Novel NOD2 Mutation in Early-Onset Inflammatory Bowel Phenotype

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Background: Nucleotide-binding oligomerization domain 2 (NOD2) is a key intracellular protein of the innate immune system. *NOD2* variants are associated with inflammatory bowel disease (IBD) and other inflammatory phenotypes. We described the case of a baby with a very early-onset IBD who is characterized by a rare homozygous variant in *NOD2,* found through whole-exome sequencing, Its pathogenic effect was investigated through bioinformatics and functional studies.

Methods: The microbicide activity of the patient's phagocytes was analyzed using *Escherichia coli*. HEK293 and Caco2 cell lines were transfected with wild-type and mutated NOD2 cDNA to evaluate the NF-kB activity and the protein distribution. The functionality of the NOD2 pathway was assessed through analysis of the expression of tumor nectrosis factor alpha (TNFα) on monocytes. The levels of various cytokines were quantified in the patient plasma by a multiplex suspension array.

Results: A missense *NOD2* mutation, c.G1277A; p.R426H in homozygosis, was found. The patient's microbicide activity was comparable to that observed in controls. HEK293 cells transfected with the mutated cDNA showed a 20-fold increase of NF-kB activation in basal condition. Moreover, Caco2 immunostaining revealed a different cytoplasmic distribution of the mutated protein compared with wild-type. A higher production of TNFα by monocytes and elevated levels of plasmatic cytokines and chemokines were evidenced in the patient.

Conclusions: This homozygous mutation is functionally relevant and shows a different NOD2 involvement in the IBD phenotype. In our patient, this mutation caused a gain of function typical of the Blau syndrome phenotype, manifesting, however, an IBD-like phenotype.

Key Words: EO-IBD, NOD2, WES, rare disease, cytokines profile

INTRODUCTION

Nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs) are a group of intracellular key receptors of the innate immune system. Along with toll-like receptors, they account for the immediate response to bacterial invasion or to any tissue damage. Abnormal activation of these sensors results in excessive inflammation. In fact, mutations in genes encoding these receptors are correlated with monogenic autoinflammatory disorders or with increased susceptibility to multifactorial inflammatory diseases, such as inflammatory bowel disease (IBD).

In particular, *NOD2* is considered a major and the first susceptibility gene.[1] It is implicated and strongly associated with IBD pathogenesis[2]; in particular, 3 known polymorphisms (R702W, G908R, and L1007PfsX2) are strongly associated with Crohn's disease (CD) in both European and American populations.

NOD2 is an intracellular receptor for the peptidoglycan fragment muramyl dipeptide (MDP), belonging to the cytosolic NLRs, and it plays an important role in the immune response against the peptidoglycan derived from bacterial lipopolysaccharides (LPS).[3] It can activate the transcription factor NF-kB, leading to the production of proinflammatory and antimicrobial molecules.

The protein of NOD2 presents a tripartite structure with 2 N-terminal caspase recruitment domains (CARDs), nucleotide-binding domain (NBD), located in the central part of the protein, and a C-terminal domain with several repeat motifs rich with multiple leucine (leucine-rich repeat domain [LRR]), which bind the product of degradation of bacterial peptidoglycan, muramyl dipeptide (MDP).[4]

It is notable that variants in different NOD2 domains have been recently described as correlated with distinct disease phenotypes. In fact, mutations in the nucleotide-binding domain (NBD) are associated with Blau syndrome

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(BS), whereas NOD2-associated autoinflammatory diseases (NAIDs)[5] and IBD are related to mutations located between the NBD and LRR domains and in the LRR domain, respectively. Thus, *NOD2* mutations may be responsible for a clinical continuum ranging from complex multifactorial diseases, such as IBD, to monogenic autoinflammatory disorders, such as BS and with NAID, likely to represent intermediate clinical and pathological disorders.[6, 7]

Here we report the case of a boy with VEO-IBD who was found homozygous for a rare *NOD2* variation in the NBD domain. This variant (rs562225614) is reported on http://exac.broadinstitute.org/ with a minor allele frequency (MAF) of 0.00005 and on https://www.ncbi.nlm.nih.gov with an MAF of 0.002.

The characterization of the patient phenotype associated with the bioinformatics and functional studies of the variant identified may further expand the genetic complexity of NOD2-associated inflammatory disorders.

CASE PRESENTATION

In this article we describe a baby male who, at 5 months, presented poor growth with hematochezia, semiformed stools with mucus. At six months, he was diagnosed with allergic proctocolitis and started a diet of nutramigen and, subsequently, of a free amino acid–based formula, yet with little benefit on the diarrhea or growth (Table 1). Moreover, after some months, in spite of a brief course of glucocorticoids, perianal abscesses occurred, raising the suspicion of an inflammatory bowel disease. The boy was thus admitted again to the hospital and underwent a colonoscopy that showed diffusely edematous mucosa without vascular distortion affected by scattered microhemorrhage areas and rare areas of deeper erosion without fibrin deposition at the bottom. These areas were more frequent in the most distal segments, the sigma and rectum.

The analysis of mucosal biopsies showed multiple hemorrhagic lesions, lymphatic hyperplasia to the sigmoid colon, and an eosinophilic infiltrate, leading to the diagnosis of an intercurrent infection in eosinophilic colitis. However, diarrhea and rectorrhagia resumed, and therapy with glucocorticoids and azathioprine was started for the diagnosis of inflammatory eosinophilic ileocolitis. Considering the worsening of the clinical condition and the poor response to drugs, an exclusive amino acid diet was started.

In addition to gastrointestinal involvement, at 2 years, he presented arthritis at the left ankle, which was treated with antibiotics and nonsteroidal anti-inflammatory drugs. He presented low growth between the third and 10th centiles and weighed less than the third centile. Due to the very early age at

TABLE 1: Clinical Characteristics of Patient

	2013			2014			2015	2016	2017
	March	April–May	August–September	January	March	May–July	March–April	June–July	September
Laboratory data	Prick test – Rast test –	CALP 1143 VES – CRP – Prick test – Rast test –	CALP 588 VES 27 Prick test – Rast test –	CALP 1092 VES 22 CRP 1.4	VES 36 CRP 1.2	CALP –	CALP –	CALP 749 VES 41 CRP 1	VES 18
Stools	Mucus	Bloody and mucus	Bloody	Slightly bloody and mucus	Slightly bloody and mucus	Normal	Normal	Normal	Slightly bloody
Clinical procedures and features	Allergic proctocolitis	Proctoscopy allergic proctocolitis, small anal fissure	Colonoscopy perianal abscess	NA	Colonoscopy, perianal fistula, eosinophilic, ileocolonic	Colonoscopy, left ankle arthritis	Abdominal sonography	Gastroscopy, colonoscopy, severe colitis	Perianal fissure
Therapy	Hypoallergenic infant formula (nutramigen)	Milk complete formula (sineall)	Rice cream, vegetables, and lyophilized meat	NA	Sineall and rice cream, vegetables, and lyophilized meat	Cefixime, ibuprofen, amino acid diet	NA	Steroid, azathioprine, mesalazine	Azathioprine, mesalazine

CALP (mg/kg); CRP (mg/dL); VES (mm/h).

Abbreviations: CALP, calprotectin; NA, data not available.

disease onset, the poor response to treatments, and the association of possible extra-intestinal manifestations, the physicians performed genetic analysis for a monogenic form of IBD, and the boy was enrolled in a program for genetic diagnostics of early-onset IBD at the IRCCS Burlo Garofolo, Trieste.

METHODS

Whole-Exome Sequencing

Genomic DNA was extracted from an EDTA blood sample using the EZ1 DNA Blood 350-µL Kit (QIAGEN, Milano, Italy), and 1 µg was employed by the Ion Proton System platform (Life Technologies) at the sequencing service of the CRIBI center (University of Padua) to capture the whole exome. Raw sequencing data were collected as unmapped reads in fastQ format. CLC Genomics Workbench version 6.5 software was employed to check the quality of reads, to map reads back to the human reference genome (hg19 version), to calculate the overall coverage, to perform local realignment and base quality recalibration, and, finally, to call single nucleotide variants (SNVs) and small insertions/deletions (INDELs), all of them collected into a standardized variant call format (VCF), version 4.1.[8] SNVs/INDELs were annotated using ANNOVAR software, referring to the known and frequent public databases.[9]

We took into consideration the SNVs/INDELs related to a list of several genes associated with and/or causative of the IBD phenotype, having an MAF lower than 0.01, as reported in 1000 Genomes Project (http://www.1000genomes.org/), NHLBI Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/), and EXAC (http://exac.broadinstitute.org/), referring to the general population. At the same time, we selected only the variants that were predicted as potentially "pathogenetic," from 2 in silico algorithm software packages (Polyphen-2; SIFT; LRT; Mutation Taster), and as phylogenetically conserved, based on GERP++ scores reported in the dbNSFP v2.0 database.

Mutation was confirmed by polymerase chain reaction amplification using the KAPA 2G Fast Hot Start Readymix (RESNOVA, Roma, Italy), and purified polymerase chain reaction products were bidirectionally sequenced using the ABI PRISM 3130XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) with Sanger sequencing in the patients and her parents. Finally, sequences were analyzed with Seqman II Software (DNASTAR I Lasergene, 7.0, Madison, WI, USA).

Neutrophil Killing Assay

The microbicide activity toward *Escherichia coli* ATCC 25922 (kindly provided by Dr. Sergio Crovella) was assessed as previously described by Decleva et al.[10] Briefly, the patient's neutrophils and neutrophils from 5 healthy controls were isolated from 5 mL of EDTA whole-blood samples by density gradient centrifugation over Percoll and suspended in Hepes buffered saline solution. Neutrophils were then incubated at 37°C with serum-opsonized *E. coli* at a neutrophil-to-bacteria ratio of 1:5. After 10 minutes or 30 minutes of incubation, neutrophils were lysed in distilled water at pH = 11, and the number of surviving bacteria was determined by counting the colony forming units (CFU; on Luria Bertani 1% agar medium).

Construction of Expression Plasmids

NOD2 wild-type human expression vector was obtained from Origene (RC215750; NM_022162), and *NOD2* mutant construct was generated by site-specific mutagenesis PCR using PfuUltra High-Fidelity DNA Polymerase (Agilent Technologies) using specific primer according to the manufacturer's protocols and thermal cycle condition (95°C 2 minutes; 20 cycle of 95°C 30 seconds, 60°C 45 seconds, 72°C 8 minutes 30 seconds; final elongation at 72°C 10 minutes).

Forward 5'- CAAGGTGGTGACCAGC CATCCGGCCGCTGTGTC-3'

Reverse 5'- GACACAGCGGCCGGAT GGCTGGTCACCACCTTG-3'

Immunofluorescence Assay

Caco-2 cells (ATCC No. HTB-37) were seeded at a density of 4×10^4cells/well into a sterile 4-well tissue culture PCA slide chamber (Sarstedt, Nümbrecht, Germany). After 48 hours, cells were transiently transfected with 0.5 µg of *NOD2* plasmids (*NOD2*_WT and *NOD2*_426H constructs) alone or in association with 10 µg/mL MDP-L18 (InvivoGen, San Diego, CA, USA) using FuGENE HD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions.

After 24 hours, cells were stained with Vybrant Dil cell-labeling solution (Molecular Probes), fixed and permeabilized with 4% PFA and 0.5% Triton X100 (Sigma-Aldrich, Milano, Italy), and successively incubated for 1 hour with primary antibodies (clone 4C5 anti DDK-antibodies; 1:1000; Origene) and for a further hour with secondary antibodies (anti-Mouse IgG Alexa Fluor 488; 1:1000; Cell Signaling Technology). The coverslips were mounted using Mowiol 40–88 (Sigma-Aldrich, Milano, Italy) and subsequently analyzed with a confocal microscope (Nikon C1si, Nikon, Tokyo, Japan) containing 488-nm argon laser and 561-nm diode lasers. Light was delivered to the sample with an 80/20 reflector. The system was operated with a pinhole size of 1 Airy disk (30 nm). Electronic zoom was kept at minimum values for measurements to reduce potential bleaching. A 60X Plan Apo objective was used, collecting a series of optical images at 0.25-µm z resolution step size. Images were processed for z-projection by using ImageJ 1.44m (NIH, Bethesda, MD, USA).

NF-kB Activation Assay

HEK293 cells were seeded at a density of $3*10^5$ in 6-well plates, and then transfected overnight with 2 µg of *NOD2* construct (WT or 426H) together with 2 µg of pNFkB-MetLuc2-Reporter vector; pMetLuc2-control Vector was used as transfection control (Ready-To-Glow Secreted Luciferase pMetLuc Vector Kit; Clontech, CA, USA). After 18 hours, cells were treated with 10 µg/mL of MDP-L18 and only the control vector with TNFα (100 ng/mL; Peprotech) for 3, 6, or 24 hours. At each time point, the luciferase activity was measured by using the Ready-to-Glow Secreted Luciferase Assay (Clontech, CA, USA), according to the manufacturer's instructions, and was normalized to control activity.

Monocyte TNFα Expression

By means of a cytometric protocol, the production of tumor necrosis factor–alpha (TNFα) triggered in monocytes by purified MDP was analyzed to depict possible alterations in the NOD2 pathway. The protocol was adapted from a clinical technique already used for the screening of a group of primary immunodeficiencies.[11] Briefly, 2×10^5 peripheral blood mononuclear cells (PBMCs) were suspended in RPMI (EuroClone, Milano, Italy) supplemented with 10% human AB serum (Sigma-Aldrich, Milano, Italy), 2-mM L-glutamine, 100-U/mL penicillin, and 0.1-mg/mL streptomycin (EuroClone, Milano, Italy). The cells were stimulated with NOD ligand (500-ng/mL L-18 MDP; InvivoGen, San Diego, CA, USA) for 4 hours at 37°C in a CO_2 incubator, in the presence of 10-µg/mL Brefeldin A (BFA; Sigma Aldrich, Milano, Italy) to inhibit the secretion of newly synthesized cytokine. FITC conjugated anti-CD14 antibody (eBiosciences) was then added for surface staining to identify the monocyte, followed by a fixation step with FACS Lysing Solution and a permeabilization step using FACS Permeabilizing Solution 2 (both from BD Biosciences, San Jose, CA, USA). After a washing step, anti-TNFα PE antibody or an isotype control antibody (PE IgG Isotype control; BD Biosciences, San Jose, CA, USA) was added to perform the intracellular staining. Finally, the cells, fixed with PBS-PFA1%, were acquired on a CyAn ADP flow cytometer (Beckman Coulter, Fort Collins, CO, USA) and analyzed using FlowJo software v 7.6 (TreeStar, Ashland, OR, USA). Results are expressed as percentage of TNFα-positive monocytes, after gating on CD14-positive cells.

Plasma Cytokine Profiles

Plasma from the patient (before and after clinical treatment) and 5 healthy controls was obtained by centrifugation of heparinized blood at 4°C at 1300xg for 10 minutes and stored at −80°C for cytokine profiling analysis.

Samples were analyzed using multiplex bead-based assay to evaluate the concentration of 17 cytokines (Bioplex human 17 plex, BioRad), including interleukin 1 beta (IL1β), IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12p70, IL13, IL17, interferon gamma (IFNγ), monocyte chemotactic protein 1 (MCP1/MCAF), macrophage inflammatory protein-1beta (MIP1β), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNFα. Plasma samples were analyzed in duplicate, following the manufacturer's instructions (Bio-Rad Laboratories), based on Luminex xMAP technology. Data were collected and analyzed using the Bio-Plex manager software (version 4.0).

RESULTS

Whole-Exome Sequencing

Whole-exome sequencing (WES) revealed the missense homozygous mutation c.1277G>A (p. R426H, rs562225614) in *NOD2* (NM_022162.1). This variant, found in heterozygosis in the boy's healthy parents, lies in the conserved NBD domain and was predicted as damaging by different bioinformatics tools (Fig. 1).

Killing Activity

The microbicide activity of the patient's neutrophils against *E. coli* (78.5% of killed bacteria at Time 10 and 83.5% at Time 30) was similar to healthy controls (85% of killed bacteria at Time 10 and 94% at Time 30) at both incubation times (Fig. 2). These results appear to rule out a major role of defective microbial killing in the pathogenesis of the disease.

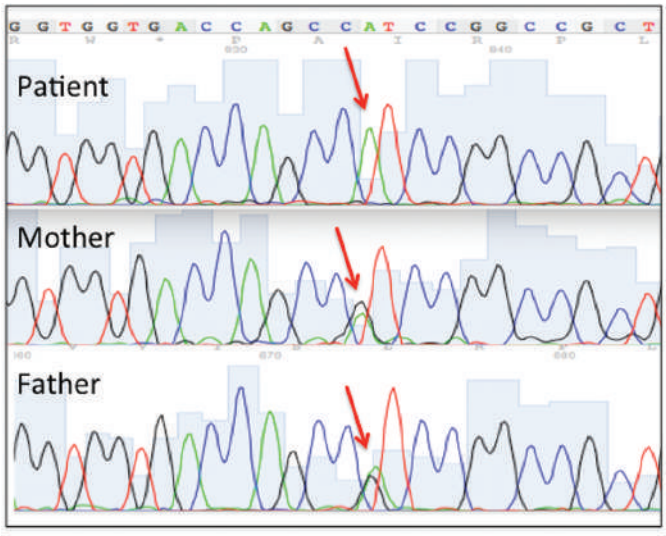
Localization of NOD2

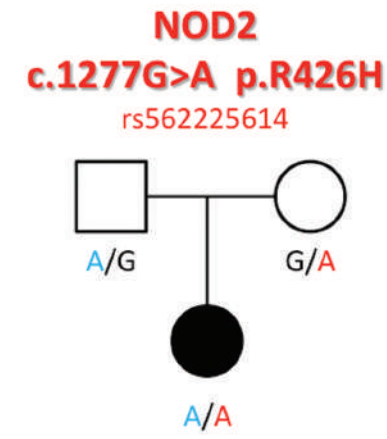
Cellular localization of NOD2 expressed in Caco-2 cells, transfected either with the wild type or the mutant vector, was assessed using immunostaining and confocal microscopy.

Immunostaining revealed a different cytoplasmic distribution of NOD2 depending on the presence of the 426H mutation and/or the MDP-L18 stimulation. Caco-2 cells transfected with *NOD2*_WT vector showed a widespread homogeneous distribution, which changed after stimulation with MDP-L18. In contrast, the NOD2_426H-mutated protein showed a spotted aggregation regardless of the treatment conditions, supporting constitutive recruitment in activated multiprotein complexes (Fig. 3).

NF-kB Activation

NOD2 is an intracellular receptor able to activate the transcriptional factor NF-kB after MDP. To test the ability of the mutated NOD2 protein (426H) to activate the NF-kB factor, luciferase activity was measured 3, 6, or 24 hours after stimulation with MDP-L18. The increment in luciferase activity for each experimental condition was determined by comparing the luminescence values measured in nontransfected (NT) cells (Fig. 4).





Chr	snp138	EXAC	1000g	PhyloP	SIFT	PP2	LRT	MutT	GERP
16	rs562225614	0.000057 7hz; 0 Hom	NA	C	D	D	D	D	4,91

FIGURE 1. Electropherogram, inheritance model of transmission, protein domain, and bioinformatics prediction of the investigated mutation. The figure shows electropherograms of the mutation (c. G1277A: p. R426H) in exon 4 of NOD2 in the genomic DNA of a patient and his parents (heterozygous). The schematic of the protein structure indicates the localization of the mutation in the NBD domain. Finally, the minor allelic frequency is shown (HZ and Hom are the number of heterozygous and homozygous cases, respectively) with reference to the EXAC and 1000*g* databases. SIFT, Polyphen2 (PP2), LRT, and Mutation Taster (MutT) describe the in silico variation pathogenicity prediction (D, deleterious). GERP and PhyloP indicate the nucleotide evolutionary conservation (C, conserved).

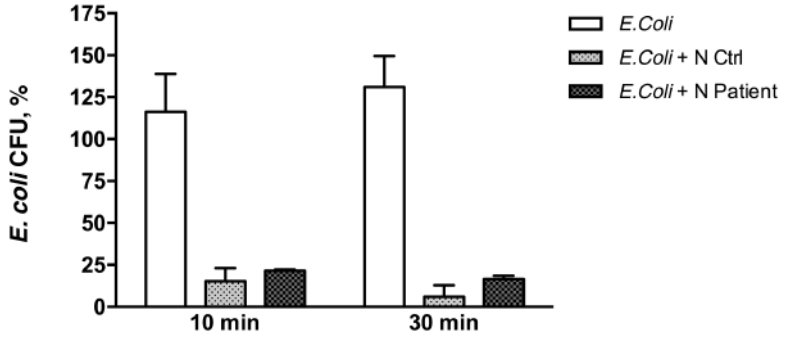


FIGURE 2. Neutrophil killing assay. Neutrophils (N) derived from a patient and from 5 healthy controls were incubated with *E. coli* for 10 or 30 minutes. The number of viable bacteria after the killing test is expressed as the percentage of CFU with respect to the count of total *E. coli* CFU at time 0.

Three hours after cell treatment, NOD2_426H induced a 20-fold increase in NF-kB activation with respect to NT, both in basal condition and after MDP stimulation. In WT cells, there was only a 10-fold increase. After 6 hours of stimulation, the level of luminescence was comparable between NOD2_WT and 426H; however, elevated levels of luminescence in basal conditions were recorded only in the presence of the 426H mutation. After 24 hours' incubation, luciferase activity in the basal condition was comparable between the WT and mutated conditions.

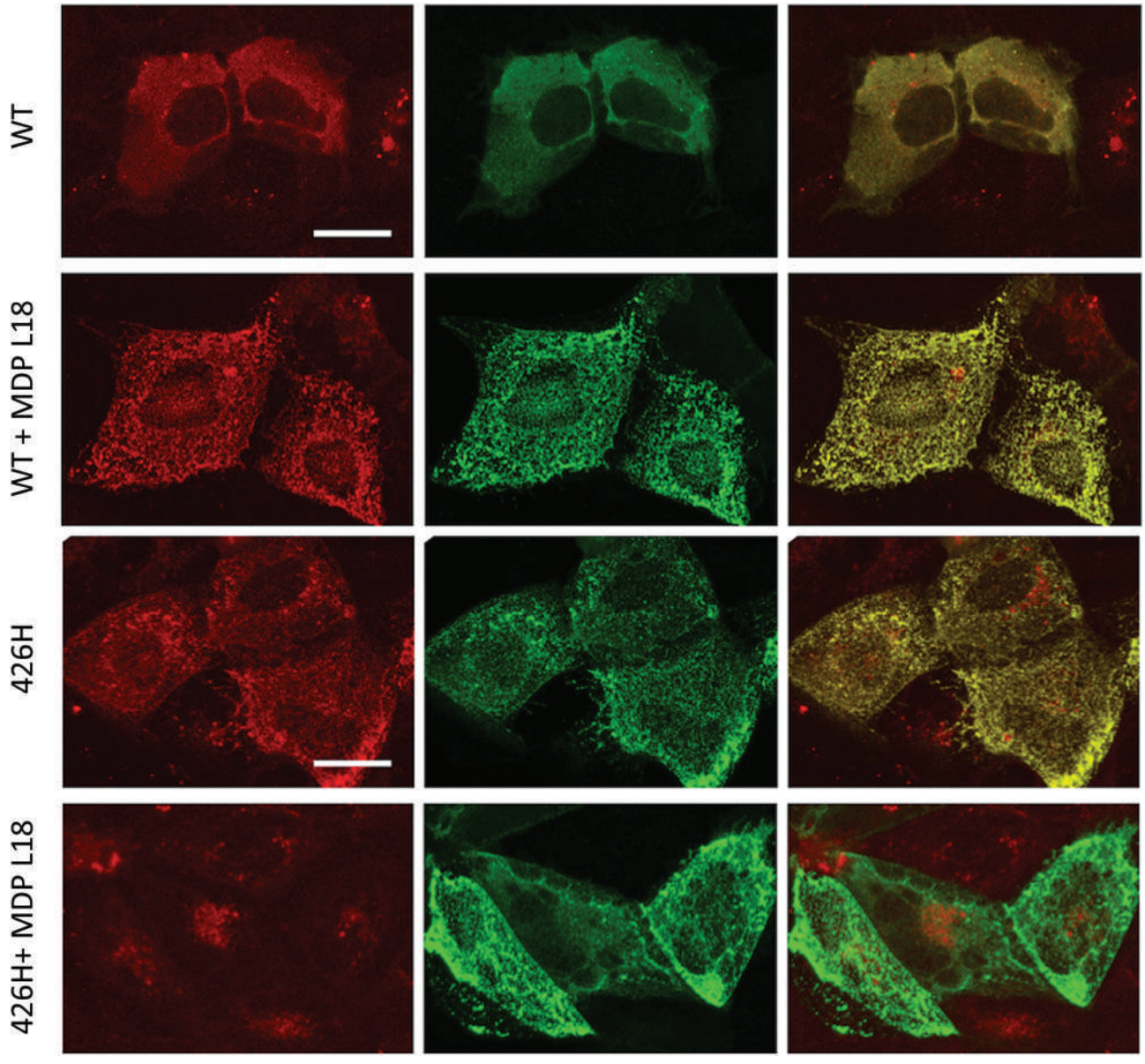


FIGURE 3. Expression and cellular localization of NOD2_WT and mutated construct. Caco2 cell line was transfected with a NOD2_WT or NOD2_426H vector; the cellular localization of the mutant vector shows a similar cytoplasmic distribution, only compared with WT construct after MDP-L18 stimulation.

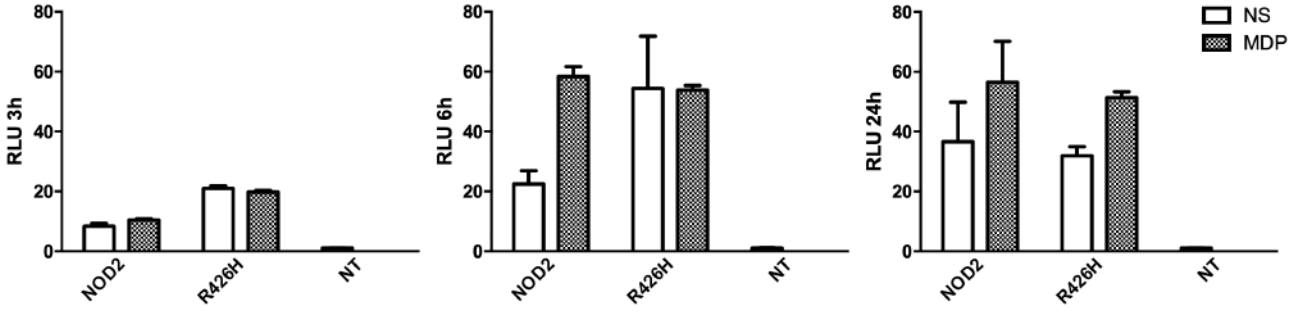


FIGURE 4. Luciferase activity in cell lines transfected with different NOD2 constructs. HEK293 cells were transfected with NOD2_WT and 426H constructs and then stimulated with MDP_L18. The luciferase activity was measured after 3, 6, and 24 hours to test the ability of the protein to correctly activate the NF-kB transcriptional factor. Abbreviations: MDP, stimulation with MDP; NS, not stimulated; NT, not transfected.

Monocyte TNFα Expression

To investigate the functionality of the innate immune pathway, lympho-monocyte cells derived from the patient and 5 healthy controls were stimulated with MDP to activate the NOD2 pathway. TNFα expression in monocytes was measured by flow cytometry after gating CD14-positive cells.

At diagnosis, the patient was characterized by an apparent inflammatory state, as evidenced by the higher production of TNFα by peripheral monocytes, compared with the average values obtained in healthy subjects. This state is detectable both in the basal condition (untreated) and after stimulation with microbial compounds (Fig. 5). Clinical treatment led to a reduction of monocyte activation, even if the percentages of the TNFα- expressing monocytes in the patient remained higher than those in controls.

With the addition of LPS, the difference was less evident, because this TLR4 ligand is able to produce a massive TNFα expression that leads to pathway saturation.

Plasma Cytokine Profiles

An increased level of plasma cytokines and chemokines, which appeared normalized during clinical remission, confirmed the presence of an important inflammatory state in the patient at diagnosis. Here we reported a representative trend of plasmatic levels of inflammatory cytokines (IL1β, IL6, and TNFα) detected in the control population and in the patient in activity and remission states of the disease (Fig. 6).

An opposite trend was observed in the case of GM-CSF, MCP1, and MIP1β; after clinical treatment, the plasma levels of these analyses were higher if compared with the first analysis, out of therapy (Table 2).

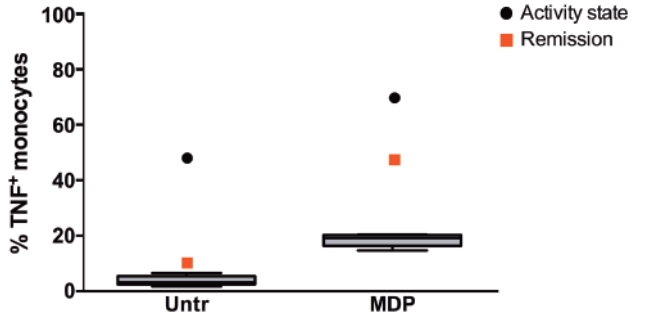


FIGURE 5. TNFα expression in peripheral monocytes. Intracellular TNFα expression in peripheral monocytes derived from a patient (activity and remission state) and 5 healthy donors (gray box plot), in basal condition (Untr) or after stimulation with MDP.

DISCUSSION

NOD2 is an intracellular receptor belonging to the cytosolic NLR family. It can recognize and bind the peptidoglycan component MDP and activate the downstream pro-inflammatory pathway mediated by NF-kB.

Among the 3 major variants described as being associated with Crohn's disease, the homozygous status of L1007PfsX2 located in the LRR domain causes a loss of function (LOF) of NOD2 protein, concerning its capacity to activate the NF-kB.[12, 13] This evidence suggests an inability of the receptor to recognize microbial patterns and activate the immune response.

Otherwise, single nucleotide polymorphisms in the NBD domain are frequently found in patients affected by BS, a disease inherited as an autosomal dominant trait. They cause a gain of function (GOF) leading to an auto-activation of NOD2-mediated NF-kB signaling.[14] Of note, BS is clinically characterized by eye, skin, and articular inflammation, but there is usually no intestinal involvement (Table 3). Our functional studies demonstrate that the consequence of the mutation is a GOF, but the patient's phenotype does not correspond to BS as it does not match the phenotype; eye exams were always negative.

In our patient, we found a homozygous missense variation in the NBD domain, leading to an amino acid change in the 426 residues. The parents of the proband were healthy and heterozygous for the mutation. A structural model of NOD2 protein has already shown that R426 locates in the Mg^{2+}/ATP patch, the site of ATP hydrolysis needed both for receptor activation and deactivation.[14] An alteration of this amino acid is thought to be crucial for the correct function and activation of NOD2.

We performed several studies that allow, for the first time, a functional characterization of this rare variant. Even if we cannot be sure that the disease of our patient is due to homozygous R426H mutations, bioinformatics

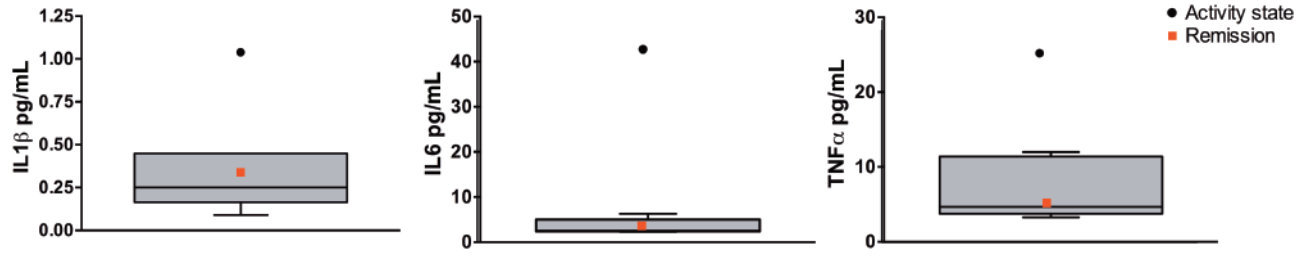


FIGURE 6. Plasma cytokine levels. Plasma levels (expressed in pg/mL) of 3 representative inflammatory cytokines (IL1β, IL6, and TNFα) quantified in a patient (activity and remission state) and in 5 healthy donors (gray box plot).

TABLE 2: Plasma Cytokine Levels

Cytokines	HC	Patient Activity State	Patient Remission State
G-CSF	6.53 (2.50–17.21)	84.86	4.06
GM-CSF	108.7 (35.09–112.7)	3.11	147.20
IFNγ	6.67 (3.18–23.11)	46.03	9.22
IL1β	0.25 (0.16–0.45)	1.04	0.34
IL2	2.88 (1.20–9.11)	130.51	0.16
IL4	0.24 (0.10–0.41)	0.90	0.40
IL5	0.17 (0.11–0.69)	1.30	0.11
IL6	2.50 (2.40–5.02)	43.40	3.63
IL7	4.07 (2.38–17.59)	5.91	1.14
IL8	2.63 (1.94–4.11)	21.78	5.09
IL10	3.76 (2.58–6.45)	34.53	3.97
IL12	4.58 (3.75–12.04)	50.73	7.66
IL13	1.31 (0.49–2.46)	6.38	1.70
IL17	15.3 (9.75–19.70)	40.27	15.30
MCP1	52.62 (47.99–69.66)	80.40	148.98
MIP1β	18.33 (15.89–23.68)	78.22	106.07
TNFα	4.66 (3.75–11.40)	26.74	5.28

Values (pg/mL) of 5 healthy controls are shown as median (interquartile range).

Abbreviation: HC, healthy control.

TABLE 3: Characteristics of Blau Syndrome, Crohn Disease, and Naid

	BS	CD	NAID	Patient
Inheritance	AD	NM	NM	AR (?)
Early onset	+	+	-	+
Eye involvement – uveitis	+	+/-	-	-
Arthritis	+	-	+/-	+
Skin rash/dermatitis	+	+/-	+	-
Gastrointestinal involvement	-	+	+	CD eosinophilic ileocolitis
Mesenteric adenitis	+/-	+/-	-	+
NOD2 gene mutation	NBD	LRR	Between NBD and LRR	NBD

"+" characteristic/present; "+/-" possible; "–" absent.

Abbreviations: AD, autosomal dominant; LRR, leucin-rich repeat domain; BS, Blau syndrome; CD, Crohn's disease; NAID, NOD2-associated autoinflammatory diseases; NBD, nucleotide binding domain; NM, non-Mendelian.

and functional data support this possibility. All our in vitro functional studies indicated that the R426H NOD2 variant is associated with auto-activation and hyperactivation of the protein.

By immunofluorescence assay, we showed that the formation of the macromolecular complex, indicative of inflammatory activation, occurs in WT NOD2 cells only after stimulation, while cells transfected with the mutated construct are present also in basal conditions, supporting a constitutive activation of the protein. This interpretation is coherent with the results from the NF-kB reporter assay, which showed a basal inflammatory activation only in the presence of the mutated NOD2.

As the patient at the diagnosis was characterized by an evident inflammatory state, we investigated the TNFα monocytes' expression and measured the cytokine profile in activity and remission states of the disease. Monocytes' TNFα expression remained elevated both in basal conditions and after stimulation with microbial compounds such as MDP and/or LPS. In addition, the plasma levels of both chemokines and cytokines, which were rather high enough at the time of diagnosis, were normalized during clinical remission.

Literature data report cases of overlapping symptoms of 2 rare diseases caused by mutations in the same gene but in different domains.[15] Overall, our results suggest that the 426H mutation may be responsible for an intermediate phenotype between autoinflammatory BS and multifactorial NOD2-associated CD, similar to other cases that have been classified in the poorly defined group of NAID.

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