

NOD2, CD14 and TLR4 mutations do not influence response to adalimumab in patients with Crohn's disease: a preliminary report

M. Barreiro-de Acosta¹, S. Ouburg², S. A. Morré², J. B. A. Crusius², A. Lorenzo¹, J. Potel³, A. Salvador-Peña² and J. E. Domínguez-Muñoz¹

¹Gastroenterology. University Hospital of Santiago de Compostela, Spain. ²Laboratory of Immunogenetics. Dept. Pathology. VU University Medical Center. Amsterdam, The Netherlands. ³Surgery. University Hospital of Santiago de Compostela, Spain

ABSTRACT

Introduction: adalimumab is a recombinant fully-human monoclonal immunoglobulin (IgG1) antibody utilized in the treatment of Crohn's disease. Unfortunately no clinical or genetic markers exist to predict response to anti-tumor necrosis factor- α (TNF) therapy. The aim of this study was to evaluate the association between selected genes involved in cytokine regulation and response to adalimumab treatment in Crohn's disease.

Methods: twenty-four patients with Crohn's disease either naïve ($n = 8$) or had lost response or were unable to tolerate the chimeric anti-TNF antibody infliximab ($n=16$) were enrolled in the study. Patients were genotyped for main polymorphisms in NOD2, CD14 and TLR4 genes. Response to adalimumab treatment was defined as a decrease of Crohn's disease activity index of at least 100 points or a closure of at least 50% of fistulas in case of fistulizing Crohn's disease.

Results: overall, 75% of patients did respond to treatment. However, no statistically significant association was found between any of the genotypes and the response to adalimumab.

Conclusions: in our small study group no association between the studied polymorphisms and response to adalimumab was apparent. Systematic studies to search for genetic markers of response to anti-TNF therapy are necessary.

Key words: Crohn's disease. Genotypes. Adalimumab.

Barreiro-de Acosta M, Ouburg S, Morré SA, A. Crusius JB, Lorenzo A, Potel J, Salvador Peña A, Domínguez-Muñoz JE. NOD2, CD14 and TLR4 mutations do not influence response to adalimumab in patients with Crohn's disease: a preliminary report. *Rev Esp Enferm Dig* 2010; 102: 591-595.

Recibido: 30-03-10.
Aceptado: 15-04-10.

Correspondencia: Manuel Barreiro-de Acosta. Gastroenterology. University Hospital of Santiago. C/ Choupana, s/n. 15704 Santiago. A Coruña, Spain. e-mail: manubarreiro@hotmail.com

INTRODUCTION

Although recent studies have identified several genes linked to Crohn's Disease (CD), the discovery of NOD2/CARD15 gene in 2001 by two independent groups (1,2) was the first CD susceptibility gene. NOD2 single nucleotide polymorphisms (SNPs) also have been associated with different clinical forms of the disease in a number of populations, including some studies in Spain (3,4). In our country a great heterogeneity among different regions has been described (5), even in a study performed in Asturias not association between NOD2 and CD was showed (6).

Toll-like receptors (TLRs) play a key role in the recognition of bacteria, virus and other pathogens. Variants of toll-like receptor-4 (TLR4) and CD14 genes (components of the lipopolysaccharide receptor complex) may also be relevant to Crohn's disease susceptibility, as has been revealed by recent phenotype-genotype TLR-4 and CD14 mutation analyses (7-10).

Adalimumab (ADA) is a recombinant fully-human monoclonal immunoglobulin (IgG1) antibody utilized in the treatment of CD, among other inflammatory and immune disorders (11). The mechanism of action of ADA relies on neutralizing the biological function of tumor necrosis factor (TNF α) by blocking its interaction with TNF receptors (12). Compared to other treatments of reference such as infliximab, the subcutaneous administration of ADA offers patients an advantage by not having to depend on infusion centers. Additionally, treatment with ADA is an option on those patients who have lost response to or are intolerant to infliximab (13-15). According to clinical trials, this represents a third of the patients under infliximab during the first year of therapy (16).

No predictive factors of response to ADA have been identified as yet (17). We hypothesized that mutations in

CD susceptibility genes might be involved in response to treatment. ADA suppresses nuclear factor κ B (NF- κ B) through the inhibition of TNF- α , whereas NOD2/CARD15 activates NF- κ B thus potentially favoring the production of TNF- α . In this way, NOD2 mutations may be involved in altered activity of the NF- κ B gene which in turn could be related to differential response to biologic treatment. TLR4 and its transmembrane co-receptor CD14 signaling pathway results in the activation of NF- κ B and triggers pro-inflammatory cytokine production.

The objective of the present study is to assess whether SNPs in NOD2 (R702W, G908R and 1007fs), in TLR4 (Asp299Gly) and in CD14 are predictive factors for differential clinical response to treatment with ADA.

METHODS

Twenty-four adult patients with CD attending the University Hospital of Santiago who had been treated with ADA were enrolled in the study. Inclusion criteria were: either naïve to ADA ($n = 8$) or had lost response or were unable to tolerate infliximab ($n=16$). All patients were stratified on the basis of age, years of disease, gender, and disease evolution according to the Montreal classification (18). From each patient, 15 ml of venous blood was collected in EDTA tubes. Samples were kept at -20°C until DNA extraction. Genotype analysis for the three main variants of the NOD2 gene associated with CD: R702W, G908R and 1007fs (also called SNP8 (dbSNP ID rs2066844), SNP12 (rs2066845) and SNP13 (rs2066847), respectively) as well as for CD14 (rs2569190) and TLR4 (rs4986790) was conducted for all patients. Genotyping was performed by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Following PCR, the amplicons were digested and products were separated on a 2% (G908R, CD14 -260 and TLR4 896) or 4% (R702W and 1007fs) agarose gel.

The NOD2 R702W SNP was assessed using the forward primer 5'-CGCACAACCTTCAGATCACA-3', and the reverse primer 5'-GGATGGAGTGGAAGTGCTTG-3'. The PCR product was digested with HpaII (5 h at 37°C). The G908R SNP was evaluated utilizing the forward primer 5'-AAGTCTGTAATGTAAAGCCAC-3', and the reverse primer 5'-CCCAGCTCCTCCCTCTTC-3', and the PCR product digested with HhaI (5 h at 37°C). The 1007fs variant was assayed according to Crane et al (19). The primers employed were 5'-GGCAGAAGCCCTCCTGCAGGGCC-3' (forward) and 5'-CCTCAAAATTCTGCCATTCC-3' (reverse) and the restriction enzyme used was ApaI (11 h at 30°C). The C/T SNP at -260 of the CD14 gene was analyzed using the forward primer 5'-CCTGCAGA-ATCCTTCCTGTT-3' and the reverse primer 5'-TCACCTCCCCACCTCTCT-3'. The PCR product was di-

gested with HaeIII (5 h at 37°C) (20). The A/G SNP at 896 in the TLR4 gene was analyzed using the forward primer 5'-AGCATACTTAGACTACCTCCATG-3' and the reverse primer 5'-TTACCCCTTCAATAGTCACACTCA-3' (21). The PCR product was digested with NcoI.

Response to treatment was assessed at 52 weeks by two independent gastroenterologists, one being unaware of the mutation status. Clinical response to ADA treatment in patients with CD was defined as a decrease from baseline CDAI of at least 100 points. For patients with fistulizing CD ($n = 21$), treatment response was defined as closure of at least 50% of the fistulas present at the beginning of treatment. Loss of response to ADA was defined as an initial good response followed by a subsequent reduction in the efficacy of treatment requiring a dosage increase to achieve the maintenance of clinical response. Adverse events were defined as those events leading to withdrawal from the study.

Statistical comparisons were conducted using the χ^2 test or the Fisher's exact test when appropriate. Statistical significance was set to $p < 0.05$. Analyses were carried out using SPSS 12.0 software v. 12.0 and Epidat software package.

This study was performed following the ethical principles of the Declaration of Helsinki for medical research involving human subjects and was approved by the Ethical Committee of Clinic Investigation of Galicia.

RESULTS

Among a group of 165 CD patients, a total of 24 patients (mean age 40.5 years, mean duration of disease 10 years, female 66.6%) received treatment with ADA. Eight patients were naïve to ADA, and 16 were intolerant to or had lost response to infliximab. A total of 33% of patients had luminal inflammatory CD (Montreal classification B1) and 66% had fistulizing CD (Montreal classification B3). Clinical data and demographic characteristics of the population are summarized in table I.

As for treatment response, 75% of patients responded to ADA, 16.7% lost response to treatment and 12.5% terminated treatment due to adverse events. No differences in response were observed according to disease evolution (inflammatory or fistulizing CD), nonetheless a higher percentage of naïve patients achieved response to treatment in comparison to non-naïve patients.

Six (25%) patients were carriers of at least one of the three NOD2 mutations. The TLR4 896 allele G was found in 4.2% of CD patients, whereas the CD14 -260 allele C was present in 62.4% of CD patients.

Response to treatment did not differ between patients with or without mutations in NOD2 (overall presence or any of the mutations separately), TLR4 896 and CD14 -260 (Fig. 1). Additionally, no differences were observed

Table I. Demographic characteristics and clinical data

	<i>Patients, n (%)</i>
Total number	24
Gender (Males/Females)	8 (33.3)/16 (66.6)
Smokers (current or ex-smokers)	15 (62.5)
Age	
Range	26-58
Mean \pm SD	40.5 \pm 9.5
Family history of IBD	2 (8.3)
Age at diagnosis	
< 40 years (A1)	23 (95.6)
> 40 years (A2)	1 (4.2)
Disease evolution - Phenotype	
Inflammatory (B1)	8 (33.3)
Stricturing (B2)	0 (0)
Penetrating (B3)	16 (66.6)
Disease location	
Terminal ileum (L1)	9 (37.5)
Colon (L2)	3 (12.5)
Ileum-colon (L3)	12 (50.0)
Upper gastrointestinal tract (L4)	0 (0)
Extraintestinal manifestations	12 (50.0)

IBD: inflammatory bowel disease.

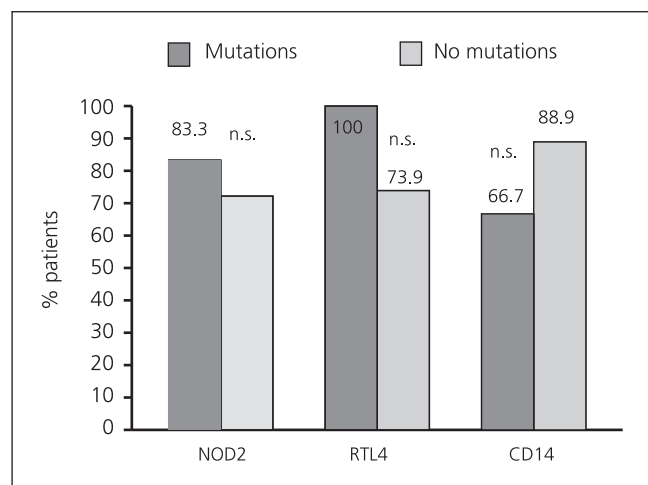


Fig. 1. Percentage of patients carrying mutations in NOD2, TLR4 and CD14 who show response to ADA treatment. No statistically significant differences of response were observed between genotypes.

with regard to adverse events or lost of response between patients who were carriers when compared with non-carriers of any of the studied polymorphisms (Table II).

DISCUSSION

Biological therapies have shown great efficacy in the treatment of CD particularly in severe cases and in patients who fail to respond to conventional therapies

Table II. Percentage of patients carrying mutations in NOD2, TLR4 and CD14 who show loss of response or adverse reactions

	<i>Loss of response</i>			<i>Adverse reaction</i>		
	Yes	No	<i>p</i>	Yes	No	<i>p</i>
NOD2	25	25	n.s.	0	28	n.s.
TLR4	0	5	n.s.	0	4	n.s.
CD14	75	60	n.s.	62	66	n.s.

such as corticosteroids (22-24). Nonetheless, although most of the patients have a good response to treatment, not all patients show a benefit from biological therapies and some patients are refractory to these novel molecules. Unfortunately, despite several studies of potential predictors to clinical response, no gene variants predictive of biological therapy response have yet been identified.

In our study, we aimed to establish an association between mutations on CARD15, TLR4 and CD14 and response to ADA treatment. Our rationale is based on the mechanism of action of newer biological therapies, such as infliximab and adalimumab, which depend on the suppression of the NF- κ B system through inhibition of TNF- α . Because Nod2 activates NF- κ B and potentially interacts with TNF- α production, mutations in these genes could result in an altered NF- κ B activation. An evaluation at the genetic level could contribute to reveal the mechanism responsible for the therapeutic response of biological therapy seen in clinical practice (25).

Indeed, in rheumatoid arthritis, an association between certain genotypes and differential response to ADA has been reported. As an example, one study has shown that patients bearing the GGC haplotype of TNF gene (-238G/-308G/-857C) in a homozygous form showed low response rate to ADA treatment (26), whereas in another study, TNF -308 homozygous (G/G) patients showed better response than heterozygous (G/A) patients (27). In other studies the development of antibodies directed against adalimumab has been shown to be associated with interleukin- (IL)10 polymorphisms (28).

Regarding CD, in previous studies from Leuven (Belgium) three polymorphisms in apoptosis genes had been identified potentially influencing the response to infliximab in luminal and fistulizing CD patients. The strongest association was seen between the Fas ligand -843 TT genotype and non-response to infliximab (29,30). With respect to the genes we studied, two independent studies with a large number of patients also excluded NOD2 gene variants as predictors for response to infliximab (31,32).

Our study supports the results that have been reported in another study (n=16) which analyzed possible associations between response to ADA and NOD2 mutations, but in our study in a higher number of patients (33). Regarding potential influence of mutations in loss of response or development of adverse events, there are not specific studies about this setting and will be necessary a higher number of patients to extrapolate conclusions. Moreover, here we show for the first time, with the limitation of the number of patients, that there is no association between biologic treatment response and TLR4 and CD14 mutations

Nonetheless, the low sample size of our population and the little number of patients with NOD2 or TLR4 mutations which may be related to the relative homogeneity of our population, may limit the extrapolation of our outcomes. Another limitation is that we also evaluated response and we have no data on remission.

In conclusion, in our Spanish Galician population response to ADA was not associated with CD susceptibility variants in the NOD2, TLR4 and CD14 genes. Subsequent studies in a larger and more heterogeneous population should be performed.

REFERENCES

- Hugot JP, Chamaillard M, Zouali H, Lesage S, Cézard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; 411: 599-603.
- Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; 411: 603-6.
- Peña AS. The significance of CARD15 mutations in Crohn's disease. The Spanish contribution. *Rev Esp Enferm Dig* 2007; 99: 563-9.
- Barreiro M, Núñez C, Domínguez-Muñoz JE, Lorenzo A, Barreiro F, Potel J, et al. Association of NOD2/CARD15 mutations with previous surgical procedures in Crohn's disease. *Rev Esp Enferm Dig* 2005; 97: 547-73.
- Barreiro-de Acosta M, Mendoza JL, Lana R, Domínguez-Muñoz JE, Díaz-Rubio M. NOD2/CARD15: Geographic differences in the Spanish population and clinical applications in Crohn's disease. *Rev Esp Enferm Dig* 2010; 102: 321-6.
- Rodrigo L, Martínez-Borra J, Garrote JA, Niño P, León AJ, Riestra V, et al. CARD15 mutations are poorly related to Crohn's disease phenotypes in Asturias. *Rev Esp Enferm Dig* 2007; 99: 570-5.
- Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 2004; 53: 987-92.
- Ouburg S, Mallant-Hent R, Crusius JB, van Bodegraven AA, Mulder CJ, Linskens R, et al. The toll-like receptor 4 (TLR4) Asp299Gly polymorphism is associated with colonic localisation of Crohn's disease without a major role for the *Saccharomyces cerevisiae* mannan-LBP-CD14-TLR4 pathway. *Gut* 2005; 54: 439-40.
- Peña AS, Peñate M. Genetic susceptibility and regulation of inflammation in Crohn's disease. Relationship with the innate immune system. *Rev Esp Enferm Dig* 2002; 94: 351-60.
- Hume GE, Fowler EV, Doecke J, Simms LA, Huang N, Palmieri O, et al. Novel NOD2 haplotype strengthens the association between TLR4 Asp299gly and Crohn's disease in an Australian population. *Inflamm Bowel Dis* 2008; 14: 585-90.
- Lichtenstein GR, Panaccione R, Mallarkey G. Efficacy and safety of adalimumab in Crohn's disease. *T. Ther Adv Gastroenterol* 2008; 1: 43-50.
- Sorbera LA, Rabasseda X, Castaner RM. Adalimumab. *Drugs Future* 2001; 26: 639-46.
- Ho GT, Smith L, Aitken S, Lee HM, Ting T, Fennell J et al. The use of adalimumab in the management of refractory Crohn's disease. *Aliment Pharmacol Ther* 2008; 24: 308-15.
- Hinojosa J, Gomollon F, Garcia S, Bastida G, Cabriada JL, Saro C, et al. Efficacy and safety of short-term adalimumab treatment in patient with active Crohn's disease who lost response or showed intolerance to infliximab: a prospective, open-label, multicentre trial. *Aliment Pharmacol Ther* 2007; 25: 409-18.
- López Palacios N, Mendoza JL, Taxonera C, Lana R, Fuentes Ferrer M, Díaz-Rubio M. Adalimumab induction and maintenance therapy for Crohn's disease. An open-label study. *Rev Esp Enferm Dig* 2008; 100: 676-81.
- Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF et al. Maintenance infliximab for Crohn's disease: The accent I randomised trial. *Lancet* 2002; 359: 1541-9.
- Koolos WM, de Jong DJ, Huizinga TWJ, Guchelaar HJ. Potential role of the pharmacogenetics in anti-TNF treatment of rheumatoid arthritis and Crohn's disease. *Drug discovery today* 2007; 12: 125-31.
- Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; 19: 5-36.
- Crane AM, Bradbury L, Van Heel DA, McGovern DP, Brophy S, Rubin L, et al. Role of NOD2 variants in spondylarthritis. *Arthritis Rheum* 2002; 46: 1629-33.
- Heesen M, Bloemeke B, Bachmann-Mennenga B, Kunz D. The CD14-260 C --> T promoter polymorphism co-segregates with the tumor necrosis factor-alpha (TNF-alpha)-308 G --> A polymorphism and is associated with the interleukin-1 beta (IL-1 beta) synthesis capacity of human leukocytes. *Eur Cytokine Netw* 2002; 13: 230-3.
- Pålsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 2004; 113: 153-62.
- Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; 337: 1029-35.
- Colombel JF, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, et al. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; 132: 52-65.
- Clark M, Colombel JF, Feagan BC, Fedorak RN, Hanauer SB, Kamm MA et al. American Gastroenterological Association consensus development conference on the use of biologics in the treatment of inflammatory bowel disease June 21-23 2006. *Gastroenterology* 2007; 133: 312-39.
- Baert FJ, D'Haens GR, Peeters M, Hiele MI, Schaible TF, Shealy D, et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology*. 1999; 116: 22-8.
- Miceli-Richard C, Comets E, Verstuyft C, Tamouza R, Loiseau P, Ravaud P et al. A single tumour necrosis factor haplotype influences the response to adalimumab in rheumatoid arthritis. *Ann Rheum Dis* 2008; 67: 478-84.
- Cuchacovich M, Soto L, Edwades M, Guterrez M, Llanos C, Pacheco D et al. Tumour necrosis factor (TNF)alpha -308 G/G promoter polymorphism and TNF-alpha levels correlate with a better response to adalimumab in patients with rheumatoid arthritis. *Scan J Rheumatol* 2006; 35: 435-40.
- Bartelds GM, Wijbrandts CA, Nurmohamed MT, Wolbink GJ, de Vries N, Tak PP, et al. Anti-adalimumab antibodies in rheumatoid

- arthritis patients are associated with interleukin-10 gene polymorphisms. *Arthritis and rheumatism* 2009; 60: 2541-2.
29. Hlavaty T, Pierik M, Henckaerts L, Ferrante M, Joossens S, van Schuerbeek N, et al. Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease. *Aliment Pharmacol Ther* 2005; 22: 613-26.
30. Hlavaty T, Ferrante M, Henckaerts L, Pierik M, Rutgeerts P, Vermeire S. Predictive model for the outcome of infliximab therapy in Crohn's disease based on apoptotic pharmacogenetic index and clinical predictors. *Inflamm Bowel Dis* 2007; 13: 372-9.
31. Vermeire S, Louis E, Rutgeerts P, De Vos M, Van Gossum A, Be-laiche J, et al. NOD2/CARD15 does not influence response to infliximab in Crohn's disease. *Gastroenterology* 2002; 123: 106-11.
32. Mascheretti S, Hampe J, Croucher PJ, Nikolaus S, Andus T, Schubert S, et al. Response to infliximab treatment in Crohn's disease is not associated with mutations in the CARD15 (NOD2) gene: an analysis in 534 patients from two multicenter, prospective GCP-level trials. *Pharmacogenetics* 2002; 12: 509-15.
33. Seiderer J, Brand S, Dambacher J, Pfennig S, Jürgens M, Göke B, et al. Adalimumab in patients with Crohn's disease--safety and efficacy in an open-label single centre study. *Aliment Pharmacol Ther* 2007; 25: 787-96.