ORIGINAL ARTICLE

# Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease

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## ABSTRACT

**Objective** The aetiology of Crohn's disease (CD) has been related to nucleotide-binding oligomerisation domain containing 2 (NOD2) and ATG16L1 gene variants. The observation of bacterial DNA translocation in patients with CD led us to hypothesise that this process may be facilitated in patients with NOD2/ATG16L1-variant genotypes, affecting the efficacy of anti-tumour necrosis factor (TNF) therapies.

**Design** 179 patients with Crohn's disease were included. CD-related NOD2 and ATG16L1 variants were genotyped. Phagocytic and bactericidal activities were evaluated in blood neutrophils. Bacterial DNA, TNFα, IFNγ, IL-12p40, free serum infliximab/adalimumab levels and antidrug antibodies were measured.

**Results** Bacterial DNA was found in 44% of patients with active disease versus 23% of patients with remitting disease (p=0.01). A NOD2-variant or ATG16L1-variant genotype was associated with bacterial DNA presence (OR 4.8; 95% CI 1.1 to 13.2; p=0.001; and OR 2.4; 95% CI 1.4 to 4.7; p=0.01, respectively). This OR was 12.6 (95% CI 4.2 to 37.8; p=0.001) for patients with a double-variant genotype. Bacterial DNA was associated with disease activity (OR 2.6; 95% CI 1.3 to 5.4; p=0.005). Single and double-gene variants were not associated with disease activity (p=0.19). Patients with a NOD2-variant genotype showed decreased phagocytic and bactericidal activities in blood neutrophils, increased TNFα levels in response to bacterial DNA and decreased trough levels of free anti-TNFα. The proportion of patients on an intensified biological therapy was significantly higher in the NOD2-variant groups.

**Conclusions** Our results characterise a subgroup of patients with CD who may require a more aggressive therapy to reduce the extent of inflammation and the risk of relapse.

## Significance of this study

### What is already known about this subject?

- Genetic susceptibility and the immunological interaction between the host and commensal flora are implicated in the aetiology of Crohn's disease (CD).
- Gut bacterial DNA translocation into blood is a frequent event in patients with CD.
- Relapse occurs during the course of the disease in patients with CD, even on immunosuppressive and/or biological maintenance treatments.
- The efficacy of different therapeutic schemes using biologicals to treat patients with CD is under discussion.

### What are the new findings?

- Nucleotide-binding oligomerisation domain containing 2 (NOD2) and ATG16L1 genetic variants are independent risk factors for bacterial DNA translocation in CD.
- Bacterial DNA translocation is directly associated with disease activity and increases the risk of relapse at 6 months.
- Patients with a NOD2 variant genotype showed significantly decreased phagocytic and bactericidal activities, increased serum tumour necrosis factor α (TNFα) levels in response to bacterial DNA and significantly decreased levels of free serum anti-TNFα.
- In vitro *Escherichia coli* DNA-stimulated neutrophils from patients with a NOD2 variant genotype show lower serum levels of anti-TNFα, suggesting faster drug consumption

### How might it impact on clinical practice in the foreseeable future?

- Our results identify a subgroup of patients with an increased risk of bacterial DNA translocation and, very likely, an increased risk of relapse.
- Classical anti-TNFα therapy regimens are likely to decrease their efficacy in controlling the risk of flare-up in these patients who might require more aggressive therapeutic programmes to reduce inflammation and episodes of bacterial DNA translocation.

## INTRODUCTION

Understanding the pathogenesis of Crohn's disease (CD), one of two major forms of inflammatory bowel disease affecting the population world-wide,[1 2] has experienced enormous progress in recent years. The aetiology of the disease is partially explained by a disturbance of the interaction between the host and intestinal microorganisms.[3–6] Accordingly, the gut epithelial barrier plays a crucial role in regulating the uptake of luminal

antigens and in controlling intestinal immune responses to luminal bacteria. Recently, we reported evidence of bacterial DNA (bactDNA) translocation into the blood of patients with CD. The associated innate immune response, which is partially affected by nucleotide-binding oligomerisation domain containing 2 (NOD2) gene status,[7 8] suggests a dysfunction in the epithelial cell layer, an intestinal bacterial overgrowth or the combination of both of these adverse events.

NOD2 encodes an intracellular receptor for the presence of muramyl dipeptide (MDP), a common component of the bacterial cell wall[9–11] and has been identified as a prominent CD susceptibility gene.[12] However, genome-wide association studies have increased the number of genes that may potentially modulate CD up to 71 loci.[13 14] Among them, the autophagy gene, ATG16L1, is very prominent.[15–17] ATG16L1 is implicated in the modulation of autophagy, an important cell survival mechanism also involved in key aspects of the innate immune response to microorganisms.[18] The CD-associated ATG16L1 variant, T300A, has been reported to result in an autophagy defect,[15 19] suggesting a role for autophagy, and ATG16L1 in particular, in the pathogenesis of CD.

Recently, ATG16L1 and NOD2 have been reported to interact in an autophagy-dependent antibacterial pathway in response to MDP that is disrupted by specific CD-associated mutations.[20] We have observed that patients with CD with a mutated NOD2 genotype cannot properly respond to bactDNA translocation.[8] However, increasing evidence has been reported linking phagocytosis, a primary mechanism of host defence against bacterial products, with the autophagy machinery.[21 22] Therefore, we hypothesise that the presence of bactDNA in the blood of patients with CD may be associated with functional impairment by the autophagy-related CD-associated gene mutation, further complicating the disease outcome.

The aim of the present study was to determine whether the presence of NOD2 and ATG16L1 mutations confer susceptibility to an impaired phagocytic activity and increased rates of bactDNA translocation into the blood of patients with CD.

## PATIENTS AND METHODS

### Patients

A total of 179 consecutive patients with CD whose condition was managed at the Inflammatory Bowel Disease (IBD) Unit of the Hospital General Universitario de Alicante and who accepted to participate in the study were included. The diagnosis of CD was established according to standard clinical, endoscopic, histological and radiographical criteria.[23] Patients who had received antibiotics in the previous 2 weeks were not considered for inclusion. All patients were classified according to the Montreal classification[24] and stratified according to the Crohn's Disease Activity Index (CDAI). All included patients received diaries to record symptoms 1 week prior to inclusion and sample collection. Intensified therapy with biologicals was defined either by an increased dose or an increase in the frequency of infusions versus dosing or schedule upon start of treatment. Twenty-five healthy controls were also included in the study. All patients and controls were Caucasian of Mediterranean ethnicity. Blood samples from patients and healthy donors were obtained for routine haematological and biochemical studies and inoculated in aerobic and anaerobic blood culture bottles, 10 ml each. Simultaneously, two separate blood samples were inoculated under aseptic conditions in rubber-sealed sterile Vacutainer SST II and K3E tubes, respectively (BD Diagnostics, Erembodegem, Belgium) that were never exposed to free air.

Patients were followed up for 6 months to evaluate the incidence of relapse. Although 'relapse' strictly refers to the clinical situation coming from remission, we wanted to evaluate whether patients' disease would turn into an active form in the next 6 months after bactDNA detection, no matter their CDAI status at admission. In the case of patients with a CDAI>150 at admission, the flare was controlled in all cases within the subsequent 2 weeks. The Ethics Committee of the hospital approved the study protocol. All controls and patients gave informed consent prior to inclusion in the study.

### Identification of bactDNA fragments and genotyping of CD-associated genes

Genomic DNA was isolated from $5\times10^6$ cells with the QIAmp DNA Blood Minikit (Qiagen, Hilden, Germany). BactDNA was identified by running broad-range PCR followed by partial nucleotide sequencing of a conserved region of the 16SrRNA gene, as previously described.[25] The three common NOD2/CARD15 allelic variants at SNP-8 (R702W, rs2066844), SNP-12 (G908R, rs2066845) and SNP-13 (L1007finsC, rs2066847) were evaluated as previously described.[26] The ATG16L1 variation rs2241880 (T300A) was genotyped by TaqMan technology (Applied Biosystems, Carlsbad, California, USA) using commercially available TaqMan SNP Genotyping Assays and TaqMan Genotyping Master Mix on a 7900HT Fast Real-Time PCR System using SDS 2.2 Software (Applied Biosystems). A variant NOD2 genotype (varNOD2) was defined as carrying any of the three variants studied either in homozygosis or heterozygosis. A variant ATG16L1 genotype (varATG16L1) was defined as carrying the rs2241880 variation either in homozygosis or heterozygosis. All genotyping results were assessed twice. The evaluators were not aware of either the patient's disease status or each other's genotype results. A Hardy–Weinberg test was performed as a quality control measure in controls. No missing genotypes were present.

### Phagocytic and bactericidal assays

The phagocytic activity of neutrophils that ingest fluorescein isothiocyanate (FITC)-labelled opsonised *Escherichia coli* and the bactericidal activity of neutrophils that oxidise the fluorogenic substrate dihydrorhodamine DHR123 to rhodamine R123 were determined using the Phagotest and Bursttest Kits (Orpegen Pharma, Heidelberg, Germany) following the manufacturer's instructions. Cells were quantified by flow cytometry using a FacsCanto (Becton Dickinson, Heidelberg, Germany) and the data were processed using the FacsDiva 12.0 software (Becton Dickinson).

### Serum cytokine and free anti-TNFα levels: presence of antidrug antibodies

ELISAs were carried out to measure tumour necrosis factor α (TNFα), interferon γ (IFNγ), interleukin (IL)-12p40 (R&D Systems, Minneapolis, Minnesota, USA), free infliximab and adalimumab levels and to detect antidrug antibodies (Matriks Biotek, Ankara, Turkey) according to the manufacturers' instructions. All samples were tested in triplicate and read in a Sunrise Microplate Reader (Tecan, Männedorf, Switzerland). The detection limit for each cytokine assay varied between 2 and 5 pg/ml and between 10 and 30 ng/ml in the case of free anti-TNFα kits. The presence of antidrug antibodies was evaluated by a cut-off value estimated by multiplying the optical density (OD) of the zero standard by 3, as indicated by the manufacturers. Samples were considered positive when the ratio sample OD/zero standard OD was higher than 3.

### Isolation and culture of human PMN cells

Polymorphonuclear leukocyte cells (PMNs) from peripheral blood samples were isolated with PolymorphPrep (Axis-Shield PoC, Oslo, Norway). After isolation of PMNs, cells were washed twice with freshly made phosphate-buffered saline at 4°C. Cell viability was evaluated by trypan blue (Sigma, Madrid, Spain). Cells were resuspended in phenol red-free RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% human serum AB (BioWhittaker, Walkersville, Maryland, USA). Neutrophils ($1\times10^6$ cells/well) were incubated with 200 ng of *E coli* DNA. Half of the supernatant volumes were collected at 24 h and TNFα and annexin V levels measured by ELISA. Cells were then treated with infliximab 100 µg/ml/$10^6$ cells (infliximab was kindly provided by Merck Sharp and Dohme of Spain MSD, Madrid, Spain). Supernatants were collected at 48 h. Total endogenous annexin V levels were evaluated as an indirect measurement of apoptotic activity in neutrophils using a human Annexin V ELISA kit from Promokine (Heidelberg, Germany), according to the manufacturer's instructions.

### Statistical analysis

Continuous variables are reported as the mean±SD and categorical variables as frequency or percentages. The Kolmogorov–Smirnov test was used to test normality of continuous variables. Statistical differences were analysed using the $\chi^2$ test for categorical data and analysis of variance (ANOVA) test for quantitative data followed by the post hoc Bonferroni correction for multiple comparisons. Quantitative data showing non-normal distribution were analysed using the Mann–Whitney U test or Kruskal–Wallis test followed by pairwise comparisons using the Mann–Whitney U test with the post hoc Bonferroni correction for multiple comparisons. Bivariate correlations were analysed using the Spearman test. Logistic regression analysis was used to determine the association between NOD2/ATG16L1 genotypes and bactDNA concentration or CDAI. We first examined variables by univariate analysis, and then significant factors at $p<0.10$ were included in multivariate analysis. The results of logistic regression are reported as OR and 95% CI. All reported p values are two sided. $p<0.05$ values were considered significant. Analyses were performed using IBM SPSS Statistics 19.

## RESULTS

### Patient characteristics

The clinical and analytical characteristics of patients are detailed in table 1, stratified according to the CDAI. No statistically significant differences were observed between groups regarding age, gender, smoking habit or disease classification by Montreal criteria. Patients with active diease showed higher erythrocyte sedimentation rate, C-reactive protein (CRP) and lower haemoglobin and albumin levels than patients with remitting disease. Steroid treatment was significantly more frequent in patients with active disease. Among healthy controls, the mean age was 32±11 years, the male/female ratio was 12 : 8 and 35% were smokers. No differences in demographic and analytical parameters between controls and patients with remitting disease were observed. All patients and controls included in the study had negative blood microbiological cultures.

**Table 1** Patient characteristics

| | CDAI | |
|---|---|---|
| | <150 (n=136) | >150 (n=43) |
| Age (years) | 40±14 | 42±15 |
| Weight (kg) | 70.12±15.76 | 68.88±20.78 |
| Gender (male/female) | 65/71 | 18/24 |
| Smoking habit, n (%) | 48 (35%) | 14 (32%) |
| Disease duration (months) | 112.52±91.66 | 106.54±98.19 |
| Resection, n (%) | 36 (26%) | 13 (30%) |
| Montreal A (age of onset), n (%) | | |
| A1 (<=16) | 6 (4.4%) | 3 (7%) |
| A2 (17–40) | 107 (78,6%) | 30 (69.7%) |
| A3 (>40) | 23 (17%) | 10 (23.3%) |
| Montreal L (location), n (%) | | |
| L1 (ileal) | 70 (51.4%) | 23 (53.4%) |
| L2 (colonic) | 32 (23.5%) | 5 (11.6%) |
| L3 (ileocolonic) | 31 (22.8%) | 12 (28%) |
| L4 (upper isolated) | 3 (2.2%) | 3 (7%) |
| Montreal B (behaviour), n(%) | | |
| B1 (non-stricturing, non-penetrating) | 68 (50%) | 19 (44.2%) |
| B1p (non-stricturing, non-penetrating, penetrating perianal disease) | 11 (8%) | 2 (4.6%) |
| B2 (stricturing) | 22 (16.3%) | 13 (30.2%) |
| B2p (stricturing, perianal disease associated) | 7 (5.1%) | – |
| B3 (penetrating) | 24 (17.6%) | 5 (13.6%) |
| B3p (penetrating, penetrating perianal disease) | 4 (2.9%) | 4 (9.3%) |
| Therapy, n (%) | | |
| Mesalazine | 35 (25.7%) | 5 (11.6%) * |
| Steroids | – | 2 (4.6%) |
| Azathioprine | 49 (36.0%) | 4 (9.3%) * |
| Infliximab | 7 (5.1%) | 5 (11.6%) |
| Adalimumab | 12 (8.8%) | 2 (4.6%) |
| Metotrexate | – | 1 (2.4%) |
| Mesalazine and steroids | 3 (2.2%) | 5 (11.6%) |
| Mesalazine and azathioprine | 7 (5.1%) | 2 (4.6%) |
| Azathioprine and steroids | 2 (1.4%) | 10 (23.2%)* |
| Infliximab and azathioprine | 8 (5.8%) | 4 (9.3%) |
| Adalimumab and azathioprine | 5 (3.6%) | – |
| Infliximab and metotrexate | 2 (1.4%) | – |
| Infliximab and steroids | 1 (0.7%) | 2 (4.6%) |
| Adalimumab and steroids | 3 (22%) | – |
| Infliximab and azathioprine and steroids | – | 1 (2.3%) |
| No treatment | 2 (1.4%) | – |
| ESR (mm) | 19.4±16.3 | 40.5±27.4* |
| CRP (mg/dl) | 0.53±1.04 | 3.43±4.99* |
| Haemoglobin (g/dl) | 13.93±3.02 | 12,49±1.66 * |
| Albumin (mg/d) | 4073.05±453.02 | 3588.2 ±573.47 * |
| Total WBCs ($mm^3$) | 6907.7±2757.24 | 7725.02 ±2642.26 |
| Temperature (°C) | 36.09±0.26 | 36.54±0.58 * |
| Pulse rate (bpm) | 70.17±5.43 | 72±7.47 |

All values shown as mean±SD or percentage.
CDAI, Crohn's Disease Activity Index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cell.
*p<0.05 compared with CDAI <150.

### NOD2/ATG16L1 genotypes and bactDNA distribution in patients with CD

The distribution of genotypes and allelic frequencies in patients with CD and controls are shown in table 2. All variants were found to be in the Hardy–Weinberg equilibrium in the controls. Overall, 69% of patients with remitting disease and 76.4% of patients with active disease showed at least one of the

**Table 2** Distribution of genotypes and allelic frequencies in patients with Crohn's disease and controls

| | Gentotype (n (%) patients/controls) | | | |
|---|---|---|---|---|
| | Homozygous wild type | Heterozygous | Homozygous variant | Variant allele frequency (%) |
| NOD2 SNP8 (R702W) | 136 (76%)/24 (96%) | 43 (24%)/1 (4%) | 0/0 | 12.0/2.0 |
| NOD2 SNP12 (G908R) | 152 (85%)/24 (96%) | 26 (14.5%)/1 (4%) | 1 (0.5%)/0 | 7.8/2.0 |
| NOD2 SNP13 (L1007finsC) | 164 (92%)/24 (96%) | 15 (8.4%)/1 (4%) | 0/0 | 4.7/2.0 |
| ATG16L1 (T300A) | 73 (41%)/22 (88%) | 84 (47%)/5 (20%) | 22 (12.3%)/0 | 35.8/10.0 |

CD-associated gene variants. A NOD2 variant, either alone or combined with the ATG16L1 variant, was present in 56 (41.2%) patients with remitting disease and in 18 (41.8%) patients with active disease. The ATG16L1 variant, either alone or combined with a NOD2 variant, was present in 82 (60.2%) patients with remitting disease and in 24 (55.8%) patients with active disease. Figure 1A shows the distribution of NOD2 and ATG16L1 variants among patients with active CD and those with remitting disease. The distribution of the genotypes under study was not statistically significant between groups (p=0.90).

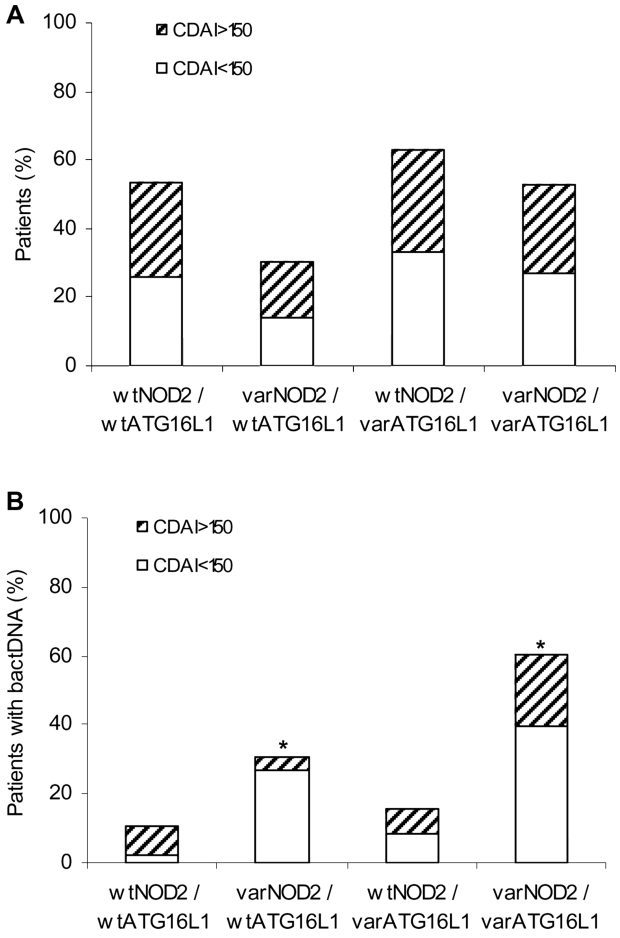


**Figure 1** (A) Distribution of NOD2/ATG16L1 combined genotypes in patients with Crohn's disease stratified by CDAI. (B) Percentage of bactDNA presence in each studied genotype distributed by CDAI; *p<0.01 compared with the wtNOD2/wtATG16L1 genotype group. bactDNA, bacterial DNA; CDAI, Crohn's Disease Activity Index; NOD2, nucleotide-binding oligomerisation domain containing 2; var, variant; wt, wild type.

From the overall series of patients, 51 of 179 (28.5%) showed the presence of bactDNA in blood samples. By groups, 19 of 43 (44.2%) patients with clinically active disease and 32 of 136 (23.5%) patients with remitting disease showed bactDNA (p=0.01). The identified bacterial species included *E coli* (n=21), *Staphylococcus aureus* (n=12), *Klebsiella pneumoniae* (n=7), *Streptococcus pneumoniae* (n=5), *Shigella* spp. (n=3), *Enterococcus faecalis* (n=2) and *Campylobacter freundii* (n=1). No statistical differences in bacterial species were observed between the studied genotypes. BactDNA was absent in the blood of healthy controls. A univariate analysis revealed that the presence of bactDNA in the blood, among others, was significantly associated with disease activity with an OR of 2.6 (95% CI 1.3 to 5.4; p=0.005) whereas single and double-gene mutations were not directly associated with disease activity (p=0.19). As expected, the use of steroids and infliximab was also significantly associated with disease activity, as they are a usual therapeutic first approach on a flare-up (see online supplementary table S1). A multivariate analysis including variables showing a p value<0.1 on the univariate analysis is detailed in table 3. Of note, bactDNA remained significantly and independently related to disease activity.

BactDNA was present in 5 of 47 (10.6%) patients with wtNOD2/wtATG16L1, 8 of 26 (30.7%) with varNOD2/wtATG16L1, 9 of 57 (15.5%) with wtNOD2/varATG16L1 and 29 of 48 (60.4%) with varNOD2/varATG16L1. Figure 1B displays the percentage of bactDNA presence in each studied genotype, distributed by CDAI. The percentage of patients with bactDNA was significantly increased in the varNOD2 genotype groups. Unlike the double wild-type genotype, single NOD2 or ATG16L1 variants were significantly associated with the presence of bactDNA on the univariate analysis (OR 4.8; 95% CI 1.1 to 13.3; p=0.03; and OR 2.4; 95% CI 1.46 to 4.5; p=0.05, respectively). This effect was more pronounced in patients with a double-variant genotype (OR 12.7; 95% CI 4.2 to 37.8; p=0.001) (see online supplementary table S2). All studied gene variants and disease activity remained significantly and independently associated with bactDNA presence on the multivariate analysis (table 3).

No differences among the three examined NOD2 allelic variants were observed for CDAI or any other clinical characteristics of patients with varNOD2. No statistically significant differences were observed, either between patients stratified by genotype or by presence of bactDNA, although CRP, total white blood cell count and CDAI were higher in patients with bactDNA (data not shown).

In 43 patients disease relapsed during this period (24%): 20 of 51 (40%) patients with bactDNA at inclusion versus 23 of 128 (18%) patients without bactDNA (p=0.01). BactDNA in the blood, among other things, was significantly associated with an increased risk of flare-up at 6 months on the initial univariate analysis, with an OR of 3.32 (95% CI 1.5 to 7.3; p=0.003)

**Table 3** Multivariate analyses of disease activity, bacterial DNA and relapse at 6 months, including variables with a p value <0.1 on univariate analysis

| Multivariate analysis | | | |
|---|---|---|---|
| **Variable** | **p Value** | **OR** | **95% CI** |
| Dependent variable: disease activity (CDAI<150/CDAI>150) | | | |
| Presence of bacterial DNA fragments (yes/no) | 0.008 | 7.422 | 1.690 to 32.596 |
| Steroid treatment (yes/no) | 0.001 | 27.303 | 4233 to 176116 |
| Infliximab treatment (yes/no) | 0.001 | 16.649 | 3.213 to 86.288 |
| C-reactive protein (mg/dl) | 0.001 | 3.833 | 1.679 to 8.752 |
| Dependent variable: bacterial DNA | | | |
| Disease activity (CDAI>150/CDAI<150) | 0.001 | 5.084 | 1.972 to 13.106 |
| Genotype | | | |
| wtNOD2/wtATG16L1 | 0.000 | | |
| varNOD2/wtATG16L1 | 0.020 | 5.117 | 1.287 to 20.346 |
| wtNOD2/varATG16L1 | 0.0401 | 2.221 | 1.394 to 5.118 |
| varNOD2/varATG16L1 | 0.000 | 12.728 | 3.738 to 43.344 |
| Dependent variable: relapse at 6 months (yes/no) | | | |
| Presence of bacterial DNA fragments (yes/no) | 0.003 | 5.132 | 1.764 to 14.926 |

CDAI, Crohn's Disease Activity Index.

(see online supplementary table S3). The multivariate analysis revealed bactDNA presence as the only independent factor associated with relapse at 6 months (table 3). Thirty-three patients (18.4%) received short-term courses of oral antibiotics during the 6-month follow-up period due to non-CD-related causes with no statistically significant effect on the risk for relapse among patients, either stratified by genotype or by the presence of bactDNA at admission. There was no statistical association between bactDNA presence at admission and the subsequent need for antibiotics, complications such as surgery or readmissions during the follow-up period among the different genotype subgroups.

### Immunological activity in response to bactDNA in patients with different NOD2/ATG16L1 genotypes

We evaluated the proinflammatory cytokine response to the presence of bactDNA in the serum of patients with CD, stratified by genotype. The results are shown in table 4. No differences were observed between patient groups without bactDNA. The proinflammatory activity was significantly increased in patients with bactDNA compared with that observed in patients without bactDNA, irrespective of genotype. However, patients with bactDNA and a variant NOD2 genotype, either alone or combined with a variant ATG16L1 genotype, showed a further statistically significant increase compared with wild-type NOD2 genotype subgroups. Furthermore, the highest proinflammatory levels were obtained in serum from patients carrying a combined NOD2/ATG16L1 double-variant genotype. An ANOVA analysis of proinflammatory cytokines including different variables and their combinations revealed that bactDNA and the interaction between bactDNA and genotype were the only statistically significant variables (data not shown).

Phagocytic and bactericidal activities were evaluated in blood neutrophils treated with a fluorochrome-labelled *E coli* from the first 12 patients with remitting disease without bactDNA of each genotype-stratified subgroup (n=48). As shown in figure 2, phagocytic and bactericidal activities were significantly decreased in the neutrophils of patients with varNOD2/wtATG16L1 and varNOD2/varATG16L1 genotypes, with no differences between these two groups. To investigate whether these results were affected by ongoing treatment, phagocytic and bactericidal activities were also evaluated in healthy controls without bactDNA carrying NOD2 (n=3) and ATG16L1 (n=5) variants or wild-type variants (n=10). As in patients, these

**Table 4** Serum cytokine levels in patients with different NOD2/ATG16L1-combined genotypes according to the presence of bacterial DNA

| | TNFα (pg/ml) | IFN-γ (pg/ml) | IL-12 (pg/ml) |
|---|---|---|---|
| Bacterial DNA– (n=128) | | | |
| wtNOD2/wtATG16L1 (n=43) | 28.08±6.89 | 30.21±4.06 | 54.19±7.70 |
| varNOD2/wtATG16L1 (n=18) | 28.71±4.91 | 31.81±4.37 | 52.31±6.45 |
| wtNOD2/varATG16L1 (n=48) | 30.09±4.50 | 34.78±7.64 | 52.64±5.71 |
| varNOD2/varATG16L1 (n=19) | 3012±3.48 | 32.04±5.70 | 50.72±5.20 |
| wtNOD2/wtATG16L1 (n=5) | 49.57±6.92* | 59.32±6.1* | 91.16±11.8* |
| Bacterial DNA+ (n=51) | | | |
| varNOD2/wtATG16L1 (n=8) | 75.67±7.80* ** | 82.86±7.48* ** | 124.01±15.5* ** |
| wtNOD2/varATG16L1 (n=9) | 50.70±8.73* | 61.43±6.6* | 98.73±12.43* |
| varNOD2/varATG16L1 (n=29) | 79.50±8.93* ** | 85.60±8.27* ** | 138.49±17.5* ** |

*p<0.01 compared with same genotype in patients who are bacterial DNA negative.
**p<0.01 compared with the wtNOD2 genotypes in patients who are bacterial DNA positive.
IFN, interferon; IL, interleukin; TNFα, tumour necrosis factor α.

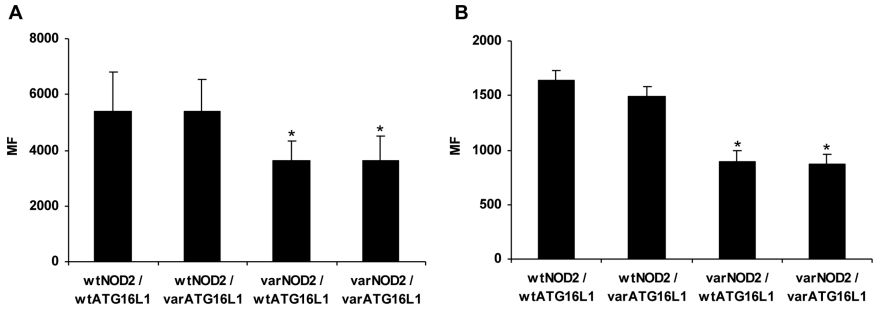


**Figure 2** Phagocytic (A) and bactericidal (B) activities in neutrophils of patients with NOD2/ATG16L1 combined genotypes (n=12/each group) without bactDNA translocation into blood; *p<0.01 compared with wtNOD2 groups. bactDNA, bacterial DNA; NOD2, nucleotide-binding oligomerisation domain containing 2; MF, mean fluorescence; wt, wild type.

activities were decreased in healthy donors carrying any NOD2 variant compared with ATG16L1 variant and wild-type genotypes (data not shown).

### Intensified anti-TNFα therapy is more frequent in patients with mutated NOD2/ATG16L1-combined genotypes

Next, we evaluated whether these combined genotypes had an impact on anti-TNFα therapy schedules. Fifty-two of the 179 patients (29%) were on anti-TNFα therapy at inclusion, either combined with immunosuppressive treatment or not, 30 on infliximab and 22 on adalimumab (see table 1). Figure 3A,B show the percentage of patients on anti-TNFα therapy and, of those, the percentage of patients on an intensified schedule of anti-TNFα therapy, either stratified by genotype or by the presence of bactDNA. As detailed, the proportion of patients on intensified therapy was higher in the groups with either a varNOD2/wtATG16L1 or a varNOD2/varATG16L1 genotype. No statistically significant differences were observed in the percentage of smokers, the time from diagnosis or the time from initiation of anti-TNFα therapy between patients on intensified treatment and those on non-intensified treatment, all of which could affect the need for intensification (see online supplementary table S4). Twenty percent of patients receiving anti-TNFα therapy showed bactDNA in blood, of which 50% were on an intensified drug schedule. By contrast, only 33% of patients without bactDNA received intensified treatment (p=ns). No differences were observed for the distribution of each anti-TNFα among patients, either grouped by genotype or by the presence of bactDNA.

Free anti-TNFα levels were measured in the serum of patients receiving therapy with biologicals and stratified according to their genotype and the presence of bactDNA. The results were restricted to samples that were negative for the presence of anti-drug antibodies (n=48) and are shown in figure 3C. Significantly decreased levels of free anti-TNFα were observed in the varNOD2/wtATG16L1 and varNOD2/varATG16L1 genotype-based subgroups, despite showing a significant number of patients on an intensified schedule. The presence of bactDNA further decreased the levels of free serum anti-TNFα, although the number of individuals was too small to report statistics.

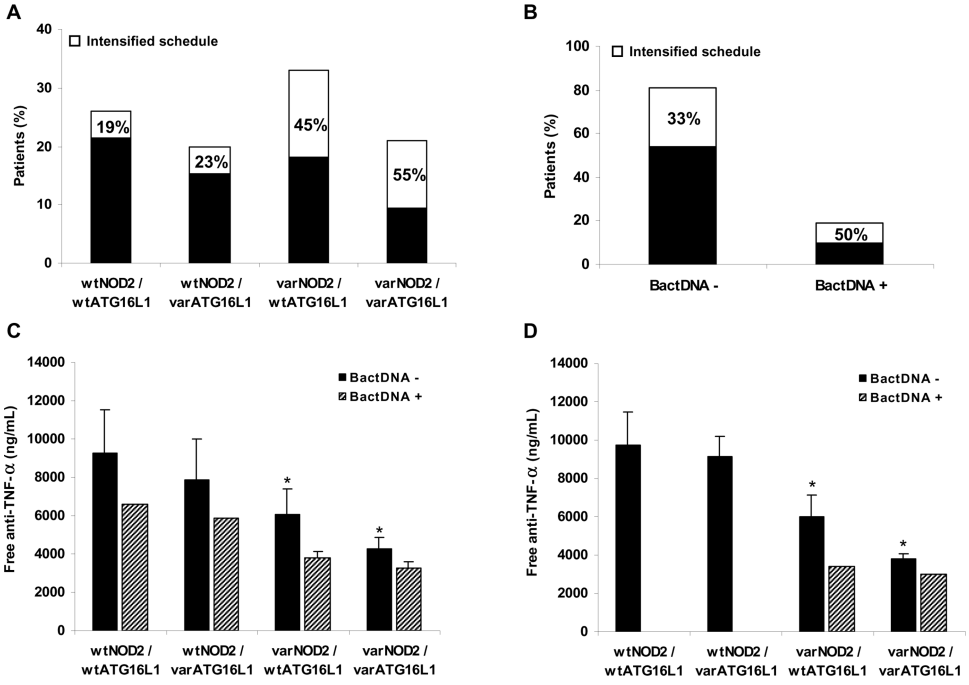


**Figure 3** (A) Percentage of patients on intensified anti-tumour necrosis factor α (TNFα) therapy stratified according to NOD2/ATG16L1-combined genotypes. (B) Percentage of patients on intensified anti-TNFα therapy according to the presence of bactDNA. (C) Free anti-TNFα levels in serum of patients receiving therapy with biologicals, either alone or combined with immunosuppressive treatment, stratified according to each genotype studied and to the presence of bactDNA; *p<0.01 compared with wtNOD2 groups. (D) Free anti-TNFα levels in serum of patients on monotherapy with biologicals, stratified according to each genotype studied and to the presence of bactDNA; *p<0.01 compared with wtNOD2 groups. bactDNA, bacterial DNA; NOD2, nucleotide-binding oligomerisation domain containing 2; var, variant; wt, wild type.

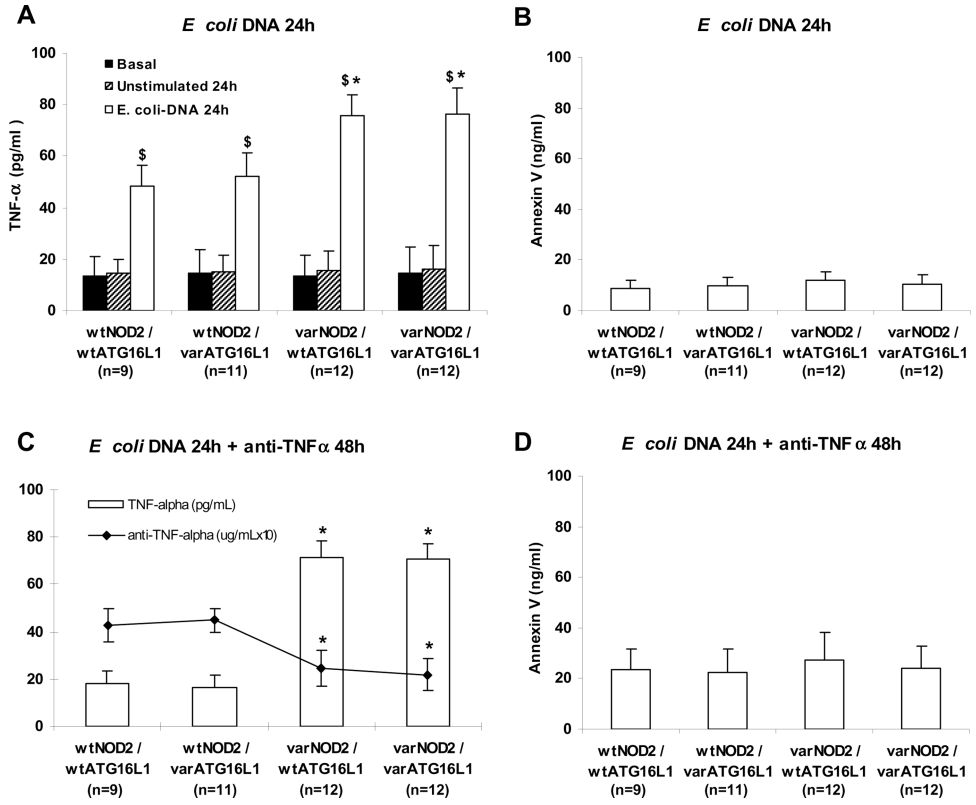


**Figure 4** (A) Tumour necrosis factor α (TNFα) levels in the supernatants of neutrophils from patients with different NOD2/ATG16L1 genotypes exposed to *Escherichia coli* DNA; $p<0.01 compared with baseline and unstimulated conditions; *p<0.01 compared with wtNOD2 groups. (B) Apoptotic activity of 24 h cultured neutrophils from patients of each genotype subgroup. (C) Free anti-TNFα levels in the supernatants of neutrophils from patients with studied NOD2/ATG16L1 genotypes exposed to *E coli* DNA and treated with infliximab; *p<0.01 compared with wtNOD2 groups. (D) Apoptotic activity of 72 h cultured neutrophils from patients of each genotype subgroup. NOD2, nucleotide-binding oligomerisation domain containing 2; var, variant; wt, wild type.

Since interactions between biologicals and azathioprine or methotrexate have been described, we repeated the analysis excluding patients on combined therapies in samples with an absence of serum antidrug antibodies (n=24) obtaining comparable results (figure 3D).

As stated above, 3 of 30 (10%) patients on infliximab and 1 of 22 (4.5%) on adalimumab showed the presence of antidrug antibodies (see methods) and were excluded from the analyses presented in figure 3C,D. None of these patients showed the presence of bactDNA in the blood. Two patients had a wtNOD2/wtATG16L1 genotype and were on combined therapy. One patient had a wtNOD2/wtATG16L1 genotype and was on monotherapy with infliximab. One patient had a wtNOD2/varATG16L1 genotype and was on monotherapy with adalimumab.

### Free anti-TNFα levels are decreased in the supernatants of neutrophils from patients with variant NOD2/ATG16L1 exposed to bactDNA

We designed an in vitro experiment with neutrophils isolated immediately before anti-TNFα infusion (trough levels) from the same 12-patient genotype-stratified subgroups without bactDNA, excluding those who showed the presence of antidrug antibodies. The results are shown in figure 4.

Levels of TNFα were significantly increased after 24 h *E coli* bactDNA stimulation in the subgroups bearing a variant versus wild-type NOD2 genotype (figure 4A). Baseline and 24 h unstimulated supernatant levels are also represented. No differences in the apoptotic activity of neutrophils at this time point were observed among groups (figure 4B). Forty-eight hours after anti-TNFα addition, levels of TNFα were significantly decreased in all subgroups with wild-type NOD2. This reduction was not observed in the supernatants of cells with a NOD2 variant. However, a significant reduction in free anti-TNFα levels was found in the supernatants of the subgroups carrying a variant NOD2 genotype, either alone or combined, compared with wild-type NOD2 (figure 4C). No differences in apoptotic activity of neutrophils between groups were observed at this time point (figure 4D).

## DISCUSSION

Patients with a NOD2-variant genotype, either alone or combined with an ATG16L1-variant genotype, show an increase in secreted TNFα levels in response to bactDNA translocation and, therefore, a faster consumption of free anti-TNFα. This finding could be clinically important since these patients may require intensified anti-TNFα therapy, have an increased risk of bactDNA translocation, and have an increased risk of relapse.

The number of IBD-associated susceptibility genes has increased in recent years to over 100.[16 27] Among them, autophagy-related genes, especially ATG16L1, have emerged as new key players[15 17] along with others that were previously identified, such as NOD2, classically associated with the development of CD.[26] Although the association between NOD2 variants and the increased risk of complicated CD has been reported in the past,[28–30] none of these genes has been linked so far with the risk of disease flare-ups. We report here that

bactDNA translocation is significantly associated with the disease activity and the presence of NOD2/ATG16L1 gene variants, suggesting that bactDNA translocation might be facilitated in patients with variant NOD2/ATG16L1-combined genotypes, increasing the risk of flare-up in these patients (figure 1). In fact, genetic interactions between polymorphisms in toll-like receptor 9, the specific receptor for immunogenic CpG motifs present in bactDNA, and CD-associated variants in NOD2 and other genes have been previously described.[31]

In our study, patients bearing a NOD2 gene variant, either alone or combined with a variant ATG16L1 genotype, showed significantly reduced phagocytic and bactericidal activity (figure 2). NOD2 genetic alterations have also been associated with a defective innate response in the past.[32] Interestingly, our data indicate that NOD2 may be involved in the phagocytic and bactericidal capacities of blood neutrophils, facilitating its ability to clear bacteria and their components from the blood of these patients. We hypothesised that the persistence of bactDNA fragments in the blood of patients with NOD2 mutation would correlate with an increased proinflammatory cytokine response. Accordingly, data shown in table 4 revealed that NOD2-mutated genotypes were associated with higher levels of these cytokines.

The role of TNFα in the pathogenesis of CD is clearly evidenced by the efficacy of anti-TNFα therapies.[33–36] However, our understanding of the effect of different NOD2/ATG16L1 genotypes and bactDNA presence on these therapies is not complete. The proportion of patients on intensified treatment was significantly higher in patients with a variant NOD2/ATG16L1-combined genotype. At the same time, free anti-TNFα levels were significantly decreased in the serum of patients with any of the NOD2 variants and especially in those with a combined NOD2/ATG16L1-variant genotype. These data are clinically relevant since low serum anti-TNFα levels have been associated with a deficient response, and therapeutic drug monitoring has been recommended in the clinical management of patients with CD.[37–39] Inter-individual variability has been a constant flaw in studies reporting pharmacokinetic data on anti-TNFα levels so far. The use of concomitant drugs such as azathioprine or methotrexate has also been reported to influence clearance of anti-TNFα and the development of immunogenicity.[40 41] To address this question, we analysed the free anti-TNFα levels in patients receiving anti-TNFα monotherapy, obtaining comparable results but with reduced variability. These results suggest that combined therapies, NOD2/ATG16L1 genotypes and the presence of bactDNA may account for the high variability observed in serum-free anti-TNFα levels and might provide an explanation for the need for intensified anti-TNFα therapy. One may think of the intensified biological schedule as the cause for the increased bacterial translocation and the subsequent bactDNA detection in the varNOD2 group. However, only 20% of patients on anti-TNFα therapy have bactDNA and, of those, 50% received intensified treatment. This percentage was not significantly different from the 33% of patients without bactDNA who were on drug intensification. Besides, if that were the case, lower bactDNA rates might be expected in patients with varNOD2 not receiving biological therapy versus those on biological therapy but bactDNA is present in 52.7% of patients with varNOD2 receiving anti-TNFα therapy versus 29.4% of those not receiving anti-TNF-α therapy (p=ns).

These results are further supported by our in vitro studies, in which neutrophils from patients with NOD2 and ATG16L1 variants showed an accelerated drug consumption rate (figure 4). Although the highest TNFα levels were observed in samples from mutated genotypes treated with *E coli* DNA and despite the fact that 24 h unstimulated cells did not show an increase in TNFα compared with baseline, the possibility of an inflammatory peak, earlier to or independent of biological therapy, is not completely ruled out. Nevertheless, these results point to the identification of a subgroup of patients with CD with the potential need for a more aggressive therapeutic approach to efficiently control their disease course or, at least, in which the current therapies are not as effective as in patients with a wild-type NOD2 genotype. Some studies have evaluated 'top-down' versus 'step-up' therapies and these strategies are presently under discussion.[42 43] In our series the presence of bactDNA was significantly associated with relapse in the 6-month follow-up period. Although evaluating risk factors for relapse was not the aim of this study, the preliminary data reported here support the design and development of new studies, including multivariate analysis of known risk factors for the clinical course of the disease, such as the lack of mucosal healing,[44] the use of non-steroidal anti-inflammatory drugs,[45–47] treatment withdrawal[48 49] and others.

In summary, combined NOD2/ATG16L1 variant genotypes identify a patient subgroup with an increased risk of bactDNA translocation. This fact may, in turn, exacerbate the inflammatory response and is directly associated with disease activity and the risk of relapse in the short term. This persistent inflammatory environment would favour the requirement for therapy intensification in patients with mutation on treatment with biologicals, who would probably be candidates for a different therapeutic strategy.

**Contributors** AG: patients' inclusion and manuscript writing. AG, LS, IA: diagnosis, treatment and follow up of patients. MS, EH: NOD2 and ATG16L1 genotyping. PZ, JMG, RF: experimental data, acquisition and analysis. JS, RW, GR: critical revision and clinical considerations. RF: study concept and design, manuscript writing.

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## REFERENCES

1 Hou JK, El-Serag H, Thirumurthi S. Distribution and manifestations of inflammatory bowel disease in Asians, Hispanics, and African Americans: a systematic review. *Am J Gastroenterol* 2009;104:2100–9.
2 Shanahan F, Bernstein CN. The evolving epidemiology of inflammatory bowel disease. *Curr Opin Gastroenterol* 2009;25:301–5.
3 Elson CO, Cong Y, McCracken VJ, *et al*. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* 2005;206:260–76.
4 Obermeier F, Dunger N, Strauch UG, *et al*. CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation. *Gastroenterology* 2005;129:913–27.
5 Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 2002;347:417–29.
6 Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am J Gastroenterol* 1997;92(12 Suppl):5S–11S.
7 Gutierrez A, Frances R, Amoros A, *et al*. Cytokine association with bacterial DNA in serum of patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2009;15:508–14.
8 Gutierrez A, Holler E, Zapater P, *et al*. Antimicrobial peptide response to blood translocation of bacterial DNA in Crohn's disease is affected by NOD2/CARD15 genotype. *Inflamm Bowel Dis* 2011;17:1641–50.
9 Girardin SE, Boneca IG, Viala J, *et al*. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003;278:8869–72.

10 Inohara N, Ogura Y, Fontalba A, *et al*. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 2003;278:5509–12.
11 Ogura Y, Inohara N, Benito A, *et al*. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 2001;276:4812–18.
12 Ogura Y, Bonen DK, Inohara N, *et al*. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;411:603–6.
13 Franke A, McGovern DP, Barrett JC, *et al*. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010;42:1118–25.
14 Lees CW, Barrett JC, Parkes M, *et al*. New IBD genetics: common pathways with other diseases. *Gut* 2011;60:1739–53.
15 Hampe J, Franke A, Rosenstiel P, *et al*. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 2007;39:207–11.
16 Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy* 2007;3:649–51.
17 Rioux JD, Xavier RJ, Taylor KD, *et al*. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 2007;39:596–604.
18 Schmid D, Munz C. Innate and adaptive immunity through autophagy. *Immunity* 2007;27:11–21.
19 Kuballa P, Huett A, Rioux JD, *et al*. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS One* 2008;3:e3391.
20 Homer CR, Richmond AL, Rebert NA, *et al*. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology* 2010;139:1630–41, 1641.
21 Sanjuan MA, Dillon CP, Tait SW, *et al*. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 2007;450:1253–7.
22 Sanjuan MA, Green DR. Eating for good health: linking autophagy and phagocytosis in host defense. *Autophagy* 2008;4:607–11.
23 Sands BE. From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology* 2004;126:1518–32.
24 Silverberg MS, Satsangi J, Ahmad T, *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(Suppl A):5–36.
25 Such J, Frances R, Munoz C, *et al*. Detection and identification of bacterial DNA in patients with cirrhosis and culture-negative, nonneutrocytic ascites. *Hepatology* 2002;36:135–41.
26 Hampe J, Grebe J, Nikolaus S, *et al*. Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 2002;359:1661–5.
27 Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;447:661–78.
28 Ahmad T, Armuzzi A, Bunce M, *et al*. The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 2002;122:854–66.
29 Economou M, Trikalinos TA, Loizou KT, *et al*. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol* 2004;99:2393–404.
30 Radlmayr M, Torok HP, Martin K, *et al*. The c-insertion mutation of the NOD2 gene is associated with fistulizing and fibrostenotic phenotypes in Crohn's disease. *Gastroenterology* 2002;122:2091–2.
31 Torok HP, Glas J, Endres I, *et al*. Epistasis between toll-like receptor-9 polymorphisms and variants in NOD2 and IL23R modulates susceptibility to Crohn's disease. *Am J Gastroenterol* 2009;104:1723–33.
32 van Heel DA, Ghosh S, Butler M, *et al*. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 2005;365:1794–6.
33 Hanauer SB, Feagan BG, Lichtenstein GR, *et al*. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002;359:1541–9.
34 Sands BE, Anderson FH, Bernstein CN, *et al*. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004;350:876–85.
35 Rutgeerts P, D'Haens G, Targan S, *et al*. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999;117:761–9.
36 Present DH, Rutgeerts P, Targan S, *et al*. Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999;340:1398–405.
37 Afif W, Loftus EV Jr, Faubion WA, *et al*. Clinical utility of measuring infliximab and human anti-chimeric antibody concentrations in patients with inflammatory bowel disease. *Am J Gastroenterol* 2010;105:1133–9.
38 Bendtzen K, Ainsworth M, Steenholdt C, *et al*. Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. *Scand J Gastroenterol* 2009;44:774–81.
39 Colombel JF, Feagan BG, Sandborn WJ, *et al*. Therapeutic drug monitoring of biologics for inflammatory bowel disease. *Inflamm Bowel Dis* 2012;18:349–58.
40 Mould DR, Green B. Pharmacokinetics and pharmacodynamics of monoclonal antibodies: concepts and lessons for drug development. *Bio Drugs* 2010;24:23–39.
41 Colombel JF, Sandborn WJ, Reinisch W, *et al*. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010;362:1383–95.
42 Burger D, Travis S. Conventional medical management of inflammatory bowel disease. *Gastroenterology* 2011;140:1827–37.
43 Lin MV, Blonski W, Lichtenstein GR. What is the optimal therapy for Crohn's disease: step-up or top-down? *Expert Rev Gastroenterol Hepatol* 2010;4:167–80.
44 De CP, Kamm MA, Prideaux L, *et al*. Mucosal healing in Crohn's disease: a systematic review. *Inflamm Bowel Dis* Published Online First: 26 April 2012. doi: 10.1002/ibd.22977
45 Bjarnason I, Hayllar J, MacPherson AJ, *et al*. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* 1993;104:1832–47.
46 Forrest K, Symmons D, Foster P. Systematic review: is ingestion of paracetamol or non-steroidal anti-inflammatory drugs associated with exacerbations of inflammatory bowel disease? *Aliment Pharmacol Ther* 2004;20:1035–43.
47 O'Brien J. Nonsteroidal anti-inflammatory drugs in patients with inflammatory bowel disease. *Am J Gastroenterol* 2000;95:1859–61.
48 Rutgeerts P, Feagan BG, Lichtenstein GR, *et al*. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004;126:402–13.
49 Rutgeerts P, Diamond RH, Bala M, *et al*. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006;63:433–42.

# Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease

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