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CARD15 Mutations in Patients With Crohn's Disease in a Homogeneous Spanish Population

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OBJECTIVES: Three mutations in *CARD15* have been repeatedly shown to be involved in Crohn's disease susceptibility, mainly in Caucasian individuals. However, those findings were not replicated in all populations studied so far. In this work, we studied the role of *CARD15* mutations in a relatively homogeneous population from the Northwest of Spain, Galicia.

METHODS: One hundred and sixty-five patients with Crohn's disease and a similar number of healthy controls were recruited from a single center in Galicia. All individuals were genotyped for the three main Crohn's disease associated *CARD15* variants (R702W, G908R, and 1007fs). Association analyses were performed to study the influence of those mutations on Crohn's disease overall and on clinical subphenotypes.

RESULTS: The allele frequencies of *CARD15* variants were lower in this population than in most of the European populations studied so far. G908R and 1007fs were significantly associated with overall susceptibility to Crohn's disease. However, these associations were lost after stratification to clinical subgroups, probably due to the small number of cases in these subgroups. Significant associations were found between G908R and 1007fs and the behavior of Crohn's disease, but they were due to the influence of years of disease on the behavior of the disease rather than being the result of a direct effect of these mutations on disease behavior.

CONCLUSIONS: The *CARD15* variants G908R and 1007fs, but not R702W, are associated with susceptibility to Crohn's disease in Galicia. Interestingly, the frequency of these mutations appears to be lower than in other Caucasian populations studied so far.

INTRODUCTION

Although the etiology of Crohn's disease (CD) is still unknown, there is significant evidence that genetic factors are involved in the susceptibility to this disease. In particular, mutations in *CARD15* gene (formerly called *NOD2*) have been repeatedly shown to be involved in CD susceptibility (1–9).

CARD15 is located on chromosome 16q12 and originally thought to be expressed mainly in monocytes (10). More recently there is evidence that it is also present in epithelial cells (11, 12). Although the exact function of this gene remains unclear, there is substantial evidence that it is involved in the activation of nuclear factor κB (NFκB) as a response to bacterial peptydoglycan (PGN) (13). Two missense variants (R702W and G908R) and a frameshift variant causing a premature stop codon (1007fs) were found in this gene in patients with CD. Supported by the functional implications of these mutations (13, 14), many studies were initiated with the aim of corroborating the initial association found between these mutations and CD. Positive associations were found in many studies; however, they were not confirmed in all the analyzed populations. Important ethnical differences were found; for example, these mutations are not found in Asian populations (15–18). Caucasian populations have been found to be more homogeneous with respect to the influence of *CARD15* mutations but some discrepancies were also observed (19–21).

Due to the existing differences among populations, it is important to know the influence of *CARD15* variants in more homogeneous populations. In this regard, the Galician population (from the Northwest of Spain) may be relevant since this represents a relatively homogeneous European population. In addition, a recent epidemiological review in Spain has shown that CD incidence in Spain ranges from 0.4 to 5.5 cases/10^5/yr, with an average rate of 1.9 (22) and the global adjusted incidence rate of CD in Spanish areas is between that of Northern and Southern Europe (23). The aim of this study was therefore to investigate the involvement of the three main mutations of *CARD15* predisposing to CD in Galician population (NW Spain), as well as their possible influence

on the different subphenotypes characteristic of this chronic disease.

MATERIALS AND METHODS

Study Subjects

A total of 165 unrelated patients with CD were recruited from the Department of Gastroenterology at the Hospital Clínico Universitario of Santiago de Compostela (Galicia, Spain). As controls, 165 healthy unrelated ethnically matched individuals with no family history of IBD were used. Blood was obtained by venipuncture in EDTA tubes from all the participants with the correspondent written informed consent. All the individuals included in this study were Galician and the residents of Galicia. Patients and controls do not statistically differ with respect to sex, age, or rural or urban origin.

Diagnosis of CD was made by clinical, radiological, endoscopic, and histological investigations. In addition, a complete form was completed for all the patients including demographic (age, sex, rural or urban origin) and clinical characteristics (subgroups of Vienna Classification (24), surgical procedures, steroid dependency, steroid resistance, infliximab treatment, and extraintestinal manifestations (EIMs)), family history, and smoking habits. Recorded surgical procedures were those directly related with CD, mainly intestinal resections and surgical treatment of fistulae. Steroid dependency was defined as a relapse within 30 days after the end of steroid treatment or after at least two attempts of tapering the steroid dose within the last 12 months. Steroid-resistant patients were defined as those who did not respond to steroid therapy (minimum 50 mg of prednisolone) for more than 7 days. EIMs were defined as chronic inflammatory conditions that involve other organ systems and included articular, skin, eye, and liver diseases. Patients who presented both stricturing and fistulizing disease according to the Vienna Classification were included in the B3 group (fistulizing disease).

Surgeries, steroid dependency, and steroid resistance were included on the basis of their possible relevance as indicators of severity of disease. To avoid different criteria for CD diagnosis, all the patients were reviewed by the same gastroenterologist.

This study was approved by the Ethical Committee of Clinic Investigation of Galicia (2002/151).

Genotyping

Genomic DNA was isolated from blood samples using a Roche DNA isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). All the study participants were genotyped for the three main variants of *CARD15* associated with CD (R702W, G908R, and 1007fs). These variants have been labeled as SNP8, 12, and 13, respectively, by Hugot *et al.* (1). The genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Once digested, the resulting products were isolated on 2% (G908R) or 4% (R702W and 1007fs) agarose gels and visualized with ethidium bromide.

The R702W variant was assayed employing the primers: 5′CGCACAACCTTCAGATCACA3′ (sense) and 5′GGATGGAGTGGAAGTGCTTG3′ (antisense). PCR conditions were: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and elongation at 72°C for 30 s, and a final elongation step at 72°C for 7 min. PCR fragments were cut with the restriction enzyme HpaII (5 h at 37°C).

The G908R variant was assayed employing the primers: 5′AAGTCTGTAATGTAAAGCCAC3′ (sense) and 5′CCCAGCTCCTCCCTCTTC3′ (antisense).

PCR conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 30 s, and a final elongation step at 72°C for 7 min. PCR fragments were cut with HhaI (5 h at 37°C).

The 1007fs variant was assayed according to Crane *et al.* (25). In short, the primers employed were 5′GGCAGAAGCCCTCCTGCAGGGCC3′ (sense) and 5′CCTCAAAATTCTGCCATTCC3′ (antisense). ApaI were used to cut PCR fragments (11 h at 30°C).

Statistical Analysis

Deviations from Hardy-Weinberg proportions (HWP) were estimated for each polymorphism with the f-statistic and significance tested using the square of the ratio of the estimate to its standard error for $f = 0$, which follows a χ^2 distribution with a 1 df (26, 27).

Haplotype frequencies in controls were calculated directly from the sample because of the absence of compound heterozygotes. In the sample of patients, expectation-maximization (EM) algorithm (28) was employed to obtain an estimate of haplotype frequencies when compound heterozygotes were present. Gametic disequilibrium was estimated employing the D' coefficient (29) as a measure of the strength of association and χ^2 tests to calculate significance.

Case-control analyses were performed to know the influence of each *CARD15* polymorphism individually as well as the influence of their combined action on CD overall. Moreover, CD cases were stratified according to several demographic and clinical characteristics, including the main groups of the Vienna Classification (age of onset [below 40 yr (A1), equal to or above 40 yr (A2)], behavior [nonstricturing, nonpenetrating (B1), stricturing (B2), penetrating (B3)], and location of disease [terminal ileum (L1), colon (L2), ileocolon (L3), upper gastrointestinal tract (L4)]), to investigate the possible effect of *CARD15* mutations on each subgroup separately. All the comparisons were studied using χ^2 statistics and employing Fisher's exact test whenever appropriate. Associations were expressed as relative risk (RR) or odds ratio (OR) with 95% confidence interval (CI). Logistic regression analyses were also performed. Statistical analyses were performed employing SPSS 11.0 and GraphPad Instat.

RESULTS

Characteristics of CD Patients

A total of 165 CD patients and a similar number of healthy control subjects were analyzed to perform a case-control study. The main demographic and clinical characteristics of patients are shown in Table 1. The average follow-up was 7.5 ± 0.5 yr (ranging from 1 to 33 yr). Family history in a first- or second-degree relative was found in 10% of the studied patients, and falls within the range found in other studied populations (30). There was a slight predominance of females among CD patients, as is generally found. There was also an increased percentage of patients from rural origin; however, this fact should not be considered as indicative of a higher disease prevalence in people from rural origin because the geographic area studied is predominantly rural. No significant differences were found between patients and controls according to sex or origin (rural or urban).

Significant associations were found between different subgroups of CD patients. Thus, people from urban origin showed an earlier development of the disease compared to people from rural origin (origin *vs* age of onset according to Vienna Classification, $p < 0.05$). Steroid dependency was found more frequently in patients who smoke and in those with a positive family history ($p < 0.05$ and $p < 0.01$, respectively).

Table 1. Demographic and Clinical Characteristics of CD Patients

Total number	165
Sex (M/F)	70 (42.4)/95 (57.6)
Origin (R/U)	91 (55.2)/74 (44.8)
Current smokers	75 (45.5)
Smokers (current and ex-smokers)	99 (60.0)
Family history	17 (10.3)
Follow-up in years	
Range	1–33
Mean ± SE	7.5 ± 0.5
Age	
Range	17–76
Mean ± SE	36.3 ± 1.0
Age of onset	
< 40 yr (A1)	135 (81.8)
≥40 yr (A2)	30 (18.2)
Disease behavior	
Nonstricturing, nonpenetrating (B1)	66 (40.0)
Stricturing (B2)	35 (21.2)
Penetrating (B3)	64 (38.8)
Location of disease	
Terminal ileon (L1)	69 (41.8)
Colon (L2)	28 (17.0)
Ileocolon (L3)	66 (40.0)
Upper gastrointestinal tract (L4)	2 (1.2)
Cortico-resistance	27 (16.4)
Cortico-dependence	35 (21.2)
Surgery	85 (51.5)
Infliximab treatment	49 (29.7)
Positive response	40 (81.6)
Extraintestinal manifestations	58 (35.2)

Percentages are shown in parentheses. M, male; F, female; R, rural; U, urban.

CARD15 and CD Susceptibility

Allele frequencies of mutant alleles of each *CARD15* variant are shown in Table 2. R702W showed the highest frequency in Galician patients (6.7%) but its frequency was also high in controls (5.8%). Thus, this polymorphism does not seem to influence on CD susceptibility in the studied population. In contrast, mutant alleles of G908R and 1007fs showed a significantly higher frequency in CD patients than in controls (4.5% *vs* 1.0 in both groups, $p < 0.01$). Both increase the risk of developing CD (OR = 5.2 95% CI 1.5–18.1). A similar result was observed when carriers (*i.e.*, individuals carrying at least one copy of the mutant allele) of each polymorphism were considered, although in this case the risk of developing CD was slightly higher for carriers of 1007fs (OR = 4.6 95% CI 1.3–16.5, $p < 0.05$; and OR = 5.0 95% CI 1.4–17.8, $p < 0.01$, for G908R and 1007fs, respectively) (Table 2).

The same analyses were then performed considering the carriers of at least one copy of mutant alleles in any variant and the carriers of two copies. A total of 27.9% of CD patients carried at least one mutant allele in any of the three considered variants, contrasting with the 15.2% of controls ($p < 0.01$). A total of 3.6% of the CD subjects carried two copies of mutant alleles (either homozygous for one variant or compound heterozygous), whereas none of the CD subjects was found homozygous for more than one mutation. In the control group, not a single subject showed two copies of rare variants, in accordance with previous studies. Carriage of at least one copy of mutant alleles of *CARD15* slightly increases the risk of developing CD (OR 2.2 95% CI 1.3–3.7, $p < 0.01$) and this risk is twofold when individuals carrying only mutations in G908R and 1007fs (the two ones involved in CD in the studied population) were considered (OR 4.7 95% CI 1.9–11.9, $p = 0.001$). The risk conferred for carriage of two mutant alleles could not be calculated because there is no subject with two copies in the sample of controls. This finding is common in all the analyzed populations, which is indicative of the high risk that possession of more than one mutant confers.

Genotype frequencies in controls and patients were in Hardy-Weinberg proportions except for G908R in the group of patients. Because haplotype frequency estimation using EM algorithm could be influenced by deviations of Hardy-Weinberg proportions, haplotype frequencies when compound heterozygotes were present were obtained employing the EM algorithm and directly from the sample after eliminating compound heterozygotes. Very similar results were obtained in both cases; the latter are summarized in Table 3. Mutant alleles of these variants were not found on the same haplotype. Differences between patients and controls were again due to G908R and 1007fs. Thus, only haplotypes containing mutant alleles in G908R and 1007fs variants were found significantly increased in CD patients in this population.

No gametic disequilibrium was found between any pair of the variants studied, as was also found in previous studies (6).

Table 2. Allelic and Carrier Frequencies of *CARD15* Variants in Controls and in Patients as a Whole and Substratified in the Main Subgroups of Vienna Classification

		Allelic Frequency (%)			Carrier Frequency (%)			Carrier Any mutant	Carrier 2 copies[1]
	N	R702W	G908R	1007fs	R702W	G908R	1007fs		
Patients	165	6.7	4.5*[a]	4.5*[a]	13.3	7.9*[b]	8.5*[c]	27.9*[d]	3.6*[e]
Controls	165	5.8	1.0	1.0	11.5	1.8	1.8	15.2	0.0
Age of onset									
A1	135	5.9	5.2	4.4	11.9	8.9	8.1	26.7	4.4
A2	30	10.0	1.7	5.0	20.0	3.3	10.0	33.3	0.0
Behavior disease									
B1	66	8.3	2.3	1.5*[f]	16.7	3.0	3.0*[g]	21.2	3.0
B2	35	7.1	2.9	5.7	14.3	5.7	11.4	28.6	2.9
B3	64	4.7	7.8*[h]	7.0	9.4	14.1*[i]	12.5	34.4	4.7
Location disease									
L1	69	5.1	4.3	5.1	10.1	8.7	10.1	27.5	1.4
L2	28	12.5	1.8	1.8	25.0	3.6	3.6	32.1	0.0
L3	66	5.3	6.1	4.5	10.6	9.1	7.6	25.8	6.1

Excepting when it is indicated, the results are considering χ^2 and Fisher's exact test.
[1] Homozygotes or compound heterozygotes.
[a] $p < 0.01$ OR = 5.19 95% CI 1.49–18.11.
[b] $p < 0.05$ OR = 4.62 95% CI 1.29–16.52.
[c] $p < 0.05$ OR = 5.01 95% CI 1.41–17.77.
[d] $p < 0.01$ OR = 2.17 95% CI 1.26–3.73.
[e] $p < 0.05$.
[f] $p < 0.05$ RR = 0.23 95% CI 0.05–1.01.
[g] $p < 0.05$ RR = 0.25 95% CI 0.06–1.08.
[h] $p < 0.05$ RR = 3.16 95% CI 1.10–9.03.
[i] $p < 0.05$ RR = 3.55 95% CI 1.14–11.06.
*Significant statistical value.
Results concerning L4 are not shown due to the small sample size (n = 2).

CARD15 and CD Subphenotypes

The stratification of CD patients according to the groups established in the Vienna Classification (age at diagnosis, behavior, and location of disease) did not show significant results (Table 2).

Thus, the age of onset does not seem to be influenced by any of these *CARD15* mutations. No significant differences were found between allele or carrier frequencies between early age of onset, A1 (< 40 yr), and late age of onset, A2 (≥ 40 yr) groups. In addition, mean age of the onset was not lower in carriers of the three mutant alleles, either carriers of single or several mutations, than in noncarriers. However, it must be emphasized that all the CD subjects carrying two copies of any rare variant had an early age of onset (average 22 yr; range 18–29), although a robust conclusion could not be obtained due to the small number of patients in this subgroup (only six). Patients with affected relatives did not show earlier age of onset.

Behavior of CD was found to be influenced by *CARD15* polymorphisms after performing univariate analyses (see Table 2). However, all the significant results obtained were lost when years of disease were considered using logistic regression analysis. Thus, the association between behavior of disease and *CARD15* mutations is due to the influence of years of disease on the behavior of CD. This result was not surprising since it is known that behavior of CD changes over time of disease (31). In fact, more detailed analyses showed a significant predominance of nonstricturing, nonpenetrating phenotype during the first years of disease and a predominance of penetrating phenotype later. Thus, 59% of patients with 5 or less years of disease showed a nonstricturing, nonpenetrating phenotype, whereas only 20.5% displayed penetrating disease. This relation was quite opposite in patients with more than 5 years of disease: 20.7% had nonstricturing, nonpenetrating disease and 57.3 had penetrating disease. That suggests that many of the cases first diagnosed as nonstricturing, nonpenetrating change over the time to more complicated forms.

Location of disease was not influenced by *CARD15* mutations. Only a slight trend toward an association with ileum, according with previous studies in which ileal disease was found to be associated with the carriage of *CARD15* variants (30), was also found in our sample. Thus, double-dose carriers showed ileal or ileocolonic CD and none of them was observed with only the colon affected. Besides, the frequency of mutant alleles of G908R and 1007fs was always higher in patients with the ileum or ileocolon affected than in colon-specific patients (although differences were not significant).

No significant associations were found among the *CARD15* mutations and family history, origin of patients, smoking

Table 3. Haplotype Frequencies in Patients and Controls

Haplotype Frequencies						
R702W	G908R	1007fs	Patients	Controls	*p* Value	OR (95% CI)
1	1	1	85.8	92.4	$p < 0.01$	0.5 (0.3–0.8)
2	1	1	6.5	5.8	NS	1.1 (0.6–2.2)
1	2	1	4.0	0.9	$p < 0.05$	4.6 (1.2–16.2)
1	1	2	3.7	0.9	$p < 0.05$	4.2 (1.2–15)

1, wild allele; 2 mutant allele.

habits, surgery, steroid dependency, or infliximab treatment (association with other treatments was not investigated because most of the patients received several different treatments). Only a positive association was shown between R702W and steroid resistance (RR = 2.4 95% CI 1.0–5.6 and RR = 2.4 95% CI 1.1–5.3, for allele and carrier frequencies, respectively, $p < 0.05$) and between the presence of EIMs and G908R (RR = 2.7 95% CI 1.1–6.3, $p < 0.05$). When arthritis, the most common EIM among the studied patients, was studied separately, no significant association was found.

We also analyzed influence of carrying mutant alleles in G908R or 1007fs, without considering R702W, which does not seem to be conferring risk to develop CD in the Galician population. However, the results obtained do not differ from the above one.

Table 4. Comparative Frequencies Among Different European Caucasian Populations

		Allelic Frequency (%)			
Population	n	R702W	G908R	1007fs	References
European range					3, 5–7, 19, 32
Patients	130–453	9.1–12.5	3.3–6	6.6–16	
Controls	81–349	3.5–6.9	1–3.0	1.0–4.4	
Scottish					20
Patients	176	5.5	2.4	4.1	
Controls	141	4.2	0	1.8	
Finnish					21
Patients	271	3.3	0.6	4.8	
Controls	300	1.8	0	1.7	
Galician					
Patients	165	6.7	4.5	4.5	
Controls	165	5.8	1.0	1.0	

n = number of patients or controls.

DISCUSSION

The three main *CARD15* polymorphisms were not equally involved in CD susceptibility in the studied Spanish population. Mutant alleles in G908R and 1007fs increase the risk of developing CD in this population but no influence was found from R702W. The risk conferred by those mutations was 4.6 and 5.0 for carriers of rare variants in G908R and 1007fs, respectively, and 4.7 when carriers of both mutations are considered together. Our findings also confirmed the independent influence of these mutations on CD susceptibility, as indicated by gametic-disequilibrium analysis.

Since Hugot *et al.* (1) reported for first time the involvement of these mutations in CD, several studies from different populations have confirmed these associations. Although important ethnical differences emerged from those studies, most of the Caucasian CD populations were found to be influenced by *CARD15* variants. However, this is not the first time that negative results are found for some of these mutations in this kind of populations. Absence of association between CD and R702W was also observed in Scottish and Finnish population (20, 21). In Finnish patients, G908R was also not involved in CD susceptibility and the same lack of association was observed in the Dutch population (19). It must be noted that the studies from Scotland, Finland, and the current study were performed with homogeneous populations. In addition, the low allele frequencies found in the Scottish and Finnish population are also to be noted, and are in accordance with those observed in our study. The frequency of mutant alleles in Galician population is below the range found in most of the European populations (3, 5–7, 19, 32) (Table 4). Of particular interest is the low frequency of the frameshift mutation since this variant showed the strongest association with CD in previous studies (5–9, 19, 21). An even lower frequency was found for this mutation in a homogeneous population from Crete (2.7% in CD patients and 1.5% in controls), although in this case the sample size was very small (33).

It is known that spurious associations can arise as a consequence of population admixture. However, it is very common to combine study results from different populations in an effort to obtain higher sample sizes. This admixture could favor the finding of positive results in many of the published studies, as well as masking particularities of single populations. Thus, the results obtained when data from different populations are pooled to develop single studies, could not be reflecting factors influencing CD susceptibility in each one of the studied populations. Therefore, the study of more homogeneous populations is very important to discard artifactual effects generated by investigators. It is also essential to detect the role of genetic factors with small influence in only one population. Thus, the results obtained in those homogenous populations could be due to peculiarities of those populations but it is also possible that those findings are not so uncommon in European populations but they are masked when different populations are studied together. In most of the works, admixture is justified because the similarity of allele frequencies in the different populations is included in the study but the possibility of different haplotype frequencies is not considered. The negative results obtained in more homogeneous populations could be indicative of the importance of studying single homogeneous populations to obtain more accurate results. Galicia is a relatively isolated region located in the Northwest of Spain. It has a border with Portugal in the South, and is separated from the rest of Spain by high mountains. The Atlantic Ocean and the Cantabric Sea form remaining limits of the region. Thus, it is characterized by a low rate of immigration. For this reason, this population represents a relatively homogeneous group, appropriate to develop association studies and this homogeneity was confirmed by the Hardy-Weinberg analyses performed in this and in previous works.

The homogeneity of this population makes it appropriate to investigate the implication of *CARD15* mutations in the different subgroups of CD because it increases the chance that individuals develop a clinical phenotype due to the same factor. CD is a heterogeneous disorder characterized by the

presence of different clinical subphenotypes. The role of genetic factors in the development of these different clinical manifestations has been extensively studied but controversial results have emerged. In this study, no association was found between the different subgroups established according to the Vienna Classification and *CARD15* variants. Only a slight trend toward the influence of these mutations to an earlier development of CD could be indicated by the early age of onset in individuals carrying two mutations (either homozygous or compound heterozygous). However, an interesting observation emerged from those analyses and that was the confusing results that can be obtained when genetic influence on the behavior of CD is studied. Louis *et al.* (31) found that behavior of CD varies dramatically over the course of the disease. According to those results, we analyzed the role of *CARD15* variants on behavior of disease paying special attention to the influence of years of disease. We observed that the frequency of penetrating complications increased over the course of the disease whereas the frequency of nonstricturing, nonpenetrating complications decreased. This observation influenced the significant associations detected between some groups of behavior of disease and the presence of mutations since after stratifying for years of disease, those significant associations disappeared. Therefore, they were due to the underlying association between years of disease and behavior. This observation emphasizes the confusing results that can emerge when years of disease are not considered.

The results obtained in this work clearly show an influence of *CARD15* on the development of CD in this Spanish population, although this influence is on CD overall and not on specific clinical subgroups. Similar to previous studies, homozygous or compound heterozygous individuals are only found among CD patients. While only 28% of patients carry at least one mutation in *CARD15*, other factors must also be involved in CD etiology in this population. First, the possibility of other polymorphisms in *CARD15* in the Galician population must be considered. In addition to the three mutations described in this work, other polymorphisms have been identified in *CARD15* gene. For example, in African-Americans, the frequency of these three main mutations was also low and new variants were found that occur solely in that population (34). On the other hand, it is likely that other genes in the same or in a different chromosome are involved in CD susceptibility. As stated before, the three *CARD15* variants considered in this work are not present in Asian populations (15–18). Thus, further research will be necessary to establish the relevance of new polymorphisms in *CARD15* or in different genes in CD in Galician population.

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