# NOD2 Is Regulated By Mir-320 in Physiological Conditions but this Control Is Altered in Inflamed Tissues of Patients with Inflammatory Bowel Disease

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**Background:** Large evidence supports the role of microRNAs as new important inflammatory mediators by regulating both the adaptive and innate immunity. In the present study, we speculated that miR-320 controls NOD2 (nucleotide-binding oligomerization domain) expression, because it contains multiple binding sites in the 3′-untranslated region of the gene. NOD2, the first gene associated to increased susceptibility to Crohn's disease, is a cytosolic receptor that senses wall peptides of bacteria and promotes their clearance through initiation of a proinflammatory transcriptional program. This study aims at demonstrating that NOD2 is a target of miR-320 as well as investigating the role of inflammation in modulating the miR-320 control on NOD2 expression and analyzing miR-320 expression in intestinal biopsies of children with inflammatory bowel disease.

**Methods:** The colonic adenocarcinoma cell line HT29 was used to assess the miR-320-mediated regulation of NOD2 expression. MiR-320 and NOD2 expression were analyzed in mucosal samples of 40 children with inflammatory bowel disease.

**Results:** During inflammation, NOD2 expression is inversely correlated with miR-320 expression in vitro and ex vivo. Exogenous miR-320 transfection in HT29 cells leads to a significant decrease of NOD2 expression, whereas the miR-320 inhibitor transfection leads to increase of NOD2 expression, nuclear translocation of nuclear factor κB, and activation of downstream cytokines.

**Conclusions:** We show for the first time that NOD2 expression is under the control of miR-320. We also show in vitro and ex vivo that inflammation induces a decrease of miR-320 and the latter correlates negatively with NOD2 expression.

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**Key Words:** inflammation, innate immunity, miRNAs, NOD2, pediatric IBD

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC). The exact cause of IBD remains unknown, although available evidence suggests that an abnormal immune response against the microorganisms of the intestinal flora is responsible for the disease in genetically susceptible individuals.[1,2] Traditionally, the adaptive immune response has been considered to play a major role in the pathogenesis of IBD; however, recent advances in immunology and genetics have clarified that the innate immune response is equally as important in inducing gut inflammation.[3]

The most compelling support for the concept of a primary defect in innate immunity leading to IBD development comes from the clear genetic association between CD and the carriage of polymorphisms within the nucleotide-binding oligomerization domain 2 (NOD2) gene.[4–7] NOD2 is a cytosolic receptor that senses wall peptides of bacteria and promotes their clearance through initiation of a proinflammatory transcriptional program and other host defense pathways, including autophagy.[8–10] NOD2 is broadly expressed in macrophages, dendritic cells, intestinal epithelial cells,[11] and even in T cells.[12] A balanced level of NOD signaling is crucial for the maintenance of the immune homeostasis.

The increasing incidence of IBD,[13] in particular the occurring at very young age,[14,15] in the developed world has catalyzed studies attempting to characterize the pathogenic mechanisms. After the advent of Genome Wide Association studies that has dramatically improved the resolution of the IBD genome, there is now strong interest in exploring epigenetic mechanisms, including the contribution of microRNAs (miRNAs).

MiRNAs are small noncoding RNAs, 18 to 23 nucleotides long, which act as posttranscriptional regulators of gene expression. They are strongly implicated in the pathogenesis of many inflammatory and immune-mediated diseases,[16–19] including IBD.[20–27]

Two recent studies suggested that miRNAs may regulate NOD2 and its signaling pathway. In particular, Chuang et al

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identified an inverse correlation between miR-512, miR-192, miR-495 and miR-671 and NOD2 expression in response to muramyl dipeptide (MDP) stimulation[28]; furthermore, Chen et al[29] showed that miR-122, targeting NOD2, inhibits the innate immune activation through nuclear factor kappa B (NF-κB) pathway.

Here, we identified a miRNA family, miR-320, that contains multiple binding sites to the 3′-untranslated region (UTR) of NOD2. Hence, in the present study the aims are to (1) demonstrate that NOD2 is a target of miR-320, (2) investigate the potential role of inflammation in altering the miR-320 control on NOD2 expression and function, (3) analyze miR-320 expression in inflamed and uninflamed tissues of a group of pediatric patients with CD and UC and find a correlation with NOD2 expression.

## MATERIALS AND METHODS

### Patients

Forty consecutive pediatric patients, 20 with CD (median age, 14.3 yr; range, 10–17 yr), and 20 with UC (median age, 14 yr; range, 5–17 yr), referred to the Pediatric Gastroenterology and Liver Unit of Sapienza University of Rome-University Hospital Umberto I and needed an ileocolonoscopy to reassess the intestinal disease, were included in this study.

Patients were under treatment with immunomodulators (azathioprine or methotrexate), mesalamine, or oral corticosteroids at low doses. Patients undergoing biological therapy were excluded from the study.

Activity in CD was measured by the PCDAI (Pediatric Crohn's Disease Activity Index) score, a multi-item measure of clinical and laboratory parameters,[30] whereas the recently validated PUCAI (Pediatric Ulcerative Colitis Activity Index) score was used to measure activity in patients with UC.[31]

The intestinal inflammation was assessed at endoscopy by using the simple endoscopic score for Crohn's disease (SES-CD) score[32] and the endoscopic Mayo subscore[33] in patients with CD and UC, respectively.

### Ethical Considerations

All patients entered into the study after informed consent from parents. The study was approved by the Ethics Committee of the Hospital.

### Biopsy Specimens

Mucosal biopsy specimens, taken from the uninflamed and inflamed colon of patients, were immediately snap-frozen in liquid nitrogen for miRNA and RNA extractions. Absorbance ratio at 260 nm/280 nm and 260 nm/230 nm were used to assess the purity of RNA samples using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

RNA integrity was evaluated by running RNA gel electrophoresis and confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Samples, included in the study, showed an RNA integrity number value >6.

### Cell Cultures

The colonic adenocarcinoma cell line HT29 and a HT29 stable cell line harboring the NOD2 3′-UTR vector were used.

HT29 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Biochrom, Berlin, Germany) at 37°C, 5% $CO_2$. The proinflammatory cytomix, containing 1 U/mL of interferon γ (Sigma-Aldrich, St Louis, MO) and 10 ng/mL of tumor necrosis factor α (TNF-α) (Sigma-Aldrich) or MDP (50 μg/mL, Sigma-Aldrich), were added to culture medium to induce inflammation. After 24 hours, cells were harvested for RNA and protein analysis by real-time PCR (RT-PCR) and western blot, respectively.

HT29 stable cell line transfection was carried out to study the 3′-UTR of NOD2 activity. The nucleotide sequence of the NOD2 3′-UTR spanning 3229 to 4485 (RefSeq# NM_022162.1) was cloned into the pLenti-UTR-Luc Vector (ABM Inc., Richmond, BC, Canada) that was transfected into HT29 cells using jetPEI DNA Transfection Reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions. The pRL-CMV Vector (Promega, Madison, WI), was co-transfected in HT29 cells as an internal control.

After transfection procedures, cells containing the exogenous 3′-UTR of NOD2 were selected using puromycin at final concentration of 1 mg/mL (Sigma-Aldrich).

### Luciferase Assay

HT29 cells transfected with the NOD2 3′-UTR were cultured in 96-well plates ($5 \times 10^3$ cells per well) and, alternately, transfected with miRCURY LNA hsa-miRNA-320 a, b, c mimics, miRCURY LNA miRNA320 family inhibitor (anti-miR-320), miRNA mimic negative control (miR-NC), miRNA320 family inhibitor negative control (anti-miR-NC), miRCURY LNA miRNA Target Site Blockers (TSBs; antisense oligonucleotides that compete with miR-320 for the 3 binding sites of the 3′-UTR of NOD2; TSB I: 5′-GAAAGCTGCTTCCTGAATTA-3′, TSB II: 5′-ATCAAAGCTGAGGTCACTAA-3′, TSB III: 5′-TTTAAAGCTGCATTTGGAGC-3′), Target Site Blocker negative control (TSB-NC) (Exiqon Inc., Woburn, MA) using INTERFERin siRNA Transfection reagent (Polyplus Transfection). Luciferase assay was performed 24 hours posttransfection using Dual-Glo Luciferase Assay System according to manufacturer's protocol (Promega).

### miRNA Mimics and miRNA Inhibitors Transfection

HT29 cells were seeded in a 6 multi-well plate ($2 \times 10^5$ cells per well) the day before to reach 50% confluence at the time of transfection. After 24 hours, cells were transfected with each hsa-miR-320 a, b and c mimics, miRNA320 family inhibitor or their controls (miR-NC and anti-miR-NC, respectively) using the INTERFERin siRNA Transfection reagent (Polyplus Transfection). Transfected cells were incubated for 24 hours at 37°C in a 5% $CO_2$ incubator. Cells exposed to cytomix or to MDP were



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cultured for further 24 hours. Cell pellets were collected for protein and RNA extraction.

## miRNA and mRNA Expression

To quantify mature miRNA expression, total RNA was isolated using the *mir*Vana miRNA Isolation Kit (Ambion, Carlsbad, CA), and 10 ng of total RNA was reverse transcribed by a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

For mRNA expression analysis, total RNA was isolated using the RNeasy kit (QiaGen GmbH, Hilden, Germany), and 1 μg of total RNA was reverse transcribed by a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RT-PCR amplification was carried out with an ABI PRISM 7300 Sequence Detection System using the TaqMan MicroRNA Assays and SYBR green technique for miRNA and mRNA amplification, respectively (Applied Biosystems). Primers for mRNA detection were identified using the Primer Express v.3.0 (Applied Biosystems) provided with the ABI Prism 7300 sequence detector: NOD2 forward primer: 5′-AAATCAGGTTGCCGATCTTCA-3′; NOD2 reverse primer: 5′-CAGCCAATCCATTCGCTTTC-3′.

GAPDH forward primer: 5′-GCACCGTCAAGGCTGAGAAC-3′; GAPDH reverse primer: 5′-GAGGGATCTCGCTCCTGGA-3′.

For miRNA detection, specific primers are contained into TaqMan assay and used according to manufacturer's protocols (Applied Biosystems).

GAPDH expression level was used to normalize NOD2 mRNA expression. Expression level of each target miRNA was normalized to U6, a ubiquitously expressed small nuclear RNA, used as internal control.

The quantity of mRNA and miRNA relative to a reference gene was calculated by the $2^{-\Delta\Delta CT}$ method.

## Total Protein Extraction

Cell pellets were suspended in ice-cold lysis buffer (50 mM Tris [pH 7.4], 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 mM sodium orthovanadate), homogenized, and incubated in ice for 20 minutes. Samples were centrifuged at 14462xg for 10 minutes, and the supernatants were collected, quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA) and analyzed by western blotting.

## Nuclear and Cytosolic Extraction

HT29 cells were seeded in T25 flasks ($1 \times 10^6$ cells). After 24 hours, cells were transfected with the miRNA320 family inhibitor or miRNA inhibitor negative control using the INTERFERin siRNA Transfection reagent (Polyplus Transfection). Then cells were exposed to MDP (50 μg/mL) and cultured for further 24 hours. The culture supernatants were collected for enzyme-linked immunosorbent assay (ELISA) to quantify the proinflammatory cytokines, TNF-α and interleukin 8 (IL-8).

For nuclear and cytosolic separation, the pellet was resuspended in 0.5 mL of fractionation buffer (10 mM HEPES [*N*-2-hydroxyethylpiperrazine-*N*-2-ethanesulfonic acid; pH 7.4], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonylfluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and homogenized. Cells were allowed to swell on ice for 30 minutes and the homogenate was centrifuged for 2 minutes at 580xg. The supernatant was collected and used for cytoplasmic protein analysis. The nuclear pellet was washed in phosphate-buffered saline, then suspended in 20 μL of ice-cold nuclear extraction buffer (20 mM HEPES [pH 7.9], 350 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and incubated on ice for 20 minutes. Samples were freeze-thawed 3 times, centrifuged for 15 minutes at 12400xg, and supernatants were collected and used for the nuclear protein analysis.

## Enzyme-linked Immunosorbent Assay

To quantify the production of TNF-α and IL-8, the media collected were measured by ELISA assay using the TNF-α and IL-8 ELISA kit, according to manufacturer's protocol (R&D Systems, Minneapolis, MN).

## Immunoblot Analysis

About 15 μg of total proteins and 3 μg of cytoplasmic/nuclear proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred in polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) and blocked with TBS-T (Tris-buffered saline with Tween-20) containing 5% nonfat dry milk. Anti-NOD2 (1:1000; Abcam, Cambridge, MA), anti-β-actin (1:5000; Sigma-Aldrich), anti-poly ADP ribose polymerase (1:2000; Sigma-Aldrich), anti-NF-κB (p65) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in TBS-T containing 3% nonfat dry milk and incubated overnight at 4°C. Membranes were incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology), washed in TBS-T and developed with ECL-Plus (Amersham, Biosciences Europe, Freiburg, Germany). Densitometrical analysis of blots was performed using the Software ImageQuant Las500 (GE Healthcare Life Science, Uppsala, Sweden).

## Immunofluorescence

HT29 cells were cultured on chamber slides ($8 \times 10^5$ cells) and exposed to MDP after transfection with miR-320 family inhibitor (anti-miR-320) or miR-320 family inhibitor negative control (anti-miR-NC). Cells were fixed with 4% paraformaldehyde for 10 minutes and then permeabilized with 0.01% Triton X-100 (Sigma-Aldrich). Blocking solution (1% bovine serum albumin in phosphate-buffered saline) was added for 30 minutes before the slides were incubated with anti-NF-κB antibody (1:100; Santa Cruz Biotechnology), followed by incubation with the secondary anti-rabbit antibody Alexa Fluor 488 labeled (1:100; Molecular Probes, Eugene, OR). DAPI (4′,6-diamidino-2-phenylindole) was used as nuclear dye. The slides were washed,



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covered with Vectashield mounting solution (Vector Laboratories, Burlingame, CA) and visualized using a Nikon Eclipse 80i digital fluorescent microscope (Nikon, Tokyo, Japan).

## Statistics

All data presented are representative of 3 experiments and are given as mean ± SEM. Statistical analysis for significance was determined using the Student's *t* test or nonparametric Mann–Whitney with GraphPad InStat software (GraphPad software, San Diego, CA). For multiple group comparisons, the nonparametric analysis of variance test was used. A $P$ value less than 0.05 was considered statistically significant.

The correlation between NOD2 mRNA and miR-320 expression in patients with IBD was assessed by the Spearman's correlation test, using GraphPad InStat software. Correlations were made between $\Delta NOD2_{(infl.-uninfl.)}$ and $\Delta miR\text{-}320_{(infl.-uninfl.)}$, assuming ΔNOD2 as the difference between NOD2 expression in the inflamed sample (infl.) and NOD2 expression in the uninflamed sample (uninfl.) of the same patient, and $\Delta miR\text{-}320_{(infl.-uninfl.)}$ as the difference between miR-320 a, -b or -c expression in the inflamed sample (infl.) and miR-320 a, -b or -c expression in the uninflamed sample (uninfl.) of the same patient.

All expression levels were normalized using the appropriate reference gene: GAPDH for NOD2 expression and U6 for miR-320 expression.

# RESULTS

## 3′-UTR of NOD2 Includes 3 Binding Sites for Each miR-320 Member

We performed a bioinformatic analysis (Targetscan and microRNA.org database) and found that 4 members of miR-320 family, miR-320 a, -b, -c and -d, showed 3 binding sites on the 3′-UTR of NOD2 (Table 1).

## Endogenous miR-320 a, -b, and -c Members are Highly Expressed in HT29 Cells

Endogenous expression of miR-320 family was assessed by qRT-PCR in HT29 cell line, a well-accepted in vitro model of intestinal epithelium. Results showed that miR-320 a, -b, and -c were highly expressed, whereas miR-320 d was very lowly expressed (Fig. 1); thus, it was not considered in the following experiments.

These results indicate that HT29 cells express endogenous miR-320.

## NOD2 Is a Target of miR-320 Family

HT29 cells, stably transfected with NOD2 3′-UTR lenti-reporter-Luc vector, were co-transfected with each hsa-miR-320 a, -b, and -c mimics and luciferase activity was measured after 24 hours. Results showed that miR-320 a, -b, and -c significantly decreased luciferase activity as compared with negative control (miR-NC) (miR-320 a: $P < 0.05$; miR-320 b: $P < 0.05$; miR-320 c: $P < 0.01$) (Fig. 2A). To further confirm these data, cells were co-transfected with a hsa-miR320 family inhibitor (anti-miR-320): a significant increase ($P < 0.01$) of luciferase activity was observed (Fig. 2B).

These results clearly suggest that NOD2 is controlled by miR-320.

Using 3 different site-blockers (Fig. 2C) competing with miR-320 for the 3 NOD2 binding sites, we showed that the second site (nucleotides 553–575 for miR-320 a, -b and nucleotides 555–575 for miR-320 c) was the only 1 functionally active; indeed, it significantly increased the luciferase activity of NOD2 ($P < 0.05$) (Fig. 2D).

## miR-320 Downregulates NOD2 mRNA and Protein Expression

Next, NOD2 mRNA and protein expression levels were assessed in HT29 cells transfected with hsa-miR-320 a, -b, -c

**TABLE 1.** Members of miR-320 Family Have 3 Binding Sites Located in the NOD2 3′-UTR

| hsa-miR-320a | hsa-miR-320b | hsa-miR-320c | hsa-miR-320d |
|---|---|---|---|
| 3' agcggGAGAGUUGGGUCGAAAa 5'<br>431: 5' cauaaUUCAGGAAGCAGCUUUc 3'<br>NOD2 3'- UTR | 3' aacggGAGAGUUGGGUCGAAAa 5'<br>431: 5' cauaaUUCAGGAAGCAGCUUUc 3'<br>NOD2 3'- UTR | 3' uggGAGAGUUGGGUCGAAAa 5'<br>433: 5' uaaUUCAGGAAGCAGCUUUc 3'<br>NOD2 3'- UTR | 3' agGAGAGUUGGGUCGAAAa 5'<br>434: 5' aaUUCAGGAAGCAGCUUUc 3'<br>NOD2 3'- UTR |
| 3' agcgggagAGUUGGGUCGAAAa 5'<br>553: 5' aauauuagUGACCUCAGCUUUg 3'<br>NOD2 3'- UTR | 3' aacgggagAGUUGGGUCGAAAa 5'<br>553: 5' aauauuagUGACCUCAGCUUUg 3'<br>NOD2 3'- UTR | 3' ugggagAGUUGGGUCGAAAa 5'<br>555: 5' uauuagUGACCUCAGCUUUg 3'<br>NOD2 3'- UTR | 3' aggagAGUUGGGUCGAAAa 5'<br>556: 5' auuagUGACCUCAGCUUUg 3'<br>NOD2 3'- UTR |
| 3' agcggGAGAGUUGGGUCGAAAa 5'<br>819: 5' aaaagCUCCAAAUGCAGCUUUa 3'<br>NOD2 3'- UTR | 3' aacggGAGAGUUGGGUCGAAAa 5'<br>819: 5' aaaagCUCCAAAUGCAGCUUUa 3'<br>NOD2 3'- UTR | 3' uggGAGAGUUGGGUCGAAAa 5'<br>821: 5' aagCUCCAAAU GCAGCUUUa 3'<br>NOD2 3'- UTR | 3' agGAGAGUUGGGUCGAAAa 5'<br>822: 5' agCUCCAAAUGCAGCUUUa 3'<br>NOD2 3'- UTR |



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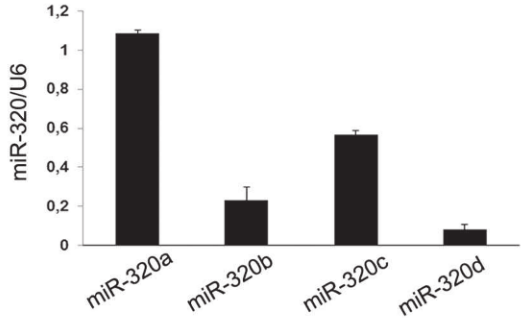


FIGURE 1. HT29 cells express endogenous miR-320. Expression of miR-320 a, -b, -c, and -d determined by qRT-PCR and normalized using U6 expression level.

mimics individually. Results showed that each miRNA significantly decreased both NOD2 mRNA and protein expression as compared with negative control (miR-NC), (miR-320 a: $P < 0.05$; miR-320 b: $P < 0.01$; miR-320 c: $P < 0.01$ and miR-320 a: $P < 0.05$; miR-320 b: $P < 0.001$, respectively) (Figs. 3A, C). To further confirm these data, HT29 cells were transfected with anti-miR-320: a significant increase of NOD2 mRNA and protein expression levels was observed ($P < 0.05$) (Figs. 3B, D).

These results show that miR-320 a, -b, and -c control NOD2 expression.

## Endogenous miR-320 Downregulation Causes NOD2 Expression Increase During Inflammation

NOD2 expression is known to be modulated during inflammation. Accordingly, HT29 cells were exposed for 24 hours to a mix of proinflammatory cytokines (cytomix: TNF-α + interferon γ), able to trigger a cytokine-mediated inflammatory response in intestinal epithelial cells, and NOD2 mRNA expression was analyzed. As expected, NOD2 expression was significantly increased ($P < 0.01$) (Fig. 4A). Afterwards, endogenous miR-320 a, -b, and -c expression was investigated after the exposure of cells to the cytomix or, alternately, to MDP, the specific agonist of NOD2 known to promote NOD2 expression.[34] Results showed a significant decrease of miR-320 a, -b, and -c ($P < 0.01$) (Fig. 4B).

These results suggest that the downregulation of endogenous miR-320 during inflammation is accountable of the NOD2 increased expression.

## Exogenous miR-320 is Able to Control NOD2 Even During Inflammation

To check the hypothesis that miR-320 would be able to control NOD2 expression even during an occurring inflammation, HT29 were transfected with exogenous hsa-miR-320 a, -b, and -c mimics and then exposed to cytomix for 24 hours. After treatments, NOD2 mRNA and protein expression levels were

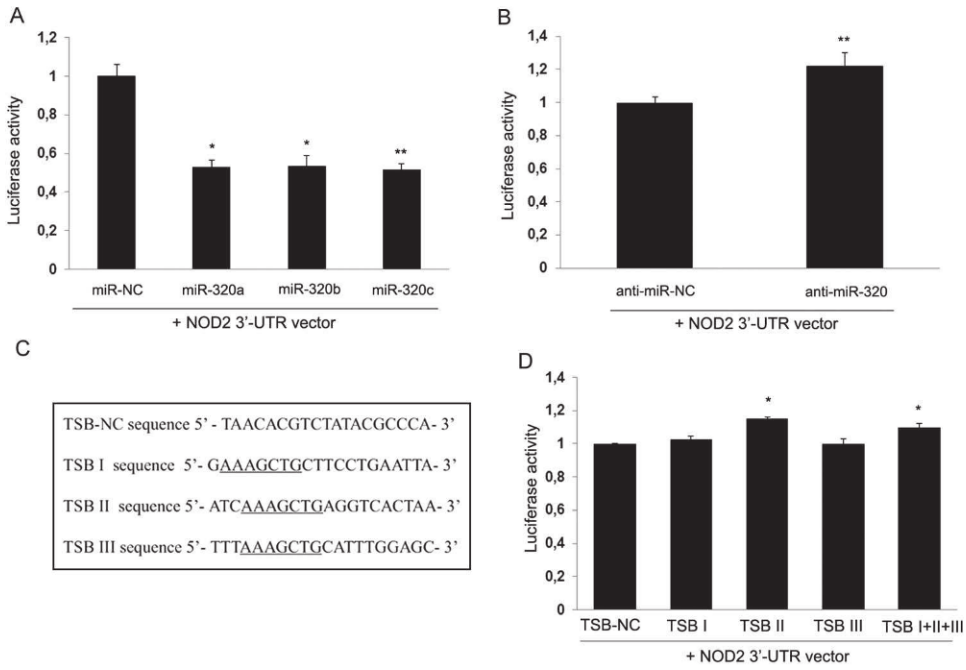


FIGURE 2. miR-320 a, -b, and -c target NOD2 3′-UTR. Luciferase assay in HT29 cells co-transfected with NOD2 3′-UTR-luc construct and control mimic (miR-NC), or miR-320 a, -b, and -c mimics (A) or miR-320 family inhibitor negative control (anti-miR-NC) or miR-320 family inhibitor (anti-miR-320) (B) or Target Site Blocker for the 3 binding sites in the NOD2 3′-UTR (TSB I, II, III: the underlined nucleotides perfectly hybridize with miR-320 target site preventing its interaction with 3′-UTR of NOD2 (C)) or Target Site Blocker negative control (TSB-NC) (D). Luciferase activity, determined 24 hours posttransfection, was normalized to Renilla luciferase activity. Values represent mean ± SEM. *$P < 0.05$; **$P < 0.01$.



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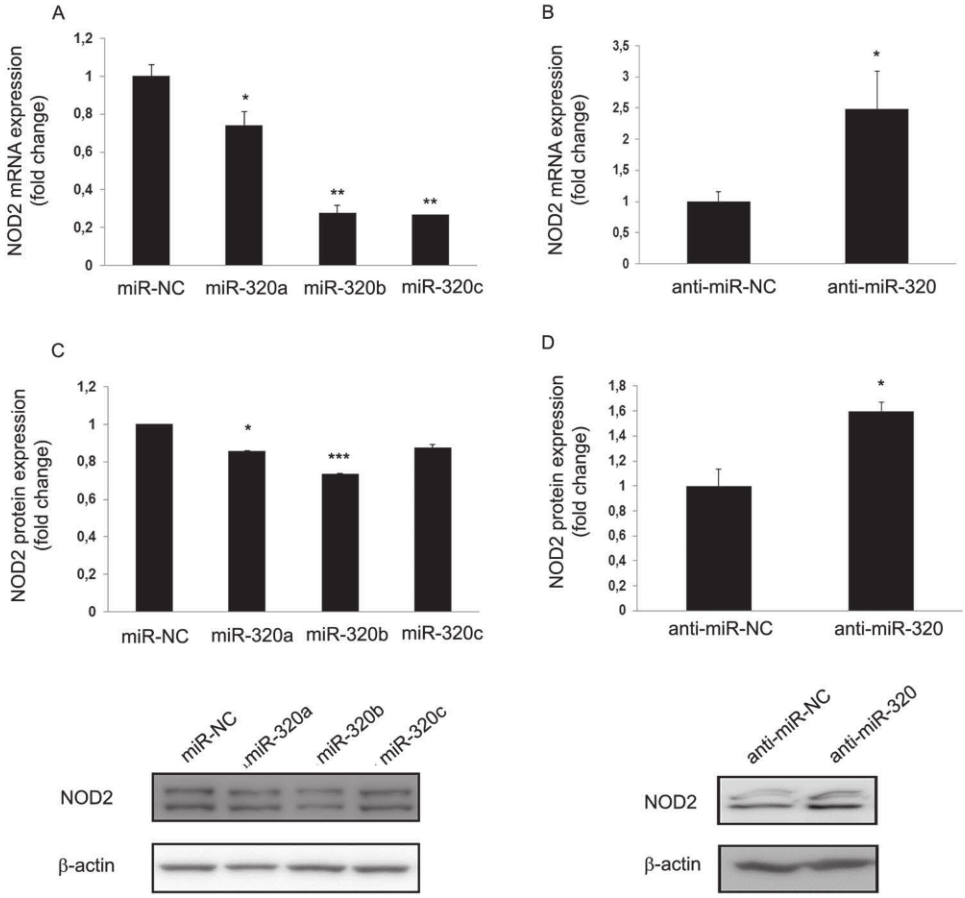


FIGURE 3. miR-320 downregulates NOD2 mRNA and protein expression. NOD2 mRNA (A and B) and protein (C and D) expression in HT29 cells after transfection with control mimic (miR-NC) or miR-320 a, -b, and -c mimics or miR-320 family inhibitor negative control (anti-miR-NC) or miR-320 family inhibitor (anti-miR-320) by qRT-PCR and western blot. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

analyzed. Results showed that each miR-320 mimic decreased NOD2 mRNA (miR-320 a: $P < 0.05$; miR-320 b: $P < 0.001$; miR-320 c: $P < 0.05$) (Fig. 5A), as well as protein expression (miR-320 a: $P < 0.01$; miR-320 b: $P < 0.05$; miR-320 c: $P < 0.05$) (Fig. 5C). Furthermore, to strengthen the data, cells, exposed to cytomix, were transfected with anti-miR-320 and a significant increase of both NOD2 mRNA and protein expression was observed ($P < 0.05$) (Figs. 5B, D).

These results show that, during inflammation, exogenous miR-320 are able to control NOD2 expression.

## Inhibition of miR-320 Upregulates the Activity of NF-κB and Transcription of Downstream Proinflammatory Cytokines

NOD2 mediates intracellular recognition of MDP and activates the transcription factor NF-κB that moves from the cytoplasm to the nucleus triggering the proinflammatory cytokines transcription. Thus, we investigated the translocation of NF-κB from the cytoplasm to the nucleus and the expression of inflammatory cytokines (TNF-α and IL-8) in HT29 cells after anti-miR-320 transfection and exposure to MDP by western blotting, immunofluorescence, and ELISA assay, respectively. We observed that, in response to increased expression of NOD2 (Fig. 6A) after treatment with MDP and transfection with anti-miR-320, the NF-κB shift to the nucleus is increased compared with anti-miR-NC transfected cells (Figs. 6B, C). Furthermore, we investigated the expression of proinflammatory cytokines, TNF-α and IL-8, after anti-miR-320-induced NF-κB activation. We found a significant increase ($P < 0.05$) of TNF-α and IL-8 in cells transfected with anti-miR-320 as compared with anti-miR-NC transfected cells (Fig. 6D).

These results show that miR-320 inhibition, causing NOD2 increased expression, promotes NF-κB activation and translocation to the nucleus, which in turn improves the transcription of proinflammatory cytokines.

## miR-320 Expression Is Significantly Decreased in the Inflamed Mucosa of Pediatric Patients with UC and CD and Inversely Correlated to NOD2 Expression

To confirm the altered expression of miR-320 under inflammatory conditions, the expression of miR-320 a, -b, and -c



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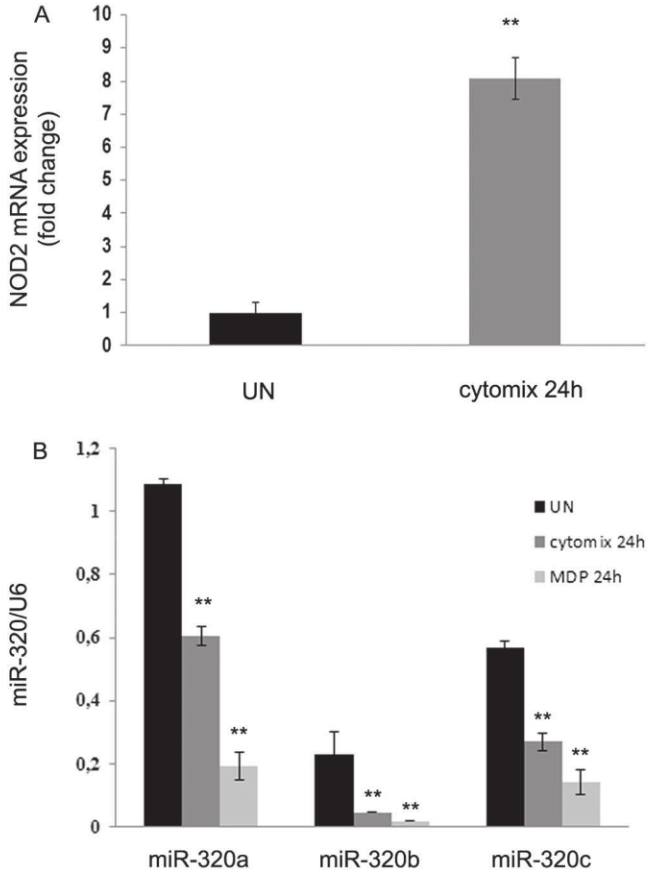


FIGURE 4. NOD2 and miR-320 expression during inflammation. Analysis of NOD2 mRNA (A) expression in HT29 cells exposed to cytomix (TNF-α and interferon γ) and miR-320 (B) expression levels in HT29 cells exposed to cytomix or MDP by qRT-PCR. $^{**}P < 0.01$.

was investigated in the inflamed colonic mucosal samples taken from 20 children with CD and 20 with UC. Results were compared with those obtained from the uninflamed mucosal areas of the same patients. Results showed that each miR-320 member was significantly decreased in the inflamed areas of all children with IBD (CD: miR-320 a: $P < 0.001$; miR-320 b: $P < 0.05$; miR-320 c: $P < 0.05$; UC: miR-320 a: $P < 0.01$; miR-320 b: $P < 0.05$; miR-320 c: $P < 0.001$) (Figs. 7A, C). Moreover, NOD2 expression was found significantly increased ($P < 0.001$) in the same samples (Figs. 7B, D).

An inverse statistical correlation was found between miR-320 a, -b, -c and NOD2 expression in patients with CD (CD: $r = -0.51$, $P < 0.01$; $r = -0.43$, $P < 0.05$; $r = -0.52$, $P < 0.01$, respectively) (Figs. 8A–C) and between miR-320 a and NOD2 expression in patients with UC ($r = -0.51$, $P < 0.01$) (Fig. 8D).

These data confirm previous in vitro results, demonstrating an inverse correlation between miR-320 and NOD2 expression and suggesting that, during inflammation, the downregulation of miR-320 is likely to be involved in the NOD2 upregulation.

## DISCUSSION

In the present study, we have identified miR-320 family as novel regulator of NOD2, a major gene of innate immunity against intracellular bacteria and inflammatory response. Our data corroborate an increasing number of studies demonstrating the role of miRNAs as negative regulators of innate immune response by targeting key signaling proteins and cytokines.

Altered expression of miR-320 has been related to several pathogenetic conditions, such as cardiac disease,[35–37] endocrinology disorders,[38–40] and cancer.[41–46] Interestingly, a recent study reported that miR-320 is also abnormally expressed in the inflamed tissues of patients with intestinal inflammation.[47]

Here, we hypothesized that miR-320 targets NOD2 and regulates its expression in intestinal epithelial cells. This hypothesis was supported by the presence of 3 different binding sites for the miR-320 family on the 3′-UTR of NOD2. To show it, we transfected HT29 cells, a well-accepted in vitro model of intestinal epithelium, and showed that in physiological conditions, miR-320 a, -b, and -c are negative regulators of NOD2 mRNA and protein expression. In particular, the effectiveness of miR-320 b and -c to downregulate NOD2 mRNA expression was showed to be approximately 70%, lower than that of miR-320 a (approximately 30%). We believe that this is an outstanding result considering that Chuang et al,[28] exploring a series of NOD2 miRNAs regulators, specifically miR-122, miR-124 miR-192, miR-495, miR-512, and miR-671, found that only miR-512 showed a similar outcome, and Chen et al,[29] analyzing the relationship between miR-122 and NOD2, showed a decrease of mRNA NOD2 expression after transfection of miR-122 precursor by approximately 75% compared with the control precursor.

In addition, to assess which of the putative miR-320 binding sites was the functional site of miR-320-based regulation, we used 3 different site blockers, that compete with miR-320 for the 3 NOD2 binding sites, and showed that the second of the 3 binding sites was the only 1 functionally active.

This is the first time that NOD2 is recognized as a functional target of miR-320. It is known that, in steady state, NOD2, similar to other innate immune receptors, is expressed at very low level in the intestinal mucosa to allow downregulation of the inflammatory response and promotion of the immunologic tolerance to luminal bacteria. However, NOD2 expression has been found to be significant upregulated in the inflamed tissues, as shown in several chronic inflammatory disorders[48–53] although, mechanisms controlling NOD2 levels have not been really investigated in depth until now. Several studies reported that changes in miRNAs are related to the onset of inflammatory diseases, particularly those controlling genes critically involved in the immune response. Thus, we analyzed the expression of endogenous miR-320 a, -b, and -c in cells previously exposed to a cytomix of proinflammatory cytokines or to MDP and found that, under inflammation, they were significantly decreased, whereas NOD2 was significantly upregulated. The relationship between these 2 events was strengthened by the evidence that after transfecting



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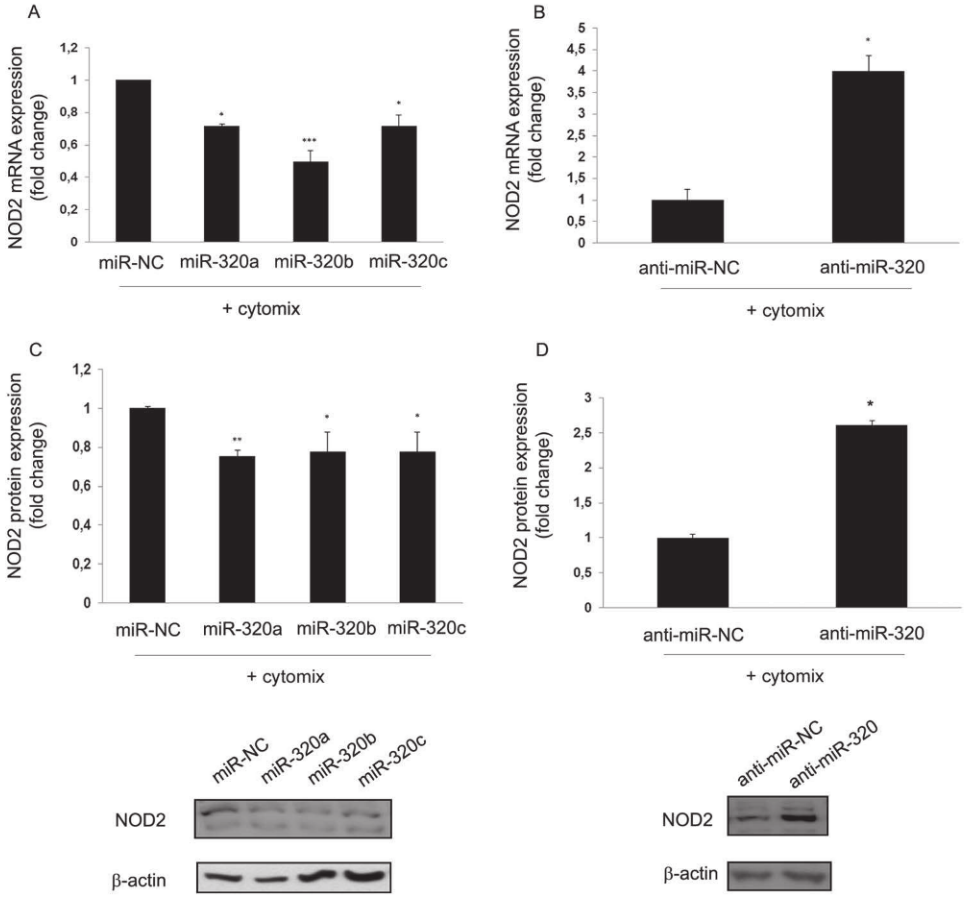


FIGURE 5. Exogenous miR-320 controls NOD2 expression during inflammation. NOD2 mRNA (A and B) and protein (C and D) expression in HT29 cells after transfection with control mimic (miR-NC) or miR-320 a, -b, and -c mimics or miR-320 family inhibitor negative control (anti-miR-NC) or miR-320 family inhibitor (anti-miR-320) and exposure to cytomix (TNF-α and interferon γ) by qRT-PCR and western blot. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

inflamed cells with exogenous miR-320 (hsa-miR-320 a, -b, and -c), NOD2 expression was significantly reduced. Otherwise, the inhibition of miR-320, through a specific anti-miR, increased NOD2 expression. The current model of NOD2 signaling proposes that on specific recognition of its ligand, NOD2 triggers an enzymatic cascade culminating in the translocation of the transcription factor NF-κB from the cytoplasm to the nucleus where it actives the proinflammatory genes transcription. Thus, to assess whether the altered NOD2 expression after miR-320 inhibitor transfection resulted in downstream regulation of its function, we analyzed the translocation of NF-κB from cytoplasm to nucleus by immunofluorescence and western blot. Interestingly, we observed that increased expression of NOD2 leads to a heightened MDP-stimulated NF-κB activation and downstream proinflammatory cytokine expression.

All these results show that miR-320 controls NOD2 expression and activity, contributing to the maintenance of intestinal homeostasis; however, during inflammation, the NOD2 miR-320-mediated control is weakened, resulting in NOD2 increase. We do believe that this event powerfully affects the magnification of the NOD2-induced proinflammatory cascade and the break down of cell homeostasis.

To deeper explore the relationship between miR-320, NOD2 and inflammation, we examined the expression levels of miR-320 a, -b, and -c in the intestinal mucosal samples taken from children with CD and UC.

In our study, we selected a pediatric population since recent epidemiological data indicate a meaningfully increased incidence of pediatric IBD over the last decades, and that the pediatric disease tends to exhibit a more extensive and severe presentations than the adult counterpart, also reflected by higher levels of humoral immune responses.[54] This has prompted the search for specific mechanisms thought to be responsible for such lifelong intestinal inflammation, also helping to explain the still ongoing rise in incidence of childhood IBD. Furthermore, the early onset may represent the "pure" form of the disease process and hence may hold secrets of the initiating events of IBD pathogenesis.[55] Since NOD2 mutations exhibit a variety of disease-predisposing mechanisms[4] (ranging from defects in viral sensing and reduced mucosal defensin production to abnormal autophagy induction)



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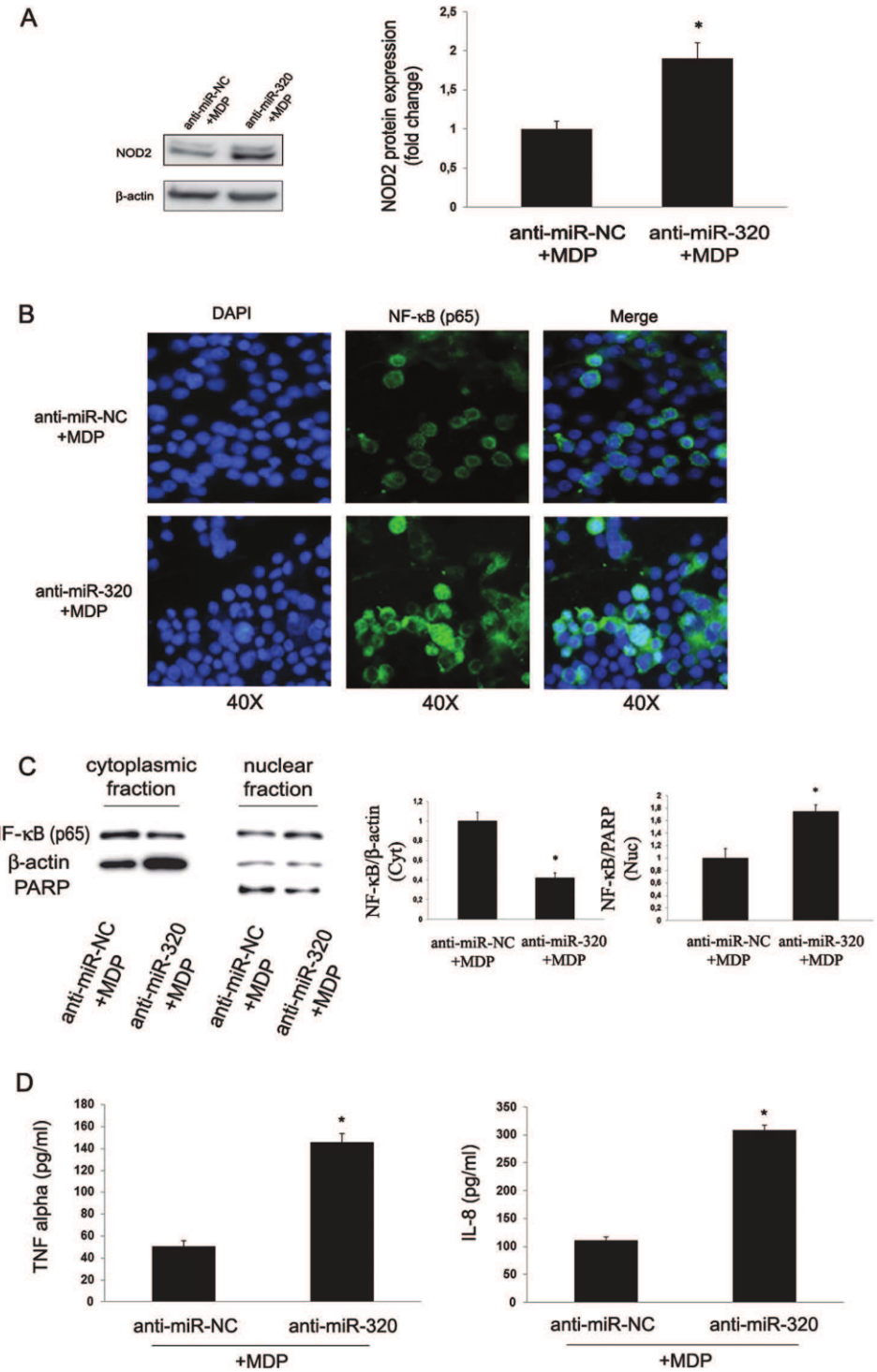


FIGURE 6. Inhibition of miR-320 increases the translocation of NF-κB from the cytoplasm to the nucleus of HT29 cells as well as the expression of downstream pro-inflammatory cytokines. NOD2 protein expression in HT29 cells transfected with miR-320 family inhibitor negative control (anti-miR-NC) or miR-320 family inhibitor (anti-miR-320) and exposed to MDP for 24 hours was assessed by western blotting (A). NF-κB nuclear translocation in HT29 cells transfected with miR-320 family inhibitor negative control (anti-miR-NC) or miR-320 family inhibitor (anti-miR-320) and exposed to MDP for 24 hours by immunofluorescence (green: NF-κB (p65) antibody; blue: DAPI [4′,6-diamidino-2-phenylindole]) for nuclear staining (B). Cytoplasmic and nuclear NF-κB analyzed by western blot; anti-poly ADP ribose polymerase and anti-β-actin were used as nuclear and cytoplasmic protein controls (C). TNF-α and IL-8 ELISA assay in HT29 cells transfected with miR-320 family inhibitor negative control (anti-miR-NC) or miR-320 family inhibitor (anti-miR-320) and exposed to MDP for 24 hours (D). *$P < 0.05$.



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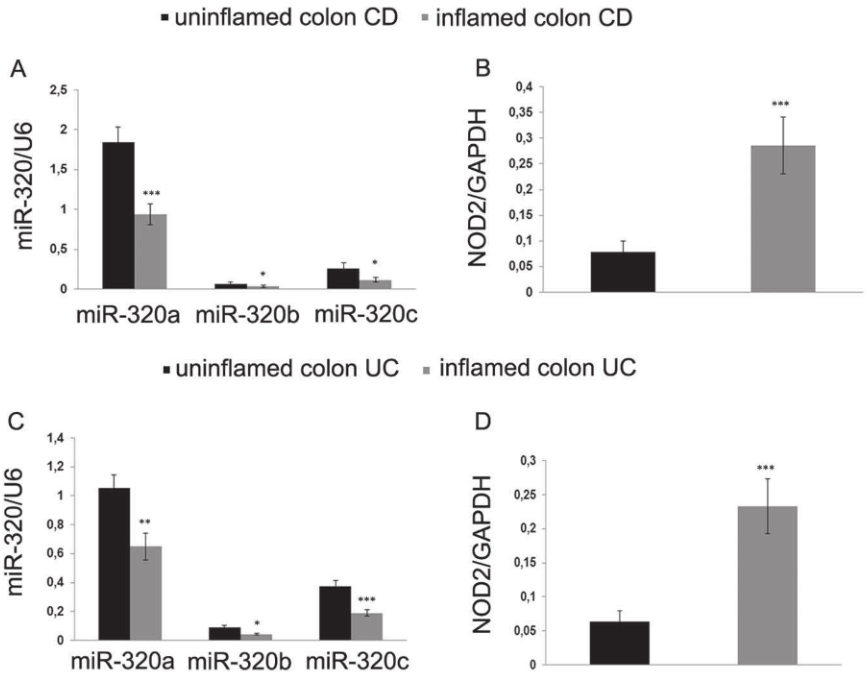


FIGURE 7. miR-320 expression is significantly decreased and NOD2 expression increased in the inflamed mucosa of pediatric patients with IBD. miR-320 a, -b, -c and NOD2 expression levels in uninflamed and inflamed mucosal samples of patients with CD (A and B) and UC (C and D) were assessed by qRT-PCR. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

that are somewhat different from those of the wild type gene, we excluded from this study the NOD2 variant carriers.

Results showed that all 3 members of miR-320 family were significantly decreased in the inflamed tissues as compared with normal tissues taken from the same subjects, whereas NOD2 was comparably increased. To link these 2 events, we performed a statistic analysis and found a significant inverse correlation between members of miR-320 and NOD2 expression in the

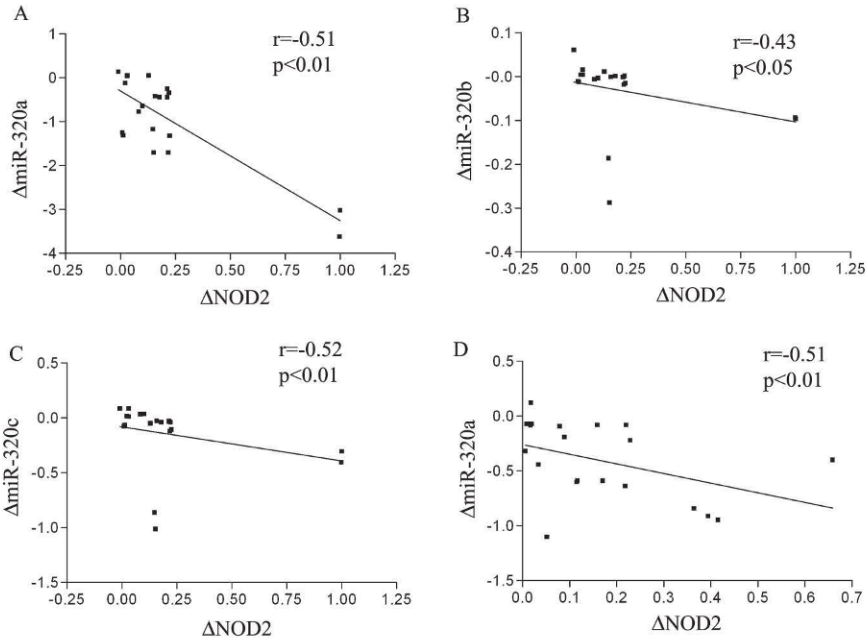


FIGURE 8. Inverse correlation between miR-320 levels and NOD2 mRNA in pediatric patients with IBD. An inverse statistical correlation was found between miR-320 a, -b, -c and NOD2 expression in patients with CD (CD: $r = -0.51$, $P < 0.01$; $r = -0.43$, $P < 0.05$; $r = -0.52$, $P < 0.01$ respectively) (A–C) and between miR-320 a and NOD2 expression in patients with UC ($r = -0.51$, $P < 0.01$) (D).



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mucosal samples of IBD children. These results confirm ex vivo that NOD2 is a target of miR-320 and its downregulation, during inflammation, contributes to the over-expression of NOD2, likely impacting on its proinflammatory cascade.

Highlighting the mechanisms of NOD2 control in a physiological or inflammatory environment is crucial to deeply understand the function of a gene with a major role in innate immunity and to identify critical elements of gene regulation that can become targets of novel therapeutic approaches. Indeed, it has recently suggested that inflammatory response, intestinal epithelial barrier and other mechanisms involved in IBD mechanisms can be regulated by targeting miRNAs, indicating the latter as therapeutic targets for IBD.[56–58]

Further studies will be focused on the role of miR-320 on NOD2 regulation in the adult population with IBD as well as on NOD2 variant form carriers.

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