
				50	1	0.40	1				
				51	40	16.13	32				
				52	3	1.21	3				
				53	4	1.61	3				
				55	3	1.21	3				
				56	2	0.81	3				
				57	7	2.82	6				
				58	3	1.21	3				

N - number of tested individuals; n - number of genes detected;
GF % - gene frequency, in percentage;
IF % - individuals positive for the gene, in percentage

5.1.2. HLA-DQA1, -DQB1 allele frequencies

The HLA-DQA1 and HLA-DQB1 allele frequencies of 124 examined healthy children are listed in Table 3.

Within DQA1 alleles, out of eleven detected, two alleles had a frequency higher than 20%: the most frequent one DQA1*01:02 (26.6%) and DQA1*05:05 (25.00%); together with the *01:01 present at 12.5%, representing jointly 64.1% of the total.

Fifteen DQB1 alleles were identified. Within DQB1 alleles, two alleles had a frequency higher than 10%: the most frequent one DQB1*03:01 (26.61%) and DQB1*05:02 (14.52%), representing jointly 41.13% of the total. The less frequent ones were DQA1*01:04, DQB1*03:04 and DQB1*06:01 which were present in only one individual.

Table 3. HLA-DQA1, -DQB1 allele frequencies in the control group

CONTROL GROUP (N=124)							
DQA1*	n	AF (%)	IF (%)	DQB1*	n	AF (%)	IF (%)
01:01	31	12.50	25	02:01	18	7.26	15
01:02	66	26.61	53	02:02	19	7.66	15
01:03	18	7.26	15	03:01	66	26.61	53
01:04	1	0.40	1	03:02	20	8.06	16
02:01	17	6.85	14	03:03	2	0.81	2
03:01	22	8.87	18	03:04	1	0.40	1
03:02	3	1.21	2	04:02	6	2.42	5
03:03	4	1.61	3	05:01	14	5.65	11
04:01	5	2.02	4	05:02	36	14.52	29
05:01	19	7.66	15	05:03	18	7.26	14
05:05	62	25.00	50	06:01	1	0.40	1
				06:02	13	5.24	10
				06:03	18	7.26	15
				06:04	10	4.03	8
				06:09	6	2.42	5

N - number of tested individuals; n - number of observed alleles;
AF % - allele frequency, in percentage;
IF % - individuals positive for the allele, in percentage

5.1.3. Two-, three- and five-locus haplotype frequencies

HLA-A,-B two-locus haplotype analysis revealed HLA-A*02-B*51 as the most frequent one found at 10.68% frequency, followed by HLA-A*02-B*18 and HLA-A*24-B*35, with a frequency of 5.43% and 4.93%, respectively. A total of 90 different haplotypes were detected, with 43 haplotypes present only once, while 8 different haplotypes occurred 5 or more times, representing 35% of a total number of haplotypes in controls.

HLA-A,-B,.DRB1 three-locus haplotype analysis revealed 141 different haplotypes, among which 95 haplotypes occured only once. Five haplotypes were present 5 or more times and together represented 18% of all haplotypes. The most frequent one was HLA-A*02-B*08-DRB1*11 (6.05%).

HLA-DRB1-DQA1-DQB1 three-locus haplotype analysis showed that HLA-DRB1*11-DQA1*05:05-DQB1*03:01 and HLA-DRB1*16-DQA1*01:02a-DQB1*05:02 were the most frequent haplotypes, with a frequency of 21% and 13%, respectively. A total of 32 different haplotypes were detected, with 14 haplotypes present only once.

Two most frequent five-locus haplotypes was found to be HLA-A*02-B*11-DRB1*18-DQA1*05:05-DQB1*03:01 and HLA-A*02-B*51-DRB1*14-DQA1*01:01-DQB1*05:03 with frequency of 6.45 % and 3.62%, respectively. A total of 156 different five-locus haplotypes were detected, with a total of 121 haplotypes that occurred only once. Six most common five-locus haplotypes represented 20% of the total. The most common two-, three- and five-locus haplotypes which occurred 5 or more times are listed in Table 4.

Table 4. Two (HLA-A, -B), three (HLA-A, -B, -DRB1 and HLA-DRB1, -DQA1, -DQB1) and
five (HLA-A, -B, -DRB1, -DQA1, -DQB1) locus haplotypes with a number of
copies ≥5 in the control group (N=124)

A*	B*	DRB1*	DQA1*	DQB1*	n	%
CONTROL GROUP (N=124)						
02	51	-	-	-	27	10.68
02	18	-	-	-	14	5.43
24	35	-	-	-	12	4.93
01	08	-	-	-	9	3.63
32	40	-	-	-	8	3.01
23	44	-	-	-	7	2.82
02	15	-	-	-	7	2.82
03	18	-	-	-	6	2.42
02	18	11	-	-	15	6.05
02	51	14	-	-	10	4.03
01	08	03	-	-	7	2.82
24	35	11	-	-	6	2.42
23	44	07	-	-	6	2.42
-	-	11	05:05	03:01	53	21.37
-	-	16	01:02	05:02	33	13.26
-	-	04	03:01	03:02	19	7.66
-	-	03	05:01	02:01	18	7.26
-	-	14	01:01	05:03	17	6.85
-	-	13	01:03	06:03	16	6.45
-	-	07	02:01	02:02	16	6.45
-	-	15	01:02	06:02	13	5.24
-	-	13	01:02	06:04	10	4.03
-	-	01	01:01	05:01	10	4.03
02	18	11	05:05	03:01	16	6.45
02	51	14	01:01	05:03	9	3.63
01	08	03	05:01	02:01	7	2.82
02	51	11	05:05	03:01	6	2.42
23	44	07	02:01	02:02	6	2.42
24	35	11	05:05	03:01	6	2.42

N - number of tested individuals; n - number of observed haplotypes;
% - haplotype frequency, in percentage

5.1.4. DQA1-DQB1 haplotype frequencies

Twenty-seven different DQA1-DQB1 haplotypes were found in the control group, eight of them showed a frequency of more than 5%, while 12 haplotypes were found only once. DQA1*05:05-DQB1*03:01 haplotype showed the highest frequency (24.19%), followed by DQA1*01:02-DQB1*05:02 and DQA1*03:01-DQB1*03:02 in 14.51% and 7.66% of controls, respectively (see Table 5).

Table 5. DQA1-DQB1 haplotype frequencies in the control group

CONTROL GROUP (N=124)			
DQA1*	DQB1*	n	(%)
01:01	05:01	14	5.64
01:01	05:03	17	6.85
01:02	05:02	36	14.52
01:02	06:02	13	5.24
01:02	06:03	2	0.81
01:02	06:04	10	4.03
01:02	06:09	5	2.02
01:03	06:01	1	0.40
01:03	06:03	16	6.45
01:03	06:09	1	0.40
01:04	05:03	1	0.40
02:01	02:02	16	6.45
02:01	03:03	1	0.40
03:01	03:01	3	1.21
03:01	03:02	19	7.66
03:02	03:01	2	0.81
03:02	03:02	1	0.40
03:03	02:02	1	0.40
03:03	03:01	1	0.40
03:03	03:03	1	0.40
03:03	04:02	1	0.40
04:01	04:02	5	2.02
05:01	02:01	18	7.26
05:01	02:02	1	0.40
05:05	02:02	1	0.40
05:05	03:01	60	24.19
05:05	03:04	1	0.40

N - number of tested individuals; n - number of observed haplotypes;
% - haplotype frequency, in percentage

5.1.5. DQ heterodimer type frequency

DQ heterodimer type frequency in controls is presented in Table 6. A total of 12 different heterodimer types were found, four occurring only once. The most frequent heterodimer types found in controls were DQ5.1 and DQ6.1, with frequency of 27.42 % and 19.35%, respectively. At less extended frequency were DQ2.5 and DQ8.3, both with the same frequency of 8.06%, followed by DQ2.2, with a frequency of 6.45%.

Table 6. Analysis of DQ heterodimer type frequency in the control group

CONTROL GROUP (N=124)		
DQ HD TYPE	n	%
DQ2.2	16	6.45
DQ2.3	1	0.40
DQ2.5	20	8.06
DQ4.3	1	0.40
DQ4.4	5	2.01
DQ5.1	68	27.42
DQ6.1	48	19.35
DQ7.3	6	2.41
DQ7.5	61	24.6
DQ8.3	20	8.06
DQ9.2	1	0.40
DQ9.3	1	1.40
DQ9.3	1	0.40

N - number of tested individuals; n - number of observed heterodimer types;
HD – heterodimer; % - HD type frequency, in percentage

5.1.6. Heterodimer genotype frequencies

The detailed heterodimer genotype distribution, as defined by the combination of HLA DQA1 and DQB1 genes and alleles in the individual genotype, is presented in Table 7. The presence of HLA-DQ2.5/DQ8/DQ2.2 genotypes was categorized according to the presence of CD predisposing HLA alleles, as described in section Methods.

Table 7. CD predisposing HLA-DQ heterodimer genotype in the control group

CONTROL GROUP (N=124)			
	DQ HD GENOTYPE	n	%
	DQ2.5/X	19	15.32
	DQ2.5*cis*	17	13.71
	DQ2.5*trans*	2	1.61
	DQ8/X	17	13.71
	DQ2.2/X	8	6.45
	DQ2.5/DQ2.5	0	0
	DQ8/DQ8	1	0.81
	DQ2.2/DQ2.2	2	1.61
	DQ2.5/DQ8	1	0.81
	DQ2.5/DQ2.2	2	1.61
	DQ8/DQ2.2	0	0.00
HD POSITIVE	DQ2.5/DQ8/DQ2.2	50	40.32
HD NEGATIVE	X/X	74	59.68

N - number of tested individuals; n - number of individuals positive for HD genotype;
HD – heterodimer; % - HD genotype frequency, in percentage
note: definition of heterodimer types is presented in section Methods

In the whole data set, 40.32 % of healthy controls were positive and 59.68 % were negative for at least one of the predisposing CD heterodimer genotype, i.e. HLA-DQ2.5/DQ8/DQ2.2 positive or negative (see Figure 5). The most prevalent HD positive genotype was DQ2.5/X with a frequency over 15.32%. Positivity for one copy of DQ2.5 heterodimer was more prevalent in *cis* position than in *trans* position. On the other hand, none of the controls was homozygous for DQ2.5, i.e. having genotype positive for two copies of DQ2.5 heterodimer. Second most prevalent genotype was single copy DQ8 genotype (DQ8/X) which was present in almost 13.71% of healthy children.
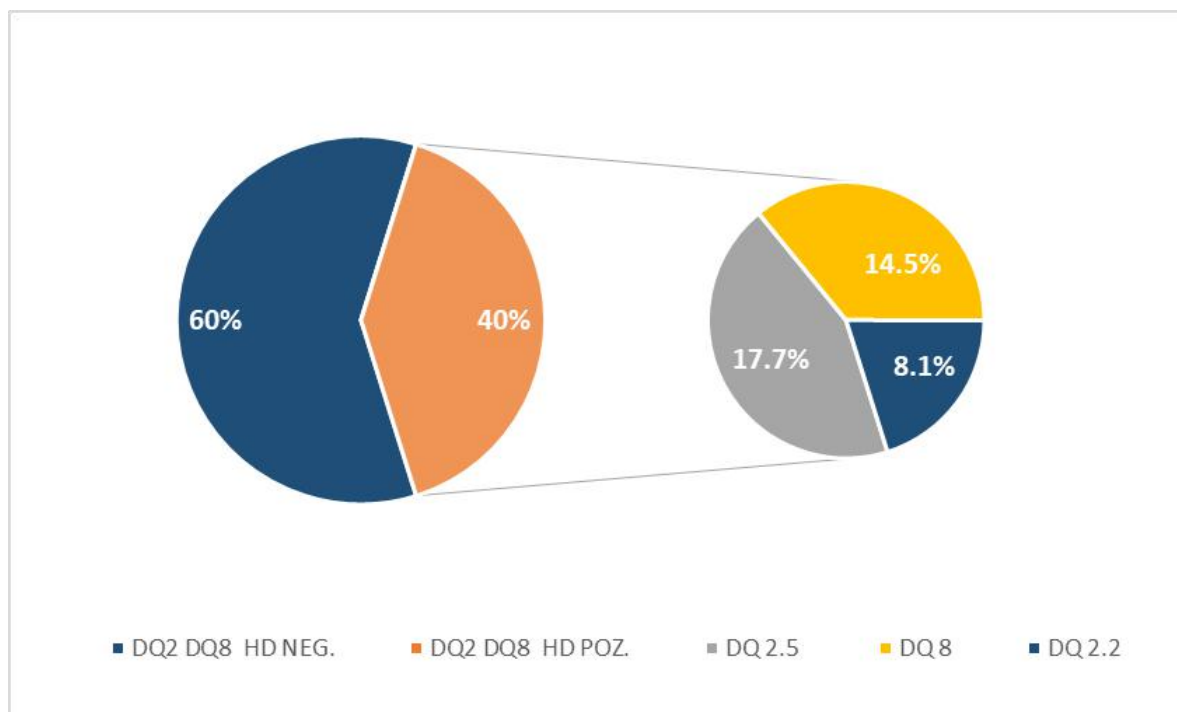
Figure 5. Predisposing CD heterodimer genotype distribution in controls

5.2. Confirmed celiac disease cases - index cases

5.2.1. General aspects

In the group of confirmed CD patients, consisting of 60 children, the gender distribution showed a female predominance, with an overall female to male ratio of 1.9:1 (see Figure 6).
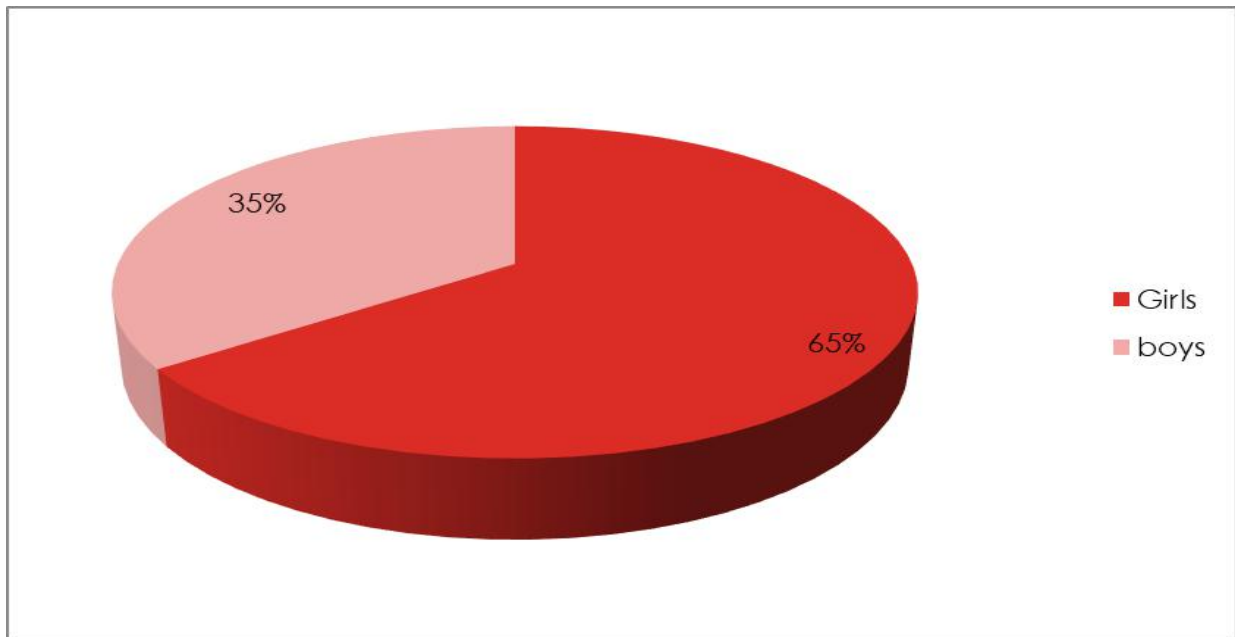
Figure 6. Gender distribution in CD patients

Patients' mean age at diagnosis was 5.5 years (SD±3.31), ranged from 17 months to 18 years. Mean age at CD onset was 3.3 years old (SD±2.02) while the delay from first symptoms indicative of CD to diagnosis was 6 months to 10 years, with a median delay of 2.4 years (SD±2.11).

Forty-one children had been breastfed as infants, while 34 of them were exclusively breastfed for the first 6 months. The overall mean duration of breastfeeding was 10.2 month. Among the cases, non-breastfed children (n=19 or 32 %) had earlier onset of symptom, at median age of 1.9 years as compared to the breastfeed group (41 or 68 %) with a median age of 3.7 years (p=0.002), as

well as earlier diagnosis (p=0.001). Anyway, there was no significant difference on delay to diagnosis between breastfed and non-breastfed children (p=0.8).

Mean age at gluten introduction was 5.2 month, one third of cases started gluten earlier than 5 months of ages. This group of children had earlier onset of symptoms and earlier diagnosis compared to those that started gluten ingestion after 5 months of ages, p<0.001. Table 8 enumerates the descriptive characteristics of all 60 cases.

Table 8. The descriptive characteristics of CD patients

Characteristic	N (%)	P value
Gender		
Female	39 (65%)	
Male	21 (35%)	
Mean age at diagnosis (y)	5.5	
Mean age at CD onset (y) and gluten introduction, <5 mo / >5 mo	2.3/3.6	0.017
Breastfeeding		
Breastfeeding, yes/no	41/19	
Mean duration of breastfeed (mo)	10.2	
Mean age at gluten introduction (mo), Bf yes/no	5.6/4.6	0.076
Mean age at CD onset (y), Bf yes/no	3.7/1.9	0.002
Mean age at diagnosis of CD (y), Bf yes/no	6.1/4.1	0.001

N - number of tested individuals; mo - months, y - years; Bf - breastfeed

Classical CD occurred in 78% of the cases (mean age at diagnosis 4.5 ± 2.36 years), while the non–classical form was found in 22% (mean age at diagnosis 9.2 ± 3.69 years) (p<0.0001). Mean age at symptom onset differed significantly between the classical and non-classical forms of CD (p<0.0001). Furthermore, there were also significant differences according to age at diagnosis and the delay to diagnosis (p <0.001 for both) (see Table 9).

Table 9. Classic versus non-classic characteristic of CD patients

Characteristics	N (%)	Mean age at CD diagnosis (y)	Mean age at CD onset (y)	Mean Delay (y)
Total Celiac disease	60 (100)	5.5	3.2	2.4
Classic CD	47 (78)	4.5	2.8	1.9
Non- classic CD	13 (22)	9.2	4.8	4.3
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

N - number of tested individuals, %-percentage, y-years

5.2.2. Clinical symptomatology

According to age groups, CD diagnoses were as follows: 60% in the first 5 years of life, 30% age 6 -10 years, and 10% 11-18 years age group.

The clinical presentation pattern showed different distributions among the three age groups (p<0.001). All signs and symptoms were significantly more prevalent in children diagnosed in the first 5 years of life (p<0.001) except for dental enamel defects, decreased bone mineralization and arthritis/arthralgia. Gastrointestinal symptoms were more prevalent in the youngest age group (1–5 years old) at 94%, whereas, the prevalence of these symptoms decreased gradually with age, presenting in 66% of the second age group and in only 16% of the third age group (see Figure 7).
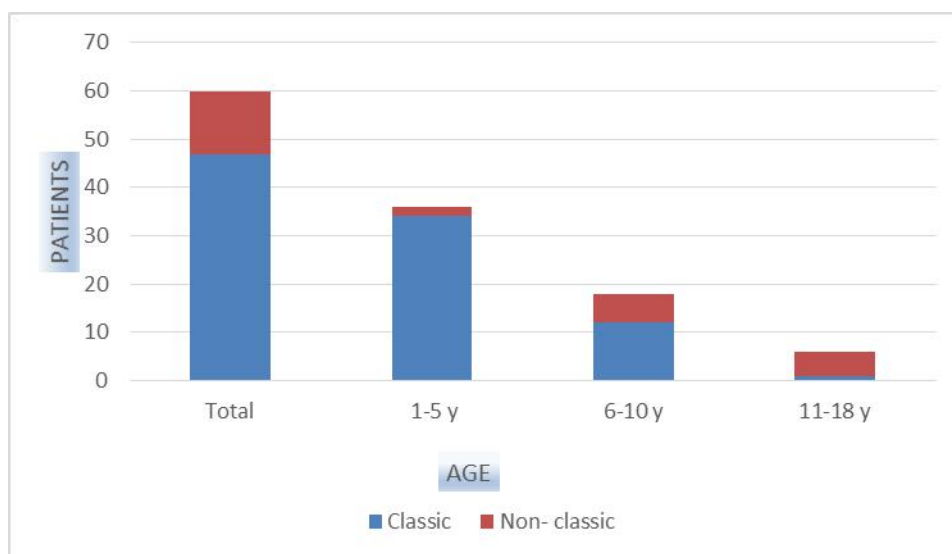
Figure 7. CD forms distribution according to age groups

The most common presenting symptoms in all 60-incident cases were anorexia and anaemia, which were found in 82% and 75% of all cases, respectively. No significant differences were noticed between the three age groups in the frequency of iron deficiency (p=0.308), but it was present in all (100%) children from the third age group. The most frequent symptom in children ages 6 to 10 was abdominal pain in 16 (89%) cases, which was also prevalent in two other age groups (p=0.898). More than two third of the patients exhibited a normal nutritional status, including most patients with classical form. Failure to thrive (FTT) and/or short stature (SS) were present in 16 and 18 cases, 27% and 30%, respectively. There were significant differences (p<0.001) between the study groups regarding FTT with or without short stature. It is noteworthy that a large number of young-age group (1-5), namely 94%, with classical malabsorption had no malnutrition at diagnosis; however, 50% of the children 6-10 years and 83% from the third age group were undernourished at diagnosis. Other extra-intestinal symptoms, rare, but mostly manifested in third age group, were arthritis, decreased bone mineralization and neuropathy. Three girls (50%) from the age group 11-18 years old had delayed puberty. The CD symptoms at presentation according to age groups are shown in Table 10.

Table 10. Clinical presentation of patients with CD according to age groups

Symptoms at Presentation	1-5y N- 36 (60 %)	6-10y N- 18 (30 %)	11-18y N- 6 (10 %)	Total N- 60 (100 %)	P- value
Gastrointestinal					
Diarrhea	34 (94 %)	12 (66 %)	1 (16 %)	47 (78 %)	<0.001
Distended abdomen	34 (94 %)	12 (66 %)	1 (16 %)	47 (78 %)	<0.001
Abdominal pain	22 (61 %)	16 (89 %)	4 (66 %)	42 (70 %)	0.898
Vomiting	21 (58 %)	6 (33 %)	1 (16 %)	28 (47 %)	0.086
Constipation	3 (8 %)	6 (33 %)	4 (66 %)	13 (22 %)	0.004
Anorexia	30 (83 %)	14 (77 %)	5 (83 %)	49 (82 %)	0.892
Extraintestinal					
Weight loss	34 (94 %)	12 (66 %)	1 (16 %)	47 (78 %)	<0.001
Failure to thrive	2 (5 %)	9 (50 %)	5 (83 %)	16 (27 %)	<0.001
Short stature	3 (8 %)	10 (55 %)	5 (83 %)	18 (30 %)	<0.004
Anemia	26 (72 %)	13 (72 %)	6 (100 %)	45 (75 %)	0.308
Irritability	16 (44 %)	9 (50 %)	5 (83 %)	30 (50 %)	0.073
Chronic fatigue	7 (20 %)	13 (72 %)	6 (100 %)	26 (43 %)	<0.001
Delayed puberty	0	0	3 (50 %)	3 (5 %)	<0.001
Neuropathy	0	1 (6 %)	2 (33 %)	3 (5 %)	<0.001
Decreased bone mineralization	1 (3 %)	2 (10 %)	2 (33 %)	5 (8 %)	0.035
dental enamel defects	3 (8 %)	3 (16 %)	1 (16 %)	6 (10 %)	0.438
Arthritis/arthralgia	1 (3 %)	2 (10 %)	2 (33 %)	5 (8 %)	0.028

N- number of tested individuals, %-percentage, y-age in years

Among others, personal and family history were investigated and the presence of any associated conditions was reported. Positive family history for CD was present for 7 children (12%), with first-degree history (siblings) in 4 children and second- or third-degree history in 3 children. Associated diseases were found in 11 patients: DMT1 in three cases (5%) while autoimmune thyroid disease, Down syndrome, Turner syndrome, and cystic fibrosis in one case each. One patient was IgA immune-deficient (see Table 11).

Table 11. Family history and associated disease frequency

Characteristics	N	%
Positive family history of CD	7	12
First degree	4	7
Second degree	3	5
Associated conditions	11	18
Type 1 diabetes	3	5
Thyroid disease	1	1.6
Bone disease	2	3.3
Down syndrom,	1	1.6
Turner syndrome	1	1.6
Fibrosis cystica	1	1.6
Radial aplasia	1	1.6
Hemiplegia	1	1.6
IgA Immune-deficiencies	1	1.6

N - number of tested individuals, %-percentage

5.2.3. Diagnostic tool

All diagnostic tools performed in confirm CD patients are presented in Figure 8.
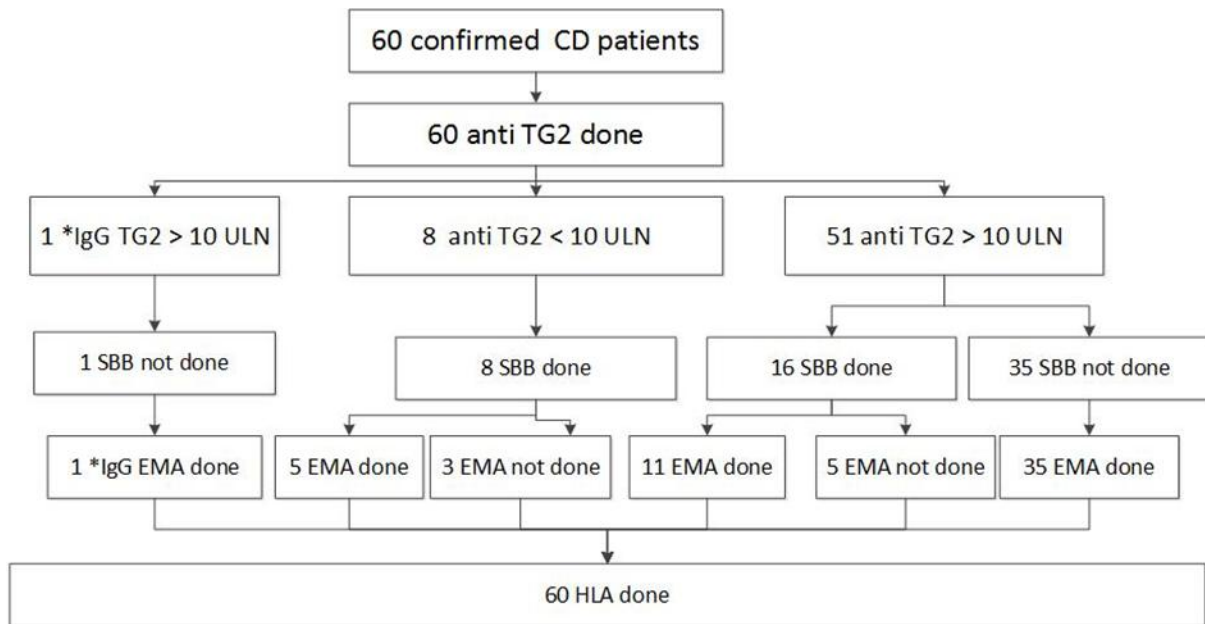
Figure 8. Algorithm for CD diagnosis conducted in 60 confirmed CD children. Anti TG2- tissue transglutaminase 2, EMA-endomysial antibody, SBB-small bowel biopsies, HLA done-heterodimer DQ2.5, DQ2.2, DQ8 testing, Not Done-not tested;

 *- One patient IgA deficient (IgG- immunoglobulin G class anti TG2 and EMA positive)

5.2.3.1. Biochemical parameters

Table 12 represent the biochemical parameters of confirmed CD patients. All 60 patients were tested for IgA anti-TG2, 59 (98.4%) resulted positive, while 51 (86%) of them had titres levels >10 times ULN. Fifty-seven patients (95%) were tested for IgA anti DGP, and 98% of them were positive. IgA EMA was performed in eighty-seven percent of patients (n-52), it resulted positive for 51 of them. In eight patients that were not tested for EMA, CD diagnosis was done according to their positive SBB. One patient with IgA deficiency, belonging to the first age group (1-5) was positive for IgG EMA and anti TG2 (>10 times ULN).

Table 12. Biochemical parameters performed in confirmed CD patients

Test	N (%)	Positive	Negative
IgA anti-TG2	60 (100%)	59 (98.4%)	1 (1.6%) *
		51 (86%)>10NUL	
		8 (14%)<10NUL	
IgA anti DGP	57 (95%)	56 (98.2%)	1 (1.8%)*
IgA –EMA	52 (87%)	51 (98.0%)	1 (2%)*
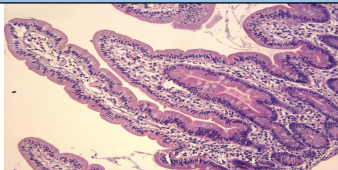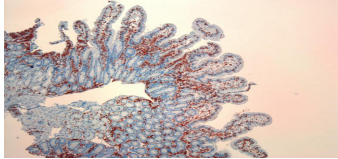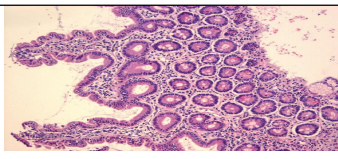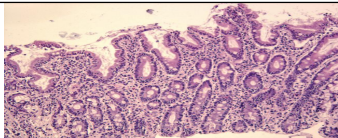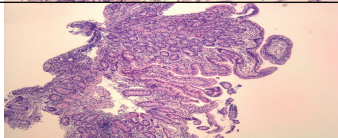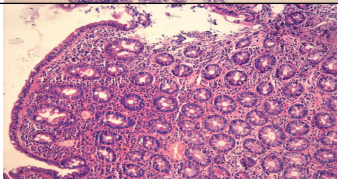
IgA anti TG2- imunoglobuline A anti tissue transglutaminase 2, DGP- deaminated gliadin peptide, EMA-anti endomysial antibody, SBB-small bowel biopsies,
*- One patient IgA deficient (IgG anti TG2 and EMA positive).

5.2.3.2. Small Bowell Biopsies

The majority of 24 performed biopsies were significant for severe enteropathy (90%, P<0.001), regardless of age, sex, or presenting symptoms. Histological examination showed villous atrophy as follows: 33% Marsh IIIa, 20% Marsh IIIb, and 38% Marsh IIIc. Increased intraepithelial lymphocytes with crypt hyperplasia (Marsh II) were found in 2 patients (see Table 13).

Table 13. Histopathological results of performed biopsies

Images	Stages *	n (%)
	Type 0 Pre-infiltrative mucosa. Normal stage of the intestinal mucosa.	0
	Type I Increased number of the non - mitotic intraepithelial lymphocytes (IELs), more than 30 per 100 enterocytes. Normal mucosal architecture.	0
	Type II Increased IEL infiltration, crypt hyperplasia (increase in crypt depth without a reduction in villous height).	2 (8 %)
	Type IIIa Classical CD lesion with partial villous atrophy	8 (33%)
	Type IIIb Classical CD lesion with subtotal villous atrophy	5 (20%)
	Type IIIc Classical CD lesion with total villous atrophy	9 (38%)

*Grading according to the revised Marsh-Oberhuber classification, as Marsh I to III, IELs - intraepithelial lymphocytes.

5.2.3.3. Genetics

5.2.3.3.1 HLA-A, B and DRB1 gene frequencies

The estimated gene frequencies for the HLA-A, -B and –DRB1 loci studied in 60 confirmed CD patients, are shown in Table 14. Fifteen HLA-A, 21 HLA-B and 12 HLA-DRB1 different genes were detected in the patient group. At HLA-A locus, the most frequent gene, A*01 was present with a frequency of 27%, while A*02 was also present with a frequency greater than 20%. These two genes together with A*03 as the third most common gene (10%) together represent more than half (57%) of all HLA-A genes detected. The less frequent genes, present in only one patient, were A*30, *31 and A*69 while four genes were not detected, HLA-A*34, A*36, A*66 and A*80.

At HLA-B locus the most prevalent gene was B*08 with a frequency of 29% while only one gene, B*51, had a frequency greater than 10%. Five different genes were present in only one patient (B*37, *47, *53, *55, *58) and 15 HLA-B genes were not found in patients (B*41, *42, *45, *46, *48, *54, *56, *57, *59, *67, *73, *78, *81, *82 and *83).

Out of 12 HLA-DRB1 genes detected, the most frequent was HLA-DRB1*03 (38.33%) followed by HLA-DRB1*07 (17.50%) and HLA-DRB1*11 (13.33%), representing jointly 69% of the total. DRB1*08, *10, *12 were the less frequent genes, detected in only one patient. Gene DRB1*09 was not detected in the patient group.

Table 14. HLA-A, B and DRB1 gene frequencies in the patient group

PATIENT GROUP (N=60)											
A*	n	GF (%)	IF (%)	B*	n	GF (%)	IF (%)	DRB1*	n	GF (%)	IF (%)
01	32	26.67	53	07	10	8.33	12	01	3	2.50	5
02	26	21.67	43	08	35	29.17	11	03	46	38.33	77
03	12	10.00	20	13	5	4.17	5	04	7	5.83	11
11	4	3.33	7	14	3	2.50	3	07	21	17.50	35
23	7	5.83	12	15	2	1.67	7	08	1	0.83	2
24	9	7.50	15	18	8	6.67	26	10	1	0.83	2
25	2	1.67	3	27	3	2.50	5	11	16	13.33	27
26	4	3.33	7	35	4	3.33	25	12	1	0.83	2
29	3	2.50	5	37	1	0.83	4	13	6	5.00	10
30	1	0.83	2	38	6	5.00	12	14	3	2.50	5
31	1	0.83	2	39	2	1.67	5	15	8	6.67	13
32	8	6.67	13	40	3	2.50	10	16	7	5.83	11
33	2	1.67	3	44	10	8.33	3				
68	8	6.67	13	47	1	0.83	11				
69	1	0.83	2	49	2	1.67	3				
				50	7	5.83	6				
				51	13	10.83	1				
				52	2	1.67	32				
				53	1	0.83	3				
				55	1	0.83	3				
				58	1	0.83	3				

N - number of tested individuals; n - number of genes detected;

GF % - gene frequency, in percentage; IF % - individuals positive for the gene, in percentage

5.2.3.3.2. HLA-DQA1 and DQB1 allele frequencies

The HLA-DQA1 and HLA-DQB1 allele frequencies of 60 examined confirmed CD patients are listed in Table 15.

Within DQA1 alleles, out of eleven detected, the most prevalent one was DQA1*05:01 (40.00%), while two alleles had a frequency higher than 10%: DQA1*02:01 (17.50%) and DQA1*01:02 (13.33%), representing jointly 70 % of the total.

Twelve DQB1 alleles were identified. DQB1*02:01 was the most frequent one (40.00%), followed by two alleles with a frequency higher than 10%: DQB1*02:02 (18.33%) and DQB1*03:01 (14.17 %), together representing jointly 73% of the total.

The less frequent ones were DQA1*03:02, DQA1*03:03, DQA1*04:01, DQB1*04:02 and DQB1*06:01 which were present in only one patient.

Table 15. HLA-DQA1, -DQB1 allele frequencies in the patient group

PATIENT GROUP (N=60)							
DQA1*	n	AF (%)	IF (%)	DQB1*	n	AF (%)	IF (%)
01:01	7	5.83	12	02:01	48	40.00	80
01:02	16	13.33	2	02:02	22	18.33	37
01:03	4	3.33	7	03:01	17	14.17	28
02:01	21	17.50	35	03:02	5	4.17	8
03:01	5	4.17	8	04:02	1	0.83	2
03:02	1	0.83	2	05:01	4	3.33	7
03:03	1	0.83	2	05:02	8	6.67	13
04:01	1	0.83	2	05:03	3	2.50	5
05:01	48	40.00	80	06:01	1	0.83	2
05:05	16	13.33	26	06:02	5	4.17	8
				06:03	4	3.33	7
				06:04	2	1.67	3

N - number of tested individuals; n - number of observed alleles;

AF % - allele frequency, in percentage; IF % - individuals positive for the allele, in percentage

5.2.3.3.3. Two-, three- and five-locus haplotype frequencies

HLA-A,-B two-locus haplotype analysis revealed HLA-A*01-B*08 as the most frequent one found at a frequency of 22.44%, followed by HLA-A*02-B*51 and HLA-A*02-B*50, with a frequency of 7.50% and 4.97 %, respectively.

A total of 56 different haplotypes were detected, with 37 haplotypes present only once, while 6 different haplotypes occurred 4 or more times, representing 45% of a total number of haplotypes in controls.

HLA-A,-B, DRB1 three-locus haplotype analysis revealed 68 different haplotypes, among which 51 haplotypes occured only once. Five haplotypes which were present 4 or more times and together represented 38% of all haplotypes. The most frequent one was HLA-A*01-B*08-DRB1*03 (22.50%).

HLA-DRB1-DQA1-DQB1 three locus haplotype analysis showed that DRB1*03-DQA1*05:01-DQB1*02:01 and HLA-DRB1*07-DQA1*02:01-DQB1*02:02 were the most frequent haplotypes, with a frequency of 38.33% and 17.50%, respectively. A total of 20 different haplotypes were detected, with 9 haplotypes present only once.

The most frequent five locus haplotypes was found to be HLA-A *01-B*08-DRB1*03-DQA1*05:01-DQB1*02:01 followed by HLA-A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 and HLA-A*02-B*51-DRB1*11-DQA1*02:0-DQB1*02:02 with a frequency of 4.17% each

A total of 71 different five locus haplotypes were detected, with a total of 55 haplotypes that occurred only once. Four most common five locus haplotypes represented 34% of the total. The most common two, three and five locus haplotypes which occurred more than 5 times are listed in Table 16.

Table 16. Two (HLA-A-B), three (HLA-A-B-DRB1 and HLA-DRB1-DQA1-DQB1) and five (HLA-A-B-DRB1-DQA1-DQB1) locus haplotypes with a number of copies ≥4 in the patient group (N=60)

PATIENT GROUP (N=60)						
A*	B*	DRB1*	DQA1*	DQB1*	n	%
01	08	-	-	-	27	22.45
02	51	-	-	-	9	7.50
02	50	-	-	-	6	4.99
68	44	-	-	-	5	4.17
03	07	-	-	-	4	3.33
24	07	-	-	-	4	3.33
01	08	03	-	-	27	22.50
02	50	07	-	-	5	4.17
68	44	07	-	-	5	4.17
02	51	11	-	-	5	4.17
24	07	15	-	-	4	3.33
-	-	03	05:01	02:01	46	38.33
-	-	07	02:01	02:02	21	17.50
-	-	11	05:05	03:01	14	11.67
-	-	16	01:02	05:02	7	5.83
-	-	04	03:01	03:02	5	4.17
-	-	15	01:02	06:02	5	4.17
01	08	03	05:01	02:01	27	22.50
02	50	07	02:01	02:02	5	4.17
02	51	11	05:05	03:01	5	4.17
68	44	07	02:01	02:02	4	3.33

N - number of tested individuals; n - number of observed haplotypes;

% - haplotype frequency, in percentage

5.2.3.3.4. DQA1-DQB1 haplotype frequencies

Sixteen different DQA1-DQB1 haplotypes were found in confirmed CD patients, four of them showed a frequency more than 5%, representing jointly 76.16% of the total. Six haplotypes were present only once. DQA1*05:01-DQB1*02:01 haplotype showed the highest frequency (40.00%), followed by DQA1*02:01-DQB1*02:02 and DQA1*05:05-DQB1*03:01 in 17.50% and 12.50% of patients, respectively (see Table 17).

Table 17. DQA1-DQB1 haplotype frequencies in the patient group

PATIENT GROUP (N=60)			
DQA1*	DQB1*	n	(%)
01:01	05:01	4	3.33
01:01	05:03	3	2.50
01:02	05:02	8	6.67
01:02	06:02	5	4.17
01:02	06:03	1	0.83
01:02	06:04	2	1.67
01:03	06:01	1	0.83
01:03	06:03	3	2.50
02:01	02:02	21	17.05
03:01	03:02	5	4.17
03:02	03:01	1	0.83
03:03	03:01	1	0.83
04:01	04:02	1	0.83
05:01	02:01	48	40.00
05:05	02:02	1	0.83
05:05	03:01	15	12.50

N - number of tested individuals; n - number of observed haplotypes;

% - haplotype frequency, in percentage

5.2.3.3.5. DQ heterodimer type frequency

DQ heterodimer type frequency in sixty confirmed CD patients is presented in Table 18. A total of 8 different heterodimer types were found, one occurring only once. The most frequent heterodimer types found in patients were DQ2.5 and DQ2.2 with frequency of 40.83% and 17.50%, respectively. At less extended frequency were DQ5.1 and DQ7.5, both with the same frequency of 12.50%, followed by DQ6.1 heterodimer, with a frequency of 10.00%.

Table 18. Analysis of DQ heterodimer type frequency in the patient group

PATIENT GROUP (N=60)		
DQ HD TYPE	n	%
DQ2.2	21	17.50
DQ2.5	49	40.83
DQ4.4	1	0.83
DQ5.1	15	12.50
DQ6.1	12	10.00
DQ7.3	2	1.67
DQ7.5	15	12.50
DQ8.3	5	4.17

N - number of tested individuals; HD – heterodimer; n - number of observed heterodimer types;
% - HD type frequency, in percentage
note: definition of heterodimer types is presented in section Methods

5.2.3.3.6. Heterodimer genotype frequencies

The detailed heterodimer genotype distribution, as defined by the combination of HLA DQA1 and DQB1 genes and alleles in the individual genotype, is presented in Table 19. The presence of HLA-DQ2.5/DQ8/DQ2.2 genotypes was categorized according to the presence of CD predisposing HLA alleles.

In the whole data set, 95 % of patients were positive and 5 % were negative for at least one of the predisposing CD heterodimer genotype, i.e. HLA-DQ2.5/DQ8/DQ2.2 positive or negative (see Figure 9). The most prevalent HD positive genotype was DQ2.5/X with a frequency of 58%. Positivity for one copy of DQ2.5 heterodimer was more prevalent in *cis* position than in *trans* position. Three patients were homozygous for DQ2.5, i.e. having genotype positive for two copies of DQ2.5 heterodimer. Second most prevalent genotype was DQ2.5/DQ2.2 genotype which was present in 20% of patients.

Table 19. CD predisposing HLA-DQ heterodimer genotype in the patient group

PATIENT GROUP (N=60)			
	DQ HD GENOTYPE	n	%
	DQ2.5/X	35	58.33
	DQ2.5*cis*	28	46.66
	DQ2.5*trans*	7	11.66
	DQ8/X	2	3.33
	DQ2.2/X	2	3.33
	DQ2.5/DQ2.5	3	5.00
	DQ8/DQ8	0	0.00
	DQ2.2/DQ2.2	0	0.00
	DQ2.5/DQ8	3	5.00
	DQ2.5/DQ2.2	12	20.00
	DQ8/DQ2.2	0	0.00
HD POSITIVE	DQ2.5/DQ8/DQ2.2	57	95.00
HD NEGATIVE	X/X	3	5.00

N - number of tested individuals; n - number of individuals positive for HD genotype; HD – heterodimer; % - HD genotype frequency, in percentage
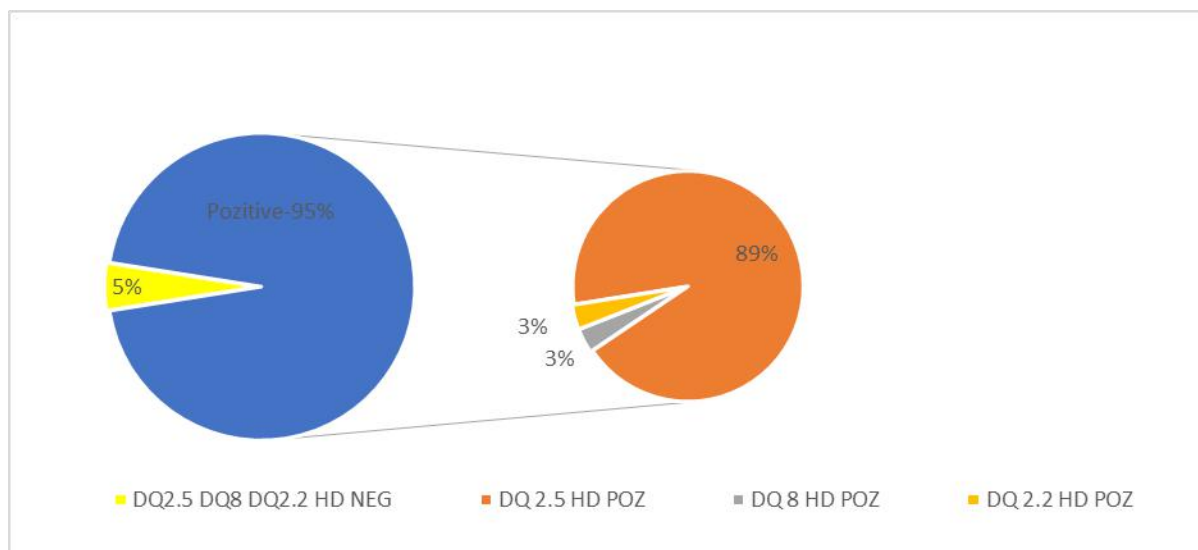
Figure 9. Predisposing CD heterodimer distribution in patients

5.3. Comparison of HLA-A, -B -DRB1, - DQA1 and –DQB1 allele and haplotype polymorphism between patients and healthy controls

5.3.1 HLA alleles and haplotypes with increased frequency in patients

Table 20 enumerates HLA alleles and haplotypes, which were found out to have statistically significant increased frequency in patients in comparison to controls. The most frequent HLA-A gene detected in patients group was HLA-A*01, with significant difference when comparing with control group (p=0.0001). At HLA-B locus, two genes were present with increased frequency in patients, B*08 (p<0.001) and B *50 (p=0.011).

As expected, the HLA-DRB1*03 and DRB1*07 alleles were more prevalent in patients in comparison to controls, with high statistically significant difference, p<0.0001 and p=0.002, respectively. Concerning the HLA-DQ alleles distribution, HLA-DQA1*05:01 and DQA1*02:01 results to be significantly more prevalent in patients (p<0.0001 and p=0.002, respectively), while

DQB1*02:01 and DQB1*02:02 alleles were more expressed in patients in comparison to controls (p<0.0001 and p=0.003, respectively).

Two-, three- and five-locus haplotype analysis also revealed several haplotypes with statistically significant increased frequency in patients.

The two locus haplotype analysis including HLA class I genes, revealed HLA-A*01-B*08 haplotype, to be more prevalent in patients in comparison to controls (22.44%, 3.62% respectively, p<0.0001), followed by HLA-A*02-B*50 to be expressed in 4.97% of patients and in 0.40 % of controls, respectively (p=0.018). On the other hand, the two locus haplotype analysis including class II genes at HLA-DQ locus showed that HLA-DQA1*05:01-DQB1*02:01 and DQA1*02:01-DQB1*02:02 haplotype were significantly more prevalent in patients in comparison to controls, p<0.0001 and p=0.001, respectively.

Three-locus haplotype analysis, including class I HLA-A and B locus and class II HLA-DRB1 locus, showed that HLA-A*01-B*08-DRB1*03, results to be much more present in patients (22.50%) in comparison to controls (2.82 %), p<0.0001. The significant difference was also detected for haplotype HLA-A*02-B*50 -DRB1*07, found in 4.16% of patients and 0.040 % of controls, p=0.031.

Three locus haplotypes analysis comprising class II HLA-DRB1, DQA1 and DQB1 loci, revealed that HLA-DRB1*03-DQA1*05:01-DQB1*02:01 and HLA-DRB1*07-DQA1*02:01-DQB1*02:02 haplotypes were significantly more prevalent in patients, p<0.0001 and p=0.001, respectively.

Finally, five locus analysis, encompassing all loci studied in this work, showed that two extended haplotypes were found in increased frequency in patients in comparison with controls. HLA-A*01-B*08-DRB1*03-DQA1*05:01-DQB1*02:01 was the most frequent haplotype (22.50%) in patients which was present in only 2.82% in controls (p<0.0001). The HLA-A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 was the second haplotype which was present with statistically significant higher frequency in patients, found in 4.2% of patients and 0.40% of controls (p=0.031).

For HLA-A*68-B*44, A*68-B*44-DRB1*07 and A*68-B*44-DRB1*07- DQA1*02:01-DQB1*02:02 haplotype, statistically significant p value was found in comparison to controls, but the calculation of OD and CI was not applicable due to 0% frequency in controls.

Table 20. HLA genes, alleles and haplotypes with statistically significant increased frequency in
patients

A*	B*	DRB1*	DQA1*	DQB1*	PATIENTS N=60	CONTROLS N=124		
					%	%	p	OD (95% CI)
01	-	-	-	-	26.67	10.48	0.0001	3.104 (1.750-5.509)
-	08	-	-	-	29.17	5.65	<0.0001	6.882 (3.530-13.418)
-	50	-	-	-	5.83	0.40	0.011	15.300 (1.860-125.842)
-	-	03	-	-	38.33	7.26	<0.0001	7.942 (4.339-14.539)
-	-	07	-	-	17.50	6.85	0.002	2.882 (1.458-5.697)
-	-	-	02:01	-	17.50	6.85	0.002	2.882 (1.458-5.697)
-	-	-	05:01	-	40.00	7.66	<0.0001	8.035 (4.438-14.547)
-	-	-	-	02:01	40.00	7.26	<0.0001	8.518 (4.661-15.567)
-	-	-	-	02:02	18.33	7.66	0.002	2.823 (1.463-5.449)
01	08	-	-	-	22.50	3.63	<0.0001	7.709 (3.493-17.013)
02	50	-	-	-	4.98	0.40	0.018	13.00 (1.547-09.244)
68	44	-	-	-	3.33	0.0	0.048	na
01	08	03	-	-	22.50	2.82	<0.0001	9.995 (4.208-23.740)
02	50	07	-	-	4.17	0.40	0.0311	10.739 (1.240-92.975)
68	44	07	-	-	4.17	0.0	0.003	na
			02:01	02:02	17.50	6.45	0.001	3.075 (1.540-6.142)
			05:01	02:01	40.00	7.26	<0.0001	8.518 (4.661-15.567)
-	-	03	05:01	02:01	38.33	7.26	<0.0001	7.942 (4.339-14.539)
-	-	07	02:01	02:02	17.50	6.45	0.001	3.075 (1.540-6.142)
01	08	03	05:01	02:01	22.50	2.82	<0.0001	9.995 (4.208-23.740)
02	50	07	02:01	02:02	4.17	0.40	0.031	10.739 (1.240-92.975)
68	44	07	02:01	02:02	3.33	0.0	0.048	na

N - number of tested individuals; % - gene/allele/haplotype frequency in percentage, p – p value;

OD - odds ratio; CI - confidence interval; na – not applicable

5.3.2. HLA alleles and haplotypes with decreased frequency in patients

Table 21 enumerates HLA alleles and haplotypes, which were found out to be statistically significant decreased in patients in comparisons to controls.

Regarding HLA-A and HLA-B locus, A*02, as the most frequent gene at HLA-A locus, and B*35, as the second most frequent gene at HLA-B locus, in healthy control individuals were more frequent than in patients. HLA-A*02 was present in 32.66 % of controls and 21.67% patients (p=0.031), while HLA-B*35 allele was present in 12.50% of controls and 3.33% of patients (p=0.009). The top three most frequent HLA-DRB1 genes in controls, DRB1*11, DRB1*13 and DRB1*16 were also more prevalent than in patients (p=0.046, p=0.010 and p=0.029, respectively).

Concerning to HLA-DQA1 and DQB1 gene distribution, the top two most frequent genes in controls at each of two loci, DQA1*01:02, DQA1*05:05 and DQB1*03:01, DQB1*05:02 were less expressed in patients than controls, p=0.004, 0.011, 0.008 and 0.034, respectively.

Two, three and five locus haplotype analysis also revealed several haplotypes with statistically significant decreased frequency in patients.

The two-locus haplotype analysis including class II genes at HLA-DQ locus revealed that DQA1*01:02-DQB1*05:02 and DQA1*05:05-DQB1*03:01 haplotype were significantly more prevalent in controls than patients, p=0.034 and 0.010, respectively.

Three locus haplotypes analysis comprising class II HLA-DRB1, -DQA1 and -DQB1 loci, showed that HLA-DRB1*11-DQA1*05:05-DQB1*03:01 and HLA-DRB1*16-DQA1*01:02-DQB1*05:02 haplotypes were more prevalent in control group, p=0.015 and 0.020, respectively.

For two different HLA-A and B haplotypes (A*02-B*18 and HLA-A*24-B*35) as well as two different A-B-DR haplotypes (HLA-A*02-B*18-DRB1*11 and HLA-A*02-B*51-DRB1*14), statistically significant p value was found in comparison to controls, but the calculation of OD and CI was not applicable due to 0% frequency in patients. Finally, there was no five-locus haplotype with statistically significant increased frequency in controls.

Table 21. HLA genes, alleles and haplotypes with statistically significant decreased frequency

        in patients

A*	B*	DRB1*	DQA1*	DQB1*	PATIENTS N=60 %	CONTROLS N=124 %	p	OD (95% CI)
02	-	-	-	-	21.67	32.66	0.031	0.570 (0.342-0.948)
-	35	-	-	-	3.33	12.50	0.009	0.241 (0.083-0.700)
-	-	11	-	-	13.33	22.18	0.046	0.539 (0.294-0.989)
-	-	13	-	-	5.00	14.52	0.010	0.309 (0.126-0.757)
-	-	16	-	-	5.83	13.71	0.004	0.295 (0.128-0.678)
-	-	-	01:02	-	13.33	26.61	0.005	0.424 (0.233-0.770)
-	-	-	05:05	-	13.33	25.00	0.011	0.461 (0.253-0.840)
-	-	-	-	03:01	14.17	26.61	0.008	0.455 (0.253-0.817)
-	-	-	-	05:02	6.67	14.52	0.034	0.420 (0.189-0.935)
02	18	-	-	-	0.0	5.43	0.004	na
24	35	-	-	-	0.0	4.94	0.008	na
02	18	11	-	-	0.0	6.05	0.002	na
02	51	14	-	-	0.0	4.03	0.018	na
-	-	-	01:01	05:03	2.50	6.86	0.088	0.348 (0.100-1.212)
-	-	-	01:02	05:02	6.67	14.52	0.034	0.420 (0.189-0.935)
-	-	-	05:05	03:01	12.50	24.19	0.010	0.447 (0.242-0.827)
-	-	11	05:05	03:01	11.67	21.37	0.015	0.485 (0.257-0.916)
-	-	16	01:02	05:02	5.83	13.26	0.020	0.403 (0.173-0.941)

N - number of tested individuals; % - gene/allele/haplotype frequency in percentage, p – p value;

OD - odds ratio; CI - confidence interval; na – not applicable

5.3.3. HLA-DRB1*04 and DQB1*03:02 (DQ8) alleles and haplotypes in patients and controls

Statistically significant difference was found between patients and controls for DQB1*03:02 (DQ8) allele, but as this is one of the alleles involved in CD genetic risk, the results are shown in Table 22. The HLA-DRB1*04 and HLA- DQB1*03:02 alleles, as well as two and three locus haplotypes comprising DRB1*04 and DQA1*03:01 alleles, were found with almost doubled frequency in controls, but the difference did not reach statistically significant p value.

Table 22. HLA-DRB1*04 and DQB1*03:02 (DQ8) alleles and haplotypes
in patients and controls

A*	B*	DRB1*	DQA1*	DQB1*	PATIENTS N=60 %	CONTROLS N=124 %	p	OD (95% CI)
-	-	04	-	-	5.83	11.29	0.100	0.486 (0.206-1.148)
-	-	-	-	03:02	4.17	8.06	0.171	0.495 (0.181-1.354)
-	-	-	03:01	03:02	4.17	7.66	0.210	0.524 (0.190-1.439)
-	-	04	03:01	03:02	4.17	7.66	0.210	0.524 (0.190-1.439)

N - number of tested individuals; % - gene/allele/haplotype frequency in percentage, p – p value;

OD - odds ratio; CI - confidence interval

5.3.4. HLA-DQ heterodimer type analysis in patients and controls

The detailed distribution of DQ heterodimer types are presented in Table 23.

In the whole data set, the frequency of DQ 2.5 HD type was almost 41% in CD patients and only 8 % in healthy controls, giving highly significant difference ($p<0.0001$). DQ2.2 was the second most prevalent HD type in patients, found at a frequency of 17.50%, and with a statistically significant higher frequency in comparison with controls ($p=0.002$).

On the other hand, although detected with a frequency greater than 10% in patients, DQ5.1 and DQ7.5 HD types were much more prevalent in the controls and the difference was statistically significant ($p=0.002$ and $p=0.008$, respectively). No statistical difference between patients and controls was observed for DQ8.3 HD type.

Table 23. HLA-DQ heterodimer type frequencies in patients and controls

	PATIENTS N=60	CONTROLS N=124		
DQ HD TYPE	%	%	p	OD (95% CI)
DQ2.2	17.50	6.45	0.002	3.075 (1.540-6.142)
DQ2.3	0.00	0.40	0.817	0.684 (0.027-16.930)
DQ2.5	40.83	8.07	<0.0001	7.867 (4.386-14.112)
DQ4.3	0.00	0.40	0.817	0.684 (0.027-16.932)
DQ4.4	0.83	2.02	0.416	0.408 (0.047-3.535)
DQ5.1	12.50	27.42	0.002	0.378 (0.205-0.695)
DQ6.1	10.00	19.35	0.025	0.463 (0.235-0.908)
DQ7.3	1.67	2.42	0.644	0.683 (0.135-3.438)
DQ7.5	12.50	24.60	0.008	0.437 (0.237-0.808)
DQ8.3	4.17	8.07	0.171	0.495 (0.181-1.354)
DQ9.2	0.00	0.40	0.817	0.684 (0.027-16.932)
DQ9.3	0.00	0.40	0.817	0.684 (0.027-16.932)

N - number of tested individuals; HD – heterodimer; % - HD type frequency, in percentage

p – p value; OD - odds ratio; CI - confidence interval

note: definition of heterodimer types is presented in section Methods

5.3.5. Heterodimer genotype analysis in patients and controls

The detailed distribution of HD genotypes, as defined by the combination of HLA-DQ haplotypes and HD types is given in Table 24.

The genotype DQ2.5/X was the most prevalent genotype in patients, with more than half of the patients carrying the single dose of DQ2.5 heterodimer, in either *cis* (on the same chromosome) or *trans* (on different chromosomes) position, with a high significant difference in comparison with controls. Presence of heterodimer in *cis* position encountered 80% (25 out of 35) of patients with a single dose of DQ2.5 heterodimer, with a frequency of 47% in the entire group of patients.

The second most frequent genotype in patients was DQ2.5/DQ2.2 which was present in 20% of patients and in less than 2% of controls, which was also high statistically significant difference (p=0.0005).

Presence of DQ2.5 heterodimer in double dose i.e. DQ2.5/DQ2.5 genotype and heterozygous DQ2.5/DQ8 genotype were present in 5% of patients, each, and without significant difference with controls. Genotypes positive for DQ8 and DQ2.2 in double dose were not present in patients, as well as heterozygous DQ8/DQ2.2 genotype, thus the calculation of OD and CI was not applicable.

In the whole data set, 95.00 % of confirmed CD patients and 40.32% of controls had a genotype that was positive for at least one of the predisposing DQ heterodimers (HLA-DQ2.5, DQ8, DQ2.2) (p<0.0001). Only three patients (5%) and at the same time almost 60% of controls were negative for HLA-DQ2.5, DQ8, DQ2.2 genotype (see Figure 10).

Table 24. DQ heterodimer genotype frequencies in patients and controls

		PATIENTS N=60	CONTROLS N=124		
	DQ HD GENOTYPE	%	%	p	OR (95%CI)
	DQ2.5/DQX	58.33	15.32	<0.0001	7.736 (3.808-15.715)
	DQ2.5*cis*	46.66	13.71	<0.0001	5.507 (2.679-11.320)
	DQ2.5*trans*	11.66	1.61	0.0108	8.056 (1.619-40.074)
	DQ8/DQX	3.33	13.71	0.046	0.217 (0.048-0.972)
	DQ2.2/DQX	3.33	6.45	0.390	0.500 (0.102-2.430)
	DQ2.5/DQ2.5	5.00	0	0.074	N/A
	DQ8/DQ8	0.00	0.81	0.814	N/A
	DQ2.2/DQ2.2	0.00	1.61	0.562	N/A
	DQ2.5/DQ8	5.00	0.81	0.109	6.474 (0.658-63.602)
	DQ2.5/DQ2.2	20.00	1.61	0.001	15.250 (3.289-70.693)
	DQ8/DQ2.2	0.00	0.00	N/A	N/A
HD POSITIVE	DQ2.5/DQ8/DQ2.2	95.00	40.32	<0.0001	28.120 (8.341-94.790)
HD NEGATIVE	DQX/DQX	5.00	59.68	<0.0001	0.035 (0.010-0.119)

N - number of tested individuals; HD – heterodimer; % - HD genotype frequency, in percentage

p – p value; OD - odds ratio; CI - confidence interval

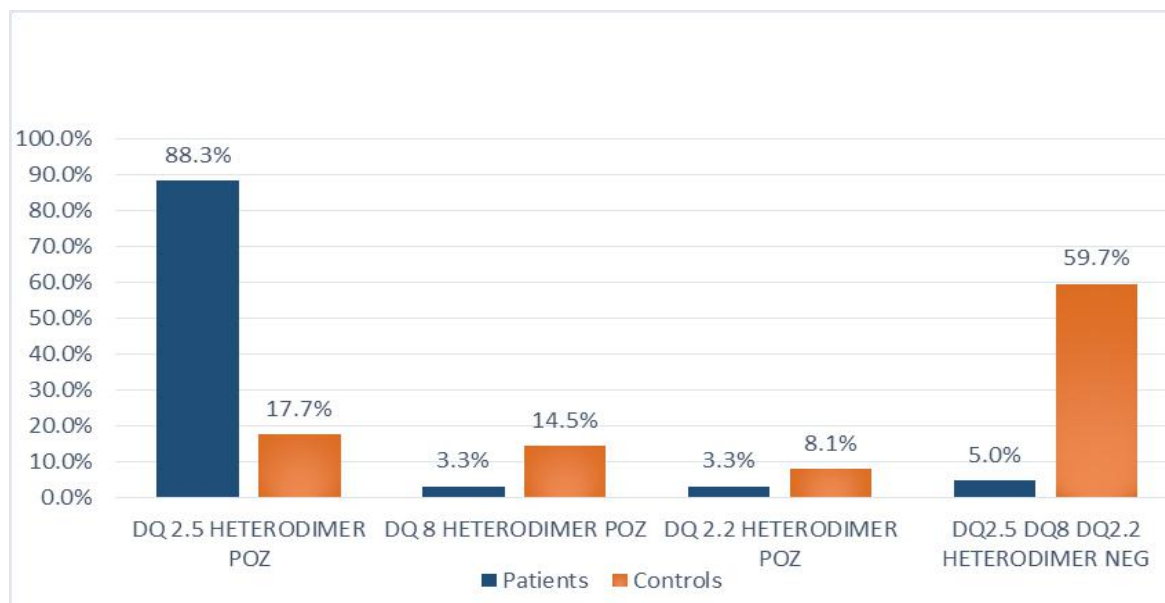note: definition of heterodimer genotypes is presented in section Methods

Figure 10. Predisposing CD heterodimer distribution in patients and controls

5.3.6. Sensitivity, specificity, positive predictive value, and negative predictive value

Sensitivity, specificity, positive predictive value, and negative predictive value have been calculated for HLA factors with extremely high statistically significant difference between patients and controls ($p<0.0001$). The results presented in Table 25 show that genotype positive for at least one of the CD predisposing heterodimers has the highest sensitivity value (95%) and highest negative predictive value (96%). The highest positive predictive value has been observed for heterozygous genotype DQ2.5/DQ2.2 (86%), but two extended five-locus haplotypes HLA-A*01-B*08-DRB1*03-DQA1*05:01-DQB1*02:01 and HLA-A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 also had high positive predictive value of 79% and 83% respectively.

Table 25. Sensitivity, specificity, positive predictive value, and negative predictive value for HLA factors with extremely high statistically significant difference between patients and controls (p<0.0001)

	SENS	SPEC	PPV	NPV
	%	%	%	%
HAPLOTYPE				
DRB1*03-DQA1*05:01-DQB1*02:01	38	7	72	76
DRB1*07-DQA1*02:01-DQB1*02:02	18	6	57	70
A*01-B*08-DRB1*03-DQA1*05:01-DQB1*02:01	23	3	79	72
A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02	4	0.4	83	68
HD TYPE				
DQ2.5	41	8	71	76
DQ2.2	18	6	57	70
HD GENOTYPE				
DQ2.5/DQX	29	8	65	73
DQ2.5/DQ2.2	10	1	86	69
DQ2.5/DQ8/DQ2.2 POSITIVE	95	40	53	96
DQ2.5/DQ8/DQ2.2 NEGATIVE	5	60	4	47

SENS – sensitivity; SPEC - specificity; PPV – positive predictive value;

NPV – negative predictive value; HD heterodimer

5.4. Patients clinically suspected (un-confirmed) for CD

5.4.1. Clinical symptomatology

All nine children treated as CD but without complete diagnostic tests, all were symptomatic, and almost all of them presented with classical tirade of symptoms (chronic diarrhoea, failure to thrive and distended abdomen) (see Table 26). Seven of them were male while mean age at the time of diagnosis was 6.2 years.

Table 26. Clinical presentation of patients clinically suspected for CD (N=9)

Symptoms at presentation	N
Gastrointestinal	
Diarrhea	8
Distended abdomen	8
Abdominal pain	6
Vomiting	4
Constipation	3
Anorexia	5
Extraintestinal	
Weight loss	8
Failure to thrive	4
Short stature	4
Anaemia	9
Irritability	6
Chronic fatigue	5

N - number of tested individuals positive for specific symptom

5.4.2. Diagnostic tools

5.4.2.1. Biochemical parameters and SBB

All nine children clinically suspected for CD had positive serology for IgA anti-TG2, while IgA EMA was performed only in 6 cases. Furthermore, none of all nine cases had confirmatory biopsy (see Table 27).

Table 27. Diagnostic tools on clinically suspected CD patients (n=9)

Diagnostic test	N
IgA anti-TG2	9
>10 NUL	6
<10 NUL	3
IgA anti-TG2 and EMA	6
IgA anti-TG2, EMA and SBB	0

N - number of tested individuals; Anti-TG2 - anti tissue transglutaminase 2; EMA-endomysium antibody; SBB-small bowel biopsies

5.4.2.2. HLA typing

HLA-A, B, DRB1, DQA1, DQB1 typing was performed for all nine cases; the typing results are presented for each patient individually in Table 28 since the group was too small for HLA allele and haplotype frequency analysis. None of the patients was positive for at least one of the three predisposing CD heterodimers. Nevertheless, five patients were positive for DQA1*05:05 allele which is a part of DQ2.5 heterodimer while the remaining four patients were completely negative for the presence of any of DQA1 or DQB1 alleles from DQ2.2, DQ8 or DQ2.2 heterodimer (heterodimer genotype DQX/DQX).

Table 28 HLA typing results of nine children clinically suspected for CD (n=9)

	HLA-A	B	DRB1	DQA1	DQB1	HD GENOTYPE
Case 1	*02, *24;	*35, *38;	*12, *16;	*01:02, *05:05;	*03:01, *05:02	DQX.5/DQX
Case 2	*02, *02;	*35, *40;	*11, *16;	*01:02, *05:05;	*03:01, *05:02	DQX.5/DQX
Case 3	*32, *32;	*07, *51;	*11, *15;	*01:02, *05:05;	*03:01, *06:02	DQX.5/DQX
Case 4	*01, *68;	*18, *37;	*11, *16;	*03:03, *05:05;	*03:01, *03:01	DQX.5/DQX
Case 5	*01, *02;	*18, *37;	*11, *16;	*01:02, *05:05;	*03:01, *05:02	DQX.5/DQX
Case 6	*01, *24;	*37, *57;	*15, *16;	*01:02, *01:02;	*05:02, *06:02	DQX/DQX
Case 7	*24, *32;	*49, *55;	*13, *13;	*01:02, *01:03;	*06:03, *06:04	DQX/DQX
Case 8	*03, *24;	*44, *51;	*01, *15;	*01:01, *01:02;	*05:01, *06:02	DQX/DQX
Case 9	*02, *26;	*51, *51;	*01, *16;	*01:01, *01:02;	*05:01, *05:02	DQX/DQX

HD - heterodimer

DQX.5/DQX – HD genotype positive for single dose of DQA1*05 allele

DQX/DQX – all other HD genotypes except DQ2.5, DQ2.2 and DQ8

5.5. CD confirmed patients, siblings of index cases

5.5.1. Clinical symptomatology and diagnostic tool

Three patients were siblings of index case patients and thus were not included in the unrelated patient group which was used for HLA allele and haplotype polymorphism analysis. All three of them were diagnosed according to New guidelines ESPGHAN criteria, presenting with classic clinical symptoms and having positive serology (TG2 titter >10 UNL and positive EMA) (see Table 29).

Table 29. Characteristics of CD confirmed patients, siblings of index cases

Case	Age	Symptoms at presentation	IgA TG2	IGA-EMA	SBB
Case 1	8	diarrhea, distended abdomen, weight loos	Done >10 NUL	Done	Not done
Case 2	6	diarrhea, distended abdomen, weight loos	Done >10 NUL	Done	Not done
Case 3	4	diarrhea, distended abdomen, weight loos	Done >10 NUL	Done	Not done

Anti-TG2-anti tissue transglutaminase 2; EMA-endomysium antibody;
SBB-small bowel biopsies

HLA typing results revealed that that two of them were identical with their index case sibling for DQ2.5/DQ2.2 genotype and one was haploidentical with index case sibling and positive for DQ2.5/DQX genotype. Altogether, all three siblings of the index case patients were heterodimer positive as well (see Table 30).

Table 30. HLA typing of CD confirmed patients and siblings index cases

	A	B	DRB1	DQA1	DQB1	HD GENOTYPE
Case 1	*02, *02	*07, *50	*03, *07	*02:01, *05:01	*02:01, *02:02	DQ2.5/DQ2.2
IC[a]	*02, *02	*07, *50	*03, *07	*02:01, *05:01	*02:01, *02:02	DQ2.5/DQ2.2
Case 2	*02, *33	*14, *27	*03, *14	*01:01, *05:01	*02:01, *05:03	DQ2.5/DQX
IC[a]	*02, *33	*14, *50	*03, *07	*02:01, *05:01	*02:01, *02:02	DQ2.5/DQ2.2
Case 3	*68, *69	*08, *44	*03, *07	*02:01, *05:01	*02:01, *02:02	DQ2.5/DQ2.2
IC[a]	*68, *69	*08, *44	*03, *07	*02:01, *05:01	*02:01, *02:02	DQ2.5/DQ2.2

IC[a] - index case

5.6. Healthy siblings of CD index cases - HLA typing

Three healthy children were siblings of index case patients and thus were not included in the unrelated control group which was used for HLA allele and haplotype polymorphism analysis. HLA typing results revealed that that one child was completely HLA different with his CD index case sibling (case 1) while two others were haploidentical with their CD index case siblings (case 2 and 3). Altogether, all three healthy siblings of the index case patients were negative for DQ2.5, DQ2.2 and DQ8 heterodimers. However, they were positive for DQA1*05:05 allele which is a part of DQ2.5 heterodimer (heterodimer genotype DQX.5/DQX) (see Table 31).

Table 31. HLA typing results of healthy siblings and celiac disease index case

	A	B	DRB1	DQA1	DQB1	HD GENOTPYE
Case 1	*32, *68	*51, *53	*11, *13	*01:02, *05:05	*03:01, *06:09	DQX.5/DQX
IC	*01, *03	*08, *38	*03, *13	*01:03, *05:01	*02:01, *06:03	DQ2.5/DQX
Case 2	*32, *68	*15, *18	*11, *15	*01:02, *05:05	*03:01, *06:03	DQX.5/DQX
IC	*01, *68	*18, *40	*03, *15	*01:02, *05:01	*02:01, *06:03	DQ2.5/DQX
Case 3	*02, *02	*18, *51	*11, *13	*01:01, *05:05	*03:01, *06:04	DQX.5/DQX
IC	*01, *02	*08, *51	*03, *13	*01:02, *05:01	*02:01, *06:04	DQ2.5/DQX

HD – heterodimer; DQX.5/DQX – HD genotype positive for single dose of DQA1*05 allele

6. DISCUSSION

To the best of our knowledge, this is the first study performed in Kosovar Albanian children with CD. The results of our study represent a general picture of celiac disease in paediatric population with a particular focus on clinical and genetic features.

In our study, the sex distribution showed a female predominance, which is in concordance with the results of other studies ranging from 1.2:1 to 2.0:1 (106, 107)

Mean age at the time of diagnosis in our studied cases was 5.5 years. Similar results were reported in several Mediterranean countries, respectively 5-9 years (108, 109). While recent studies from Sweden, America, and Finland confirm the tendency towards a higher age at diagnosis, varying from 6.7 to 9.3 years (110-112). Furthermore an upward shift in age at diagnosis was reported by Namatovu et al. (113) in a Swedish population-based follow-up study, from 1.1 years, to 4.6 and 6.7 years in the periods 1973-1994, 1995-2003 and 2004-2009, respectively.

Since the classical form of CD tends to occur at earlier ages, the high prevalence of classical CD (78%) in our study is not surprising given the young age of our patients. In contrast, only a small number of patients showed the atypical manifestation, and only a few of these were diagnosed as belonging to the risk group. Regarding the classical form domination, similar results (70.9%), were reported from a prospective Spanish study, conducted in 2005–2006 (114). Furthermore, our finding is in striking contrast to the results of several recent studies from other European countries, which have shown a decrease in the relative percentage of classical forms of CD. In the last few years, it has been reported that classical forms represent only 15% to 44% of all cases (115, 116). Classical form domination of CD cases in our study may be due to the lack of screening tests and a better awareness among health professionals about atypical or mild presentations of CD in Kosovo.

Our study showed that breastfed children had older age at the diagnosis of CD but, simultaneously, the number of children that were not breastfed was small. In addition, the breastfed children had delayed gluten introduction as compared to the non-breastfed group. Epidemiological studies suggest that early infant feeding practices may be important environmental risk factors for the subsequent development of CD. Radlovic et al. (117)

69

concluded that mean age at diagnosis was significantly higher in infants, who had been breastfed at the time of gluten introduction. In addition, Vajpayee et al. (118) conclude that delayed gluten introduction to infant's diet along with continuing breastfeeding, delays symptomatic CD (103). While Akobeng et al. (119), in a meta-analysis, concluded that children being breastfed at the time of gluten introduction had a 52% reduction in the risk of developing CD. All these studies, like ours, showed that continuing breastfeeding at the time of gluten introduction delayed the onset of the CD in early crucial years of rapid growth and development. However, it is not clear whether breastfeeding provides permanent protection, or merely delays the disease.

In our study, 78% of patients had GI symptoms, similar to a study in Switzerland showing 71% of children with these symptoms over a five-year study period (2001–2006) (120). Earlier studies from Turkey reported that the classical form was the dominant mode of presentation in 2005, whereas, the majority of cases changed to the atypical form by 2012 (121, 122).

FTT and short stature were present in approximately 30% of the patients in our study almost same frequency was reported recently by Nurminen et al. (123) in a Finish study (27%). Even thought our results differ from a Swedish study (124) conducted in 2000, where FTT was observed in 11% of children with CD, while a study from Greece showed that children with CD tend to suffer less often from short stature in recent years, at only 6% (116).

Iron deficiency anaemia was frequently seen in our study population, especially in adolescents. Multiple diseases can cause anaemia itself, but it can sometimes be the only presenting symptom of CD. This has been well evaluated by Çekın AH et al. (125) who found a CD prevalence of 8.33% among patients with iron deficiency anaemia of obscure origin, and nearly the same prevalence (7.8%) was previously reported by Uçardağ et al. (126).

Our results show that IgA antibodies to human TG2 and EMA are reliable as a test for both the diagnosis and follow-up of childhood CD. Our results for these markers in untreated CD are similar to those of previous studies using human TG2 with an ELISA test showing both high sensitivity and specificity (127).

Having a family member with celiac disease significantly increases the chance of celiac disease in other family members. An estimated pooled prevalence in first-degree relatives is 3-7.5% varying according to the relationship, gender, and geographic location (128-130). Our results (of 7 % first-degree relatives) are highly in correlation with other authors' reports (131, 132).

The association between CD and autoimmune disorders is well-known, due to shared pathological and genetical mechanisms. There is a possibility of genetic susceptibility to autoimmunity through IL-2 and IL-21 locus, both inside and outside of HLA region. In our study in 18% of children CD was associated with other autoimmune disorders, Type 1 diabetes mellitus (5%) was the leading immunological disorder, which is in accordance with reports from other studies (5-15%) (133).

We believe that our study is representative of the clinical and epidemiological trends for paediatric CD in Kosovo, as our hospital is a tertiary centre and thus, covers diverse areas - both rural and urban ones. Nonetheless, we are aware that our study may have some limitations. First, it is not feasible to measure the true incidence of CD due to the nature of the disease, its symptomatology and the lack of a nationwide screening program. Second, a possible limitation when reporting symptoms inherent in a study of young patients is that they may be less able than older individuals to describe subjective symptoms, while parents may exaggerate or minimise the symptomatology.

In the HLA part of this study, the first step was to analyse HLA data in healthy control group, as no adequate previous HLA data for Kosovar Albanian population has been available. For example, until the date, there are no reports according to HLA- DQA1 allele distribution in Albanian population from Kosovo and consequently no data about DQA1-DQB1 haplotype, DQ heterodimer type and DQ heterodimer genotype frequencies.

Healthy control group consisted of 124 unrelated healthy children, age and sex matched with our CD patient group. HLA-A, B and DRB1 gene frequencies and DQA1 and DQB1 allele frequencies have been analysed, as well as haplotype frequencies. The results obtained were compared with the results from two studies by Sulcebe (134, 135). There was no difference in HLA-A, B, DRB1 and DQB1 allele frequencies, neither between our control group and group of 120 Albanian individuals from Kosovo (134), nor with group of 432 Albanian healthy individuals from Albania (135), except for HLA DRB1*11 allele which was slightly more prevalent in this study (p=0.042). Both Sulcebes' studies have the data for HLA-C locus allele frequencies, while our study has the HLA-DQA1 locus allele frequencies, thus the comparison could not be performed for these two loci.

The analysis of haplotype frequencies revealed concordant result about HLA-A*02-B*18-DRB1*11 as the most frequent haplotype in all of these three populations, being the only

haplotype exceeding the frequency of 5%. The haplotype HLA-A*01-B*08-DRB1*03 was among first three most common haplotypes in all three populations, present at a frequency of 2-3%. The most striking difference was found for haplotype HLA-A*02-B*51-DRB1*14 which is the second most common haplotype in our healthy control group with a frequency of 3.6%, but was not reported as a haplotype exceeding frequency of 1% (0.96%) in both Sulcebes' studies. In Allele*Frequencies worldwide populations database (136) this haplotype is furthermore found with a frequency also lower than 1% in several populations (Macedonia, Italy-Lombardia, Bosnia and Herzegovina, Croatia, Turkey).

It can be summarized that the HLA allele and haplotype frequencies in healthy control group used in this study are not significantly different from the allele and haplotype frequencies found in two other Albanian populations studied so far, although some differences are found which can be of interest for further population studies of Kosovar Albanian population.

In this study, we provide data, for the first time in Albanian paediatric population, supporting a strong association between CD and HLA-class II DRB1, DQA1 and DQB1 alleles and haplotypes, as well as DQ heterodimers. Overall, we found that 95% of children with CD were DQ2 and/or DQ8 positive, as compared with 40.32% of the healthy individuals. Our results are in accordance with those reported from the other studies, even though the frequency of HLA-DQ2 and DQ8 heterodimer positivity among patients diagnosed with CD varies among different populations. This differences in the best way has been described in large HLA-DQ study conducted in France, Italy, Finland, Norway, and England by Karell et al. They report the range of HLA DQ heterodimer frequency from the lowest one of 89.4% in Italy to the highest one of 96.7% in England (137). Such heterodimer frequency differences between populations were also observed in a prospective study in 13 Mediterranean countries (with different percentage of patients with HLA typing results). As conclusion they report that 77.4% of cases carried the DQ2 heterodimer, 20% the DQ8 heterodimer, whereas 1.9% didn`t carry either of these molecules (138).

Our results are similar to study performed in a Greek pediatric population, which found out that 95.8% of individuals with CD were DQ2 and/or DQ8 positive, as compared with 32.5% of the healthy individuals (139). Furthermore, similar results have been reported from some other European countries (140-144). Anyway, some studies reported even higher heterodimer frequency in patients. A study from Croatia, reports that 98% of patients with CD were carriers of

HLA-DQ2 and DQ8 heterodimers (145), while the recent study by Delgado et al. (146) reported the following incidences: 96.7% of patients were HLA-DQ2 and 4.4% HLA-DQ8 positive, giving a 100 % HLA-DQ2 and/or HLA-DQ8 positivity in CD patients.

According to Karhus et al. (147) general Danish population has high frequency of either HLA DQ2 and/or DQ8 allele related (47.7%) and consecutively all individuals with CD were HLA DQ2 and/or HLA DQ8 positive, with the majority being HLA DQ2.5 positive. The authors are giving a speculation that the likelihood of DQ2/DQ8 positivity could be attributed to the high frequency of alleles associated with CD in patients as compared with controls.

On the other hand, the low frequency (76.9%-81.2%) of these alleles in the patients from several regions of Russia and from Kazakhstan (148-150) can be related also with the other studies speculations that in general population high frequencies of susceptibility alleles decline from West to East with low frequencies in populations in Southeast Asia and the virtual absence of DQ2/DQ8 in Japan.

Low frequency of HLA-DQ2 and/or DQ8 was reported also in two studies performed in CD patients from Turkey, 76% and 84.7% respectively (151, 152).

In the present study, 18 (30%) patients had two DQ heterodimer copies. HLA epidemiological studies have shown that HLA-DQ gene dose has a strong quantitative effect on the magnitude of gluten-specific T cell responses, and these individuals have the highest risk of developing CD (153). Clerget-Darpoux et al. estimated the risk for developing celiac disease to be 6.8 times greater for those having a double dose of DQB1*02:01 (154). Vader et al. (155) showed that HLA-DQ2.5/2.2 heterozygous individuals could express two HLA-DQ2 dimers on antigen-presenting cells that bind and present all characterized gluten epitopes.

Interesting, the incidence of HLA-DQ8 alone, in double dose or with other heterodimers, in our CD cases (8.33 %) was slightly lower than in controls (15.3%), with no significant differences, which is in line with the result reported in literature. According to Murad et al (156) the frequency of the DQ8 heterodimer (DQB1*03:02 allele) was elevated in patients versus controls, but uncorrected P value was borderline significant (10.2% vs. 8.6%), while Cabrera et al. (157) reports slightly higher frequency with no significant differences between patients and control individuals, 11.22% and 15.31%, respectively. Lower frequency but in line with our results were Koskinen`s et al. (158) reports of DQ8 frequency of 2.8 %, 6.2% and 6.7% in Hungarian, Finish and Italian CD patients, respectively.

In our study, we have found a pronounced increase of the DQA1*02:01, DQA1*05:01, DQB1*02:01, DQB1*02:02, DRB1*03, and DRB1*07 alleles. These alleles are classically considered to be associated with CD, as it is shown in other population's studies as well (159, 160). However, surprisingly, we found no association with DRB1*04 and DQB1*03:02 because, although 4.16% of the patients carry the DQ8 haplotype, the distribution in the CD population was lower than in the control group (7.66%). This is an observation that is comparable with results reported by some other authors (161). The main associated haplotype was DRB1*03–DQB1*0201–DQA1*0501 (DR3–DQ2) (38.8%), followed by DRB1*07–DQB1*02:02–DQA1*02:01 (DR7–DQ2) (17.5%) haplotype.

Regarding the three HLA-DQ2 and DQ8 negative patients, two of them carried the haplotype DRB1*11-DQB1*03:01-DQA1*05:05 (DR5-DQ7), one carrying it in homozygosis. The third patient, who lacked all HLA-CD susceptibility factors, was a girl with positive serological test and partial villous atrophy, who responded to a gluten-free diet.

According to the data, originating from different countries the percentage of patients with celiac disease that are HLA-DQ2 and HLA-DQ8 negative is between 0 and 10%, but there are also other studies reporting higher percentages (162, 163). In the multicentre study by Karell et al. (137), out of 6.0% of CD cases lacking the DQ2 or DQ8 heterodimer, 5.6 % of them carried one half of the DQ2 heterodimer, and 4 (0.4%) were DQ2, DQ8, α5, β2 negative. On the other hand, Fernández-Bañares et al. (164) showed that 3% of CD patients in Spain might be non-HLA-DQ2.5/8, half than the frequency observed in the European Genetics Cluster on CD while the frequency of lacking HLA-DQ8 and the two alleles encoding HLA-DQ2 was 1%. In addition more recent study performed in Brazilian CD children (165) reports that 3 % of tested patients were negative for DQ2/DQ8 heterodimer, 2 % showed low risk predisposing alleles and 1% tested negative for all of predisposing alleles. These results show that the incidence of patients diagnosed with celiac disease, who are HLA-DQ2, and HLA-DQ8 negative varies among different populations.

As expected, the most common five-locus haplotype in patients was A*01-B*08-DRB1*03-DQA1*05:01-DQB1*02:01 with an extremely high frequency (22%). This haplotype is regarded as a very common in healthy individuals (2.8%), because due to the high polymorphism, haplotypes in general population are not very often found at a frequencies greater than 5%. This is an extended, highly conserved haplotype, named AH8.1. It is also a risk factor for several other

autoimmune diseases (lupus, myasthenia, autoimmune hepatitis, diabetes type 1 etc.) (166), and in that sense can be regarded as an autoimmune haplotype. As it is a DQ2.5 heterodimer positive haplotype, it contributes to the most DQ2.5 heterodimer positivity in *cis* position in patients.

The most frequent haplotype in controls A*02-B*18-DRB1*11 with a frequency of over 6%, was not found in patients, thus making the calculation of odds ratio not applicable. However, this is a finding that needs to be clarified by further studies, as this data suggest a protective role for CD in Kosovar Albanian population. Less pronounced, but similar results are found for haplotype A*02-B*51-DRB1*14 (4% in controls, 0% in patients). On the other hand, the haplotype A*02-B*51-DRB1*11 was common in patients as well as in controls, 4.1% (third most common haplotype) and 2.4% (fourth most common haplotype) respectively.

Interestingly, the incidence of 5-loci extended haplotype A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 was observed in 4.1 % of our CD cases and in only 0.4% of controls. This haplotype has been not previously reported as haplotype associated with CD. It is most probable haplotype of Euro-Asiatic origin, as it is present in Mongolians (HF: 3.2%), in Chaouya population from Morocco (HF: 2.9%), Manchu (HF: 2.2%), Turks and Kurds (HF: 1.3%), Spaniards (HF: 1.2%) and Italians (HF: 0.5%) (167-169). Another haplotype that was found in four patients and not at all found in the control group was A*68-B*44-DRB1*07-DQA1*02:01-DQB1*02:02.

In Europe, this haplotype has been reported only in populations from Portugal-Azores Island, Turkey, Italy and Norway-ethnic Norwegians. It clearly shows that this haplotype was migrating from south to north of Europe or vice-versa. It is interesting to note, that both of these rare haplotypes, are the ones carrying DQ2.2 heterodimer, which can be of interest for further studies in CD patients from Kosovo, both paediatric and adult ones.

Our results show that genotype positive for at least one of the CD predisposing heterodimers has high sensitivity value (95%) in contrast to quite low specificity (40%). On the other hand, high negative predictive value (96%) was associated with the highest positive predictive value observed for heterozygous genotype DQ2.5/DQ2.2 of (86%). Our results are in high accordance with literature results reporting that absence of the corresponding genes virtually excludes a diagnosis of CD with a very high negative predictive value, while the positive predictive value of HLA DQ2/DQ8 is however limited as they can be present in approximately 40% of the general population, most of whom will never develop CD (170).

Among nine patients clinically suspected (un-confirmed) for CD, all DQ2/DQ8 negative, the DQA1*05 allele was found in 5 cases, showing the possible association between CD and DQA1*05 phenotype. There was not found out any correlation according to clinic symptomatology and HLA heterodimer lacking, but it was shown that 3 out of 4 children negative for HLA DQ2/DQ8 missed two diagnostic tests (SBB and EMA) while TG2 titres was less than 10 time of NUL. This may be the explanation of HLA DQ2/DQ8 negativity for these nine children. To all of them was proposed a gluten challenge with biopsies, because there is doubt about the initial diagnosis since HLA-DQ2 and HLA-DQ8 were negative and the children were placed on a GFD without proper testing (32).

We assessed CD-associated HLA-DQ-DR frequencies in Kosovar Albanian patients and controls to evaluate possible effects of different genetic substrates. Even if many theories and environmental risk factors are subject to ongoing verification - like gluten introduction in populations, timing of gluten introduction in infants, breastfeeding, composition of microbiota, metabolic profiles, vaccination schedule, infections, use of antibiotics, etc. the high-risk HLA genotypes at present remain the most important factor affecting CD onset. This study is giving the evidence to this finding in Kosovar Albanian population, as well.

It must be also stressed that our study had some limitations regarding the sample size; thus, further studies must be done to overcome these drawbacks, including a broader population, both in sample size and patients age range.

7. CONCLUSION

- The diagnosis was primarily suspected on its presentation with classical gastrointestinal manifestations

- The proportion of cases with diarrhoea 78%, suggest that maybe a sizeable proportion of cases with atypical presentations are not recognized

- Anorexia and anaemia were the most prevalent extraintestinal manifestation especially in school children and adolescents

- Girls were almost twice as often affected as boys; gender ratio was 1.9:1

- Quite young age at the time of diagnosis (5.5 years) and the relatively short delay to diagnosis, appears to be the result of the classic clinical manifestation

- Positive family history for CD was present for 12% of cases, 60% of them siblings, that shows a significantly increased chance of celiac disease in first and second-degree relatives

- Anti-tissue transglutaminase 2 (TG2) antibody tests was the most used serological marker in CD diagnosis

- New 2012`s ESPGHAN criteria was shown to be usefully for CD diagnosis in Kosovo as in many other resource limited countries due to its cost-effectiveness and the possibility of reducing the time of diagnosis

- HLA allele and haplotype analysis revealed ancestral HLA-A*01-B*08-DRB1*03-DQA1*05:01-DQB1*02 as the most frequent haplotype in patients with high statistically significant increased frequency compared with controls (p<0.0001, OR=10)

- The extended haplotype HLA-A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 is present in patients with high statistically significant increased frequency compared with controls (p<0.0001, OR=11) and might be a Kosovar Albanian population specific risk factor for CD

- The extended haplotype HLA-A*02-B*18-DRB1*11-DQA1*05:05-DQB1*03:01 was the most frequent haplotype in healthy control group and not at all present in patients, thus might be a Kosovar Albanian population specific protective factor for CD

- HLA-DQ heterodimer type analysis revealed DQ2.5 and DQ2.2 heterodimers as two most frequent heterodimers in patients, representing 58,3% of all heterodimers detected, and being statistically significantly increased in comparison with controls. Heterodimer DQ8.3 did not differ in frequency between patients and controls

- The two most frequent heterodimers in controls were DQ5.1 and DQ7.5 representing 52% of heterodimers detected and both with a statistically significant increased frequency in comparison with patients

- Heterodimer genotype DQ2.5/X was the most prevalent one (58%), while the second most frequent genotype was DQ2.5/DQ2.2 (20%), together being present in 78% of patients and in 17% of controls.

- Genotype positive for DQ2.5 either in a single or double dose was present in 88% of patients, while genotypes positive for any of two other risk heterodimers were present in additional 7% of patients. In total, 95.00 % of patients and 40.32% of controls had a genotype that was positive for at least one of the predisposing DQ heterodimers (HLA-DQ2.5, DQ8, DQ2.2) (p<0.0001).

- CD was diagnosed in three DQ heterodimer negative patients, two carried DQA1 allele of DQ2.5 heterodimer (DQA1*05:05) and one was completely negative for any of risk heterodimer alleles

- Among all detected HLA allele, haplotype and heterodimer polymorphisms as the CD risk factors, presence of at least one of DQ2.5, DQ8, DQ2.2 heterodimers had the highest sensitivity (95%) and highest negative predictive value (96%), absence of DQ2.5, DQ8, DQ2.2 heterodimers in an individual genotype had a highest specificity (60%) and DQ2.5/DQ2.2 genotype had highest positive predictive value (86%). Haplotype A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 also had very high positive predictive value (83%)

- Kosovar Albanian pediatric CD patients present similar distribution of predisposing HLA-DQ2 and DQ8 heterodimers as in other European and non-European populations but some HLA genetic factors specific for Kosovar Albanian population have also been determined

8. ABSTRACT

Clinical, biochemical and immunogenetical characteristics of celiac disease in Albanian pediatric patients from Kosovo, Atifete Ramosaj-Morina, 2019

Introduction: Celiac disease is an immune-mediated chronic inflammatory disorder triggered after gluten ingestion in genetically susceptible individuals. HLA-DQ2 and HLA-DQ8 heterodimers have been recognized as necessary - but not sufficient – genetic factors for the occurrence of celiac disease.

Aim: To present the clinical and biochemical characteristics in celiac disease Kosovar Albanian children as well as to analyse HLA class I and class II allele and haplotype polymorphism in patients and matched controls.

Materials and methods: A total of 72 patients treated for celiac disease from 2005 to 2016 at Pediatric Clinic, University Clinical Center of Kosovo and 124 age matched, unrelated healthy children as control group were enrolled in the study. Serum antibody testing and small bowel biopsies were performed in Kosovo while molecular HLA typing was done in Tissue Typing Center, UHC Zagreb.

Results: Celiac disease was diagnosed due to classical form of presentation in 78% of patients. Mean age at the time of diagnosis was 5.5 years, sex distribution showed a female predominance (1.9:1). Among 60 confirmed celiac disease patients, 95.00 % carried at least one of the risk DQ heterodimers (HLA-DQ2.5, DQ8, DQ2.2) in comparison with 40.32% of controls (p<0.0001, OD=28; negative predictive value 96%). None of the nine clinically unconfirmed patients carried the predisposing DQ heterodimer. The HLA-A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 was found as population specific celiac disease predisposing haplotype with a positive predictive value of 83%.

Conclusion: Kosovar Albanian pediatric celiac disease patients show the same distribution of predisposing HLA-DQ2 and DQ8 heterodimers as in other European and non-European populations but some HLA genetic factors specific for Kosovar Albanian population have also been determined.

Key words: Celiac disease, HLA, DQ heterodimer, Kosovo Albanian children.

9. SAŽETAK

Uvod: Celijakija je imunološki posredovana kronična upalna bolest tankog crijeva koja u genetski predisponiranih osoba, nastaje uslijed prehrane glutenom. Heterodimeri HLA-DQ2 i HLA-DQ8 su neophodan, ali ne i dostatan, genetički čimbenik za razvoj celijakije.

Cilj: U albanske djece s Kosova s celijakijom prikazati klinička i biokemijska obilježja te analizirati i usporediti polimorfizam alela i haplotipova HLA razreda I i razreda II u bolesnika i u zdravoj kontrolnoj skupini.

Materijali i metode: U istraživanje je bilo uključeno 72 djece liječeno s dijagnozom celijakije u razdoblju od 2005-2016 godine u Klinici za pedijatriju, Sveučilišnog kliničkog centra Kosovo te 124 zdrave, nesrodne djece sukladne dobi, kao kontrolna skupina. Testiranje antitijela te biopsije sluznice tankog crijeva provedene su na Kosovu, a molekularna tipizacija HLA u Odjelu za tipizaciju tkiva Kliničkog bolničkog centra Zagreb.

Rezultati: Ukupno je 78% bolesnika dijagnosticirano temeljem očitovanja s klasičnom slikom bolesti, srednja dob pri postavljanju dijagnoze bila je 5,5 godina, s raspodjelom spolova 1,9:1 u korist djevojčica. U skupini od 60 bolesnika s klinički potvrđenom dijagnozom, 95% ih je bilo nositelj barem jednog od rizičnih DQ heterodimera (HLA-DQ2.5, DQ8, DQ2.2), a u kontrolnoj skupini 40.32% (p<0.0001, OD=28; negativna prediktivna vrijednost 96%). Niti jedan od devet bolesnika koji su imali klinički nejasnu sliku nije bio nositelj rizičnog heterodimera DQ. HLA-A*02-B*50-DRB1*07-DQA1*02:01-DQB1*02:02 haplotip je otkriven kao rizični genetski čimbenik za celijakiju u populaciji Kosova, s pozitivnom prediktivnom vrijednošću 83%.

Zaključak: U albanske djece s Kosova s celijakijom utvrđena je istovjetna učestalost rizičnih heterodimera HLA-DQ2 i DQ8 kao i u drugim europskim i ne-europskim populacijama, ali uz to otkriveni su i genetički čimbenici HLA koji su specifični za albansku populaciju s Kosova.

Ključne riječi: celijakija, HLA, DQ heterodimer, albanska djeca s Kosova

10. LIST OF REFERENCES

1. Vogten AJ, Peña AS. Coeliac disease: one century after Samuel Gee (1888). Neth J Med. 1987;31(5–6):253-5.

2. Mearin ML. Celiac disease among children and adolescents. Curr Probl Pediatr Adolesc Health Care. 2007;37(3):86–105.

3. Seah PP, Fry L, Rossiter MA, Hoffbrand AV, Holborow EJ. Anti-reticulin antibodies in childhood coeliac disease. Lancet Lond Engl. 1971;25;2 (7726):681–2.

4. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. J Exp Med. 1989;169(1):345–50.

5. Palavecino EA, Mota AH, Awad J, Derosa S, Herrera M, Chertkoff L, et al. HLA and celiac disease in Argentina: involvement of the DQ subregion. Dis Markers. 1990;8(1):5–10.

6. Schuppan D, Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, et al. Identification of the Autoantigen of Celiac Disease. Ann N Y Acad Sci. 1998;859(1):121–6.

7. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. Arch Dis Child. 1990;65(8):909–11.

8. Mustalahti K, Catassi C, Reunanen A, Fabiani E, Heier M, McMillan S, et al. The prevalence of celiac disease in Europe: results of a centralized, international mass screening project. Ann Med. 2010;42(8):587–95.

9. Cataldo F, Pitarresi N, Accomando S, Greco L, SIGENP, GLNBI Working Group on Coeliac Disease. Epidemiological and clinical features in immigrant children with coeliac disease: an Italian multicentre study. Dig Liver Dis Off J Ital Soc Gastroenterol Ital Assoc Study Liver. 2004;36(11):722–9.

10. Vanciková Z, Chlumecký V, Sokol D, Horáková D, Hamsíková E, Fucíková T, et al. The serologic screening for celiac disease in the general population (blood donors) and in some high-risk groups of adults (patients with autoimmune diseases, osteoporosis and infertility) in the Czech Republic. Folia Microbiol (Praha). 2002;47(6):753–8.

11. Altobelli E, Paduano R, Petrocelli R, Di Orio F. Burden of celiac disease in Europe: a review of its childhood and adulthood prevalence and incidence as of September 2014. Ann Ig. 2014;26(6):485-98.

12. Rostami K, Kerckhaert J, Tiemessen R, von Blomberg BM, Meijer JW, Mulder CJ. Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. Am J Gastroenterol. 1999;94(4):888–94.

13. Mäki M, Mustalahti K, Kokkonen J, Kulmala P, Haapalahti M, Karttunen T, et al. Prevalence of Celiac disease among children in Finland. N Engl J Med. 2003;348(25):2517–24.

14. Fasano A, Berti I, Gerarduzzi T, Not T, Colletti RB, Drago S, et al. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. Arch Intern Med. 2003;163(3):286–92.

15. Cook HB, Burt MJ, Collett JA, Whitehead MR, Frampton CM, Chapman BA. Adult coeliac disease: prevalence and clinical significance. J Gastroenterol Hepatol. 2000;15(9):1032–6.

16. Comba A, Eren NB, Demir E. Prevalence of celiac disease among school-age children in Çorum, Turkey. Turk J Gastroenterol. 2018;29(5):595-600.

17. Darada K, Bitar A, Mokadem MA-R, Hashash JG, Green P. Celiac disease in Middle Eastern and North African countries: a new burden? World J Gastroenterol. 2010;16(12):1449–57.

18. Bai JC, Ciacci C. World Gastroenterology Organisation Global Guidelines: Celiac Disease February 2017. J Clin Gastroenterol. 2017;51(9):755–68.

19. Lionetti E, Catassi C. New clues in celiac disease epidemiology, pathogenesis, clinical manifestations, and treatment. Int Rev Immunol. 2011;30(4):219–31.

20. Bodé S, Gudmand-Høyer E. Incidence and prevalence of adult coeliac disease within a defined geographic area in Denmark. Scand J Gastroenterol. 1996;31(7):694–9.

21. Hawkes ND, Swift GL, Smith PM, Jenkins HR. Incidence and presentation of coeliac disease in South Glamorgan. Eur J Gastroenterol Hepatol. 2000;12(3):345–9.

22. Collin P, Reunala T, Rasmussen M, Kyrönpalo S, Pehkonen E, Laippala P, et al. High incidence and prevalence of adult coeliac disease. Augmented diagnostic approach. Scand J Gastroenterol. 1997;32(11):1129–33.

23. López-Rodríguez MJ, Canal Macías ML, Lavado García JM, Sánchez Belda M, Robledo Andrés P, Pedrera Zamorano JD. Epidemiological changes in diagnosed coeliac disease in a population of Spanish children. Acta Paediatr Oslo Nor. 1992. 2003;92(2):165–9.

24. Hoffenberg EJ, MacKenzie T, Barriga KJ, Eisenbarth GS, Bao F, Haas JE, et al. A prospective study of the incidence of childhood celiac disease. J Pediatr. 2003;143(3):308–14.

25. Corrao G, Usai P, Galatola G, Ansaldi N, Meini A, Pelli MA, et al. Estimating the incidence of coeliac disease with capture-recapture methods within four geographic areas in Italy. J Epidemiol Community Health. 1996;50(3):299–305.

26. Magazzú G, Bottaro G, Cataldo F, Iacono G, Di Donato F, Patane R, et al. Increasing incidence of childhood celiac disease in Sicily: results of a multicenter study. Acta Paediatr Oslo Nor. 1992. 1994;83(10):1065–9.

27. Singh P, Arora S, Singh A, Strand TA, Makharia GK. Prevalence of celiac disease in Asia: A systematic review and meta-analysis. J Gastroenterol Hepatol. 2016;31(6):1095–101.

28. Ludvigsson JF, Card TR, Kaukinen K, Bai J, Zingone F, Sanders DS, et al. Screening for celiac disease in the general population and in high-risk groups. United Eur Gastroenterol J. 2015;3(2):106–20.

29. Dixit R, Lebwohl B, Ludvigsson JF, Lewis SK, Rizkalla-Reilly N, Green PHR. Celiac disease is diagnosed less frequently in young adult males. Dig Dis Sci. 2014;59(7):1509–12.

30. Rashid M, Cranney A, Zarkadas M, Graham ID, Switzer C, Case S, et al. Celiac disease: evaluation of the diagnosis and dietary compliance in Canadian children. Pediatrics. 2005;116(6):754-759.

31. Walker MM, Murray JA, Ronkainen J, Aro P, Storskrubb T, D'Amato M, et al. Detection of celiac disease and lymphocytic enteropathy by parallel serology and histopathology in a population-based study. Gastroenterology. 2010;139(1):112–9.

32. Husby S, Koletzko S, Korponay-Szabó IR, Mearin ML, Phillips A, Shamir R, et al. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. J Pediatr Gastroenterol Nutr. 2012;54(1):136–60.

33. Rostom A, Murray JA, Kagnoff MF. American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. Gastroenterology. 2006;131(6):1981–2002.

34. Molberg O, Mcadam SN, Körner R, Quarsten H, Kristiansen C, Madsen L, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat Med. 1998;4(6):713–7.

35. Ludvigsson JF, Leffler DA, Bai JC, Biagi F, Fasano A, Green PHR, et al. The Oslo definitions for coeliac disease and related terms. Gut. 2013;62(1):43–52.

36. Fasano A. Clinical presentation of celiac disease in the pediatric population. Gastroenterology. 2005;128(4 Suppl 1): S68-73.

37. Fasano A, Catassi C. Coeliac disease in children. Best Pract Res Clin Gastroenterol. 2005;19(3):467–78.

38. Kagnoff MF. Celiac disease: pathogenesis of a model immunogenetic disease. J Clin Invest. 2007;117(1):41–9.

39. Megiorni F, Pizzuti A. HLA-DQA1 and HLA-DQB1 in Celiac disease predisposition: practical implications of the HLA molecular typing. J Biomed Sci. 2012; 19:88.

40. Ontiveros N, Hardy MY, Cabrera-Chavez F. Assessing of Celiac Disease and Nonceliac Gluten Sensitivity. Gastroenterol Res Pract. 2015; 2015:72395.

41. Louka AS, Sollid LM. HLA in coeliac disease: unravelling the complex genetics of a complex disorder. Tissue Antigens. 2003;61(2):105–17.

42. Mazzarella G, Maglio M, Paparo F, Nardone G, Stefanile R, Greco L, et al. An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. Gut. 2003;52(1):57–62.

43. Fleckenstein B, Molberg Ø, Qiao S-W, Schmid DG, von der Mülbe F, Elgstøen K, et al. Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Role of enzyme specificity and pH influence on the transamidation versus deamidation process. J Biol Chem. 2002;277(37):34109–16.

44. Fleckenstein B, Qiao S-W, Larsen MR, Jung G, Roepstorff P, Sollid LM. Molecular characterization of covalent complexes between tissue transglutaminase and gliadin peptides. J Biol Chem. 2004;279(17):17607–16.

45. Hüe S, Mention J-J, Monteiro RC, Zhang S, Cellier C, Schmitz J, et al. A direct ro5le for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity. 2004;21(3):367–77.

46. Meresse B, Chen Z, Ciszewski C, Tretiakova M, Bhagat G, Krausz TN, et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity. 2004;21(3):357–66.

47. Meresse B, Curran SA, Ciszewski C, Orbelyan G, Setty M, Bhagat G, et al. Reprogramming of CTLs into natural killer-like cells in celiac disease. J Exp Med. 2006;203(5):1343–55.

48. Benelli E, Zin A, Martelossi S. Celiac disease in children. Minerva Pediatr. 2018

49. Green PHR, Krishnareddy S, Lebwohl B. Clinical manifestations of celiac disease. Dig Dis Basel Switz. 2015;33(2):137–40.

50. Ferrara M, Coppola L, Coppola A, Capozzi L. Iron deficiency in childhood and adolescence: retrospective review. Hematol Amst Neth. 2006;11(3):183–6.

51. NIH Consensus Development Conference on Celiac Disease. NIH Consens State Sci Statements. 2004;21(1):1-23.

52. Parzanese I, Qehajaj D, Patrinicola F, Aralica M, Chiriva-Internati M, Stifter S, et al. Celiac disease: From pathophysiology to treatment. World J Gastrointest Pathophysiol. 2017;8(2):27–38.

53. Rostom A, Dubé C, Cranney A, Saloojee N, Sy R, Garritty C, et al. Celiac disease. Evid Rep Technol Assess (Summ). 2004;(104):1–6.

54. Bonamico M, Pasquino AM, Mariani P, Danesi HM, Culasso F, Mazzanti L, et al. Prevalence and clinical picture of celiac disease in Turner syndrome. J Clin Endocrinol Metab. 2002;87(12):5495–8.

55. Korponay-Szabó IR, Dahlbom I, Laurila K, Koskinen S, Woolley N, Partanen J, et al. Elevation of IgG antibodies against tissue transglutaminase as a diagnostic tool for coeliac disease in selective IgA deficiency. Gut. 2003;52(11):1567–71.

56. Ch'ng CL, Jones MK, Kingham JGC. Celiac disease and autoimmune thyroid disease. Clin Med Res. 2007;5(3):184–92.

57. Gobbi G. Coeliac disease, epilepsy and cerebral calcifications. Brain Dev. 2005;27(3):189–200.

58. Anania C, De Luca E, De Castro G, Chiesa C, Pacifico L. Liver involvement in pediatric celiac disease. World J Gastroenterol. 2015 May 21;21(19):5813–22.

59. Eigner W, Bashir K, Primas C, Kazemi-Shirazi L, Wrba F, Trauner M, et al. Dynamics of occurrence of refractory coeliac disease and associated complications over 25 years. Aliment Pharmacol Ther. 2017;45(2):364-372.

60. Byström IM, Hollén E, Fälth-Magnusson K, Johansson A. Health-related quality of life in children and adolescents with celiac disease: from the perspectives of children and parents. Gastroenterol Res Pract. 2012;2012:986475.

61. Durham J, Temples HS. Celiac Disease in the Pediatric Population. J Pediatr Health Care. 2018;32(6):627-631.

62. Wessels MMS, de Rooij N, Roovers L, Verhage J, de Vries W, Mearin ML. Towards an individual screening strategy for first-degree relatives of celiac patients. Eur J Pediatr. 2018;177(11):1585-1592.

63. Marginean CO, Meliţ LE, Mareş R-C, Mărginean MO, Voidăzan S, Dobreanu M. Clinical and biological correlations in celiac disease in children: the prospective single experience of a romanian tertiary center: A case-control study (Strobe-Compliant study). Medicine (Baltimore). 2017;96(20):6936.

64. 10 Am J Gastroenterol. 2010;105(12):2520–4.

65. Hawamdeh H, Al-Zoubi B, Al Sharqi Y, Qasrawi A, Abdelaziz Y, Barbar M. Association of Tissue Transglutaminase Antibody Titer with Duodenal Histological Changes in Children with Celiac Disease. Gastroenterol Res Pract. 2016;2016:6718590.

66. Lewis NR, Scott BB. Systematic review: the use of serology to exclude or diagnose coeliac disease (a comparison of the endomysial and tissue transglutaminase antibody tests). Aliment Pharmacol Ther. 2006;24(1):47–54.

67. Lewis NR, Scott BB. Meta-analysis: deamidated gliadin peptide antibody and tissue transglutaminase antibody compared as screening tests for coeliac disease. Aliment Pharmacol Ther. 2010;31(1):73–81.

68. Giersiepen K, Lelgemann M, Stuhldreher N, Ronfani L, Husby S, Koletzko S, et al. Accuracy of diagnostic antibody tests for coeliac disease in children: summary of an evidence report. J Pediatr Gastroenterol Nutr. 2012;54(2):229–41.

69. Lebwohl B, Rubio-Tapia A, Assiri A, Newland C, Guandalini S. Diagnosis of celiac disease. Gastrointest Endosc Clin N Am. 2012;22(4):661–77.

70. Rashid M, MacDonald A. Importance of duodenal bulb biopsies in children for diagnosis of celiac disease in clinical practice. BMC Gastroenterol. 2009; 9:78.

71. Marsh MN. Grains of truth: evolutionary changes in small intestinal mucosa in response to environmental antigen challenge. Gut. 1990;31(1):111–4.

72. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. Eur J Gastroenterol Hepatol. 1999;11(10):1185–94.

73. Dickson BC, Streutker CJ, Chetty R. Coeliac disease: an update for pathologists. J Clin Pathol. 2006;59(10):1008–16.

74. Plaza-Izurieta L, Fernandez-Jimenez N, Bilbao JR. Genetics of Celiac Disease. HLA and Non-HLA Genes. In Arranz E, Fernández-Bañares F, Rosell CM, Rodrigo L, Peña AS, editors. Advances in the Understanding of Gluten Related Pathology and the Evolution of Gluten-Free Foods. Barcelona, Spain: OmniaScience; 2015. p. 79-104.

75. Murray JA, Moore SB, Van Dyke CT, Lahr BD, Dierkhising RA, Zinsmeister AR, et al. HLA DQ gene dosage and risk and severity of celiac disease. Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc. 2007;5(12):1406–12.

76. Mubarak A, Spierings E, Wolters V, van Hoogstraten I, Kneepkens CM, Houwen R. Human leukocyte antigen DQ2.2 and celiac disease. J Pediatr Gastroenterol Nutr. 2013;56(4):428-30.

77. Monsuur AJ, de Bakker PIW, Zhernakova A, Pinto D, Verduijn W, Romanos J, et al. Effective detection of human leukocyte antigen risk alleles in celiac disease using tag single nucleotide polymorphisms. PloS One. 2008;3(5):2270.

78. Megiorni F, Mora B, Bonamico M, Barbato M, Nenna R, Maiella G, et al. HLA-DQ and risk gradient for celiac disease. Hum Immunol. 2009;70(1):55-9.

79. Pallav K, Kabbani T, Tariq S, Vanga R, Kelly CP, Leffler DA. Clinical utility of celiac disease-associated HLA testing. Dig Dis Sci. 2014;59(9):2199–206.

80. Peña-Quintana L, Torres-Galván MJ, Déniz-Naranjo MC, Ortigosa-Castillo L, Ramos-Varela JC, Calvo-Hernández F, et al. Assessment of the DQ heterodimer test in the diagnosis of celiac disease in the Canary Islands (Spain). J Pediatr Gastroenterol Nutr. 2003;37(5):604–8.

81. Margaritte-Jeannin P, Babron MC, Bourgey M, Louka AS, Clot F, Percopo S, et al. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. Tissue Antigens. 2004;63(6):562–7.

82. Piancatelli D, Ben E, Barhdadi I, Oumhani K, Sebastiani P, Colanardi A et al. HLA Typing and Celiac Disease in Moroccans. Med Sci (Basel). 2017, 6;5(1).

83. Dolinšek J, Micetik-turk D, Urlep-Žužej D, Zagradišnik B, Haimilia K, Holopainen P. Importance of Celiac Disease Patients Lacking HLA DQ2 or DQ8 Heterodimer in Slovenia. J Pediatr Gastroenterol Nutr 2006; 42(5): 18-19.

84. Smigoc Schweiger D, Mendez A, Kunilo Jamnik S, Bratanic N, Bratina N, Battelino T, et al. High-risk genotypes HLA-DR3-DQ2/DR3-DQ2 and DR3-DQ2/DR4-DQ8 in co-occurrence of type 1 diabetes and celiac disease. Autoimmunity. 2016;49(4):240-7.

85. Alarida K, Harown J, Di Pierro MR, Drago S, Catassi C. HLA-DQ2 and -DQ8 genotypes in celiac and healthy Libyan children. Dig Liver Dis. 2010; 42:425-7.

86. Stanković B, Radlović N, Leković Z, Ristić D, Radlović V, Nikčević G, et al. HLA genotyping in pediatric celiac disease patients. Bosn J Basic Med Sci. 2014 Aug 16;14(3):171-6.

87. Rostami-Nejad M, Romanos J, Rostami K, et al. Allele and haplotype frequencies for HLA-DQ in Iranian celiac disease patients. World J Gastroenterol 2014; 20: 6302-8.

88. Sumník Z, Kolousková S, Cinek O, et al. HLA-DQA1*05DQB1*0201 positivity predisposes to celiac disease in Czech diabetic children. Acta Paediatr 2000; 89: 1426-30.

89. Neuhausen SL, Weizman Z, Camp NJ, et al. HLA DQA1-DQB1 genotypes in Bedouin families with celiac disease. Hum Immunol 2002; 63: 502-7.

90. Selleski N, Almeida LM, Almeida FC, Pratesi CB, Nóbrega YKM, Gandolfi L. Prevalence of celiac disease predisposing genotypes, including HLA-DQ2.2 variant, in Brazilian children. Arq Gastroenterol. 2018 Jan-Mar;55(1):82-85.

91. Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, et al. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: Results from the European Genetics Cluster on Celiac Disease. Hum Immunol. 2003;64(4):469–77.

92. Bascuñán KA, Vespa MC, Araya M. Celiac disease: understanding the gluten-free diet. Eur J Nutr. 2017;56(2):449–59.

93. Kurppa K, Collin P, Mäki M, Kaukinen K. Celiac disease and health-related quality of life. Expert Rev Gastroenterol Hepatol. 2011;5(1):83–90.

94. Sugai E, Nachman F, Váquez H, González A, Andrenacci P, Czech A, et al. Dynamics of celiac disease-specific serology after initiation of a gluten-free diet and use in the assessment of compliance with treatment. Dig Liver Dis Off J Ital Soc Gastroenterol Ital Assoc Study Liver. 2010;42(5):352–8.

95. Nachman F, del Campo MP, González A, Corzo L, Vázquez H, Sfoggia C, et al. Long-term deterioration of quality of life in adult patients with celiac disease is associated with treatment noncompliance. Dig Liver Dis Off J Ital Soc Gastroenterol Ital Assoc Study Liver. 2010;42(10):685–91.

96. Raehsler SL, Choung RS, Marietta EV, Murray JA. Accumulation of Heavy Metals in People on a Gluten-Free Diet. Clin Gastroenterol Hepatol. 2018;16(2):244-251.

97. Elli L, Rossi V, Conte D, Ronchi A, Tomba C, Passoni M, et al. Increased mercury levels in patients with celiac disease following a gluten-free regimen. Gastroenterol Res Pract. 2015; 2015: 953042

98. Rajpoot P, Sharma A, Harikrishnan S, et al. Adherence to gluten-free diet and barriers to adherence in patients with celiac disease. Indian J Gastroenterol. 2015; 34:380–386.

99. Glissen Brown JR1, Singh P. Coeliac disease. Paediatr Int Child Health. 2018; 13:1-9

100. Stein J, Schuppan D. Coeliac Disease - New Pathophysiological Findings and Their Implications for Therapy. Viszeralmedizin. 2014;30(3):156–65.

101. Leffler DA, Kelly CP, Abdallah HZ, Colatrella AM, Harris LA, Leon F, et al. A randomized, double-blind study of larazotide acetate to prevent the activation of celiac disease during gluten challenge. Am J Gastroenterol. 2012;107(10):1554–62.

102. Ciacci C, Maiuri L, Russo I, Tortora R, Bucci C, Cappello C, et al. Efficacy of budesonide therapy in the early phase of treatment of adult coeliac disease patients with malabsorption: an in vivo/in vitro pilot study. Clin Exp Pharmacol Physiol. 2009;36(12):1170–6.

103. Lobatón T, Vermeire S, Van Assche G, Rutgeerts P. Review article: anti-adhesion therapies for inflammatory bowel disease. Aliment Pharmacol Ther. 2014;39(6):579–94.

104. Jüse U, Arntzen M, Højrup P, Fleckenstein B, Sollid LM. Assessing high affinity binding to HLA-DQ2.5 by a novel peptide library based approach. Bioorg Med Chem. 2011;19(7):2470–7.

105. Alex Lancaster, Richard M Single, Owen D Solberg, Mark P Nelson, and Glenys Thomson. 2007b. "PyPop update - a software pipeline for large-scale multilocus population genomics". Tissue Antigens. 69 Suppl 1. 192-197.

106. Steens RFR, Csizmadia CGDS, George EK, Ninaber MK, Hira Sing RA, Mearin ML. A national prospective study on childhood celiac disease in the Netherlands 1993-2000: an increasing recognition and a changing clinical picture. J Pediatr. 2005;147(2):239–43.

107. Ress K, Luts K, Rägo T, Pisarev H, Uibo O. Nationwide study of childhood celiac disease incidence over a 35-year period in Estonia. Eur J Pediatr. 2012;171(12):1823–8.

108. Tucci F, Astarita L, Abkari A, Abu-Zekry M, Attard T, Ben Hariz M, et al. Celiac disease in the Mediterranean area. BMC Gastroenterol. 2014;14:24.

109. Fortunato F, Martinelli D, Cozza V, Ciavarella P, Valente A, Cazzato T, et al. Italian family paediatricians' approach and management of celiac disease: a cross-sectional study in Puglia Region, 2012. BMC Gastroenterol. 2014;14:38.

110. Tanpowpong P, Broder-Fingert S, Katz AJ, Camargo CA. Age-related patterns in clinical presentations and gluten-related issues among children and adolescents with celiac disease. Clin Transl Gastroenterol. 2012;3:e9.

111. McGowan KE, Castiglione DA, Butzner JD. The changing face of childhood celiac disease in north america: impact of serological testing. Pediatrics. 2009;124(6):1572–8.

112. Savilahti E, Kolho K-L, Westerholm-Ormio M, Verkasalo M. Clinics of coeliac disease in children in the 2000s. Acta Paediatr Oslo Nor 1992. 2010;99(7):1026–30.

113. Namatovu F, Sandström O, Olsson C, Lindkvist M, Ivarsson A. Celiac disease risk varies between birth cohorts, generating hypotheses about causality: evidence from 36 years of population-based follow-up. BMC Gastroenterol. 2014;14:59.

114. Cilleruelo ML, Roman-Riechmann E, Sanchez-Valverde F, Donat E, Manuel-Ramos J, Martín-Orte E, et al. Spanish national registry of celiac disease: incidence and clinical presentation. J Pediatr Gastroenterol Nutr. 2014;59(4):522–6.

115. Garampazzi A, Rapa A, Mura S, Capelli A, Valori A, Boldorini R, et al. Clinical pattern of celiac disease is still changing. J Pediatr Gastroenterol Nutr. 2007;45(5):611–4.

116. Roma E, Panayiotou J, Karantana H, Constantinidou C, Siakavellas SI, Krini M, et al. Changing pattern in the clinical presentation of pediatric celiac disease: a 30-year study. Digestion. 2009;80(3):185–91.

117. Radlovic NP, Mladenovic MM, Lekovic ZM, Stojsic ZM, Radlovic VN. Influence of early feeding practices on celiac disease in infants. Croat Med J. 2010;51(5):417–22.

118. Vajpayee S, Sharma SD, Gupta R, Goyal A, Sharma A. Early Infant Feeding Practices May Influence the Onset of Symptomatic Celiac Disease. Pediatr Gastroenterol Hepatol Nutr. 2016;19(4):229–35.

119. Akobeng AK, Ramanan AV, Buchan I, Heller RF. Effect of breast feeding on risk of coeliac disease: a systematic review and meta-analysis of observational studies. Arch Dis Child. 2006;91(1):39–43.

120. Lurz E, Scheidegger U, Spalinger J, Schöni M, Schibli S. Clinical presentation of celiac disease and the diagnostic accuracy of serologic markers in children. Eur J Pediatr. 2009;168(7):839–45.

121. Demir H, Yüce A, Koçak N, Ozen H, Gürakan F. Celiac disease in Turkish children: presentation of 104 cases. Pediatr Int Off J Jpn Pediatr Soc. 2000;42 (5):483–7.

122. Dinler G, Atalay E, Kalayci AG. Celiac disease in 87 children with typical and atypical symptoms in Black Sea region of Turkey. World J Pediatr WJP. 2009;5 (4):282–6.

123. Nurminen S, Kivelä L, Huhtala H, Kaukinen K, Kurppa K. Extraintestinal manifestations were common in children with coeliac disease and were more prevalent in patients with more severe clinical and histological presentation. Acta Paediatr. 2018.

124. Ivarsson A, Persson LA, Nyström L, Ascher H, Cavell B, Danielsson L, et al. Epidemic of coeliac disease in Swedish children. Acta Paediatr Oslo Nor 1992. 2000;89(2):165–71.

125. Çekın AH, Çekın Y, Sezer C. Celiac disease prevalence in patients with iron deficiency anemia. Turk J Gastroenterol Off J Turk Soc Gastroenterol. 2012;23(5):490–5.

126. Uçardağ D, Güliter S, Ceneli O, Yakaryilmaz F, Atasoy P, Cağlayan O. Celiac disease prevalence in patients with iron deficiency anemia of obscure origin. Turk J Gastroenterol Off J Turk Soc Gastroenterol. 2009;20(4):266–70.

127. Baudon J-J, Johanet C, Absalon YB, Morgant G, Cabrol S, Mougenot J-F. Diagnosing celiac disease: a comparison of human tissue transglutaminase antibodies with antigliadin and antiendomysium antibodies. Arch Pediatr Adolesc Med. 2004;158(6):584–8.

128. Singh P, Arora S, Lal S, Strand TA, Makharia GK. Risk of Celiac Disease in the First- and Second-Degree Relatives of Patients with Celiac Disease: A Systematic Review and Meta-Analysis. Am J Gastroenterol. 2015;110(11):1539-48.

129. Altamimi E. Celiac Disease in South Jordan. Pediatr Gastroenterol Hepatol Nutr. 2017; 20(4):222-226.

130. Rubio-Tapia A, Van Dyke CT, Lahr BD, Zinsmeister AR, El-Youssef M, Moore SB et al. Predictors of family risk for celiac disease: a population-based study. Clin Gastroenterol Hepatol 2008;6: 983–987.

131. Bonamico M, Ferri M, Mariani P, Nenna R, Thanasi E, Luparia RP, et al. Serologic and genetic markers of celiac disease: a sequential study in the screening of first degree relatives. J Pediatr Gastroenterol Nutr 2006;42:150–154.

132. Castro-Antunes MM, Magalhães R, Nobre JMM Duarte BP, Silva GA. et al. Celiac disease in first-degree relatives of patients. J Pediatr (Rio J) 2010;86:331–336.

133. Oliveira GN, Mohan R, Fagbemi A. Review of celiac disease presentation in a pediatric tertiary centre. Arq Gastroenterol. 2018;55(1):86-93.

134. Sulcebe G, Cuenod M, Sanchez-Mazas A, Tiercy JM, Zhubi B, Shyti E, et al. Human leukocyte antigen-A, -B, -C, -DRB1 and -DQB1 allele and haplotype frequencies in an Albanian population from Kosovo. Int J Immunogenet. 2013;40(2):104-7.

135. Sulcebe G, Shyti E. HLA-A, -B, -C, -DRB1 and -DQB1 allele and haplotype frequencies in a population of 432 healthy unrelated individuals from Albania. Hum Immunol. 2016;77(8):620-621.

136. Gonzalez-Galarza FF, Takeshita LY, Santos EJ, Kempson F, Maia MH, Silva AL, Silva AL, Ghattaoraya GS, Alfirevic A, Jones AR and Middleton D Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations Nucleic Acid Research 2015 28: D784-8.

137. Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, et al. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European Genetics Cluster on Celiac Disease. Hum Immunol. 2003;64(4):469–77.

138. Tucci F, Astarita L, Abkari A, Abu-Zekry M, Attard T, Ben Hariz. Celiac disease in the Mediterranean area. BMC Gastroenterol. 2014;11;14:24

139. Krini M, Chouliaras G, Kanariou M, Varela I, Spanou K, Panayiotou J et al. HLA class II high-resolution genotyping in Greek children with celiac disease and impact on disease susceptibility. Pediatr Res. 2012; 72(6):625-30.

140. Hernández-Charro B, Donat E, Miner I, Aranburu E, Sánchez-Valverde F, Ramos-Arroyo MA. Modifying effect of HLA haplotypes located trans to DQB1*02-DRB1*03 in celiac patients of Southern Europe. Tissue Antigens 2008;71:213–8.

141. Margaritte-Jeannin P, Babron MC, Bourgey M, Louka AS, Clot F, Percopo S, et al. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. Tissue Antigens. 2004;63(6):562–7.

142. Piccini B, Vascotto M, Serracca L, Luddi A, Margollicci MA, Balestri P, et al. HLA-DQ typing in the diagnostic algorithm of celiac disease. Rev Esp Enferm Dig. 2012;104(5):248-54.

143. Wroblova K, Kolorz M, Pav I, Horakova Z, Filipova P, Bartos M, et al. Frequencies of HLA-DQ2 and HLA-DQ8 haplotypes in Czech and Slovak coeliac patients and the healthy population. Acta Biochim Pol. 2014;61(1):191-3.

144. Donat E, Planelles D, Capilla-Villanueva A, Montoro JA, Palau F, Ribes Koninckx C. Allelic distribution and the effect of haplotype combination for HLA type II loci in the celiac disease population of the Valencian community (Spain). Tissue Antigens 2009;73:255–61

145. Žunec R, Grubić Z, Jurčić Z, Peršić M, Kaštelan A, Kerhin-Brkljačić V. HLA-DQ2 heterodimer in the diagnosis of celiac disease. Biochemia medica. 2004; 14:119-124.

146. Delgado JF, Amengual MJ, Veraguas A, Rodríguez E, de Los Santos MM, Guallarte MP. Paediatric celiac patients carrying the HLA-DR7-DQ2 and HLA-DR3-DQ2 haplotypes display small clinical differences. Acta Paediatr 2014;103: 238-42.

147. Kårhus LL, Thuesen BH, Skaaby T, Rumessen JJ, Linneberg A. The distribution of HLA DQ2 and DQ8 haplotypes and their association with health indicators in a general Danish population. United European Gastroenterol J. 2018;6(6):866-878.

148. Savvateeva LV, Erdes SI, Antishin AS, Zamyatnin AA Jr. Overview of Celiac Disease in Russia: Regional Data and Estimated Prevalence. J Immunol Res.2017:2314813.

149. Kondratyeva E. I., Yankina G. N. Incidence of polymorphic variants of HLA DQA1 and DQB1 genes in patients with celiac disease in Tomsk and Krasnodar. Pediatric Nutrition. 2012;10:11–14.

150. Kurtanov H. A., Danilova A. L., Yakovleva A. E., Savvina A. D., Maximova H. P. Genetic research of HLA genes I and II class—DRB1, DQA1, DQB1 in patients with celiac disease. The Bulletin of Hematology. 2015;11:44–47

151. Basturk A, Artan R, Yilmaz A. The incidence of HLA-DQ2/DQ8 in Turkish children with celiac disease and a comparison of the geographical distribution of HLA-DQ. Przeglad Gastroenterol. 2017;12(4):256–61.

152. Kuloğlu Z, Doğanci T, Kansu A, Demirçeken F, Duman M, Tutkak H, et al. HLA types in Turkish children with celiac disease. Turk J Pediatr 2008;50: 515-20

153. Louka AS, Nilsson S, Olsson M, Talseth B, Lie BA, Ek J, et al. HLA in coeliac disease families: a novel test of risk modification by the 'other' haplotype when at least one DQA1*05-DQB1*02 haplotype is carried. Tissue Antigens.vol. 2002; 60:147-54.

154. Clerget-Darpoux F, Bouguerra F, Kastally R, Semana G, Babron MC, Debbabi A, et al. High risk genotypes for celiac disease. C R Acad Sci III 1994;317:931–936

155. Vader W, Stepniak D, Kooy Y, et al. The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. Proc Natl Acad Sci USA. 2003;100:12390-5.

156. Murad H, Jazairi B, Khansaa I, Olabi D, Khouri L. HLA-DQ2 and -DQ8 genotype frequency in Syrian celiac disease children: HLA-DQ relative risks evaluation. MC Gastroenterol. 2018;18(1):70.

157. Cabrera CM, Méndez-López IM, Caballero A. Risk variation in celiac disease in a population from Southern Spain: evaluating the influence of the DQB1*02:02 allele frequency. Scand J Gastroenterol. 2018;53(3):266-272.

158. Koskinen L, Romanos J, Kaukinen K, Mustalahti K, Korponay-Szabo I, Barisani D, et al. Cost-effective HLA typing with tagging SNPs predicts celiac disease risk haplotypes in the Finnish, Hungarian, and Italian populations. Immunogenetics. 2009;61(4):247-56

159. Michalski JP, McCombs CC, Arai T, Elston RC, Cao T, McCarthy CF, et al. HLA-DR, DQ genotypes of celiac disease patients and healthy subjects from the West of Ireland. Tissue Antigens 1996;47:127–33.

160. Tighe MR, Hall MA, Ashkenazi A, Siegler E, Lanchbury JS, Ciclitira PJ. Celiac disease among Ashkenazi Jews from Israel. A study of the HLA class II alleles and their associations with disease susceptibility. Hum Immunol 1993;38:270–6.

161. Johnson TC, Diamond B, Memeo L, Negulescu H, Hovhanissyan Z, Verkarre V, et al. Relationship of HLA-DQ8 and severity of celiac disease: comparison of New York and Parisian cohorts. Clin Gastroenterol Hepatol 2004:2:888–94.

162. Vidales MC, Zubillaga P, Zubillaga I, Alfonso-Sánchez MA. Allele and haplotype frequencies for HLA class II (DQA1 and DQB1) loci in patients with celiac disease from Spain. Hum Immunol. 2004;65(4):352-8.

163. Pallav K, Kabbani T, Tariq S, Vanga R, Kelly CP, Leffler DA. Clinical utility of celiac disease-associated HLA testing. Dig Dis Sci 2014; 59: 2199-206

164. Fernández-Bañares F, Arau B, Dieli-Crimi R, Rosinach M, Nuñez C, Esteve M. Systematic Review and Meta-analysis Show 3% of Patients With Celiac Disease in Spain to be Negative for HLA-DQ2.5 and HLA-DQ8. Clin Gastroenterol Hepatol. 2017;15(4):594-596.

165. Selleski N, Almeida LM, Almeida FC, Pratesi CB, Nóbrega YKM, Gandolfi L. Prevalence of celiac disease predisposing genotypes, including HLA-DQ2.2 variant, in Brazilian children. Arq Gastroenterol. 2018;55(1):82-85.

166. Candore G, Lio D, Colonna Romano G, Caruso C. Pathogenesis of autoimmune diseases associated with 8.1 ancestral haplotype: effect of multiple gene interactions. Autoimmun Rev. 2002;1(1-2):29-35.

167. Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T, eds.HLA 1991.Vol I. Oxford: Oxford University Press, 1992:1065–1220.

168. Arnaiz-Villena A, Karin M, Bendikuze N, Gomez-Casado E, Moscoso J, Silvera C et al. HLA alleles and haplotypes in the Turkish population: relatedness to Kurds, Armenians and other Mediterraneans. Tissue Antigens. 2001;57(4):308-17.

169. Canossi A, Piancatelli D, Aureli A, Oumhani K, Ozzella G, Del Beato T. Correlation between genetic HLA class I and II polymorphisms and anthropological aspects in the Chaouya population from Morocco (Arabic speaking). Tissue Antigens. 2010;76(3):177-93.

170. Tye-Din JA., Cameron DJ, Daveson AJ, Day AS, Dellsperger P, Hogan C, et al. Appropriate clinical use of human leukocyte antigen typing for coeliac disease: an Australasian perspective. Internal medicine journal, 45(4), 441-50.

11. CURRICULUM VITAE

Atifete Ramosaj-Morina was born on October 25, 1980, in Decan, Kosovo, where she completed primary school and gymnasium.

She has graduated in Faculty of Medicine, University of Prishtina, Kosovo in 2005.

In 2014 she has finished successfully the specialization in field of Pediatric, at Pediatric Clinic, University Clinical Center of Kosova, were she has started working as Pediatrician.

Starting from 2007 she works as teaching assistant in Department of Anatomy, Faculty of Medicine, University of Prishtina.

In the academic year 2011/2012, she enrolled in the PhD Program on "Biomedicine and Health Sciences" at the University of Zagreb, in Croatia.

She is author of several scientific papers published in peer reviewed journals as well as presented in international and national conferences.

She has attended many professional training and seminars.

She is married mother of two children.