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ORIGINAL ARTICLE

NOD2 Polymorphism Predicts Response to Treatment in Crohn's Disease—First Steps to a Personalized Therapy

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Abstract
Background and Aims Great efforts have been made to predict disease behavior over time and the response to treatment in Crohn's disease (CD). Such understanding could personalize therapy. Early introduction of more aggressive therapies to patients at high risk and no introduction of predictable refractory treatments could become possible. We hence tested the influence of the *NOD2* carrier status on treatment response.
Patients and Methods In 185 CD patients (age 45 ± 9.8 years, female $n = 108$, minimum disease duration 10 years), the three most common polymorphisms (*p.Arg702Trp*, *p.Gly908Arg*, *p.Leu1007fsX1008*) of *NOD2* were tested by polymerase chain reaction and sequencing. Detailed clinical and medical history were obtained with a standardized questionnaire and by reviewing the medical charts. Treatments introduced were chosen by physicians blinded to genotype data.
Results The frequency of the *NOD2* variant allele was about one-third (67, 30.2%) of CD patients. *NOD2* carriers were more often treated with systemic and locally active steroids and with an immunosuppressant (Azathioprine/6-MP). *NOD2* mutation carrier status was more often associated with systemic steroid [8.9% vs. wild-type (WT) 1.2%, $P = 0.0086$] and local-steroid refractory (14.9% vs. WT 3.5%; $P = 0.001$). The WT patients were significantly higher refractory to immunosuppressant (12.8% vs. *NOD2* carriers, 0.5%, $P = 0.03$). Most WT patients were treated with TNF-α antagonists and remission rates were significantly higher in this group after 1 year of treatment (84% vs. *NOD2* carriers, 33%, $P = 0.07$).
Conclusions The study presents first hints for the *NOD2* carrier status to be predictive for response to therapy. A higher percentage of CD patients with *NOD2* mutation carrier status was steroid refractory but could be treated well with immunosuppressants. The WT status showed a higher response to steroids and remission rates within 1 year of anti-TNF-α therapy. On the way to personalized medicine, this approach should be further investigated in larger studies.

Keywords *NOD2* · Crohn's disease · TNF-α antagonist · Top-down · Step-up

Abbreviations

6-MP	6-Mercaptopurine
AZA	Azathioprine
CARD	Caspase recruitment domain
CD	Crohn′s disease
CDAI	Crohn′s disease activity index
IBD	Inflammatory bowel disease
NOD	Nucleotide oligomerization domain
SNP	Single-nucleotide polymorphism
TNF	Tumor necrosis factor
WT	Wildtype

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Introduction

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. It is a multifactorial, polygenic disease with genetic heterogeneity. In addition to genetic predisposition, various host (e.g., epithelial, immune, and nonimmune) and environmental factors play a major role in the pathogenesis of CD [1, 2]. Guidelines recommend that most patients with active disease should be treated initially with corticosteroids [3, 4]. Although this approach is usually effective for control of symptoms, many patients become refractory to, or dependent on, these drugs [5, 6]. For this reason, a treatment with corticosteroid-sparing drugs, such as azathioprine, mercaptopurine, or methotrexate, should be initiated [7–9]. Recent published data underline the concept of an early combined immunosuppression in CD, the so-called "top-down" strategy [10]. A combined immunosuppressive therapy with antimetabolites/methotrexate and TNF-α antagonists is associated with a higher risk of opportunistic infections and hepatosplenic T cell lymphoma [11]. Therefore, great efforts have been made to predict disease behavior over time and the response to treatment in CD. Early introduction of more aggressive therapies to patients at high risk of disabling disease, and no introduction of predictable refractory treatments to reduce side effects of therapies, could become possible [12]. Attempts have been made to define clinical subgroups on the basis of age at onset, disease location, extent (diffuse or localized), and behavior (primary inflammatory, fistulizing, or fibrostenotic disease). Mucosal TNF-α transcripts in steroid-refractory CD patients receiving immunosuppressive therapy may have predictive values [13].

Crohn's disease has a strong genetic component, with a lifetime risk of 10–20% to develop CD in the presence of an affected first-degree relative, thus defining subgroups based on genetic mutations might be a helpful marker [14, 15]. To date, genome-wide meta-analysis has identified at least 71 loci that confer susceptibility to CD [16]; the first, and most consistently replicated, critical mutations were found in the *CARD15/NOD2* gene on chromosome 16 (IBD1) [17]. The physiological role of the NOD2 protein remains under detailed examination. Variant *NOD2* alleles are associated with reduced (alpha)-defensin release from Paneth cells in response to bacteria [18]. Of particular importance is the C-terminus leucine-rich repeat domain, reportedly the major structural motif that functions as a pattern-recognition receptor for the microbial component muramyl dipeptide [19].

Two single-nucleotide polymorphisms of *NOD2* (*p.Arg702Trp and p.Gly908Arg*) and a frame-shift mutation (*p.Leu1007fsX1008*) were shown by independent groups to be associated with susceptibility to CD [20–22]. The presence of 1 variant allele increases the risk of developing CD from 1.5- to 4.3-fold; the presence of 2 copies increases the risk to 20- to 40-fold [23–25]. CD patients with *NOD2* mutations exhibit early onset of the disease, mainly ileal involvement and increased risk of surgical intervention after developing complications such as strictures, fistulas and stenosis [14, 17, 26]. *NOD2* mutation carrier status does currently not allow the predicting of disease progression and the need of immunosuppressive therapies such as steroids, azathioprine or biologicals (i.e. TNF-α antagonists).

Based on these observations, we aimed to test a possible influence of the *NOD2* carrier status on response to standard medical treatments. Such understanding could personalize therapy.

Patients and Methods

Study Population and Disease Phenotype

Written, informed consent was obtained from all patients prior to the study. The study was approved by the Ethics committee of the Ulm University and adhered to the ethical principles for medical research involving human subjects of the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm). For the diagnosis of CD, established diagnostic guidelines including endoscopic, radiological, and histopathological criteria were used [27]. Patients with CD were assessed according to the Montreal classification based on age at diagnosis (A), location (L), and behavior (B) of the disease. Patients with colonic inflammatory bowel disease unclassified (IBDU) were excluded from the study. Phenotypic characteristics included demographic data and clinical parameters (behavior and anatomic location of IBD, disease-related complications, previous surgery or immunosuppressive therapy) which were recorded by investigation of patient charts and a detailed questionnaire including an interview at the time of enrolment. All phenotypic data were collected blind to the results of the genotypic data.

DNA Extraction and Genotyping of the *NOD2* Variants

Blood samples were taken from all study participants, and genomic DNA was isolated from peripheral blood leukocytes using the DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer's guidelines. DNA was amplified by PCR with primer pairs flanking the *p.Arg702Trp*, *p.Gly908Arg*, and *p.Leu1007fsX1008* variants as described [28]. After purification, PCR products were analyzed with the ABI PRISM Dye Terminator Cycle Sequencing KIT (Applied Biosystems, Darmstadt,

Germany) on an ABI 373A DNA-sequencer using the same primers applied for amplification.

Definitions of Response to Therapy

All patients were treated according to the German clinical practice guidelines on the diagnosis and treatment of CD [27] blinded to the genotype data. Patients received budesonide (9 mg/day), prednisolone (2 mg/kg up to 60 mg), immunomodulators (2.5 mg/kg for AZA and 1–1.5 mg/kg for 6-MP), infliximab (5 mg/kg at weeks 0, 2, 6 and every 8 weeks) or adalimumab (80 mg starting dose followed by 40 mg every second week). When patients were treated with steroids, remission was defined by a decrease of the CDAI score to 150 or less. Patients who responded to prednisolone but relapsed upon steroid withdrawal were defined as steroid-dependent. Patients who did not respond to steroids, defined by decrease of the CDAI score of at least 70 within the first 4 weeks, were defined as steroid-refractory [27]. When immunomodulators (AZA/6-MP) were given, clinical remission was defined by a decrease of the CDAI score to 150 or less after steroid withdrawal for more than 3 months. When TNF-α antagonists (infliximab/adalimumab) were used, remission was defined as a decrease of the CDAI score to 150 or less after 2–3 infusions (infliximab, weeks 3–7) or after 3 injections (adalimumab, week 6).

Statistical Analyses

All data given in the text and figures are expressed as mean values ± SEM. The data were analyzed using non-parametric two-tailed Mann–Whitney U test with $P \leq 0.05$ considered as an indicator of significance. In addition, a multivariate assessment of the relationship between the independent variables "group [wild-type (WT) vs. NOD2]," "localization (ileus, colon, etc.)," "stricture (yes vs. no)," "fistula (yes vs. no)," "surgery (yes vs. no)" and the dependent outcome variable "therapy response to steroids (yes vs. no)" was carried out. Due to the binary characteristic of the dependent variable "therapy response," a multivariate logistic regression model has been chosen as statistical method for analyzing the data.

Results

Demographic Characteristics of the Study Population

One hundred and eighty-five patients were included in our retro-perspective study. *NOD2* carrier status was found in 77 patients including 1 homozygous *NOD2* carrier. The demographic characteristics and disease location according to the Montreal classification are depicted in Table 1. More patients with *NOD2* variants had disease location at the ileal site; significantly more patients with *NOD2* carrier

Table 1 Demographic characteristics of the study population

	$NOD2^{-/-}$	$NOD2^{+/-}$	$NOD2^{+/+}$	Significance P value
Male *n* (%)	41 (53%)	35 (45%)	1 (1.3%)	0.634
Median age at diagnosis (year)	30.1 (14–59)	26.4 (15–48)	18	0.712
Disease location (*n* and %) (Vienna)				
Ileal disease: L1	26/118 (22.1%)	21/68 (30.9%)		0.1076
Colonic disease: L2	10 (8.2%)	3 (4.1%)		0.09
Ileocolonic disease: L3	59 (50.8%)	33 (48.8%)	1	0.7966
Upper gastrointestinal involvement	15 (13.1%)	2 (3.1%)		<0.05
Anal involvement[a]	24 (21.1%)	10 (14%)	1	0.134
Disease behavior (*n* and %) Vienna				
Inflammatory (B1)	48 (41%)	34 (50.5%)	1	0.356
Stricturing (B2)	16 (14%)	19 (28.2%)	1	0.08
Penetrating (B3)	53 (45%)	20 (29%)	1	<0.05
Need for IBD surgery	52 (44%)	49 (72%)	1	<0.05
History of smoking	63 (53%)	31 (45%)		Not significant
Extraintestinal manifestations	39 (33%)	24 (35%)		Not significant
p.Arg702Trp		31		
p.Gly908Arg		34	1	
p.Leu1007fsX1008		18		

[a] (L1) + (L3) + (L4 − patients with ileal involvement)

Table 2 Medication of the study population including 67 patients with *NOD2* variants

Medication	Study collective[a]	WT *NOD2* status[b]	*NOD2* carrier[c]
Budesonide	50.8% (94/185)	44.1% (52/118)	62% (42/67)
Prednisolone	75.7% (140/185)	66.1% (78/118)	92.5% (62/67)
Immunomodulators	36.7% (68/185)	39.7% (35/118)	49.2% (33/67)
Anti-TNF-α	13.5% (25/185)	15.3% (18/118)	10.4% (7/67)

[a] Percentage of patients of the total study collective receiving the indicated medication. The numbers in parentheses indicate the total numbers of patients within the study collective of 185 patients treated by the indicated medication

[b] Percentage of patients with the WT *NOD2* status treated by the indicated medication. The numbers in parentheses indicate the total numbers of 118 patients with WT *NOD2* status receiving the indicated medication

[c] Percentage of patients with *NOD2* variants receiving the indicated medication. The numbers in parentheses indicate the total numbers of 67 patients with *NOD2* variants treated by the indicated medication

status developed stricturing and/or penetrating disease behavior as compared to *NOD2* WT patients confirming previous reports (Table 1).

Ninety-four patients were initially treated with budesonide including 42 *NOD2* carriers, 140 patients including 62 *NOD2* carriers were treated with prednisolone, 68 patients were treated with immunosuppressants(AZA/6-MP), including 33 *NOD2* carriers, and 25 patients received TNF-α antagonists (infliximab or adalimumab, respectively) (Table 2).

More *NOD2* Carriers are Refractory to Treatment with Budesonide

First, we determined the response to budesonide. Seventy-one percent of *NOD2* WT patients responded to the treatment with budesonide, 21% were budesonide-dependent and 8% were refractory to budesonide. Budenoside treatment of *NOD2* WT patients was more effective in patients with ileal-cecal disease location. When *NOD2* carriers were analyzed, 33% of *NOD2* carriers responded to budesonide, 19% were steroid-dependent and 48% were refractory to treatment with budesonide. Budesonide-treated patients with *NOD2* variants were significantly impaired in response to budesonide (Fig. 1). Together, this data indicated that patients with *NOD2* WT status show better response rates to budesonide as compared to patients with *NOD2* variants.

Impaired Response to Prednisolone in CD Patients with *NOD2* Carrier Status

Next, we determined the response to prednisolone in our study collective. Fifty-seven percent of patients with *NOD2* WT status were sensitive to the treatments with prednisolone, 39% were steroid-dependent, and 2% were refractory to prednisolone treatment. When *NOD2* carriers were analyzed, 46% of *NOD2* carriers were sensitive to prednisolone, 35% were steroid-dependent, and 17% were refractory to treatment with prednisolone. Again, significantly more patients with *NOD2* variants were refractory to prednisolone as compared to patients with *NOD2* WT status, but this difference was not as pronounced as with budesonide (Fig. 1). Because more patients with *NOD2* variants had disease location at the ileal site and developed more likely stricturing and/or penetrating disease behavior, we tested in a multivariate logistic regression model if the independent variables localization (ileal site), stricturing or internal fistulizing disease behavior and surgery influence the therapy success of the treatment with steroids. The success of treatment with steroids did not depend on disease location, the development of stricturing and/or penetrating disease behavior and not on surgery. The success of treatment with prednisolone depended in our model and patient collective only on the *NOD2* carrier status (Table 3).

Response of *NOD2* Carriers to Immunomodulators (AZA/6-MP)

Because the percentage of patient refractory to the treatment with prednisolone was significantly increased among the patients with *NOD2* variants as compared to patients with *NOD2* WT status, we next analyzed the response of patients with *NOD2* variants to immunomodulators (AZA/6-MP). Sixty-five percent of patients with *NOD2* WT status went into remission under treatment with AZA/6-MP, whereas 34% of patients with *NOD2* WT status were refractory to treatment with AZA/6-MP. Eighty-eight percent of patients with *NOD2* variants went into remission under treatment with AZA/6-MP, and 12% of patients with *NOD2* variants were refractory to treatment with AZA/6-MP. The percentage of patients with *NOD2* variants in remission under treatment with AZA/6-MP was significantly increased as compared to patients with *NOD2* WT status (Fig. 1). In contrast, significantly more patients with *NOD2* WT status were refractory to treatment with AZA/6-MP as compared to patients with *NOD2* variants.

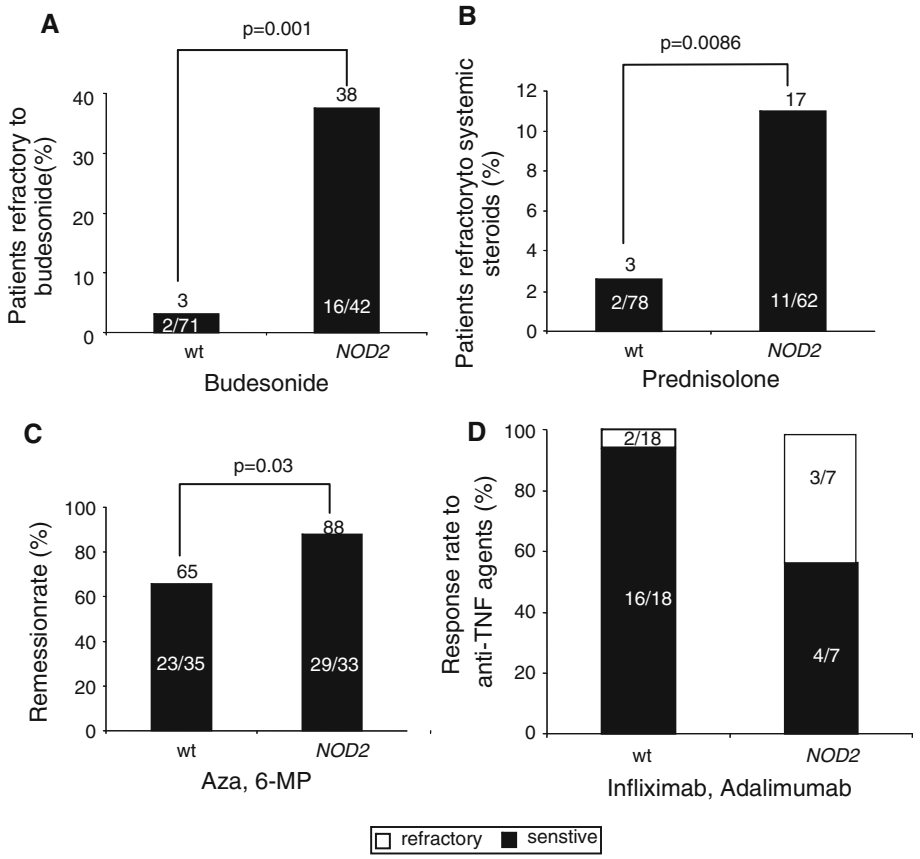

Fig. 1 **a** The percentage of patients with *NOD2* WT status responding to treatment with budesonide is increased as compared to patients with *NOD2* variants. The percentage of patients refractory to budesonide treatment is presented. **b** The percentage of patients with *NOD2* carrier status refractory to prednisolone treatment is increased. In the non-parametric two-tailed Mann–Whitney *U* test, $P \leq 0.05$ was considered statistically significant; *WT*, *NOD2* wild-type status; *NOD2*, patients with *NOD2* variants. **c** The percentage of patients with *NOD2* carrier status in remission under treatment with immunomodulators (AZA/6-MP) is increased as compared to patients with *NOD2* wild-type status. The percentage of patient in remission under treatment with AZA/6-MP with *NOD2* carrier status was compared with WT *NOD2* patients. **d** Patients with *NOD2* wild-type status respond to treatment with TNF-α antagonists. *Black area* indicates the percentage of patients in remission under treatment with TNF-α antagonist, and the *white area* indicates the percentage of patients responding to treatment with TNF-α anatgonist. *Numbers* within the area indicate the numbers of patients within the total numbers of patients per indicated group; *numbers on top of the bars* indicated the percentage of patients in the respective group. A non-parametric two-tailed Mann–Whitney *U* test was used; $P \leq 0.05$ was considered as an indicator of significance; *WT*, *NOD2* wild-type status; *NOD2,* patients with *NOD2* variants

Response of *NOD2* Carriers to Treatment with TNF-α Antibodies (Infliximab/Adalimumab)

We next analyzed the response of the patients to the TNF-α antagonists infliximab or adalimumab. Eighty-nine percent of patients with *NOD2* WT status went into remission under treatment with TNF-α antagonists. Eleven percent of patients with *NOD2* WT status were refractory to treatment with TNF-α antagonists. Fifty-seven percent of patients with *NOD2* variants were sensitive to TNF-α antagonists, and 43% of patients with *NOD2* variants were refractory to TNF-α antagonists (Fig. 1).

All together, our results demonstrate that CD patients with *NOD2* WT status differ in response to standards of medication as compared to *NOD2* carriers.

Discussion

Treatment algorithms and the drug history in CD in dependence on the *NOD2* mutation carrier status have not yet been investigated. We have shown that patients with CD and *NOD2* carrier status were more refractory for steroids but could be treated well with immunosuppressives. The patients with WT *NOD2* status, who were steroid-dependent, showed a significantly lower response to treatment with immunomodulators (AZA/6-MP).

In our patients, more CD patients with *NOD2* variants were refractory to treatment with budesonide and/or prednisolone. In a multivariate logistic regression model, treatment success with prednisolone was independent of disease localization (ileal site), stricturing or internal

Table 3 Effects of the independent variables localization (ileal site), stricturing or internal fistulizing disease behavior and surgery on therapy success with systemic steroids in a multivariate logistic regression model

	P value
Localization (ilelal site L1 + L3)	0.4682
Stricturing (B2)	0.1015
Internal fistulizing (B3)	0.8845
Surgery	0.8992

In the multivariate logistic regression model, a *P* value <0.05 was considered as statistically significant

fistulizing disease behavior, and the need for surgery. Another study could not find an association of *NOD2* carrier status and response to steroids [29]. In contrast to the study of Weiss et al., median age of disease is >18 years in our study cohort. Carrying out the analysis of treatment responses in dependence of *NOD2* variants may differ significantly between patient cohorts with pediatric and adult CD patients.

Associations between polymorphism in the *TNF-α* gene, but not in the multidrug resistance gene 1 (MDR-1), and response to treatment with steroids has been described in an Italian pediatric IBD cohort [30]. High expression of glucocorticoid receptors by mononuclear cells in the peripheral blood of CD patients may predict the response to treatment with steroids [31]. Data of glucocorticoid receptor expression of CD patients with or without *NOD2* mutation carrier status are still missing. In patients receiving TNF-α antagonists gene expression profiling and *IL-23R* variants may predict treatment response to TNF-α antagonists [32, 33]. It might be possible that patients with a reduced glucorticoid receptor expression could have benefited from early combination therapy with immunosuppressants [12].

In our study, CD patients with *NOD2* WT status responded to steroids. The percentage of patients with *NOD2* WT status refractory to budesonide or prednisolone was decreased as compared to patients with *NOD2* carrier status.

More patients with *NOD2* carrier status were treated with AZA/6-MP, and the percentage of patients going into remission under treatment with AZA/6-MP was increased as compared to patients with *NOD2* WT status. AZA/6-MP metabolites and TPMT activity were not detected on a routine basis in our study [34, 35]. Because most patients received remission under treatment with AZA/6-MP, the discrepancy between patients with *NOD2* WT status and patients with *NOD2* variants may be associated with genetic and biochemical factors that need to be defined in future studies.

The AZA/6-MP refractory patients with *NOD2* WT showed response to TNF-α antagonists. Several studies have investigated the influence of *NOD2* polymorphism on response to TNF-α antagonists. *NOD2* polymorphism is not predictive for the outcome of treatment with infliximab [36, 37]. We included in our study patients treated with infiximab and adalimumab which may explain differences to previous studies.

NOD2 carriers are characterized by early onset of CD associated with strictures and penetrating disease behavior and increased need for surgery as previously reported [38–40]. In our patients, *NOD2* carriers are characterized by early onset of disease, but increased need for surgery could not be confirmed.

Disease phenotype and location are considered to predict disabling disease. Young age, smoking habits, perianal lesions and severe ulcerations are clinical predictors of risk for progressive disease [15, 41]. All treatment regimens in our study were chosen by an algorithmic approach based on national guidelines [27]. Selection of medication depended on the interpretation of the clinical data by the individual physician blinded to the *NOD2* genotype status.

Interpretation of the results of our study is limited by the facts that analysis of remission rates depending on treatment regimen was carried out in a retrospective manner, and not in a prospective controlled clinical trial at a single IBD study center only, and not in a multicentre approach. In our study with a limited sample size, a replication cohort is missing. Carrying out genome-wide association studies (GWAS) could be particularly interesting to identify additional variants associated with disease behavior and response to treatment with standard medication.

The task to choose the right medication for an individual IBD patient will likely become more complex in future. Although the prediction of treatment response by phenotype, genotype and serological parameters is still in its infancy, the individual choice of the treatment regimen may help to maximize efficacy, minimize delays to effective treatment, and improve safety and tolerability.

In conclusion, our data show that CD patients without *NOD2* mutations suffering from a steroid-dependent or refractory course have significantly less chance to reach steroid-free remission by a treatment with immunosuppressive agents as compared to CD patients with *NOD2* mutations, which was independent disease localization (ileal site), stricturing or internal fistulizing disease behavior, and the need for surgery. Otherwise, these *NOD2* WT status patients were very sensitive to anti-TNF-α antibodies and all patients reached steroid-free remission. Although this group of patients was small in our study, our results give a first hint that a top-down therapy strategy could be effective especially in these patients. Further studies are needed to prove this concept. This could be an important step toward a personalized therapy in CD patients.

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