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

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Infliximab induces remission in 30–40% of patients with active Crohn's disease. Treatment response is a stable trait over repeated doses yet the clinical predictors of response are still unknown. Recently, three variants in the CARD15 gene have been identified as major genetic risk factors for Crohn's disease. Single nucleotide polymorphisms (SNPs) 8, 12 and 13, have been shown to be independently associated with Crohn's disease susceptibility. The aim of the present study was to investigate these variants in relation to the therapeutic efficacy of infliximab. SNPs were genotyped (TaqMan) in two cohorts ($n = 90$ and $n = 444$ (ACCENT I)) of active Crohn's disease patients (CDAI 220–450). The patients were recruited from independent multicenter trials conducted according to GCP. At the start of both trials, patients received a single infusion of open label infliximab (5 mg/kg bodyweight). The genotypic and allelic frequencies of each SNP were significantly associated with Crohn's disease in comparison to 370 healthy controls as reported previously. Response to infliximab (drop in CDAI > 70 points or remission, respectively) was not associated with the genetic variants in the CARD15 gene in either cohort. The subsequent negative findings in a two-cohort model exclude SNPs 8, 12 and 13 of the CARD15 gene as predictors for therapeutic response to infliximab treatment.
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Introduction

Crohn's disease is a complex disorder characterized by chronic relapsing intestinal inflammation [1]. Clinical features include the development of abdominal pain, diarrhea, intestinal stenoses, abdominal abscesses and enteric fistulae. Lifetime prevalence of Crohn's disease is estimated to be as high as 0.5% in some areas of Western industrialized countries [2]. Although glucocorticoids represent an effective short-term treatment of acute relapse in most patients [3,4], long-term maintenance of remission proves difficult in many patients. It is estimated that at least 50% of patients develop glucocorticoid-refractory or glucocorticoid-dependent disease.

Monoclonal antibodies that bind specifically to tumor necrosis factor-α (TNF-α; infliximab, CDP571) have been shown to be an effective treatment for active, therapy-refractory, Crohn's disease [5–7]. Treatment with a single infusion of infliximab has been demonstrated to induce clinical remission, accompanied by healing and restitution of the intestinal microarchitecture (as determined by endoscopy), in approximately 30–40% of Crohn's disease patients. Groups receiving different doses of infliximab (5, 10 and 20 mg/kg bodyweight) do not show any significant differences in the frequency of response [6,8]. Furthermore, this selective response appears to be a stable phenotype even after repeated dosing. These features, together with a lack of clinical markers for the prediction of response, plus the potential (although rare) side effects [9–11] and the high price of the therapy, indicate the need for a pharmacogenetic approach to predicting therapeutic efficacy.

The first major Crohn's disease susceptibility gene (*IBD1* on chromosome 16q12) has been recently identi-

fied through a positional cloning strategy [12]. CARD15 (NOD2) is a member of the Apaf-1/Ced-4 super-family of apoptosis regulators, which also includes NOD1, CIITA, NAIP, DefCap, Nalp2 and Mater [13]. These proteins all possess a nucleotide-binding oligomerization domain, and many also have N-terminal caspase recruitment domains (CARD) and a C-terminal leucine rich region (LRR). Unlikely other members of the family such as NOD1 and Apaf-1 that are expressed, at varying levels, in all adult tissues [14–16], CARD15 is expressed primarily in monocytes [17]. The CARD15 protein has been shown to activate nuclear factor (NF)-κB through interactions with its CARD domains and these may be also involved in NF-κB activation in response to bacterial lipopolysaccarides (LPS) [13,17–19].

Three variants in the CARD15 gene, SNP8, SNP12 and SNP13 (following the nomenclature of Hugot *et al.* [12]), have been shown to be independently associated with Crohn's disease susceptibility in several populations [12,19,20]. The rare alleles of these three variants were not found on the same haplotype [12]. SNP8 and SNP12 are non-conservative variants in the LRR (SNP12: G881R [12]/G908R [20]; SNP8: R675W [12]/R702W [20]). SNP13 (980fs [12]/3020insC [20]) is a cytosine insertion at nucleotide position 3020 in exon 11. This results in a frameshift and a predicted protein truncation in the LRR [12,19,20]. The rare allele frequency of SNP8, SNP12 and SNP13 in several healthy control populations has been shown to be approximately 4%, 1–2% and 2–4% respectively. In Crohn's disease patients the frequency is elevated to approximately 11%, 6% and 12% respectively [12,19, 20]. It appears that these variants alter the CARD15-mediated induction of NF-κB by LPS [17–19,21] and hence, may influence the regulation of TNF expression. An influence of these CARD15 variants on the response to anti-TNF therapies therefore appears possible.

Pharmacogenetic studies aim to investigate the putative genetic background in differential responses to therapies or of adverse drug reactions [22]. Previous studies have included investigations into genes coding for drug-metabolizing enzymes such as the cytochrome P450 [23,24] and thiopurine methyl transferase (TPMT) [25–28]. In addition to polymorphisms, which may alter drug metabolism, genetic variability can influence the biological effect of drugs. For example, the 3–6 tandem repeat of the Sp-1 binding motif in the promoter of the 5-lipoxygenase (ALOX5), which diminishes gene transcription, is associated with a lack of improvement in asthmatic patients after treatment with drugs inhibiting ALOX5 [29].

Since variants of the CARD15 gene are clearly associated with a genetic risk of developing Crohn's disease and are linked to the NF-κB–TNF-α pathway, we investigated the potential association between disease relevant variants and treatment response to infliximab. The study has been designed to investigate two independent cohorts of patients from clinical trials in order to minimize the risk for type I errors and to maximize the power to detect biological effects. The results clearly exclude any association between the Crohn's disease causative variants in the CARD15 gene and response to treatment with infliximab.

Materials and methods

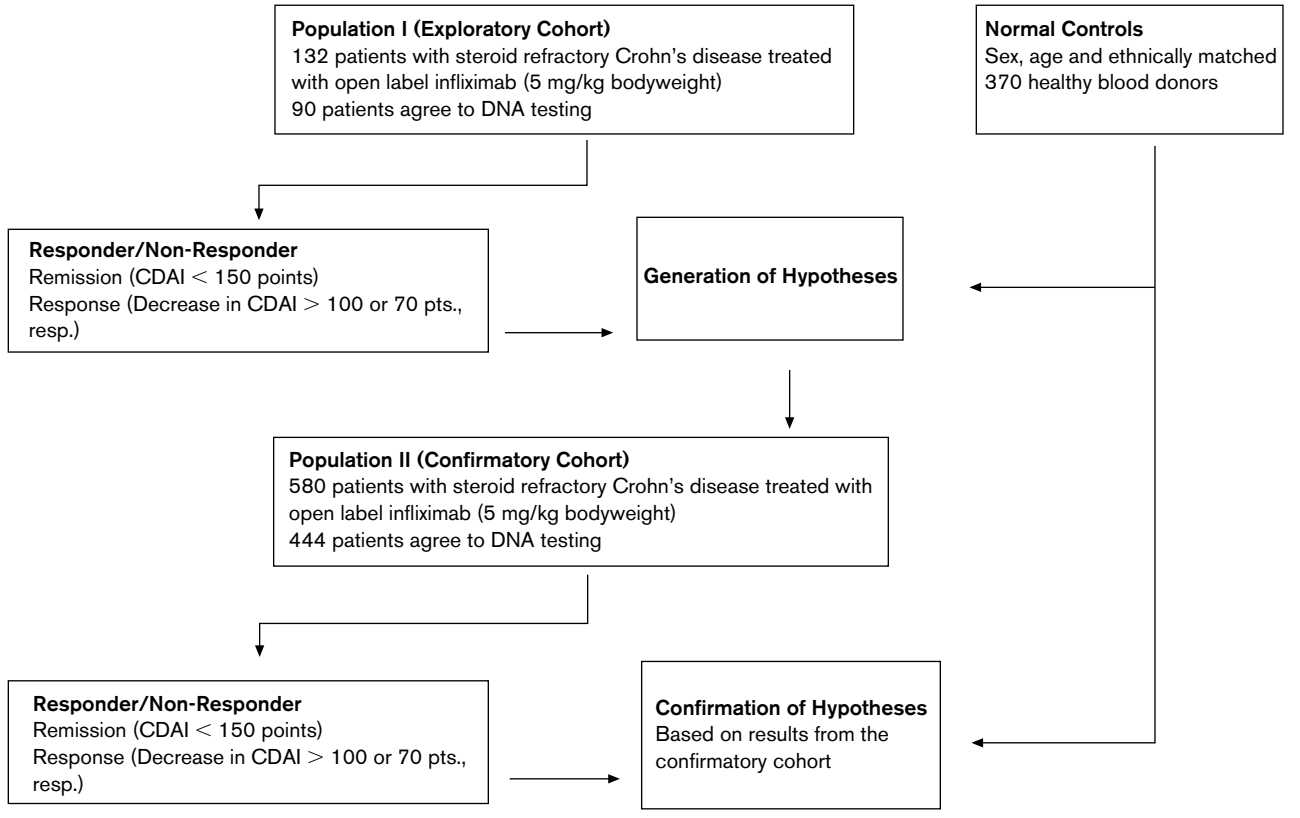
Prospective clinical trials

Patients from two prospective, multicenter clinical trials were investigated (Fig. 1). The first trial was conducted in 39 German centers with the support of a grant from Schering Plough/Essex Pharmaceutical GmbH (Munich, Germany) and monitored under 'Good Clinical Practice'/'Convention of Helsinki' (GCP/ICH) standards [30]. A total of 154 patients were screened. Of the 132 patients who matched the inclusion and exclusion criteria, 90 (57 women and 33 men) volunteered to take part in the pharmacogenetic sub-study. Infliximab (5 mg/kg bodyweight) was administered open label to all patients. The second study (ACCENT I) was conducted by Centocor Pharmaceuticals Inc. (Malvern, PA and Leiden, The Netherlands) in 45 centers across North America, Europe, Canada and Israel. The study was performed under the United States Federal Drug Administration's investigational new drug status, using GPC/ICH level procedures. Of the 580 patients enrolled in the clinical trial, 444 patients agreed to participate in the genotyping study. In this trial, a single open label infusion of infliximab was administered to all patients with chronic active Crohn's disease at the beginning of the study. Patients were later randomized to a placebo controlled re-infusion protocol (which was not part of our assessment). The clinical protocols and the genetic test procedures received prior approval by the ethics committees/institutional review boards of all participating institutions. All patients gave written informed consent to the study, and to the pharmacogenetic substudy, before treatment and genotyping.

Patient selection

Inclusion criteria were similar to those used in previously published studies performed to establish the clinical efficacy of infliximab in Crohn's disease [6]. For the first trial, patients were required to have chronic, active Crohn's disease (CDAI 220–450) in the ileum and/or colon (>6 months since diagnosis) with, or without, fistulae. Previous unsuccessful therapies had to include at least one of the following: (i) glucocorticoids (>10 mg/day for at least 3 months with changes <20 mg (in the average daily dose) during the last 4

Fig. 1



Study design

weeks and < 5 mg (average daily dose) during the last 2 weeks); (ii) azathioprine (> 3 months, ⩾ 2 mg/kg bodyweight per day); (iii) 6-MP (> 3 months, ⩾ 1 mg/kg bodyweight and day); (iv) methotrexate (> 3 months). Age limits were 18–65. All patients received infliximab at a dose of 5 mg/kg bodyweight at week 0, with repetitions at weeks 2 and 6 for those patients having fistulae (44 of 90). The primary definition of a therapeutic response was a drop in the CDAI by at least 70 points 4 weeks after the first infusion. Remission, as a secondary end point, was defined as a CDAI value less than 150 points at week 4 [3,4].

The inclusion criterion for the second trial was chronic, active Crohn's disease (CDAI 220–400) in the ileum and/or colon (> 3 months since diagnosis by radiography or endoscopy) without draining fistulae. Concomitant medications such as aminosalicylates, corticosteroids, azathioprine, 6-mercaptopurine, mycophenolate mofetil and methotrexate were allowed at a stable dose before and during the trial. Crohn's disease-specific antibiotics had to be discontinued at least two weeks prior to prescreening. Minimum age was 18 years. All patients received infliximab in a dose of 5 mg/kg bodyweight at week 0. The primary definition of a therapeutic response was a drop in the CDAI of at least 70 points 2 weeks after the first infusion. Remission, as a secondary end point, was defined as a CDAI value less than 150 points [3,4].

Exclusion criteria in both trials were pregnancy, severe other disease (including irritable bowel syndrome and gastrointestinal infections), a foreseeable need for surgery, a documented or suspected fixed stenosis and prior treatment with anti-TNF-α. Other investigational drugs were not allowed within 30 days. Further laboratory exclusion parameters were leukocyte counts $>3.5 \times 10^9$/l, hemoglobin < 8.5 g/l, neutrophil counts $< 1.5 \times 10^9$/l and /or thrombocyte counts $< 100 \times 10^9$/l.

Genotyping

Single nucleotide polymorphisms 8, 12 and 13, corresponding to R702W, G908R and 3020insC, were submitted previously to GenBank [20]. The detection methods, sequence context and allele frequencies can be viewed at http://www.ncbi.nlm.nih.gov/SNP/snp_search.cgi?searchType=byPub&pub_id=601. These three SNPs were genotyped in the Crohn's disease patients included in the two clinical trials and additionally in 370 healthy individuals from a German popu-

lation (blood donors from Kiel). Genotyping was performed using allelic discrimination by TaqMan technology (ABI 7700, Applied Biosystems, Foster City, CA) [31]. The following primers and probes were used: SNP8 forward primer 5′ TTCCTGGCAGGGCTGTT GTC 3′, reverse primer 5′ AGTGGAAGTGCTTGCG GAGG 3′, 1st probe 5′ CCTGCTCCGGCGCCAGGC 3′, 2nd probe 5′ CCTGCTCTGGCGCCAGGCC 3′; SNP12 forward primer 5′ ACTCACTGACACTGTC TGTTGACTCT 3′, reverse primer 5′ AGCCACCTC AAGCTCTGGTG 3′, 1st probe 5′ TTCAGATTCT GGCGCAACAGAGTGGGT 3′, 2nd probe 5′ TTTT CAGATTCTGGGGCAACAGAGTGGGT 3′; SNP13 forward primer 5′ GTCCAATAACTGCATCACCTAC CTAG 3′, reverse primer 5′ CTTACCAGACTTC CAGGATGGTGT 3′, 1st probe 5′ CCCTCCTGCA GGCCCTTGAAAT 3′, 2nd probe 5′ CCTCCTGCA GGCCCCTTGAAAT 3′ [20]. Primers and probes were designed with Primer Express (Aplera, Foster City, Ca, USA) and synthesized through Eurogentec (Liege, Belgium). The amplification conditions involved two pre-PCR steps of 2 min at 50 °C and 10 min at 95 °C, followed by 35 cycles including a denaturation step at 95 °C for 15 s and an annealing step of 1 min at 62 °C in a final volume of 5 μl.

Statistics

All samples were recoded in a central laboratory and genotypes were assigned without knowledge of individual treatment response. The software packages SPSS and SAS were used for statistical data analysis [32,33]. For the evaluation of the response rates, a polynomial χ^2 test was used. The influence of the infusion schema in the first study was evaluated by logistic regression. The frequency of the SNPs in relation to clinical outcome was evaluated using Fisher's Exact and χ^2 tests. A multivariate analysis was conducted using the combined genetic variants and key clinical characteristics (sex, age of onset, disease location).

Results

Patients

By definition, the inclusion criteria in the first trial selected only patients with therapy-refractory disease. Although the inclusion of patients receiving only aminosalicylates was actually possible in the second trial, in reality very few patients had uncomplicated active disease. In the second cohort, 90.4% of all randomized patients were receiving some type of concomitant medication (i.e. gluccocorticoids, immunomodulatory agents, aminosalicylates, antibiotics, antidiarrheals, narcotic and opioid analgesics), with 51.1% of all randomized patients receiving glucocorticoids and 29.3% receiving immunomodulating agents (azathioprine, 6-mercaptopurine, methotrexate). In the first cohort (90 patients), 60% of patients showed a clinical response (reduction of CDAI of at least 70 points) and a remission rate (CDAI < 150 points) of 33% were observed at week 4. No significant differences between infusion schemas ($P = 0.262$) were found. The median drop in the CDAI was 110 points. In the second cohort (444 patients), the response rate was 62% (reduction of CDAI of at least 70 points) and the remission rate was 28%. The median drop in CDAI was 93 points. Population demography was not different between the cohorts with the exception of a small non-Caucasian admixture in the second cohort (2.1% Blacks, 0.9% Asian and 1.2% qualified as 'Others').

CARD15 variants

Single nucleotide polymorphisms 8, 12 and 13 (following Hugot *et al.* [12]) were investigated. In comparison between the Crohn's disease patients, which comprised a mostly therapy refractory population, and the 370 unrelated healthy controls these markers demonstrated a statistically significant association with Crohn's disease – thus confirming previous results [12,19,20]. The rare allele frequencies of SNP 8, 12 and 13 were 4%, 2% and 3% in the healthy controls and 8%, 5% and 8% in the Crohn's disease patients ($P = 0.002$, $P = 0.013$ and $P = 0.00012$), respectively. Frequencies for the homozygote variant, heterozygote and homozygote wild-type genotypes for SNP8 were 0.3%, 8% and 91.7% in the healthy controls against 2%, 13% and 85% in the Crohn's disease patients. For SNP12 these frequencies were 0%, 4% and 96% in the healthy controls and 0%, 8% and 92% in the patients, and for SNP13 the frequencies were 0%, 7% and 93% in the controls against 1%, 13% and 86% in the patients.

The combination of multiple variants could have additive effects in modulating clinical response. Table 1 shows the frequency of the different variants in the samples. Four main combinations of the SNPs 8, 12 and 13 were found: 2–2–2, 3–2–2, 2–2–3 and 2–3–2 (Table 1). They account for 93.2% of the patients, indicating that most patients carry no, or only one, variant.

CARD15 variants and response to infliximab

The allelic and genotypic frequencies of the three SNPs were tested in both cohorts for association with response to infliximab (drop of CDAI of at least 70 points), or for achievement of remission (CDAI < 150 points). None of the SNPs showed a significant association (all $P > 0.5$) with response, or remission, in the first cohort. The proportion of patients responding and not responding to the treatment was similarly distributed among the CARD15 genotypes (Table 2A and B). As a gene-dosage effect may play a role [12], the presence of combined variants was investigated (Table 1). Again, no association with treatment response was seen. It should also be noted that the number of these patients is very small. In the second cohort the number

Table 1 Genotype distribution and combinations of the CARD15 variants SNP8, SNP12 and SNP13 in the patients investigated

SNP8	SNP12	SNP13	Cohort 1	Cohort 2
1	2	2	1.16	1.56
1	2	3	0	0.26
2	2	1	3.49	0.52
2	2	2	63.95	69.61
2	2	3	16.28	7.53
2	3	2	2.33	5.71
2	3	3	1.16	2.08
3	2	1	0	0.26
3	2	2	8.14	10.39
3	2	3	3.49	1.3
3	3	2	0	0.78
			Total: 100%	Total: 100%

The table shows population frequencies (%) of combined genotypes of the three variants (SNP8, SNP12 and SNP13) in the CARD15 gene in the two cohorts ($n = 90$ and $n = 444$).
Genotypes were coded as '1' = variant homozygote, '2' = wild type homozygote and '3' = heterozygote

of hypotheses was therefore reduced to test whether the presence of any of the three CARD15 variants could be associated with treatment response (Table 2A and B). Again, a P value $\geqslant 0.5$ was obtained for every test. A multivariate analysis was conducted to investigate whether genetic variants may have an influence in patient subgroups, i.e. those defined by sex, age of onset or disease location (ileum, colon or both). Population and disease characteristics showed no direct influence on treatment response and no significant association between genetic variants and response was observed in any of the subgroups.

Discussion

Infliximab is a potent new therapy for refractory Crohn's disease. However, some patients demonstrate a sustained benefit from the therapy while others respond significantly less well [6,30,34]. Clinical predictors for efficacy are presently unknown. Therapeutic response to infliximab is a stable trait, with repeated administration inducing only very limited benefit in primary non-responders [6,30,35]. Therefore, the therapeutic response to infliximab appears suitable for a pharmacogenetic exploration.

In a complex disorder, such as Crohn's disease, different responses to medications might be due to different combinations of the primary disease genes in particular patients. The SNPs 8, 12 and 13 in the CARD15 gene, which have been described as major susceptibility factors for Crohn's disease, represent a suitable target for the pharmacogenetic investigation of response to infliximab. The rare alleles of these 3 SNPs in this gene have been shown to be independently associated with a risk for Crohn's disease [12,19,20].

Treatment with infliximab leads to a reduction in mucosal TNF expression and NF-κB activation [34,36]. It has been also suggested that the immunology of non-response to infliximab is characterized by a persistent activation of the NF-κB system and a rebound of TNF production [34]. Accumulation of NF-κB p65 in the colonic mucosa, preceding clinical relapse after treatment with infliximab, suggests that lack of response

Table 2A Genotype frequencies (%) of SNP8, SNP12 and SNP13 in responders versus non-responders (drop of CDAI of at least 70 points)

Genotype	Responders	Non-responders	Responders	Non-responders
	Cohort 1, $n = 90$		Cohort 2, $n = 444$	
SNP8 (–/–)	93	87	85	83
SNP8 (+/–)	7	13	14	14
SNP8 (+/+)	0	0	1	3
SNP12 (–/–)	93	97	91	92
SNP12 (+/–)	7	3	9	8
SNP12 (+/+)	0	0	0	0
SNP13 (–/–)	78	69	88	88
SNP13 (+/–)	20	22	11	11
SNP13 (+/+)	2	9	1	1

Table 2B Genotype frequencies (%) of SNP8, SNP12 and SNP13 in patients reaching remission (CDAI < 150 points) versus those not reaching remission

Genotype	Remission	No remission	Remission	No remission
	Cohort 1, $n = 90$		Cohort 2, $n = 444$	
SNP8 (–/–)	96	88	85	85
SNP8 (+/–)	4	12	13	14
SNP8 (+/+)	0	0	2	1
SNP12 (–/–)	93	95	93	91
SNP12 (+/–)	7	5	7	9
SNP12 (+/+)	0	0	0	0
SNP13 (–/–)	74	77	90	87
SNP13 (+/–)	23	18	10	12
SNP13 (+/+)	3	5	0	1

may be due to an early re-activation of the inflammatory cascade including the NF-κB pathway [34]. Mutations in the LRR of the CARD15 gene appear to alter NF-κB activation in response to bacterial (LPS) [13,17–19]. Therefore, polymorphisms in the CARD15 gene are attractive functional candidates to explore clinical hyporesponsiveness to the blockade of the TNF/NF-κB pathway by administration of infliximab.

In the study of two parallel cohorts of patients with Crohn's disease, who were treated with infliximab, we do not see any association between the three variants of the CARD15 gene and response. The distribution of the genotypes did not differ significantly between the patients who responded and those who did not respond, or between those achieving or failing to achieve remission. The response phenotype also appears not to be influenced by a dosage effect (i.e. double heterozygotes or presence of combined mutations). Multivariate analysis rejected the hypothesis that an association between CARD15 variants and response to infliximab could have been restricted to specific patient subgroups.

The two-cohort design used in this study maximized the power to detect association between a polymorphism and response to infliximab. Assuming a frequency in the study population of 20% for a genotype predisposing to non-response, and assuming a relative risk of 2, the power of this design to detect an effect in at least one of the two studies at an alpha level of 0.05 is 97% (the power is 90% for a genotype frequency of 10%). This implies that a frequent polymorphism can be ruled out as a strong causative mutation, or being in strong linkage with such a mutation, for treatment response, if no association is detected with $P \leqslant 0.05$ in at least one of the studies.

In the two cohorts under investigation the three SNPs in the CARD15 gene appeared, as expected, significantly associated with Crohn's disease itself. As these patients have not been involved in any of the genetic studies published previously [12,19,20], they represent an important, independent confirmation of the disease association in an international cohort, one that has been collected in more than 80 centers world-wide.

In the study presented here, we could demonstrate that disease associated variants in the CARD15 gene can be excluded as predictors for clinical efficacy of an anti-TNF therapy with infliximab. It therefore appears unlikely that an interference between immunological pathways regulated by the CARD15 gene and the mechanisms of TNF blockade with infliximab exist. Although most patients investigated received infliximab for refractory disease it appears likely that this conclusion also applies to patients receiving the drug to close fistulae. No apparent association between the CARD15 genotype and occurrence of side effects was seen. Further pharmacogenetic work will focus on variants of genes, which are more directly involved in the TNF-receptor/NF-κB pathway.

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