SEROLOGIC SCREENING AND GENETIC TESTING AMONG BRAZILIAN PATIENTS WITH CELIAC DISEASE AND THEIR FIRST DEGREE RELATIVES

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ABSTRACT – Context - Celiac disease susceptibility has been shown to be associated with the HLA alleles DQA1*0501 and DQB1*0201 (together encoding the DQ2 heterodimer) that are present in practically all celiac disease patients. The DQ8 heterodimer (coded by DQA1*03-DQB1*0302), which is carried on a DRB1*04 (DR4) haplotype, is commonly encoded for by the few celiacs who do not carry the DQ2 heterodimer. Only a few celiac disease patients have been reported without these known risk alleles. Objective - To assess the prevalence of celiac disease in a group of first degree relatives of celiac patients, and the frequency of HLA predisposing alleles both in the group of celiac patients and in their first degree relatives, identifying those first degree relatives who would need further screening for celiac disease. Methods - Ninety celiac disease patients and 207 first degree relatives underwent serologic screening for celiac disease (endomysial and transglutaminase antibodies) followed by intestinal biopsy in positive patients. The HLA-DQA1*0501, DQB1*0201 and DRB1*04 frequencies of celiac patients and their first degree relatives were determined utilizing the PCR method. Results - All the celiac disease patients (n = 90) with the exception of one (1.1%) carried at least one of the alleles investigated. Altogether 11 (5.3%) of the investigated first degree relatives did not carry any of the alleles studied. Fourteen (6.7%) new cases of celiac disease were found among the 207 celiac disease patients first degree relatives. Conclusions - Considering the cost-benefit of the HLA typing of all the first degree relatives of celiac patients, their HLA status should be decided on an individual basis, taking account of their profile and preferences, and the existence of other medical conditions.

INTRODUCTION

Celiac disease (CD) is an autoimmune disorder triggered by the ingestion of gluten and related cereals, resulting in jejunal villous atrophy and flattening of mucosa. The disease has a strongly genetic basis that has been confirmed by a concordance rate among monozygotic twins and a higher prevalence among family members of celiac patients⁴⁻⁸. CD was initially considered a rare malabsorption syndrome in childhood affecting predominantly Europeans or individuals of European ancestry. Today CD is recognized as a common disorder with an increasing prevalence in the general population, being described in several different non-European countries¹¹⁻¹³. It manifests at any age, with a large proportion of new cases being diagnosed in patients with extraintestinal manifestations. Prevalence in Brazil is apparently similar to those found in European countries. Screening studies performed during the last decade showed a prevalence varying from 1:213 to 1:681 in presumably healthy blood donors⁵⁻⁹, 31, 34 and from 1:53 to 1:417 in the general population in different Brazilian regions¹⁵⁻¹⁷, 35, 38, 44. Although CD is one of the most common autoimmune lifelong diseases, most affected individuals remain undiagnosed¹². This is probably due to the fact that many patients have atypical symptoms or none at all. CD aggregates in families and this is the first observable clue to its genetic susceptibility. Concordance in monozygotic twins is high at approximately 75%²¹, whereas prevalence among first-degree relatives (FDR) varies from 5% to 22.5%²⁰. CD has a strong genetic association with the human leucocyte antigen (HLA) that is estimated to account for up to 40% of genetic load⁴. Practically all patients with CD are positive for the DQA1*0501 and DQB1*02 alleles which, either in cis or in trans, form the DQ2 heterodimer⁹. Most CD patients who lack the entire DQ2 complex have at least one of the alleles coding for it,
either DQA1*0501 or DQB1*02. The DQ8 heterodimer (coded by DQA1*03-DQB1*0302), which is carried on a DRB1*04 (DR4) haplotype, is commonly encoded for by the few celiacs who do not carry the DQ2 heterodimer. Only a few CD patients have been reported without these known risk alleles.\(^{25, 37}\)

Considering that as many as 20%-30% of healthy subjects in the general population are also carriers of these alleles, the value of HLA typing in CD is mainly considered for its negative predictive value since the disorder very rarely develops unless a person carries alleles that encode for HLA-DQ2 or DQ8.\(^{22, 23}\) Despite the fact that its results are not absolutely certain, HLA typing for CD is generally considered as an important tool in the definition of dubious cases and in the screening strategy for asymptomatic individuals who belong to at-risk groups such as the case of FDR of celiac patients.

The HLA alleles are lifelong stable markers and their typing may detect genetically susceptible or non-susceptible subjects long before the possible appearance of the disorder.\(^{25, 30}\)

Studies on the prevalence of HLA alleles predisposing to CD in the general population and studies on the risk among FDR of celiac patients remain scarce in Brazil. In view of the largely mixed racial background of the Brazilian population, it is likely that such studies will show significant regional variations. Consequently, the aims of the present study were to assess: 1. the frequency of undiagnosed CD among FDR of celiac patients applying highly sensitive serologic tests (IgA-EMA and IgA-tTG), and 2. the frequency of HLA predisposing alleles DQA1*0501 and DQB1*0201 and DRB1*04 in a group of CD patients, and their FDR.

## METHODS

### Population and design

Between January 2004 and December 2008, FDR (parents, siblings and offspring) of 90 CD patients diagnosed according to the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPghan) criteria who attended the Brasília University Hospital Gastroenterology Clinics, Brasília, DF, Brazil, were invited to take part in the study. None of these index cases were related to each other. The group was composed of 55 (61.1%) females and 35 (38.9%) males (mean age at diagnosis, 15.5 years; age range, 1 to 55 years).

Of the initial group of 298 identified FDR, 91 could not be located or refused to participate. The remaining group of 207 family members included 122 parents (44 fathers and 78 mothers, aged 18-75 years; mean age 40 years; median 42 years), 63 siblings (29 brothers and 34 sisters, aged 1-48 years; mean age 16 years; median 14 years) and 22 offspring (11 males and 11 females, aged 1-32 years; mean age 10 years; median 11 years).

The Brasília University Hospital is a general hospital attending mainly low-income population originating from the outskirts and suburban regions of the city of Brasília (Federal District). Racially, this is a predominantly mixed-blood population in which a considerable contribution of European ancestry can be detected in association with a variable parcel of other races, mainly Africans and, to a lesser extent, Amerindians.

The study protocol was approved by the institution Ethics Committee of the Brasília University School of Health Sciences (protocol CEP-FM 051/2003). Eligible patients and healthy family members received written and oral information concerning the use of their blood in this study, and all provided signed consent. All participating family members were on a gluten-containing diet when the serologic tests were performed. On each participant relative was applied a standardized questionnaire focusing on the presence of gastrointestinal signs and symptoms, and the presence of other symptoms, not related to the gastrointestinal tract but frequently seen in association with CD (atypical form).

### Serologic markers

Blood samples were centrifuged to separate the serum and stored at -20°C for further use. All sera were previously tested by turbidimetric immunoquantification (COBAS MIRA, Roche Diagnostic Systems) to determine immunoglobulin A level, to exclude IgA deficiency. The serologic screening included: (1) Serum IgA endomysial antibody test (IgA-EMA) determined by indirect immunofluorescence method using sections from the distal portion of monkey’s esophagus (The Binding Site, Birmingham, UK) as substrate. The patient’s serum was diluted 1:5 in phosphate buffer at pH 7.4. The final reaction was detected with fluorescein isothiocyanate rabbit anti-human IgA conjugate (Biosystems, Barcelona, Spain).

Two independent observers examined all the slides and results were considered positive when bright green fluorescence of the reticulin like staining of smooth muscle was seen under fluorescence microscope; (2) IgA human recombinant antitransglutaminase antibody (IgA-tTG) evaluated with ELISA, according to manufacturer instructions (INOVA Diagnostic Inc., San Diego, California, USA).

### Genetic markers

Genomic DNA was extracted from peripheral venous blood using a commercial kit (Illustrati\(^{34}\) Blood genomic Prep Mini Spin, GE Gelathecure, Buckinghamshire, UK). Celiac patients and relatives were typed for the presence of HLA DQ2 (DQA1*0501,DQB1*0201) and DRB1*04 by PCR applying the method described by Sacchetti et al.\(^{41}\) PCR products were separated by pre-stained ethidium bromide agarose gel electrophoresis and subsequently visualized by UV illumination as separated bands of 177 bp (DRB1*04), 144 bp (DQA1*05) and 110 bp (DQB1*0201). Equivocal results were restested and confirmed using the kit Eu-DQ (Eurospital, Trieste, Italy).

### Small bowel histology

Intestinal biopsy was suggested to all IgA-EMA or IgA-tTG positive subjects. In addition, serologic follow-up was advised to all relatives who were carriers of the predisposing HLA alleles. Children underwent small intestinal biopsy with a Watson pediatric capsule, samples being taken at the ligament of Treitz. In adults, biopsy samples were obtained through an endoscopy procedure, using an Olympus gastroscope (Tokyo, Japan) with four to five fragments being taken from

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the second portion of the duodenum. All biopsy specimens were blindly evaluated by a pathologist and a gastroenterologist and classified according to Marsh as typical normal (0), infiltrative (I), infiltrative hyperplastic (II), flat destructive (III), and atrophic hypoplastic (IV).

**RESULTS**

Increased levels of IgA-tTG were present in 18 of the 207 CD relatives (8.7%), whereas 14 of 207 (6.7%) were both IgA-tTG and IgA-EMA positive. Three of the four subjects who were tTG positive and EMA negative, respectively aged 6, 13, 34 years, were carriers of the DQ2 heterodimer and underwent jejunal biopsy with normal results. The fourth subject, an asymptomatic mother aged 48 years, that had disclosed only a slightly increased level of IgA-tTG was negative for predisposing alleles and was consequently considered as non-celiac. Intestinal biopsy was suggested and subsequently carried out in all the 14 subjects positive in both serological tests, and reported compatible with the diagnosis of CD in all cases.

Eleven (78.5%) of these 14 patients were carriers of the HLA-DQ2 heterodimer, 2 carried concomitantly the DQ2 heterodimer and the DRB1*04 allele. Only 1 of the CD diagnosed relatives was negative for the DQ2 heterodimer, being a carrier of a single DQB1*0201 allele in association with the DRB1*04 allele. Consequently, the strict prevalence of biopsy proven celiac disease among the 1st degree relatives of CD patients was 6.7% (14/207). Among this group of 14 relatives diagnosed as celiac, 6 (4.9%) were parents (2 mothers, 4 fathers), 6 (9.5%) were siblings (5 sisters, 1 brother) and 2 (9%) were offsprings (1 girl, 1 boy) (Table 1).

Only one relative showed a silent form of the disease, despite the fact that she was positive in both serologic tests, was a carrier of a DQ2 allele, and her biopsy sample disclosed Marsh III jejunal lesions. The remaining 13 disclosed a variety of signs and symptoms, including diarrhea, anemia, abdominal distension, fatigue, recurrent abdominal pain, joint pain and depression.

Among the 90 index cases, the DQ2 heterodimer was present alone in 60 (66.6%), and in 18 (20%) it was detected in association with the DRB1*04 allele. Amid the remaining celiac patients, 2 (2.2%) carried only the DRB1*04 allele while in 5 (5.5%) this allele was detected in association with one of the two alleles (DQA1*0501 or DQB1*0201) that compose the DQ2 heterodimer. Furthermore, in 2 patients (2.2%), a single DQA1*0501 was present and in another 2 (2.2%), a single DQB1*0201 was found. In 1 (1.1%) celiac patient, none of the predisposing HLA alleles was detected.

Table 2 shows the results of the HLA typing of 90 celiac disease patients, their 14 celiac positive relatives and 193 non-celiac relatives on the basis of disease status and kinship degree.

### TABLE 1. Serologic, molecular, histologic and clinical findings in 14 first-degree relatives identified as celiac

<table>
<thead>
<tr>
<th>N</th>
<th>Age</th>
<th>Gender</th>
<th>Kinship</th>
<th>tTG</th>
<th>EMA</th>
<th>HLA</th>
<th>Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>female</td>
<td>daughter</td>
<td>184.8</td>
<td>Positive</td>
<td>DQB1 &amp; DRB1</td>
<td>Marsh III</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>female</td>
<td>mother</td>
<td>91.7</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>male</td>
<td>brother</td>
<td>246.5</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>female</td>
<td>sister</td>
<td>94.2</td>
<td>Positive</td>
<td>DQ2 &amp; DRB1</td>
<td>Marsh III</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>female</td>
<td>sister</td>
<td>82.8</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>female</td>
<td>sister</td>
<td>213.2</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>7</td>
<td>59</td>
<td>male</td>
<td>father</td>
<td>109.3</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh II</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>female</td>
<td>mother</td>
<td>37.4</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh I</td>
</tr>
<tr>
<td>9</td>
<td>35</td>
<td>male</td>
<td>father</td>
<td>31.3</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>10</td>
<td>54</td>
<td>male</td>
<td>father</td>
<td>71.85</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh I</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>male</td>
<td>son</td>
<td>92.54</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>12</td>
<td>75</td>
<td>male</td>
<td>father</td>
<td>43.4</td>
<td>Positive</td>
<td>DQ2 &amp; DRB1</td>
<td>Marsh II</td>
</tr>
<tr>
<td>13</td>
<td>45</td>
<td>female</td>
<td>sister</td>
<td>63.61</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
<tr>
<td>14</td>
<td>19</td>
<td>female</td>
<td>sister</td>
<td>85.6</td>
<td>Positive</td>
<td>DQ2</td>
<td>Marsh III</td>
</tr>
</tbody>
</table>

DQ2 = DQA1*0501, DQB1*0201; DRB1=DRB1*04; DQA1= DQA1*0501; DQB1= DQB1*0201

### TABLE 2. HLA status of the probands (n = 90) and their first-degree relatives (n = 207)

<table>
<thead>
<tr>
<th>Celiac relatives</th>
<th>Non-celiac relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLA status</strong></td>
<td><strong>HLA status</strong></td>
</tr>
<tr>
<td><strong>Probands</strong></td>
<td><strong>Parents</strong></td>
</tr>
<tr>
<td>DQ2</td>
<td>60</td>
</tr>
<tr>
<td>DRB1</td>
<td>2</td>
</tr>
<tr>
<td>DQ2 &amp; DRB1</td>
<td>18</td>
</tr>
<tr>
<td>DQA1 &amp; DRB1</td>
<td>1</td>
</tr>
<tr>
<td>DQB1 &amp; DRB1</td>
<td>4</td>
</tr>
<tr>
<td>Only DQA1</td>
<td>2</td>
</tr>
<tr>
<td>Only DQB1</td>
<td>2</td>
</tr>
<tr>
<td>Absence of DQ2/DRB1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>90</strong></td>
</tr>
</tbody>
</table>

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Among the 193 non-celiac FDR, the heterodimer DQ2 was found in 81 (42%), and in 25 (13%), was concomitantly seen associated with the allele DRB1*04. DRB1*04 was present alone in 9 (4.6%), associated with DQA1 in 7 (3.6%), and with DQB1 in 10 (5.2%). Isolated DQA1 and DQB1 were respectively observed in 21 (10.9%) and 29 (15%) subjects. The absence of any CD predisposing alleles was ascertained in 11 (5.7%) subjects.

**DISCUSSION**

Although previous studies have focused on the prevalence of celiac disease among first-degree relatives of celiac patients, surveys of prevalence of CD predisposing HLA alleles in the general population and particularly in FDR of patients with CD are extremely rare in Brazil. Silva et al., in a first report on the association of HLA and CD in Brazilian population, concluded that susceptibility alleles observed in Brazilian patients were similar to those observed in European studies. However, most of the patients studied by these authors were of Italian ancestry; it is likely that due to the remarkably mixed racial background of the Brazilian population, the CD predisposing allele frequency in Brazil will show significant regional variations.

In this study, we defined an individual as celiac when found to be serologically positive and when the individual’s biopsy result is consistent with CD. Although we recommend and obtained small bowel biopsy from all subjects disclosing predisposing alleles and positivity for one or both serologic tests, CD was not confirmed in four exclusively tTG positive patients. Our clinical experience with our serologic assays agrees with that of the literature whereby the IgAanti-tTG test is more sensitive and the IgA-EMA is more specific. False positive EMA results are rare and IgA-EMA testing is nearly 100% specific. False negative results can be obtained in older age groups (>35 years) and in children younger than 2 years.

In our series, the prevalence of CD among relatives was 14/207 (6.7%) and the CD predisposing alleles, DQ2 (DQA1*0501 and DQB1*02) and/or DRB1/DQ8, alone or in association were present in all newly diagnosed celiac relatives. In contrast among the non-celiac relatives, at least one of the risk alleles was found in 182 (94.3%) and the heterodimer DQ2 was found alone or in association with the DRB1*04 allele in 106 (54.9%).

It has been demonstrated that practically all CD patients carry HLA-DQ2 and/or HLA-DQ8 molecules or one chain of the DQ2 heterodimer, coded by DQA1 *05 (α5 chain) or DQB1 *02 (β2 chain) alleles, and that CD only exceptionally occurs in the absence of at-risk DQ factors. A variable risk gradient does exist, depending on which susceptible DQ alleles are present, ranging from 1:7 in subjects carrying both alleles DQ2 and DQ8 to 1:2518 in individuals who do not display any predisposing allele. A significant increased risk of developing CD is also determined by the presence of one (risk of 1:35) or two copies (risk of 1:10) of the allele DQB1*02. The homozigosis of this allele is associated with an earlier onset, increased anti-tTG antibody levels, presence of complications and increased severity of the disease. On the other hand, the presence of DQA1*05 allele alone is associated with extremely low odds of developing gluten intolerance (risk of 1:1842).

The prevalence of 6.7% found in the present study is in agreement with the findings of several previous studies in which the prevalence of CD among FDR varied between 4.5% and 9.5%. In a more recent study of 158 adult FDR, Biagi et al. found a prevalence of 17.7%, confirming that CD relatives are at high risk of developing gluten intolerance, reinforcing the need for extensive screening policy in these subjects. Genetic testing before serologic screening has been proposed to reduce the number diagnostic testing of relatives that, being negative for HLA-DQ2 or DQ8, have a minimum risk of developing CD. In this way, between 10% to 20% FDR could be excluded from serologic testing. However, in a recent study, Chang and Green concluded that the use of HLA typing before serologic screening for CD, at the current cost of serologic assays, would not reduce screening expenses. A substantial reduction in the cost of HLA typing would be required before it could become an affordable option. In situations in which a high-throughput approach is needed, HLA-tagging single-nucleotide polymorphisms (SNPs), which has shown high specificity and sensitivity in the identification of celiac-disease-associated HLA risk haplotypes, could be used with significant cost and time savings.

On the other hand, HLA typing of serologically negative relative would be justifiable to determine their future risk of developing CD. The fact that HLA positive subjects can be classified as celiac at any time during their lifetime would imply the need to periodically test those relatives. Nevertheless, the appropriateness and timing of further screening of asymptomatic HLA positive FDR of celiac patients is still an open question. Several follow-up studies advocate the periodic reevaluation of relatives who have been negative on initial screening but a close analysis of these studies show that the number of subjects was low, the study designs were different, and the incidence figures were not necessarily comparable. However, it can be seen that within a follow-up from 7 to 12 years, about 2% to 5% of seronegative relatives developed the disease. In a 7-year follow-up study of 158 adult FDR, concluded that the annual incidence of CD in his study group was 0.4%. They did not perform genetic studies due to high cost considerations and also cited that almost 50% of the serologically negative FDR refused a second test. These authors concluded that, in view of the low incidence rate, further studies are necessary before deciding whether adult FDR should be followed up over time and which is the best screening strategy in terms of the cost-benefit relationship. They stressed that their data are valid for adult FDR but not for children. Similar studies are necessary among children in whom the greater life expectancy makes the need for follow-up more important.

In conclusion, we found 14 (6.7%) new cases of DC among 207 FDR of 90 biopsy-proven celiac patients. The prevalence...
of DC and the frequency of predisposing HLA alleles in this FDR group are similar to those previously described in other studies. HLA typing excluded 11 (5.7%) FDR from further follow-up and identified other 21 (10.9%) with a low grade risk of developing CD in the future (carriers of a single DQA1 allele).

In line with Chang et al.\(^{(13)}\) we did not find HLA typing to be cost-effective, considering that it is a time consuming process and that it would be beneficial solely to a relatively small percentage of FDR. In our opinion, considering the high negative predictive value of the absence of the HLA DQ2 and DQ8 predisposing alleles, its determination would be only justified in those cases in which the definitive diagnosis of CD is questionable, allowing the exclusion of negative HLA patients from further serologic testing.

**RESUMO** – Contexto - A susceptibilidade para a doença celiaca está principalmente associada à presença dos alelos HLA DQA1*0501 e DQB1*0201 (que conjuntamente codificam o heterodímero DQ2), os quais são praticamente encontrados em todos os pacientes celiacos. O heterodímero DQ8 (codificado pelos alelos DQA1*03 e DQB1*0302) associado ao haplotipo DRB1*04 (DR4) é comumente encontrado nos poucos pacientes celiacos que não carregam o heterodímero DQ2. **Objetivos** - Determinar a prevalência de doença celiaca em grupo de parentes de primeiro grau de pacientes celiacos e a frequência da presença de alelos HLA predisponentes tanto nos pacientes celiacos como em seus parentes de primeiro grau, identificando neste último grupo, os que iriam necessitar de futuros testes diagnósticos. **Métodos** - Noventa pacientes celiacos e 207 dos seus parentes de primeiro grau foram submetidos a testes sorológicos para a doença celiaca (pesquisa de anticorpos antiendomísio e antitransglutaminase) sendo a seguir efetuada biopsia intestinal nos pacientes sorologicamente positivos. Tanto nos pacientes celiacos como em seus parentes foi determinada a frequência de alelos HLA-DQA1*0501, DQB1*0201 e DRB1*04 pela técnica de PCR. **Resultados** - Todos os pacientes celiacos (n = 90), com exceção de um (1.1%) eram portadores de pelo menos um dos alelos investigados. Onze (5.3%) dentre os 207 parentes de celiacos não carregavam nenhum dos alelos predisponentes para DC. Quatorze (6.7%) novos casos de doença celiaca foram identificados entre os 207 parentes investigados. **Conclusões** - Considerando o fator custo-benefício da tipagem rotineira dos alelos HLA predisponentes em todos os parentes de primeiro grau de pacientes celiacos, é opinião dos autores que a identificação do perfil HLA deverá ser decidida em bases individuais, levando-se em conta as características de cada indivíduo, suas preferências e a comitante existência de outras patologias médicas.

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