

## ONLINE MUTATION REPORT

Promoter polymorphisms of the *CD14* gene in Italian patients with coeliac disease

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Coeliac disease (CD) is an autoimmune enteropathy triggered by ingestion of wheat gluten or related protein from rye and barley, and is one of the most frequently occurring, treatable, lifelong disorders.<sup>1,2</sup> Undetected or untreated CD may cause other, more severe, complications later, such as autoimmune diseases, osteoporosis, neurological disorders, and infertility.<sup>3,4</sup>

Several studies have shown CD clusters in families with a sibling relative risk of 20–60 and high concordance between monozygotic twins (75%),<sup>5–8</sup> which indicates a strong genetic component to coeliac disease. As expected in complex autoimmune diseases, human leukocyte antigen (HLA) linked genes are the main genetic factor involved in CD. More than 90% of patients suffering from CD share the major histocompatibility complex II class HLA-DQ2 haplotype. Most of the remainder present HLA-DQ8. However, there are patients who present neither HLA-DQ8 nor HLA-DQ2 on their antigen-presenting cells. These genetic findings strongly indicate the involvement of other genes in the pathogenesis of coeliac disease. So far, various genome-wide linkage analyses have been performed in order to identify non-HLA linked genes responsible for CD. A recent study by Greco *et al.*<sup>9</sup> on Italian coeliac families demonstrated the existence of a genetic risk factor on chromosome 5 (5q31–33), which has been reported in other linkage studies in different populations.<sup>10–11</sup> This genomic region contains several candidate genes for CD, including the interleukin (*IL12B*) gene, the polymorphisms of which do not seem to be associated with CD,<sup>12</sup> and the gene encoding for *CD14*.<sup>13</sup>

*CD14* is a multifunctional receptor involved in the innate immune response. In fact, it is thought to be one of the most important receptors for lipopolysaccharide and other bacterial wall-derived components.<sup>14–16</sup> However it is well known that *CD14* has several other functions,<sup>17,18</sup> including the clearance of apoptotic cells.<sup>19</sup> It is constitutively expressed, primarily on the surface of monocytes, macrophages, and neutrophils (m*CD14*); a soluble form, s*CD14*, has been isolated from serum and is derived both from secretion of *CD14* and from enzymatically cleaved glycosyl-phosphatidylinositol anchored m*CD14*.<sup>20,21</sup>

The serum levels of *CD14*, and thus the levels of m*CD14*, are genetically regulated. Four single nucleotide polymorphisms have been identified in the promoter region of the gene, and are thought to regulate the *CD14* gene expression.<sup>22</sup> Recently, different studies have correlated a C→T transition at position –159 in the promoter region of the *CD14* gene, with an increased risk of developing Crohn's disease and ulcerative colitis;<sup>23,24</sup> Furthermore, a study by Holla *et al.*<sup>25</sup> demonstrated an association between a G→T transversion at position –1359 and the severity of periodontitis.

In our study the frequency of the two promoter polymorphisms was assessed in three selected populations in order to explore the influence of *CD14* in coeliac disease, because the *CD14* gene is located in a "hotbed" region for CD and is involved in the clearance of apoptotic cells, which

## Key points

- Coeliac disease is a gluten dependent, immune mediated enteropathy caused, in genetically susceptible individuals, by a T-cell response to a new epitope generated by the transglutaminase-driven deamination of dietary gliadin. This disease is one of the most common multifactorial autoimmune disorders in the white population (1 in 200 individuals).
- *CD14* is a multifunctional receptor involved in innate immune response. In fact, it is thought to be one of the most important receptor for lipopolysaccharide and other bacterial wall-derived components. However, it is well known that *CD14* has several other functions, including the clearance of apoptotic cells.
- As the *CD14* gene is located in a "hotbed" region for CD and is involved in the clearance of apoptotic cells, the aim of this study was to assess the frequency of two promoter polymorphisms in three selected populations in order to explore the influence of *CD14* in this common autoimmune disorder.
- In our study the allelic and genotype frequencies of C(–159)T did not differ among 115 coeliac patients carrying the typical HLA haplotype (DQ2 or DQ8), 37 coeliac patients with an atypical HLA haplotype, and 180 selected healthy controls of the same ethnic origin. There was a significant skewing in coeliac patients with for the genotype frequencies at position –1359 compared with healthy controls. This difference is mainly due to the higher frequency of (–1359)TT subjects in coeliac patients carrying the atypical HLA haplotype.
- Several hypotheses could explain the involvement of *CD14* in coeliac disease; our findings show the importance of apoptosis regulation. Furthermore, they indicate that the genes encoding proteins, which play a key role in this process, are potential candidates for CD.

could be important in CD, as already demonstrated by the findings of Boniotta *et al.*<sup>26</sup>

## METHODS

## Patients and controls

For this study 115 Italian coeliac patients with typical HLA haplotypes (either DQ2 or DQ8), 37 Italian coeliac patients with atypical HLA haplotypes, and 180 pan-ethnic controls (healthy blood donors with no history of either gastrointestinal or autoimmune diseases) were enrolled. Diagnosis of coeliac disease was performed following the ESPGHAN

indications.<sup>27</sup> The study was approved by the ethics committee at the Burlo Garofolo Children's Hospital, Trieste. The importance of a positive result was explained to all participants, and verbal consent was obtained from the patients.

### CD14 genotyping

DNA was extracted from peripheral whole blood using standard laboratory protocols.<sup>28</sup>

The detection of the bi-allelic polymorphism at position -159 was performed following the PCR-RFLP technique already described by Baldini *et al.*<sup>29</sup>

The detection of the bi-allelic polymorphism at position -1359 was performed with a modified version of the PCR-RFLP technique described by Holla *et al.*<sup>25</sup> Briefly, PCR was performed with the forward primer 5'-AGCACITTTGAGAGGCCAAGG-3' and the reverse primer 5'-CCACATCCCTAGACCTCTGGG-3', both designed on the basis of the published CD14 human sequence with the software Primer Express 1.5 (Applied Biosystems, Foster City, CA, USA). After an initial denaturation step at 95°C for 10 min, 35 cycles with a denaturation step at 95°C for 30 s, an annealing step at 62°C for 30 s, and an elongation step at 72°C for 30 s were carried out. The PCR reaction mix was 1.5 mmol/l MgCl<sub>2</sub>, 800 μmol/l dNTPs, 200 μmol/l for each primer and contained 1 U of AmpliTaq Gold (Applied Biosystems). PCR products were digested for 2 h at 37°C with the endonuclease FokI (New England Biolabs, Beverly, MA, USA) and subsequently separated on a 2% agarose gel. The G allele showed one fragment of 303 bp, while the T allele showed two fragments of 215 and 88 bp.

### Statistical analysis

Gene frequencies were calculated from the observed number of genotypes. The significance of differences in allelic frequencies was calculated by Fisher's exact test. To test the significance of differences in genotype frequencies a  $\chi^2$  test was used. Holm's correction for multiple tests was performed and only corrected p values ( $p_{\text{corr}}$ ) < 0.05 were considered to be significant. All the statistical analyses were carried out with SPSS software (Version 6.1.3; SPSS Inc., Chicago, IL, USA).

## RESULTS

Allelic frequencies and genotype frequencies for the position -159 in the three populations studied are shown in Table 1. The frequency of the (-159)C allele was 53%, 46%, and 50% in typical HLA coeliac patients, atypical HLA coeliac patients, and healthy controls, respectively. No differences were observed in genotype frequencies between the three populations.

Table 2 shows the allelic and genotype frequencies for the G to T transversion at position -1359. The frequency of the (-1359)G allele did not differ in the three populations; it was 80% in typical HLA coeliac patients, 77% in atypical HLA coeliac patients, and 84% in healthy controls (Fisher's exact test,  $p = 0.631$  for typical compared with atypical HLA coeliac patients;  $p = 0.251$  for typical HLA coeliac patients compared with healthy controls, and  $p = 0.172$  for atypical HLA coeliac patients compared with healthy controls). No differences were observed in genotype frequencies when healthy controls and typical HLA coeliac patients were compared ( $\chi^2 = 4.19$ ,  $p = 0.123$ ). However an increased frequency of (-1359)TT homozygotes were observed in the latter population (odds ratio TT v GT and GG genotypes: 3.82; 95% confidence interval (CI) 0.97 to 15.10). In fact the frequencies of (-1359)GG, (-1359)GT, and (-1359)TT genotypes were 70%, 28%, and 2% in controls, and 67%, 27%, and 6% in typical HLA coeliac patients, respectively. The genotype

**Table 1** Allele and genotype frequencies for the G to T transition at position -159 in the promoter region of the CD14 gene

Base	Coeliac patients		
	HLA- DQ2 or DQ-8 n = 115	Neither HLA- DQ2 nor DQ-8 (n = 37)	Healthy controls (n = 180)
*C	0.53 123/230	0.46 34/74	0.50 181/360
T	0.47 107/230	0.54 40/74	0.50 179/360
§C/C	0.33 38/115	0.24 9/37	0.27 48/180
C/T	0.41 47/115	0.43 16/37	0.47 85/180
T/T	0.26 30/115	0.33 12/37	0.26 47/180

\*Allelic frequency (Fisher's exact test): typical v atypical HLA coeliac patients:  $p = 0.320$ ; typical HLA coeliac patients v healthy controls:  $p = 0.500$ ; atypical HLA coeliac patients v healthy controls:  $p = 0.581$ .  
§Genotype frequency (3x2 contingency tables): typical v atypical HLA coeliac patients:  $\chi^2 = 1.13$ ,  $p = 0.567$ ; typical HLA coeliac patients v healthy controls:  $\chi^2 = 1.61$ ,  $p = 0.446$ ; atypical HLA coeliac patients v healthy controls:  $\chi^2 = 0.62$ ,  $p = 0.733$ .

**Table 2** Allele and genotype frequencies for the G to T transversion at position -1359 in the promoter region of the CD14 gene

Base	Coeliac patients		
	HLA- DQ2 or DQ-8 n = 115	Neither HLA- DQ2 nor DQ-8 (n = 37)	Healthy controls (n = 180)
*G	0.80 185/230	0.77 57/74	0.84 304/360
T	0.20 45/230	0.23 17/74	0.16 56/360
§G/G	0.67 77/115	0.67 25/37	0.70 127/180
G/T	0.27 31/115	0.19 7/37	0.28 50/180
T/T	0.06 7/115	0.14 5/37	0.02 3/180

\*Allelic frequency (Fisher's exact test): typical v atypical HLA coeliac patients:  $p = 0.631$ ; typical HLA coeliac patients v healthy controls:  $p = 0.251$ ; atypical HLA coeliac patients v healthy controls:  $p = 0.172$ .  
§Genotype frequency (3x2 contingency tables): typical HLA coeliac patients v atypical HLA coeliac patients:  $\chi^2 = 2.680$ ,  $p = 0.262$ ; typical HLA coeliac patients v healthy controls:  $\chi^2 = 4.19$ ,  $p = 0.123$ ; atypical HLA coeliac patients v healthy controls:  $\chi^2 = 12.640$ ,  $p = 0.0018$ .

frequencies in the atypical HLA coeliac patients were 67%, 19%, and 14% for the genotype (-1359)GG, (-1359)GT, and (-1359)TT, respectively. This distribution did not differ when compared with that of typical HLA coeliac patients ( $\chi^2 = 2.680$ ,  $p = 0.262$ ), but it was significantly different from that of the healthy controls ( $\chi^2 = 12.640$ ,  $p = 0.0018$ ,  $p_{\text{corr}} < 0.05$ ), mainly due to the difference in the frequency of (-1359)TT homozygous individuals. Odds ratio for TT genotype in atypical HLA coeliac patients was 9.22 (95% CI 2.10 to 40.50).

## DISCUSSION

Coeliac disease is one of the most common multifactorial autoimmune disorders in the white population (1 in 200 individuals). The principal genetic (HLA-linked genes) and environmental (wheat gluten) factors responsible for CD are well known, but few data are available for non-HLA-linked genes.

In this case-control study we have assessed the frequency of two polymorphisms in the promoter region of the *CD14* gene, which regulate the expression of the protein in three selected populations.

LeVan *et al.*<sup>30</sup> demonstrated that (-159)T allele promotes *CD14* gene transcription and that homozygous subjects for this allele have increased CD14 serum levels.<sup>29</sup> In our study the allelic and genotype frequencies of C(-159)T did not differ between the 115 coeliac patients carrying the typical HLA haplotype (DQ2 or DQ8), the 37 coeliac patients with an atypical HLA haplotype, and the 180 selected healthy controls of the same ethnic origin. Interestingly, there was a significant skewing in coeliac patients with atypical HLA for the genotype frequencies at position -1359 compared with healthy controls. This difference is mainly due to the higher frequency of (-1359)TT subjects in coeliac patients carrying the atypical HLA haplotype. The frequency of (-1359)TT patients was also greater in coeliac patients with typical HLA, even though our results do not reach statistical significance. As demonstrated by Vercelli *et al.*<sup>31</sup> (-1359)TT subjects have lower serum levels of CD14. The odds ratio of developing coeliac disease for (-1359)TT atypical HLA coeliac patients is 9.22 (95% CI 2.10 to 40.50).

Several hypotheses could explain the involvement of CD14 in coeliac disease. Firstly, individuals with low levels of sCD14, and thus mCD14, might be more prone to Gram-negative bacterial infections. These infections may disrupt the permeability of intestinal epithelia, leading to an increased concentration of gluten in the lamina propria, where most of the gluten-reactive lymphocytes are localised. Such individuals may be more susceptible to developing coeliac disease. Unfortunately, no studies linking the G(-1359)T polymorphism to a risk of infection have been carried out. Moreover, a recent study by Agnese *et al.*<sup>32</sup> showed a significant correlation between TLR4 polymorphisms and an augmented risk of gram negative bacterial infection: no association was found between CD14 C(-159)T polymorphism and the incidence of infection or patients' outcome.

Another possibility, and in our opinion the most interesting, may derive from the role of CD14 in apoptosis regulation. The defective clearance and removal of apoptotic cells has been closely linked to autoimmune and persistent inflammatory diseases.<sup>33</sup> Recently Boniotta *et al.*<sup>26</sup> found a significant association between allelic variants within the first exon of the mannose-binding lectin 2 (*MBL2*) gene and an increased risk of CD, and discussed the importance of mannose-binding lectin in the clearance and removal of apoptotic cells. Several studies demonstrate that CD14 is able to bind several apoptotic-cell-associated molecular patterns (ACAMPs),<sup>19, 34, 35</sup> such as ICAM-3 on apoptotic leukocytes,<sup>36</sup> and may either act as a phagocyte pattern-recognition receptor or bind directly to ACAMPs to initiate engulfment of apoptotic cells. Moreover, it is known that MBL binds to apoptotic cells and initiates their uptake by interacting with phagocyte calreticulin receptors on the macrophage cell surfaces.<sup>37</sup> In the same way, CD14 is expressed mainly on the surface of these "professional" phagocytes that, unlike dendritic cells, are also able to present apoptotic-cell-derived antigens to T cells in a tolerogenic manner, providing functional inactivation or deletion of potentially autoreactive T cells.<sup>38, 39</sup> Furthermore, sCD14 is able to bind and switch off activated T cells.<sup>40</sup> Taken together, these data explain why subjects with lower levels of CD14 (both mCD14 and sCD14) are more prone to develop autoimmune diseases, such as CD.

At the moment, linkage disequilibrium of the G(-1359)T allele with the real causative gene for CD in the genomic region 5q31-33 cannot be excluded.

In conclusion, a significant association between (-1359)TT genotype and an increased risk for CD was shown in coeliac

patients with atypical HLA haplotype. There was a positive trend for a higher frequency of (-1359)TT individuals in typical HLA coeliac patients, although it did not reach statistical significance. To our knowledge, this is the first study that has found a significant association between CD and a polymorphism in a gene located in the candidate genomic region 5q31-33.

Finally, our findings demonstrate the importance of apoptosis regulation. They indicate that the genes encoding proteins, which play a key role in this process, are potential candidates for CD. A larger cohort of typical HLA coeliac patients should probably be studied, and functional studies carried out, to clarify the role of CD14 in coeliac disease.

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