HLA class II and III in Crohn’s disease

Associations between HLA-DR1 and TNF microsatellites
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Abstract

Genomewide scans performed in Crohn’s Disease patients have failed to find a major susceptibility locus, and have prompted the general agreement that CD is a polygenic entity in which several genes may contribute to susceptibility.

This study was initiated with the fact that the HLA-DR1/TNFa2b1 haplotype is reported to confer a higher susceptibility to Crohn’s disease. To confirm this hypothesis the TNF microsatellites as well as HLA class II genes will be studied in Spanish CD patients and controls.

The study group consisted of 244 adult unrelated Spanish patients recruited from a single centre, 343 healthy white unrelated subjects from the Madrid region were randomly selected as controls. DNA was purified from peripheral blood samples and typed by a sequence specific oligonucleotide polymerase chain reaction (SSO-PCR) method for HLA-DRB1 alleles (IBD3) and by allele-specific PCR for TNF microsatellite polymorphisms.

No positive association was found with any of the TNF ab microsatellite combinations or with any of the HLA-DR alleles. In examining the HLA-DR1 subtypes, only HLA-DRB1*0103, the less frequent HLA-DR1 allele, was found to be significantly increased in patients with Crohn’s disease.

The haplotype HLA-DRB1*0101/TNFa2b1 confers susceptibility to Crohn’s disease in the Spanish population. In addition, TNFa2b1 is found to modulate the susceptibility of HLA-DRB1*0102, although the mechanism remains unclear. Finally, HLA-DRB1*0103 is associated with Crohn’s disease, in absence of TNFa2b1. This finding supports the concept of disease heterogeneity.
Abbreviations

CD - Crohn's Disease
IBD - Inflammatory Bowel Disease
UC - Ulcerative Colitis
CARD - Caspase Recruitment Domain
NF-κB - Nuclear Factor-κB
HLA - Human Leukocyte Antigen
MHC - Major Histocompatibility Complex
TNF-α - Tumor Necrosis Factor-α
LT-α - Lymphotoxin-α
AH - Ancestral Haplotype
PCR - Polymerase Chain Reaction
LD - Linkage Disequilibrium
OR - Odds Ratio
Introduction in Crohn’s Disease

Characteristics
Crohn’s Disease (CD) and Ulcerative Colitis (UC) are the two major forms of Inflammatory Bowel Diseases (IBD). Crohn, Ginsberg and Oppenheimer described Crohn’s disease for the first time in 1932 [1]. The bowel inflammation is transmural and discontinuous with diseased segments alternating with normal mucosa. The symptoms vary according to both localization and pattern of involvement [2-4]. CD can affect the whole gastro-intestinal tract. The most commonly involved localization is the ileocecal area; symptoms referable to this area include right lower quadrant abdominal pain, diarrhoea and low grade fever. A combination of oedema, bowel wall thickening, fibrosis and spasm results in the classic ‘string sign’ of a narrowed terminal ileum [2, 3]. Thirty percent of patients have disease limited to the small intestine. Manifestations include abdominal pain, diarrhoea fever weight loss and obstructive symptoms [2, 3].

Classification of Crohn’s Disease
The estimation of disease severity in CD is difficult. The most recent developed classification standard for CD is the Vienna classification.[5, 6]. In the Vienna classification, the definitions are confined to age at diagnosis (A), location of disease involvement (L) and behaviour of the disease (B). A1 defines patients with age at diagnosis of Crohn’s disease before 40 years of age and A2 defines those diagnosed thereafter [5-8]. L1 corresponds to the disease located in the terminal ileum (possibly involving the caecum), L2 involves the colon, L3 the ileocolon and the patient is defined L4 when the upper gastrointestinal tract is involved. B1 corresponds to a non-stricturing non-penetrating inflammatory disease. B2 defines a stricturing disease and B3 involves penetrating disease [5-8]. This clinical classification may permit the development of comparative databases and provincial registries for future studies including therapeutic trials with more homogeneous populations [5].

Epidemiology
The prevalence of inflammatory bowel diseases varies greatly between distinct populations. Understanding of the differences between and within different groups may provide insight into possible causative factors [9-11]. Variation in distribution is related by geography, racial and social economic status [10]. IBD’s occur world-wide and have a north south slope in their incidence, with higher frequencies in the northern part of the world, observed in Europe, but also in the American continent. Where the incidence of
HLA class II and III in Crohn’s disease-Introduction

UC is high, so is also the incidence of CD [10]. Northern countries, such as the United Kingdom, Norway, Sweden and the United States, have the highest rates of the diseases. The inflammatory Bowel Diseases are less common in Central and southern Europe [9-11].

Affliction is approximately the same in both sexes. There is a slight preponderance of UC in men, while CD is somewhat more dominant in women [11]. This may be due to the role of autoimmune affection in CD, because all autoimmune diseases have a female predominance.

Pathogenesis

The pathogenesis of the inflammatory bowel diseases is complex, just as the factors causing the diseases [10].

The current hypothesis for the pathogenesis of IBD holds that the basis for their heterogeneity is at the primary genetic level, and the expression of genetic susceptibility is triggered by environmental factors [12].

Pathogenic agents

A long search for an infectious cause has failed to confirm a direct pathogenic role of a specific infectious agent in the pathogenesis of IBD [12]. Several bacterial species, including *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* and *Mycobacterium tuberculosis* have been investigated as a possible cause of IBD, whereas *Clostridium difficile* has been associated with disease exacerbation [3, 12, 13]. It is well recognised that the intestinal sites most frequently affected in IBD -the distal ileum and the colon- are those with the highest bacterial concentrations. Several experimental animal models have reported that bacterial flora have to be present for the inflammation to occur. Duchmann et al have suggested that the normal state of immune tolerance toward autologous micro flora is lost in animal models and in individuals with IBD [14, 15].

Immunology in Crohn’s Disease

The gastrointestinal immune system has to perform two tasks: on one hand it has to offer the host protective mechanisms against invasion by potential pathogens, on the other hand, it has to permit oral tolerance against a wide array of harmless products of normal digestion and the normal intestinal flora, of which many are potentially immunogenic [10].

Changes in cell type number and/or function, including the release of soluble mediators have been associated with the development of CD and UC. Evidence also indicates
that the type of inflammatory response occurring in the intestine of patients with CD differs from that in UC. In CD mucosa a Th1 response occurs, while in UC a humoral immunity appears to be dominant [16, 17]. CD4+ T-cells are widely believed to play an essential role in the pathogenesis of mucosal inflammation. The antigens that trigger the initial T cell activation and expansion in the intestinal mucosa may or may not be of bacterial origin, but they remain unknown [10]. In active CD lesions, there is a higher proportion of Fas-negative and CD45RO-negative cells compared to UC and controls, suggesting that the presence of apoptosis resistant T cells in CD mucosa may prolong inflammation and contribute to chronicity [10, 12, 16]. In addition, macrophages and endothelial cells have a determining role in the initiation of the immune modulated inflammatory process [4]. The Th1 response is manifested by an increased production of IFN-γ and TNF-α. These cytokines promote a delayed-type macrophage activation and the development of hypersensitivity responses [18, 19].

**Genetic contribution**

The question of whether the cause of IBD is genetic or environmental remains open. However, it is likely that the cause is a combination of both: predisposition (susceptibility) is inherited and when exposed to an environmental agent, it promotes the development of these diseases.

Although the genetic susceptibility remains only partially defined, both UC and CD are strongly influenced by genetic factors. There is an increased incidence in Jewish populations, strong familial aggregation and increased concordance among monozygotic twins [10, 18, 20-25]. Both UC and CD exist in the same families with a higher frequency than might be expected by chance alone, suggesting an etiologic relationship between UC and CD [2]. It is clear that neither form of IBD can be explained by a simple Mendelian inheritance pattern. The genetic model, which is most widely accepted at the current time, is that Crohn’s Disease and ulcerative colitis are related polygenic disorders, sharing some, but not all susceptibility loci. The disease phenotype is likely to be determined by the interaction between different allelic variants in a number of genes, and the environment [26].

**Candidate genes**

There are different approaches in finding a genetic association in diseases like the inflammatory bowel diseases. One of them is a hypothesis free method, relying on a systematic screen of polymorphic markers distributed across the human genome. In large panels of multiply affected families, regions are scanned to show linkage to
disease susceptibility [27, 28]. Regions of linkage are those where the affected sibling or relative pairs show significantly greater allele sharing than expected by chance alone [28]. At least seven full genome scans have been published in which strong candidate regions have been identified for IBD.

The first genome scan was published in 1996 by Hugot et al, found a susceptibility locus on chromosome 16 [29]. This linkage in Crohn’s disease has been almost universally replicated throughout the world [23, 30]. The refinement of this localisation of the later called IBD1 susceptibility locus, was carried out by fine mapping the candidate region and performing positional candidate gene studies. This lead to the identification of a single gene, which was identified as the caspase recruitment domain gene (CARD 15) which is related to the NOD1/Apaf superfamily of apoptosis regulators, expressed in monocytes. A number of polymorphisms within the NOD-2 gene were shown to be associated with Crohn’s disease susceptibility. One of the polymorphisms affects a repeat, which is strongly homologous with the Toll-like receptor gene superfamily. These toll-like receptors represent an integral part of the innate immune response to bacterial infection and the repeat sequence encodes that region of the molecule that interacts with bacterial antigens [31]. Another polymorphism of the NOD 2 gene is associated with NF-κB activation in response to bacterial LPS, only the deletion of the leucine rich region leads to increased activation of NF-κB[17].

Hampe et al have provided evidence for association of a NOD2 frameshift mutation with Crohn’s disease [32]. This mutation is caused by a cytosine insertion, 3020insC, and this allele is expected to encode a truncated protein. The normal function of NOD2 is to activate NF-κB, making it responsive to bacterial lipopolysaccharides. This function is deficient in mutant NOD2 and suggests a link between the innate immune response and development of disease [32, 33]. Mutations within the NOD2-gene are found in only 30% of the cases of Crohn’s Disease [32].

Shortly after the discovery of IBD1, Satsangi et al reported evidence for further susceptibility loci in both CD and UC. Strong evidence for linkage was found for a region on the long arm of chromosome 12, named IBD2 [34]. Although the chromosome 12 linkages have been strongly replicated in a number of databases, replication has not been universal. Analysis of data from different groups reveals that the IBD2 locus may be uniquely involved in the pathogenesis of ulcerative colitis [26, 33].

Increasing evidence also implicates the subchromosomal region on chromosome 14q11-12 as being involved in susceptibility to Crohn’s Disease [35-38]. The major histocompatibility region on chromosome 6p was also identified as a susceptibility locus for IBD; this locus was later called IBD3. This locus contains the
Human leukocyte antigen (HLA) genes, which are candidates genes in the pathogenesis of IBD because their products play a central role in the immune response[39].

**The HLA complex**

The major histocompatibility complex (MHC) region on chromosome 6 contains more than 200 genes, many of them participating in the regulation of immune and inflammatory responses and thus provide ideal candidate for etiologic investigations. The HLA genes are divided in three classes. The class II genes play a central role in the immune response. The gene products consist of an α and a β chain which form a groove in which the antigenic peptide, after digestion of the antigen by antigen presenting cells, is presented to the T cell receptor [39-41]. The three different HLA class II molecules are HLA-DP, -DQ and -DR. Subunits of HLA-DP and DQ are each encoded by a polymorphic α and β chain gene. In the case of HLA-DR, there is a non-polymorphic α chain gene and up to three distinct highly polymorphic β chain genes. One of these genes, DRB1, is always present in all individuals and is by far the most polymorphic. Therefore, molecular and serological analysis of DRB1 polymorphisms has become an important tool in studies of the relation between HLA class II genes and disease [42]. Besides the fact that the alleles can be used to study the relationship between HLA class II and diseases, polymorphic sequences may have functional implications. Different alleles have different peptide binding characteristics and polymorphisms that are located outside the binding site of the molecules may affect interaction with T cells or expression of the HLA molecule [43].

**HLA and susceptibility to CD**

Although overall association with disease susceptibility is relatively weak, in White Europeans strong association between disease behaviour and HLA allelic variation have been described, particularly in ulcerative colitis. It is noteworthy that ethnic differences in the contribution of the HLA region are very apparent [34, 44]. Positive and negative associations with HLA class II have been described. Positive associations have been reported for HLA-DRB1*01 [43, 45], later attributed to the DRB1*0103 subtype [46], DRB1*1302 [23, 24] and DRB1*07 [42, 43, 46, 47]. Also an increase in the DQB1*0201 allele frequency was found. Negative associations with HLA-DR2, attributed to the DRB1*1501 subtype [42, 46, 48], HLA-DRB1*03 [42, 43, 47] and with DQB1*06[43], have been reported. A decreased amount of DQB*0101 alleles was found, just as DRB1*0501 and a strong decreased number of DRB1*03 alleles, which suggest that DRB1*03 mediates resistance to Crohn’s disease [42, 43, 46]. Other
genes or markers in addition to HLA-DR have also been reported to be associated with Crohn’s disease but in general they are in linkage disequilibrium with the aforementioned HLA-DRB1 alleles [49].

Of the number of genes within the HLA class III region, the genes encoding tumour necrosis factor-α (TNF-α) require particular mention. Increasing clinical evidence supports the pivotal role of TNF-α in the development and progression of Crohn’s disease. In both Japanese and European populations, allelic variation within the promoter region of the TNF-α gene has been associated with susceptibility to and progression of Crohn’s disease [50].

A variety of therapeutic approaches has been used to inhibit TNF-α in patients with IBD. These include the monoclonal antibodies against TNF-α, such as infliximab and CDP571. The mechanisms of action for these monoclonal antibodies include neutralization of both soluble and transmembrane TNF-α. Other approaches to inhibit TNF-α are blocking the production and secretion of TNF-α. To achieve a block against leukocyte adhesion, TH1 polarization and T cell activation (and T cell depletion), monoclonal antibodies are designed to α4 β7 integrin and antisense to intercellular adhesion molecule-1, so cellular interaction is blocked [51-53].

Ancestral Haplotypes and Microsatellites

In 1996 Plevy et al found a Crohn’s Disease-associated group of markers on chromosome 6. They determined TNF microsatellite allele frequencies at five loci and identified a combination of TNF microsatellite alleles on chromosome 6 that is associated with CD [49]. Microsatellites are repeats of a DNA base motif with a size of 1-6 bp, up to 100 times. They are distributed regularly all over the genome. Many of them are polymorphic and their high polymorphism is based upon a variable number of repeats. They are widely used for genetic mapping, linkage analysis, population genetics and in forensic medicine. This markers have been described in the HLA region since 1991 [54]. Microsatellites don’t have a gene product, but can represent a gene located nearby in the locus. Using the polymerase chain reaction (PCR), these repeats can be easily amplified. One group of microsatellites in the HLA class III region are TNF a, b, c, d and e. The TNF a and b microsatellites are located 3.5 kilobases upstream of the Lymphotoxin-α (LTA) gene. TNFc is located in the first intron of LTA gene and the TNF d and e loci are located 8-10 kilobases downstream of the TNF-α gene, the loci of the TNF microsatellites are visualized in figure 1.
Polymorphisms of these 5 alleles are often in linkage disequilibrium. Plevy et al found linkage disequilibrium for the 5 TNF microsatellites, the haplotype TNFa2b1c2d4e1, this haplotype was significantly more frequently found in patients with Crohn’s disease [49].

The MHC complex contains many immune related genes and it had now become clear that the different alleles are strongly linked. MHC alleles are usually inherited en bloc without recombination and, hence, can be grouped into several haplotypes. One group of these haplotypes seems to be conserved by some evolutionary advantage and are called ancestral haplotypes (AH), which are designated by their HLA-B allele [55]. AHs extend from HLA-B to HLA-DR. These haplotypes and recombinants between any two of them account for 73% of unselected haplotypes in the Caucasian population [55, 56].
Aim of the Study

In this study of the genetic background of Crohn’s Disease, we will focus on polymorphisms in the HLA Class II and III genes.

In several studies positive and negative associations with HLA class II and Crohn’s disease have been described. The HLA class II genes are candidates for a role in the pathogenesis of CD because their products play a central role in the immune response[39]. A class II allele often associated with susceptibility to CD is HLA-DR1, this has been described by many groups concerning the Northern European and American population [42, 43, 57].

The HLA class III alleles have also been described as being associated with Crohn’s Disease. Of the number of genes within the HLA class III region, the genes encoding tumour necrosis factor-α (TNF-α) require particular mention. In both Japanese and European populations, allelic variation within different regions of the TNF-α gene, like the promoter region and TNF microsatellites has been associated with susceptibility to and progression of Crohn’s disease [49, 50, 58].

Plevy et al have described a group of TNF microsatellites to be associated with susceptibility to CD [49]. In addition, this group of microsatellites is described together with Class II HLA genes to be associated to TNF-α secretion, with a possible link to insulin dependent diabetes mellitus [59]. This group of microsatellites, TNFa2b1, is found in linkage disequilibrium with HLA-DR1/DQ5. The haplotype with both TNFa2b1 and HLA-DR1 is described to have a higher frequency in patients with Crohn’s disease and this combination occurs together in AH 35.2 and 35.3, two ancestral haplotypes which do not arise often in Spain [55, 56].

In this work association of HLA class II and III with Crohn’s disease will be investigated in the Spanish population. Special attention will be paid to HLA-DR1 and the TNF-microsatellites. This study was prompted with the fact that the HLA-DR1/TNFa2b1 haplotype confers a higher susceptibility to Crohn’s disease, and our objective was therefore to find out if this is due to TNFa2b1 or HLA-DR1 or one of the HLA-DR1 subtypes.

To confirm this association, the TNF microsatellites as well as HLA class II alleles and DR-1 subtypes will be studied in Spanish CD patients and controls.
Materials and Methods

Study objects
The study group consisted of 244 adult unrelated Spanish patients recruited from a single centre. Diagnosis of Crohn’s Disease was based on standard clinical, radiological, endoscopic, and histological data following Leonard Jones criteria [60]. Healthy white unrelated subjects (343) from the Madrid region were randomly selected as controls.

DNA isolation and HLA class II genotyping
The DNA was extracted from fresh peripheral blood leukocytes by a ‘salting out’ procedure with 6 M NaCl after overnight incubation with proteinase K [61]. The HLA genotyping for DR and DQ subtypes was performed by amplification of the highly variable exons of the HLA-DR, DQ-α and DQ-β loci. Amplification was performed using the appropriate primers from the 11th International Histocompatibility Workshop [62]; used primers are shown in table 1A. PCR mix per sample consisted of 1 µl Buffer Gold, 0.5 µl dNTP’s and water to a final volume of 10 µl. For each sample 500 µg of genomic DNA was added and 0.05 µl of AmpliTaq Gold DNA polymerase. The PCR protocol performed by the PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) was as followed: preheating at 95 °C for 11 min, followed by 39 cycles of denaturing at 95°C for 20s; annealing at 55°C for 20s and extension at 72°C for 30s. Final incubation at 72°C for 7min, ending with cooling at 15°C ‘forever’. The quality of the PCR product was assessed by agarose gel electrophoresis; 5 µl of amplification product was electrophoresed on agarose gel (1.5%), visualised by ethidium bromide staining and exposure to UV light.

To perform the genotyping 10 µl from the PCR products were slot blotted onto nylon membranes. Cross linkage of PCR products to membranes was achieved by exposure to UV light. Hybridisation was performed at specific temperatures with digoxygen in-labeled allele-specific oligonucleotide probes from the 11th International Histocompatibility Workshop [62]. Hybridisation temperatures and sequences of used probes are shown in table 1b-I, -II and -III.

The results were visualised by exposing the membranes to a radiographic film for 5-15 minutes.
### Table 1A: Sequences of primers used to type HLA class II

<table>
<thead>
<tr>
<th>Allele</th>
<th>Primers</th>
<th>Sequence</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR</td>
<td>DRB-AMP-A</td>
<td>5’-CCCCACACGCAGCTTTCTTG-3’</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>DR</td>
<td>DRB-AMP-B</td>
<td>5’-CGGCTGCAGCACTGAGCTCT-3’</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>DQ-α</td>
<td>DQA-AMP-A</td>
<td>5’-ATGGTGTAACCTGGACTGAGT-3’</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>DQ-α</td>
<td>DQA-AMP-B</td>
<td>5’-TTGGTAGCAACGCGTACGGA-3’</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>DQ-β</td>
<td>DQB-AMP-A</td>
<td>5’-CATGTCGGCTACTCCACCAACG-3’</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>DQ-β</td>
<td>DQB-AMP-B</td>
<td>5’-CTGTTAGTTGGTCGACACAC-3’</td>
<td>0.2 µM</td>
</tr>
</tbody>
</table>

**Polymerase chain reaction to subtype DR1**

For subtyping DR1, a PCR was performed with the primers shown in table 1c-I under the same conditions as mentioned in the HLA class II general typing. After amplification, the DOT-blot technique was used to further subtype, using specific probes for subtype 101, 102 and 103, shown in table 1c-II.

### Table 1B-I Primers used for DR1 subtyping

<table>
<thead>
<tr>
<th>Allele</th>
<th>Primers</th>
<th>Nucleotide sequences</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR</td>
<td>DRB-AMP-A</td>
<td>5’-CCCCACAGCACGTTCTTG-3’</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>DR1</td>
<td>DRB specific</td>
<td>5´-TTCTTGTGAGCAGTTTAAG-3´</td>
<td>0.2µM</td>
</tr>
</tbody>
</table>

### Table 1B-II Sequences of probes used for DR typing and hybridisation temperatures

<table>
<thead>
<tr>
<th>Allele</th>
<th>Probe</th>
<th>Nucleotide sequences</th>
<th>Hybridisation temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1001</td>
<td>5’-TAAAGTTGAATGCTATT-3’</td>
<td>37</td>
</tr>
<tr>
<td>3, 6, 11</td>
<td>1003</td>
<td>5’-GTACTCTACGTTCTGAGTG-3’</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>1004</td>
<td>5’-AGCAGGGTTAAGCTTGAG-3’</td>
<td>45</td>
</tr>
<tr>
<td>8, 12</td>
<td>1005</td>
<td>5’-GGTACGAGTCCTAAGAT-3’</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>1006</td>
<td>5’-GAAGCAGGTAAGTATAAG-3’</td>
<td>37</td>
</tr>
<tr>
<td>9</td>
<td>1007</td>
<td>5’-GAGGAGGTTAAGTTPGAG-3’</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>1008</td>
<td>5’-GAGGAGGTTAAGTTPGAG-3’</td>
<td>37</td>
</tr>
<tr>
<td>51</td>
<td>1009</td>
<td>5’-CGAGGAGGATAAGTGAAG-3’</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>2802</td>
<td>5’-GGTACTGGAGAAGACT-3’</td>
<td>48</td>
</tr>
<tr>
<td>53</td>
<td>2810</td>
<td>5’-GCGAGTGAGGACCTGAT-3’</td>
<td>45</td>
</tr>
<tr>
<td>11</td>
<td>5703</td>
<td>5’-GCCTGATGAGGAGTACTG-3’</td>
<td>45</td>
</tr>
</tbody>
</table>
Polymerase Chain Reaction to type TNFa and b microsatellites

Primers used to amplify the microsatellites are shown in table 1c. Primer pairs at final condition were added in a total reaction volume of 10 µl. Added was: 1 µl Buffer Gold, 0.5 µl dNTP’s. For each sample 50 µg of genomic DNA was added and 0.05 µl of AmpliTaq Gold DNA polymerase. All used solutions were derived from Roche. The PCR protocol performed by the PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) was as followed: preheating at 95°C for 11 minutes and then denaturing at 95°C for 20s; annealing at 55°C for 20s and extension at 72°C for 30s, for 39 cycles. A final incubation at 72°C for 7min, ending with cooling at 15°C completed the preparation of the samples.

The primers were supposed to amplify three fragments: the TNFa microsatellite, the TNFb microsatellite and the third, a joined amplicon of TNF a and b. This is because the TNF a and b microsatellites are close enough to be jointly amplified, and this haplotypes are directly inferred. In this case, IR2 and IR1 form a pair of primers to amplify the whole fragment. Location of the primers are visualised in fig 2.

One microliter of PCR product was added to 20 microliters of Formamide (Roche) this was heated at 95°C. After denaturing the PCR products were subsequently run in an ABI prism 310 automatic sequencer (Perkin Elmer, Norwalk, CT). The microsatellite samples were analysed using Genescan software with the TAMRA 500 bp marker and Local Southern size-calling method.
Table 1C-I Primer sequences of microsatellites

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>Primer</th>
<th>Sequence</th>
<th>Label</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFa-1</td>
<td>IR2</td>
<td>5'-GCCTCTAGATTTCATCCAGCCACA-3'</td>
<td>HEX</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>TNFa-2</td>
<td>IR4</td>
<td>5'-CCTCTCTCCCCTGCAACACACACA-3'</td>
<td></td>
<td>0.1 µM</td>
</tr>
<tr>
<td>TNFb-1</td>
<td>IR1</td>
<td>5'-GCACTCCAGCCTAGGCACAGA-3'</td>
<td></td>
<td>0.1 µM</td>
</tr>
<tr>
<td>TNFb-2</td>
<td>IR5</td>
<td>5'-GTGTGTGTTGAGAGAGAGAGA-3'</td>
<td>FAM</td>
<td>0.1 µM</td>
</tr>
</tbody>
</table>

**Statistical analysis**

The allele frequencies in patients and controls were compared by the $\chi^2$ test or Fishers exact test when an expected value was <5. For both HLA-DRB1 and TNFab microsatellite genotyping, p values were corrected for the numbers of alleles determined. P-values were considered significant at a level of <0.05.
Results

**TNF microsatellites**

When the TNF microsatellites TNF a and b were analyzed (data shown in table 2A-1), only one allelic combination was found with a small negative association with CD, TNFa10b4 was slightly decreased in CD patients (53 out of 244), compared to Spanish controls (100 out of 343), p=0.04; OR=0.67. After correction, none of the p-values were significant.

No positive association was found in any of the TNF ab combinations, including the allelic combination previously described by Plevy et al, TNF a2b1 (53 out of 244 vs 64 out of 343).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients (244)</th>
<th>Controls (343)</th>
<th>p-value</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1b5</td>
<td>23</td>
<td>30</td>
<td>0.78</td>
<td>1.09</td>
</tr>
<tr>
<td>a2b1</td>
<td>53</td>
<td>64</td>
<td>0.36</td>
<td>1.21</td>
</tr>
<tr>
<td>a2b3</td>
<td>25</td>
<td>30</td>
<td>0.54</td>
<td>1.19</td>
</tr>
<tr>
<td>a2b5</td>
<td>16</td>
<td>23</td>
<td>0.94</td>
<td>0.98</td>
</tr>
<tr>
<td>a6b5</td>
<td>55</td>
<td>71</td>
<td>0.59</td>
<td>1.11</td>
</tr>
<tr>
<td>a7b4</td>
<td>50</td>
<td>64</td>
<td>0.58</td>
<td>1.12</td>
</tr>
<tr>
<td>a10b4</td>
<td>53</td>
<td>100</td>
<td>0.04*</td>
<td>0.67</td>
</tr>
<tr>
<td>a11b4</td>
<td>54</td>
<td>74</td>
<td>0.87</td>
<td>1.03</td>
</tr>
<tr>
<td>a13b4</td>
<td>12</td>
<td>24</td>
<td>0.30</td>
<td>0.69</td>
</tr>
</tbody>
</table>

* = p<0.05; Pc = n.s.

**HLA class II**

When the allelic distribution of the HLA class II genes was examined, no significant positive association of any of the HLA-DR genes was observed (after Bonferroni correction for multiple comparisons). As expected, DR2 is less frequent in C-patients, and it will not be discussed further, data are shown in table 2B-I.

DR1 in particular, was found at a similar frequency in CD patients compared to controls (p=0.41, OR 1.19).
**Table 2B-I** Frequency of HLA alleles in Spanish CD patients and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients (244)</th>
<th>Controls (343)</th>
<th>p-value</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1</td>
<td>59</td>
<td>73</td>
<td>0.41</td>
<td>1.18</td>
</tr>
<tr>
<td>DR2</td>
<td>39</td>
<td>89</td>
<td>0.004*</td>
<td>0.54</td>
</tr>
<tr>
<td>DR3</td>
<td>53</td>
<td>82</td>
<td>0.54</td>
<td>0.88</td>
</tr>
<tr>
<td>DR4</td>
<td>60</td>
<td>74</td>
<td>0.39</td>
<td>1.19</td>
</tr>
<tr>
<td>DR6</td>
<td>69</td>
<td>103</td>
<td>0.65</td>
<td>0.92</td>
</tr>
<tr>
<td>DR7</td>
<td>82</td>
<td>92</td>
<td>0.08</td>
<td>1.38</td>
</tr>
<tr>
<td>DR8</td>
<td>9</td>
<td>28</td>
<td>0.03*2</td>
<td>0.43</td>
</tr>
<tr>
<td>DR11</td>
<td>50</td>
<td>81</td>
<td>0.37</td>
<td>0.83</td>
</tr>
</tbody>
</table>

*1: Pc < 0.05  
*2: p < 0.05; Pc n.s.

**HLA-DR1 subtypes**

In examining the HLA-DR1 subtypes (table 2B-II), only HLA-DRB1*0103, the less frequent DR1 allele, was found to be significantly increased in patients with Crohn’s disease (15 out of 244 vs 7 out of 343; p= 0.01, OR=3.14). In contrast, HLA-DRB1*0101 and HLA-DRB1*0102 were not found to be significantly associated (p=0.93 resp 0.94).

**Table 2B-II** Frequency of HLA-DR subtypes in Spanish CD patients and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients (244)</th>
<th>Controls (343)</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1*0101</td>
<td>30</td>
<td>43</td>
<td>0.93</td>
<td>0.98</td>
</tr>
<tr>
<td>DRB1*0102</td>
<td>16</td>
<td>22</td>
<td>0.94</td>
<td>1.02</td>
</tr>
<tr>
<td>DRB1*0103</td>
<td>15</td>
<td>7</td>
<td>0.001*</td>
<td>3.14</td>
</tr>
</tbody>
</table>

**HLA / TNF linkage disequilibrium**

When the HLA-DR genes were studied together with TNF alleles, HLA-DR1 was found to be in linkage disequilibrium with TNFa2b1 in controls (p=0.005, OR=2.28). LD was found in controls between DRB1*0102 and TNFa2b1 (P=0.0001, OR =6.07) and DRB1*0103 and TNF a2b1 (p=0.01, OR=6.01). However, DRB1*0101, the most frequently found DR1 subtype in the Spanish population, was not found to be in LD with TNF a2b1. DRB1*0101, together with TNFa2b1 forms a haplotype which occurs frequently in Northern Europe and USA, but the results found in this study show that this combination is very rare in the Spanish population (6 out of 343). An association of
this TNFa2b1-DRB1*0101 combination with Crohn’s Disease is found, despite the small numbers obtained (P=0.049; OR=2.65).

When HLA-DRB1*0102 distribution was examined, it was found that the relative frequency of TNFa2b1 among DRB1*0102+ individuals is higher in patients than in controls. However, in absolute terms, the DRB1*0102+/TNFa2b1−-haplotype is not significantly increased in patients (p=0.13; OR=1.81). The DRB1*0102+/TNFa2b1−-haplotype is significantly decreased in patients (p=0.02; OR=0.14). The positive association of DRB1*0103 is found specifically in patients without TNFa2b1 (2 vs 13; p=0.001; OR=3.14).

Table 2C-I Frequency of TNFa2b1 in Spanish CD patients and controls carrying the DR1 subtypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Patients (244)</th>
<th>Controls (343)</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1*101+/TNFa2b1+</td>
<td>11</td>
<td>6</td>
<td>0.049*</td>
<td>2.65</td>
</tr>
<tr>
<td>DRB1*102+/TNFa2b1+</td>
<td>15</td>
<td>12</td>
<td>0.13</td>
<td>1.81</td>
</tr>
<tr>
<td>DRB1*103+/TNFa2b1+</td>
<td>2</td>
<td>4</td>
<td>1.0</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 2C-II Frequency of HLA DR1 subtypes in Spanish CD patients and controls, without TNFa2b1

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Patients (244)</th>
<th>Controls (343)</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1*101+/TNFa2b1+</td>
<td>19</td>
<td>37</td>
<td>0.2</td>
<td>0.70</td>
</tr>
<tr>
<td>DRB1*102+/TNFa2b1+</td>
<td>1</td>
<td>10</td>
<td>0.02*</td>
<td>0.14</td>
</tr>
<tr>
<td>DRB1*103+/TNFa2b1+</td>
<td>13</td>
<td>3</td>
<td>0.001</td>
<td>3.14</td>
</tr>
</tbody>
</table>

Finally, the TNF alleles a2b1 in a haplotype with none of the of the DR1 subtypes does not have an association with CD (data not shown).
Discussion

In this study we have not found any specific role for HLA-DR alleles or TNF microsatellites in CD susceptibility, but it has confirmed that there are interactions between HLA class II and III genes. Earlier data had described a susceptibility allele lying in a haplotype containing HLA-DR1 and TNFa2b1 [49]. In order to find out whether this is due to TNFa2b1 or HLA-DR1 alone, we studied the frequency of both alleles in Spanish controls and patients. We found no significant association for any of the TNFab microsatellites combinations, nor for HLA-DR1. An association of a haplotype containing TNFa2b1 and HLA-DR1 with CD was not confirmed in our study. However, we found an association of the disease with the haplotype HLA-DRB1*0101/TNFa2b1.

**HLA-DR1 / TNFα2b1 haplotypes**

**HLA-DRB1*0101**

We can speculate that in Northern European countries and USA a high proportion of DR1 haplotypes are HLA-DRB1*0101/TNFα2b1. The overall association with HLA-DR1 and with TNFα2b1 could be due to the association of the haplotype containing the HLA-DR1 subtype DRB1*0101 and TNFα2b1. In contrast, in Spain, the HLA-DRB1*0101/TNFα2b1 haplotype is not the predominant DR1 haplotype. This lower representation can be the explanation for the lack of an overall association with DR1/TNFα2b1 in the Spanish population, although the HLA-DRB1*0101/TNFα2b1 haplotype seems to be in fact associated with the disease (P=0.049; OR=2.65).

**HLA-DRB1*0102**

Our data also show that there is a different distribution of TNFα2b1 among HLA-DRB1*0102 carriers, when patients were compared to controls. The relative frequency of TNFα2b1 among DRB1*0102 individuals is lower in controls (3.5% in controls vs 6.13% in patients). This can be explained in 2 different ways: HLA-DRB1*0102/TNFα2b1 could be a susceptibility haplotype, or HLA-DR*0102 in the absence of a2b1 could be a marker of protection. Alternatively both explanations could be true, or a combination of both. At the present time it is difficult to distinguish between those two main possibilities, although data tend to favour the first.

**HLA-DRB1*0103**

The frequency of the HLA-DRB1*0103 allele is increased in patients compared to controls, in the absence of TNF α2b1 (p=0.001; OR=3.14). In the Spanish control
population, this allele is associated with TNF a2b1 (p=0.01; OR=6.01), but in the patient group most of the times HLA-DRB1*0103 is found in the absence of a2b1. This suggests that there is a DRB1*0103 haplotype, which does not contain TNFa2b1, that carries a susceptibility allele. In connection with this point it is important to stress that the DRB1*0103 gene has been associated with UC [42] and with a strictly colonic disease in CD patients[63]. In UC patients, HLA-DR*0103 is usually in linkage disequilibrium with the IKBL-C polymorphism[64]. Whether HLA-DRB1*0103/IKBL-C or another haplotype is important in CD susceptibility remains to be studied.

In summary, our results show that the haplotype HLA-DRB1*0101/TNFa2b1 gives susceptibility to Crohn’s disease in the Spanish population. In addition, TNFa2b1 is found to modulate the susceptibility of HLA-DRB1*0102, although the mechanism remains unclear. Finally, HLA-DRB1*0103 gives susceptibility to Crohn’s disease especially in absence of TNF a2b1, so the susceptibility is probably due to an other haplotype, containing HLA-DRB1*0103 and not TNFa2b1.
Acknowledgements

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References


