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# Lymphotoxin alpha gene in Crohn's disease patients: absence of implication in the response to infliximab in a large cohort study

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**Abstract:** A haplotype in the lymphotoxin alpha (LTA) gene has been associated with a lack of response to infliximab in a small cohort of Crohn's disease (CD) patients. The present study aimed to confirm the implication of this haplotype in the response to infliximab in a larger cohort of Caucasian patients. The response to the first infusion with infliximab was evaluated in 214 Caucasian patients with either luminal (*n*= 150) or fistulising (*n* = 64) CD. Clinical response was based on the decrease in CD Activity Index (luminal) or on the evolution in the fistula discharge (fistulising). Biological response was assessed in 139 patients who had elevated C-reactive protein (CRP) before treatment and for whom CRP values were also available after treatment. A positive biological response was defined as a decrease in CRP of at least 25%. The patients were genotyped for six polymorphisms in the LTA gene. A positive clinical response was present in 65.4% of the patients and a positive biological response was observed in 80.6% of the patients. No association was found with any of the studied polymorphisms, nor with the previously published LTA haplotype and the response to infliximab. We could not confirm an association between the LTA locus and clinical or biological response to infliximab in a large cohort of CD patients.

**Keywords:** Crohn's Disease; infliximab; lymphotoxin alpha; pharmacogenetics

## Introduction

Infliximab, an anti-tumor necrosis factor-alpha (TNF) antibody, has a high therapeutic efficacy in refractory Crohn's disease (CD) [1], leading to a fast and considerable improvement of quality of life, and to a rapid improvement of the intestinal lesions as assessed by endoscopic and histological analysis [2]. After a single infusion, 60-80% of the patients improve their clinical condition and 33% of the patients are in clinical remission [3]. Therapeutic response seems to be partially determined by genetic factors. Considering the cost and potential side-effects of this drug a genetic targeting of the responding patients is certainly of great interest [4].

Several findings indicate a potential role of the lymphotoxin alpha (LTA) gene as a genetic marker for the response to infliximab. The LTA gene is located in the major histocompatibility complex (MHC) locus on chromosome 6p21 which is implicated in CD susceptibility (IBD3) [5]. Furthermore the MHC complex might include genetic determinants of response to infliximab in rheumatoid arthritis patients [6]. Taylor *et al.* [7] have described a significant association between a haplotype in the LTA region and the response to this anti-TNF antibody in CD patients. In their study, patients carrying the LTA 1_1_1_1 haplotype (corresponding to Nco1-TNFc-aal3-aa26) did not show any response to the treatment. However, this study was performed on a relatively small cohort of North American patients treated with infliximab (*n*=59). The studied LTA haplotype comprised an *Nco*1 restriction fragment length polymorphism in intron 1 (rs909253), a microsatellite (rs5875327) also in intron 1 (TNFc) and two amino acid polymorphisms [aal3 (rs2857713), aa26 (rsl041981)], respectively, in exon 2 and in exon 3. The aa26 change corresponds to a single nucleotide polymorphism (SNP) (rsl041981) in exon 3 leading to a modification of the sixtieth amino acid of the protein. Given that there exist no independent studies investigating this LTA haplotype in CD with respect to response to infliximab, the present study aimed to confirm the association between the LTA Nco1-TNFc-aal3-aa26 haplotype and a clinical or biological response to infliximab in a large cohort of Caucasian patients.

## Materials and methods

### *Study population and treatment*

Two hundred and sixty-four patients with CD treated by infliximab for the first time and included in the Belgian Infliximab Expanded Access Program (Schering-Plough NV/SA, study 011246-1) were studied. Approval from the Ethic Committee was obtained in January 2004. To be included in the Expanded Access Program, patients had to provide their informed consent and to fulfil one of the three following criteria: (i) single or multiple entero-cutaneous draining fistula(s) as a complication of CD resistant to conventional treatment for at least 3 months; (ii) moderately to severely active CD for at least 6 months, with colitis, ileitis, or ileocolitis, confirmed by radiography or endoscopy, and refractory or dependent on oral corticosteroid therapy ( > 8 mg/day prednisone equivalent); or (iii) patients refractory or intolerant to immunosuppressive agents (methotrexate, azathioprine, 6-mercaptopurine, or cyclosporine).

Patients with refractory luminal disease received a single infliximab infusion at week 0. For fistulising disease, three consecutive infusions at weeks 0, 2 and 6 were administered. Infliximab (Remicade; Centocor Inc., Malvern, Pennsylvania, USA) was given as an intravenous infusion of 5 mg/kg in all patients. Both luminal ($n$ = 150) and fistulising ($n$ = 64) forms were scored for the response to treatment. Luminal CD patients were followed prospectively for 12 weeks with clinical examination, CD activity index (CDAI) calculation and C-reactive protein (CRP) measurement at weeks 0, 4, 8 and 12. Fistulising CD patients were followed prospectively for 18 weeks with physical examination including fistulous track drainage and CRP measurements at weeks 0, 2, 6, 10, 14 and 18.

### *Patient classification*

Four patients were excluded because of their ethnic group (no Caucasian) and 46 for incomplete clinical data, particularly insufficient data regarding the clinical response at week 4 for the luminal disease or at week 10 for the fistulising disease. The response to infliximab was evaluated on the basis of both clinical and biological data. Clinically, the patients with luminal CD were classified as responders at week 4 according to a decrease of 70 points in CDAI from baseline in accordance with the classification used by Taylor *et al.* [7] and with controlled trials [8]. Patients were considered as complete responders at week 4 according to a decrease of the CDAI below 150. In fistulising disease, patients were considered as responders at week 10 according to a decrease of at least 50% of the fistula drainage at two consecutive visits [9].

The biological response was evaluated at week 4 for luminal disease and at week 10 for fistulising disease on the basis of CRP evolution. Only the patients who presented an elevated CRP before treatment (more than twice the upper limit of the normal range) were considered ($n$=139). Patients were categorized as responders after a decrease of at least 25% from the baseline level [10].

### *LTA genotyping*

DNA was extracted using a conventional phenol/chloroform technique. Patients were genotyped for the polymorphisms previously described (rs909253, rs2857713, rs5875327, rsl041981) and two other SNPs (rs746868, rs3093543) in the LTA gene.

The LTA gene was amplified by polymerase chain reaction (PCR) (primers: LTA1F: 5'- CCCGTGCTTC GTGCTTTGG-3', LTA2R: 5'-GAGATCAGGGGTCTG GATCA-3'; annealing temperature: 65°C, 30 cycles; Faststart polymerase [Roche, Basel, Switzerland); $MgCl_2$ (1.7 mM)]. Direct sequencing was performed using the same primers and the Big Dye Terminator sequencing kit v3.1 (Applied Biosystems, Foster City, California, USA). The TNFc microsatellite was studied by fluorescent PCR (primers: TNFIR6 5'-GGTTTCTCTGACTGCATCT TGTCC-3', TNFIR7: 5'-FAM TCATGGGGAGAAC CTGCAGAGAA-3'; annealing temperature: 60°C, 30 cycles; Gold polymerase [Applied Biosystems, Applied Biosystems); $MgCl_2$ (1.5 mM)]. Data were treated by the Genescan software v3.7 (Applied Biosystems). The haplotypes were rebuilt using Phase version 2.1 software [11-12].

### *Statistical analysis*

The clinical response rates according to the different haplotypes were compared using Fisher's exact test or the

chi-square test when appropriate. To be in accordance with the work of Taylor *et al* [7], we also compared the frequencies of homozygous patients of the most frequent haplotypes between responders and nonresponders patients, using Fisher's exact test. Comparisons were also performed considering both genotypes and alleles for each SNP taken separately using a chi-square test or Fisher's exact test when appropriate. The threshold for statistical significance was lowered to $\alpha = 0.008$ after Bonferroni's adjustment for multiple test analysis. The CRP values were normalized and the CRP level variation before and after treatment were compared. The biological response according to the different haplotypes and genotypes (for each SNP) were compared by Fisher's exact test or chi-squared. The relative reduction of the CRP between weeks 0 and 4 was calculated in both responders and nonresponders and the comparison between both groups was performed using a Mann-Whitney *U*-test.

## Results

Patient characteristics are shown in Table 1. A clinical response was observed in 65.4% and a biological response in 80.6% of CD patients when considering the complete cohort. Three haplotypes are preponderant and the LTA 1_1_1_1 haplotype accounts for 13% (27/214), as described in the previous study. The integration of the genotype data of the two additional SNPs (rs746868, rs3093543) did not show any new haplotypic variation in this region. For example, all the patients homozygous for the LTA 1_1_1_1 (rs909253_TNFc_rs2857713_ rsl041981) haplotype were also homozygous 1_1_2_1_1_1 (rs909253_TNFc_rs746868_rs2857713_ rsl041981_ rs3093543). The studied SNPs were found in our cohort at the same frequency as described in the public database (http://www.ncbi.nlm.nih.gov).

We did not find any association between the LTA 1_1_1_1 haplotype or any of the other haplotypes and the clinical response to infliximab (Table 2). Considering the homozygous LTA 1_1_1_1 patients, we could not confirm the results of Taylor's study on the complete cohort (Fisher's exact test, $n = 214$, $P = 0.215$) nor on luminal or fistulising forms of the disease (Table 3). We also evaluated the patients homozygous for the frequent allele of the rs2857713 and rsl041981 SNPs as performed by Taylor's group without confirming their results. Considering the biological response, no significant association was found between either the proportion of patients with a decrease of at least 25% in CRP level or a difference in the mean relative change in CRP and the studied haplotypes on the complete, luminal or fistulising cohorts. Similarly, we could not demonstrate any association between clinical or biological response to infliximab and individual SNPs studied separately on the complete cohort, as well as on the luminal and fistulising CD patients. These statistical analyses were performed considering the frequencies of the alleles (Table 4) as well as the frequencies of the three genotypes (data not shown).

**Table 1.** *Patients characteristics (n = 214)*

| **Characteristics** | | |
|---|---|---|
| Patients | Median age (interquartile range) | 38 (21-55) |
| | Median age at diagnosis (interquartile range) | 22 (11-33) |
| | Sex ratio: F/M (%) | 139 (65)/75 (35) |
| Disease | Luminal/fistulising (%) | 151 (70.8)/63 (29.2) |
| | Ileon (%) | 31 (14.7) |
| | Colon (%) | 62 (29.1) |
| | Ileo-colonic (%) | 99 (46.4) |
| | Upper gastrointestinal involvement (%) | 11 (5.1) |
| | Anal involvement (%) | 77 (36.2) |
| | Crohn's Disease Index Activity (mean±SD) | 241 (±97) |
| | Normalized C-reactive protein (mg/dl) (mean±SD) | 11.9 (±17.5) |
| Concomitant treatment | 5-Aminosalicylates (%) | 52 (24.5) |
| | Methotrexate (%) | 13 (6.2) |
| | Steroids (%) | 59 (27.6) |
| | Azathioprine/6-mercaptopurine (%) | 121 (56.8) |

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***Table 2.*** *Observed haplotype frequencies (%) and numbers of alleles (n) in responding and nonresponding patients (clinical response)*

| Haplotype | Nonresponders | Responders | *P* |
|---|---|---|---|
| 1_1_1_2_1_1 (*n*) | 2.2 (3) | 4.3 (11) | 0.2146 |
| 1_1_2_2_1_1 (*n*) | 1 7.8 (24) | 1 8.6 (47) | 0.8072 |
| 1_1_2_2_2_1 (*n*) | 5.9 (8) | 5.1 (13) | 0.7637 |
| 1_2_1_1_1_1 (*n*) | 38.5 (52) | 38.4 (97) | 0.9604 |
| 1_2_2_1_1_1 (*n*) | 1.5 (2) | 2.7 (7) | 0.3336 |
| 2_1_1_1_1_2 (*n*) | 31.8 (43) | 29.6 (75) | 0.7045 |
| 2_1_2_1_1_2 (*n*) | 2.2 (3) | 1.2 (3) | 0.3533 |
| Total | 135 | 253 | 0.3533 |

*The haplotypes (noted 1 for the major allele, considering, respectively, rs909253_ rs746868_ rs5875327_ rs2857713_ rs3093543_ rs1041981) were rebuilt with Phase software version 2.1. For each subgroup of patients (responders/nonresponders), the frequencies of the haplotypes and the number of alleles (n) are reported. P-values were calculated using the chi-square test or Fisher's exact test when appropriate, considering a total of 388 alleles.*

***Table 3.*** *Polymorphisms allele frequencies and P-values considering the clinical response*

| | | Minor allele frequency percentage (number of alleles) | | P |
|---|---|---|---|---|
| Microsatellite or SNP ID | Disease type | Nonresponders | Responders | Alleles |
| TNFc $(GA)_{8/10}$ | All | 28.4 (40) | 28.2 (79) | 0.145 |
| | Luminal | 28 (31) | 29.6 (54) | 0.303 |
| | Fistulising | 30 (9) | 24.5 (24) | 0.099 |
| rs909253 (A/G) | All | 35.1 (53) | 30.7 (86) | 0.186 |
| | Luminal | 32.2 (39) | 30.2 (55) | 0.188 |
| | Fistulising | 46.7 (14) | 31.6 (31) | 0.362 |
| rs2857713 (A/G) | All | 25 (37) | 30 (84) | 0.202 |
| | Luminal | 26.3 (31) | 32.4 (59) | 0.414 |
| | Fistulising | 20 (6) | 25.5 (25) | 0.099 |
| rs1041981 (A/C) | All | 35.8 (53) | 31.8 (89) | 0.421 |
| | Luminal | 33 (39) | 31.9 (58) | 0.297 |
| | Fistulising | 46.7 (14) | 31.6 (31) | 0.321 |
| rs746868 (C/G) | All | 37.8 (56) | 39.6 (111) | 0.370 |
| | Luminal | 38.1 (45) | 37.4 (68) | 0.505 |
| | Fistulising | 20 (6) | 41.8 (41) | 0.026 |
| rs3093543 (A/C) | All | 5.4 (8) | 5.7 (16) | 0.535 |
| | Luminal | 6.8 (8) | 6.6 (12) | 0.567 |
| | Fistulising | 0(0) | 4(4) | 0.347 |

*For each polymorphism, the minor allele frequencies and allele number are noted for each subgroup of patients (complete cohort, luminal or fistulising form, n = number of patients) and the clinical response (nonresponders and responders). P-values were obtained after comparison of allele frequencies between responders and nonresponders, on all (n=214), luminal (n=150) or fistulising patients (n = 64).*

***Table 4.*** *LTA 1_1_1_1 halotpype frequencies (%) in responding and nonresponding patients for clinical response*

| | Clinical response | | | Biological response | | |
|---|---|---|---|---|---|---|
| | Responders | Nonresponders | *P* | Responders | Nonresponders | *P* |
| Complete cohort *(η)* | 1 6.9 (20) | 10.4 (7) | 0.22 | 3.8 (1) | 10.6 (12) | 0.40 |
| Luminal disease *(η)* | 5.2 (9) | 5.3 (6) | 0.58 | 5(1) | 7(6) | 0.78 |
| Fistulising disease *(n)* | 12.6 (11) | 3.4 (1) | 0.16 | 0(0) | 22.2 (6) | 0.22 |

*For each subgroup of patients, the frequencies of homozygous patients for the LTA 1_1_1_1 haplotype (respectively rs909253_rs5875327_rs2857713_rs1041 981, noted 1 for the major allele) and the number of homozygous patients (η) are reported. P-values considering both clinical and biological response were obtained after comparison of each haplotype frequency in both groups of responding and nonresponding patients. The clinical response was evaluated on 214 patients (150 with a luminal disease and 64 with a fistulising disease), whereas 139 patients were evaluated for the biological response (106 with a luminal disease and 33 with a fistulising disease).*

## Discussion

Genetic factors may be implicated in the response rate to infliximab. Several genes have already been studied to associate genetic variation with clinical or biological response to this drug [13]. In the present study, we focused on the previously described association between a LTA haplotype and the lack of response to infliximab.

The LTA gene is localized on the short arm of chromosome 6 within the region of the major histocompatibility complex, and this region has been associated with variation in immune response and with predisposition to CD [14]. Recent studies have also demonstrated a correlation between this location and the response to infliximab in rheumatoid arthritis [6]. Several genes controlling the immune response are located within this region, such as the TNF and LTA genes, encoding for two closely related cytokines. Several polymorphisms in the TNF promoter have already been studied on large CD cohorts without any evidence for an association between these polymorphisms and the response to infliximab [15,16]. However, the LTA 1_1_1_1 haplotype was shown to be significantly associated with a lack of response to infliximab [7]. A genetic variation in this region, which would be in linkage disequilibrium with the described haplotype, could be suspected to influence the response rate to infliximab. The implication of a genetic variation in the LTA gene itself or in the TNF gene is attractive considering the role of these cytokines in immunity and inflammation. However, before any further study of this genetic region, a confirmation of the implication of the LTA 1_1_1_1 haplotype was required considering the small size of the previously studied cohort.

The present study included 214 Caucasian patients who were treated with infliximab. The response to infliximab was evaluated considering both clinical and biological data. We studied the complete cohort as well as the luminal or fistulising forms separately. Despite the same clinical classification as used in Taylor's study, we did not find any association between the previously described haplotype and the response to infliximab. Considering also other criteria of response as fistula discharge for fistulising disease or a biological response based on the CRP level, no association was found between response to the drug and the studied LTA haplotype. The studied polymorphisms are in linkage disequilibrium with TNF microsatellite haplotypes, consisting of five microsatellites which encompass the region, including the LTB, TNF and LTA genes. By extension, the lack of association observed in the present study could also be considered for these haplotypes, with a diminution of power. Other candidate genes involved in TNF signalling pathways should be studied in reasonably large cohorts, with a strict definition of drug response.

The cohort of Taylor *et al.* [7] was relatively small and reliable results could not be expected without a larger confirmatory cohort. Considering our cohort size, the haplotype frequency and various degrees of influence of the LTA haplotype on the treatment response rate, we evaluated the probability of detecting a significant association between the haplotype and the response to infliximab. If we consider a decrease in the response rate of 20, 40 or 70% (associated with the LTA haplotype), the probability of finding an association with Taylor's cohort was, respectively, of 0.094, 0.314 and 0.675. When considering an effect of 40% and the size of our cohort, the risk of missing a real association is less than 10% and this drops to less than 1% if the effect is greater than 50%. These results suggest that the size of our cohort is sufficient to exclude a response rate reduction of at least 40% associated with the LTA 1_1_1_1 haplotype. However, the probability of detecting an effect of 20% or less with our cohort falls to 0.346 and the cohort size should be increased to 600 or 1500 patients to exclude a potential effect of 20 and 10%, respectively, of this haplotype on the prevalence of response.

It is likely that the response to infliximab is a multifactorial mechanism involving both environmental and genetic factors. The implication of genetic determinants on the response is probably weak. Therefore, independent pharmacogenetic studies should carefully evaluate clinical and biological response in large cohorts of patients. As very few groups will be able to study such cohorts, large multicenter studies or meta-analysis will need to be performed to provide definitive evidence for the presence or absence of any association between the LTA haplotypes and infliximab response. Negative and positive studies should thus be reported or collected to be included in such meta-analysis.

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