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Association between genotypes of rs34436714 of *NLRP12* and serum tumor necrosis factor-alpha in inflammatory bowel disease

A case-control study

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Abstract

We aimed to investigate the impact of the single nucleotide polymorphisms of rs34436714 of the NOD-like receptor protein 12 gene on the production of tumor necrosis factor-alpha (TNF α) in patients with inflammatory bowel disease (IBD)

In a matched case-control study 90 patients with IBD, 56 with Crohn disease (CD) and 34 with ulcerative colitis, were genotyped and compared to 98 healthy comparators matched for age and gender. Expression level of TNF α , interleukin (IL)-6, IL-12, and soluble triggering receptor expressed on myeloid cells were measured in patients' sera. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated for TNF α production.

Serum TNF α was greater among carriers of GT/TT genotypes than GG genotypes of rs34436714. Stimulated TNF α production was also higher in carriers of GT/TT genotypes. The frequency of CD with fistulizing behavior and with CD involving the small intestine was greater among carriers of GT/TT genotypes than of the GG genotype. Distribution of the GG, GT, and TT genotypes of rs34436714 were in Hardy-Weinberg equilibrium in both groups. The genotype distribution was the same in both groups.

Carriage of minor frequency alleles of rs34436714 was accompanied by greater circulating levels of TNF α and by greater capacity for stimulated TNF α production by PBMCs. These alleles had an impact on the phenotype of patients with CD.

Abbreviations: CD = Crohn disease, IBD = inflammatory bowel disease, IL = interleukin, LPS = lipopolysaccharide, NLRP12 = NOD-like receptor protein 12, NOD = nucleotide binding domain, PBMCs = peripheral blood mononuclear cells, SNP = single nucleotide polymorphism, sTREM-1 = soluble triggering receptor expressed on myeloid cells-1, TNF α = tumor necrosis factor-alpha, UC = ulcerative colitis.

Keywords: inflammatory bowel disease, NLRP12, single nucleotide polymorphisms, TNF α

1. Introduction

The interaction of gut microbiota with tissue macrophages is more and more recognized as a key component in the pathogenesis of inflammatory bowel disease (IBD). This interaction is mediated by the recognition of pathogen-associated patterns of bacterial flora by the nucleotide binding domain (NOD)-like intracellular receptors of macrophages. Carriage of

single nucleotide polymorphisms (SNPs) in any of these receptors may modulate the susceptibility of the host for the development of IBD.^[1]

One well-recognized receptor is NOD-like receptor protein 12 (NLRP12). NLRP12 is associated with the protein adaptor ASC inducing the formation of an active IL-1 β maturing inflammatory. It also acts as a negative regulator of pro-inflammatory cytokine production via inhibition of non-canonical NF- κ B

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activation.^[3] The NLRP12 has been shown to play a role in the maintenance of intestinal homeostasis and protection against tumorigenesis in animal models.^[4] Recent data revealed decreased expression of *NLRP12* in patients with active ulcerative colitis (UC) as compared to healthy controls and patients with inactive UC.^[5] Since NLRP12 is a negative regulator of inflammation, it may be hypothesized that it also downregulates the production of tumor necrosis factor- α (TNF α). TNF α is recognized as a key molecule in the pathogenesis of IBD and factors affecting its production may participate in pathogenesis.

NLRP12 is located on chromosome 19. One common SNP of *NLRP12* is rs34436714 where guanine is substituted by thymine leading to the missense substitution of glycine by valine. Taking into consideration the above described role of NLRP12 to downregulate inflammation in the gut and the lower *NLRP12* expression among patients with IBD, we questioned if carriage of SNP alleles of rs34436714 may modulate the production of TNF α .

2. Patients and methods

2.1. Study design

This was a case-control study of patients with IBD and healthy comparators. The study was approved by the Ethics Committee of the ATTIKON University Hospital, in Athens, Greece.

Inclusion criteria for patients were:

- (1) Caucasian origin;
- (2) age equal to or above 18 years;
- (3) both genders;
- (4) written informed consent provided by the patients; and
- (5) diagnosis of IBD with clinical, endoscopic, and histological documentation.

Inclusion criteria for the comparators were to be of the same age and gender and to provide written informed consent as the patients and not to have any known chronic disorder. Exclusion criteria were:

- (1) age below 18 years;
- (2) denial to consent;
- (3) any other chronic inflammatory disorder;
- (4) infection by the human immunodeficiency virus;
- (5) chronic hepatitis B or C; and
- (6) any fungal infection.

Ten millilitres of peripheral blood was sampled from each patient or comparator after venipuncture of the forearm vein under aseptic conditions; 5 ml were collected into EDTA-coated tubes (Vacutainer, BD) to isolate genomic DNA by standard methods; another 5 ml were collected into sterile and clean tubes free of anticoagulant. After centrifugation, serum was stored at -80°C until assayed.

Genomic DNA was isolated from whole blood using standard procedures. The genotype for the *NLRP12* polymorphism rs34436714 in the patients was screened by the TaqMan SNP assay C_25927410_10, on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems, CA).

In 1 out of 5 patients, another 20 ml of whole blood was sampled and collected into heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated after gradient centrifugation over Ficoll (Merck, Darmstadt, Germany). After

3 consecutive washings in ice-cold phosphate buffered saline (pH: 7.2), PBMCs were counted in a Neubauer chamber with trypan blue exclusion of dead cells. Cells were distributed in duplicate in wells of 96-well plates and incubated at a density of $5 \times 10^6/\text{ml}$ without/with 10 ng/ml of lipopolysaccharide (LPS) of *Escherichia coli* O55:B5 (Sigma, St. Louis). The growth medium was RPMI1640 enriched with 5% glutamate (Merck). Plates were incubated for 24 hours at 37°C in 5% CO_2 atmosphere. At the end of the incubation plates were centrifuged and supernatants were collected and stored at -80°C until assayed for TNF α .

TNF α , interleukin (IL)-6, IL-12, and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) were measured in serum and supernatants of patients by an enzyme immunoassay according to the instructions of the manufacturer (R&D Inc, Minneapolis). The lower limit of detection was 5.6 pg/ml for TNF α and IL-6; 31.3 pg/ml for IL-12; and 15.1 pg/ml for sTREM-1.

The primary study endpoint was the impact of the genotype of rs34436714 on TNF α levels. Secondary study endpoints were:

- (1) the impact of the genotype of rs34436714 on the other measured serum cytokines;
- (2) the impact of the genotype of rs34436714 on the clinical characteristics of Crohn disease (CD); and
- (3) the difference in genotype distribution of rs34336714 between patients and comparators.

2.2. Study power

The study was powered for the primary endpoint. It was hypothesized that

- (1) 30% of patients would be carriers of at least 1 minor frequency allele and 70% of patients of major frequency alleles; and
- (2) the mean difference of serum TNF α would be 50 pg/ml and the standard deviation would vary by 70 pg/ml.

In order to demonstrate this difference at the 10% level of significance with 80% power at least 80 patients should be enrolled.

2.3. Statistical analysis

Cytokine concentrations were expressed as means \pm SE and compared by the Mann-Whitney *U* test. Genotype and allele distribution were expressed as frequencies and compared by the Fisher exact test. Any value of *P* below .05 was considered significant.

3. Results

A total of 90 patients with IBD, 34 with UC and 56 with CD, and 98 healthy comparators were enrolled. The study flow chart is shown in Figure 1. The demographics of patients and comparators are provided in Table 1.

The primary study endpoint was the modulation of TNF α by SNPs of rs34436714. As it can be seen in Figure 2A, circulating concentrations of TNF α were greater among patients carrying at least 1 T allele of rs34436714. In order to elaborate this further, we isolated PBMCs from 20 patients, 10 with UC and 10 with CD. After stimulation with bacterial LPS, PBMCs of patients carrying T alleles of rs34436714 produced more TNF α than PBMCs of patients carrying only the major frequency allele (Fig. 2B). However, it is suggested that treatment with chemical or biological

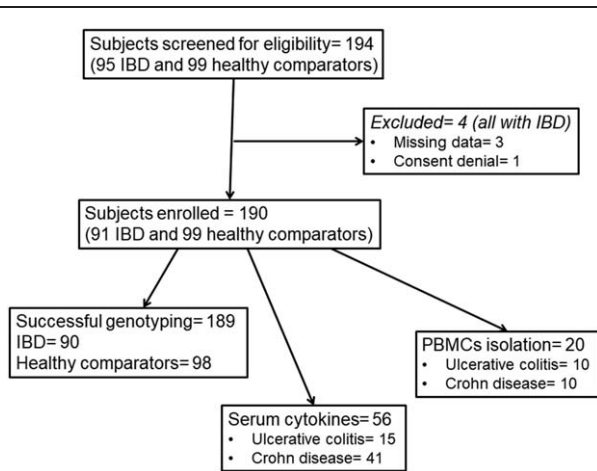


Figure 1. Study flow chart. IBD=inflammatory bowel disease, PBMCs= peripheral blood mononuclear cells.

disease response modifiers may modulate the ability of the host for TNF α production.^[6] To this end, circulating concentrations of TNF α were compared separately between patients carrying major and minor frequency alleles of rs34436714 in relation to treatment with at least 1 drug and with at least 2 or more drugs. Circulating TNF α was greater among patients under treatment with 2 or 3 drugs who were carriers of at least 1 minor frequency T allele of rs34436714 (Fig. 2C).

Expression levels of sTREM-1, IL-6, and IL-12 did not differ between carriers of at least 1 T allele of rs34436714 and carriers of only major frequency alleles (Fig. 3).

We hypothesized that carriage of minor frequency T alleles that was associated with greater production capacity for TNF α might also be associated with the clinical phenotypes of CD. Analysis showed that carriage of GT and TT genotypes was associated with greater frequency of CD with fistulizing behavior (Fig. 4A) and with CD located only at the small intestine (Fig. 4B).

Among the comparators, 66 (67.3%), 28 (29.5%), and 4 (4.1%) had GG, GT, and TT genotypes, respectively. Among IBD patients, 59 (65.6%), 28 (31.1%), and 3 (3.3%) had GG, GT, and TT genotypes, respectively. No deviation from Hardy-Weinberg equilibrium was detected in either comparators (HWE: 0.01; *P*: .976) or patients (HWE: 0.02; *P*: .884). No difference in the genotype distribution was found between patients and comparators (*P*: .907); 81.6% (n=160) of comparators were carriers of major frequency G alleles and 18.4% (n=36) were carriers of minor frequency T alleles; this distribution was 81.8% (n=146) and 80.2% (n=34), respectively among patients.

4. Discussion

Our study shows how patients suffering with IBD who are carrying minor frequency T alleles of rs34436714 of *NLRP12* have greater circulating levels of TNF α and greater potential for the production of TNF α after PBMCs stimulation. It also shows how carriage of these alleles may have an impact on the phenotype of patients with CD.

Table 1
Demographic and clinical characteristics of patients enrolled in the study.

	Ulcerative colitis (n=34)	Crohn disease (n=56)	Healthy comparators (n=98)
Male gender (n, %)	18 (52.9)	20 (35.7)	45 (45.9)
Age, yr (mean \pm SD)	49.5 \pm 17.2	42.3 \pm 18.9	43.5 \pm 17.8
History of operation (n, %)	2 (5.9)	19 (33.9)	
Extraintestinal manifestations (n, %)	2 (5.9)	12 (21.4)	
Extent of GI involvement (n, %)			
Up to sigmoid	4 (11.8)		
Pancolitis	30 (88.2)	11 (19.6)	
Colon and terminal ileum		8 (14.3)	
Colon and small intestine		16 (28.6)	
Terminal ileus only		9 (16.1)	
Small intestine only		12 (21.4)	
Behavior (n, %)			
Inflammatory	0	29 (51.8)	
Fibrostenotic	0	12 (21.4)	
Fistulizing	0	15 (26.8)	
Activity [†] (n, %)			
Nonactive	19 (55.9)	40 (71.4)	
Mild	8 (23.5)	5 (8.9)	
Moderate	4 (11.8)	7 (12.5)	
Severe	3 (8.8)	4 (7.1)	
Medications (n, %)			
5-amino salicylate	28 (82.4)	16 (28.6)	
Immunosuppressants [‡]	9 (26.5)	19 (33.9)	
Anti-TNFs [‡]	0	20 (35.7)	
Corticosteroids	2 (5.9)	11 (19.6)	

TNF=tumor necrosis factor alpha, SD = standard deviation.

^{*} Mayo score for ulcerative colitis and Harvey-Bradshaw score for Crohn disease.

[†] Azathioprine or cyclosporine.

[‡] Infliximab or adalimumab.

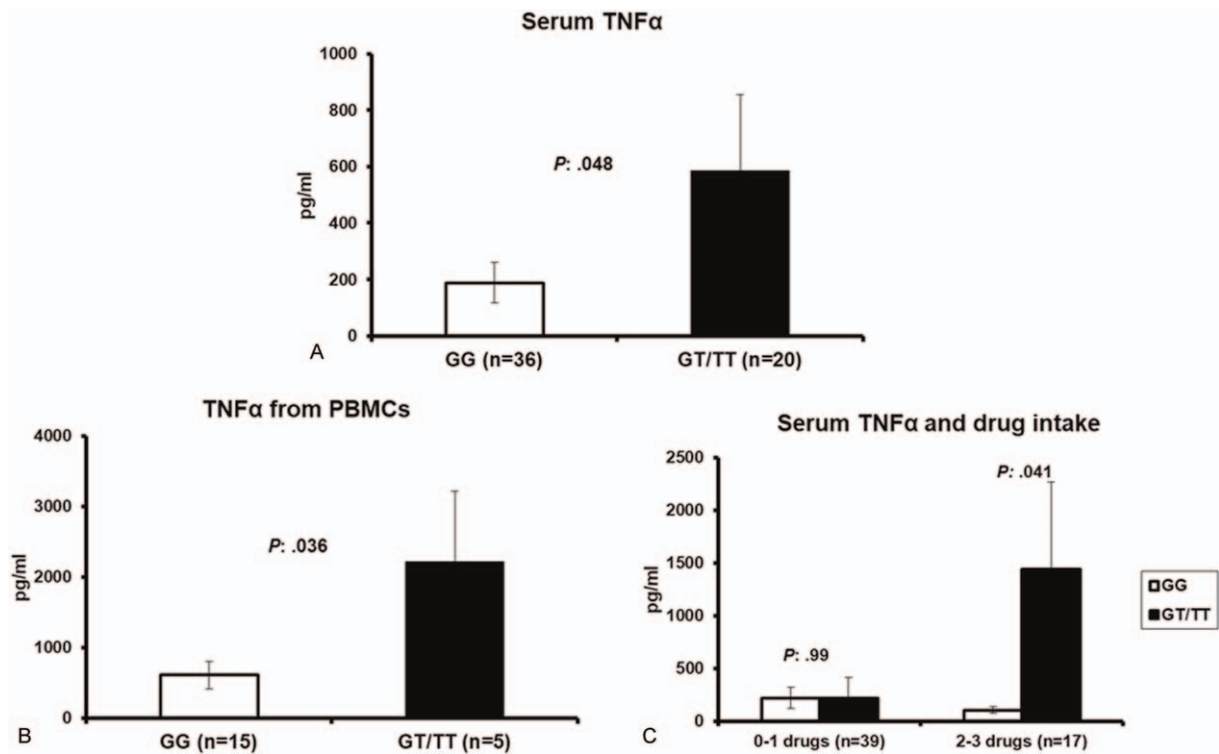


Figure 2. Impact of rs34436714 genotype of *NLRP12* on TNF α . Results are provided for (A) serum TNF α ; (B) production of TNF α by PBMCs of patients after stimulation with bacterial lipopolysaccharide; and (C) serum TNF α in relation to the intake of disease-modifying drugs (see Table 1). *P* values refer to the indicated statistical comparisons. Production of TNF α by PBMCs without stimulation was below the limit of detection. NLRP12 = NOD-like receptor protein 12, PBMCs = peripheral blood mononuclear cells, TNF α = tumor necrosis factor-alpha.

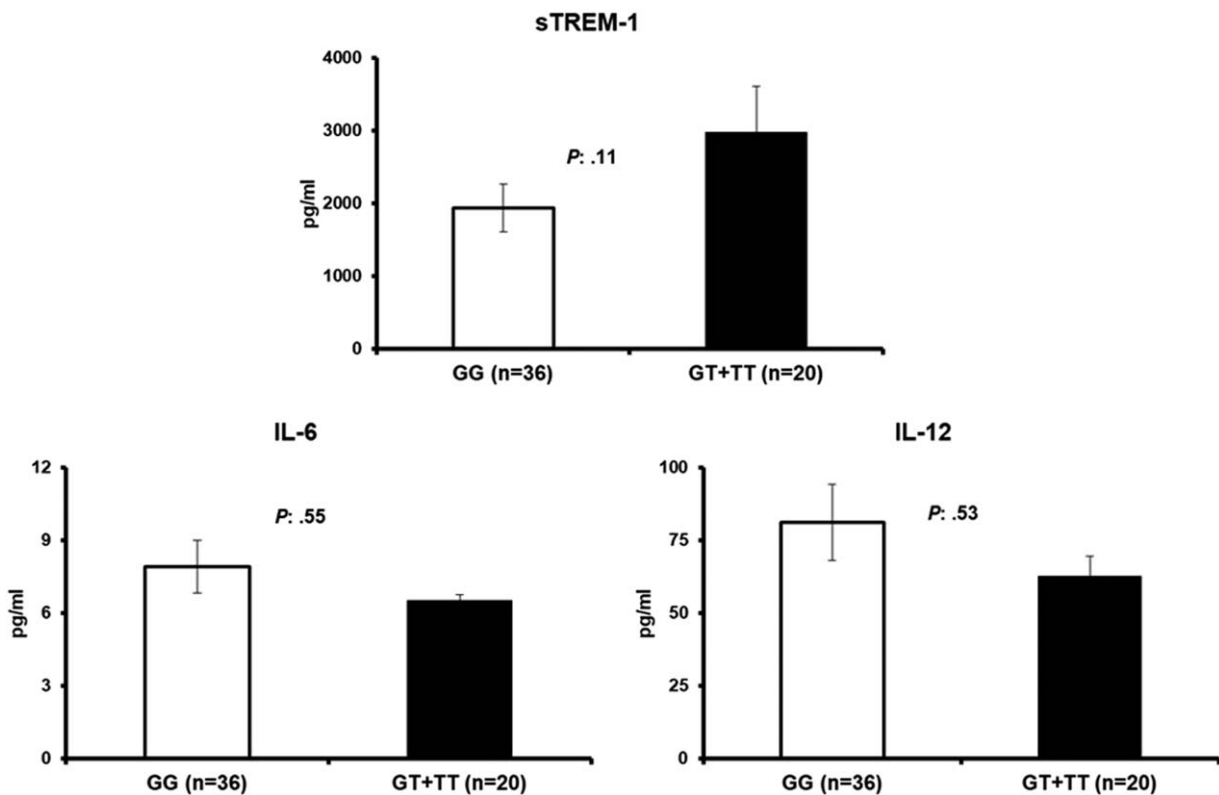


Figure 3. Serum concentrations of sTREM-1 and of IL-6 and -12 in relation to the rs34436714 genotype of *NLRP12*. *P* values refer to the indicated statistical comparisons. IL = interleukin, NLRP12 = NOD-like receptor protein 12, sTREM-1 = soluble triggering receptor expressed on myeloid cells-1.

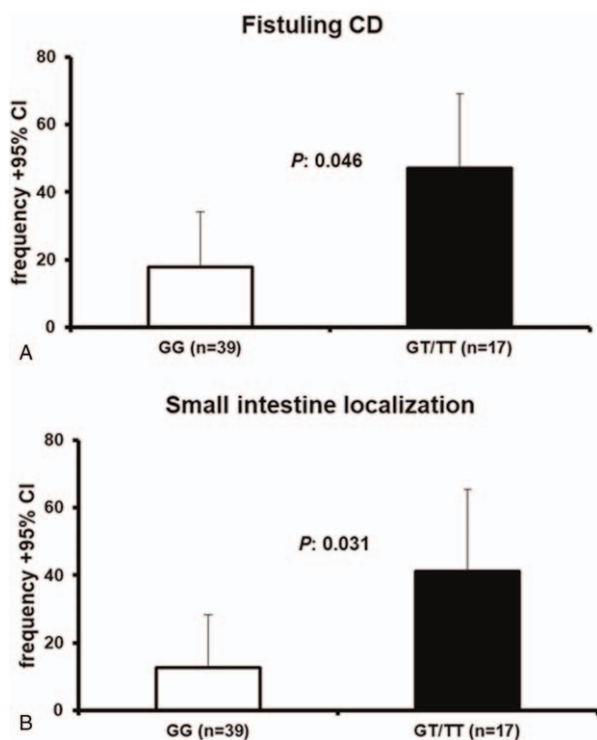


Figure 4. Association of genotypes of rs34436714 of *NLRP12* with the phenotype of Crohn disease (A) frequency of fistulizing CD among patients carrying the GG and the GT/TT genotypes; and (B) frequency of CD localized only at the small intestine among patients carrying the GG and the GT/TT genotypes. *P* values of the indicated statistical comparisons are provided. CD = Crohn disease, *NLRP12* = NOD-like receptor protein 12.

A thorough literature search identified few studies on the impact of the function of *NLRP12* gene for the pathogenesis of IBD. To our knowledge there is only clinical study trying to investigate the role of the expression of *NLRP12* in IBD: in a paired comparison that involved 10 pairs of monozygotic UC twins, healthy twins, and 7 UC patients, gene-profiling showed reduced expression of *NLRP12* in active UC.^[5] However, the role of rs34436714 was not explored.

Our results point towards an impact of SNPs of rs34436714 on the production of TNF α . Indeed, IBD has been associated with increased expression of TNF α in intestinal tissues.^[7,8] The lack of measurements of TNF α in at the gut tissue level may, at first glance, appear as a limitation of our study. However, 2 points may be argued to strengthen the impact of the analyzed SNPs of rs34436714 on TNF α expression as performed in this study through the use of serum TNF α measurements:

- (1) increased serum TNF α has been described by others in patients with IBD^[8,9]; and
- (2) carriage of minor frequency T alleles of rs34436714 was associated with greater serum TNF α but not with other circulating cytokines.

This is in accordance with the current knowledge that IBD is a disease hallmarked by elevated TNF α ^[10] but not by elevated IL-6 and IL-12.

It has been shown that circulating monocytes of patients with CD produce more TNF α than healthy volunteers or patients with UC.^[11] Although it is anticipated that greater production of

TNF α may be associated with the behavior of CD and refractoriness to treatment, such data are not published. To this end, only indirect evidence is available. More precisely, it has been shown in patients with IBD that carriage of minor frequency A alleles at the -308 gene position increases by 6- to 7-fold the gene transcription of *TNF α* and the subsequent TNF α deposition at the tissue level. This influences the behavior of CD towards less steroid-dependent disease.^[12-14] To this end, it was interesting to find that carriage of minor frequency T alleles of rs34436714 that are associated with elevated circulating TNF α and with high stimulated production of TNF α by PBMCs was also associated with the behavior of CD and with response to treatment. These carriers presented commonly with CD with fistulizing behavior involving the small intestine and necessitated 2 or 3 drugs. Although this may be considered of importance from a pharmacogenomics perspective, a recent genome-wide association study of 359 patients using the Illumina immunochip and covering 196,524 SNPs did not report any association between durability of response to anti-TNFs and rs34436714.^[15]

The production of TNF α in the gut is stimulated by microbiota where *NLRP12* may function as a modulator. This is supported by studies describing *NLRP12* as an antagonist of pro-inflammatory signals induced by Toll-like receptor ligands and *Mycobacterium tuberculosis*^[16] and downregulation of TLR-dependent NF κ B after gene silencing of *NLRP12*.^[3] The diversity of gut microbiota is decreased when mice that are deficient for *NLRP12* become obese after being fed high-fat diet; this is associated with greater systemic inflammation.^[17] Similar findings were shown in mice subject to experimental colitis.^[5]

The increased production of sTREM-1 that was found in the IBD patients of our study is in agreement with the findings of 1 previous study of our group that identified sTREM-1 as a mediator in IBD positively correlated with IBD activity.^[18] Despite the elevation of sTREM-1 in CD, this was not affected by the carriage of minor frequency alleles of *NLRP12*. This underscores the importance of the pathway linking *NLRP12* to TNF α and not to other pro-inflammatory mediators.

The small study population is also a limitation of our study. However, the primary endpoint, that is, the association of serum TNF α with the carriage of minor frequency T alleles of rs34436714 was shown even under that limitation.

Our findings showed that carriage of minor frequency alleles of rs34436714 of *NLRP12* was accompanied by greater circulating levels of TNF α and by greater capacity for stimulated TNF α production by PBMCs. These alleles had an impact on the phenotype of patients with CD. Although our results pinpoint some significance of SNPs of rs34436714 in the pathogenesis of IBD, they cannot prove if they drive or not a direct modulation of the production of blood TNF α .

Author contributions

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