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Genetic predictors of long-term response and trough levels of infliximab in crohn's disease

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ABSTRACT

Introduction: Several factors, such as trough serum anti-TNF levels, have been associated with response to therapy in Crohn's disease. However, this association is observed after initiation of treatment. Identifying DNA variants may prove useful for predicting long-term response or failure to these drugs before initiation of treatment.

Objective: To identify genetic variants associated with long-term response to infliximab and trough levels in Crohn's disease.

Patients and methods: An observational, longitudinal study was conducted. We analyzed blood samples from 132 infliximab-treated patients diagnosed with Crohn's disease from 2 hospitals. We genotyped 21 polymorphisms previously related to anti-TNF response in genes involved in the NFkB-mediated inflammatory response, TNFα-signaling and cytokines regulated by NFkB, using real-time PCR. Trough infliximab levels were measured using ELISA. The association between SNPs and time-to-failure (defined as the time from the initiation of induction therapy to the date of treatment withdrawal due to a primary or secondary failure) was analyzed using log-rank test. The association between SNPs and supra-(> 7 μg/mL) or infratherapeutic (< 3 μg/mL) infliximab trough levels was analyzed using a linear-by-linear association chi-squared test.

Results: Two SNPs in TLR2, rs1816702 and rs3804099, and 1 SNP in TNFRSF1B, rs1061624, were associated with long-term response (up to ten years follow-up) to infliximab (HR, 0.13 [95%CI, 0.02–1.00], $p < 0.05$; HR, 0.39 [95%CI, 0.18–0.88], $p < 0.05$; and HR, 0.04 [95%CI, 0.18–0.92] $p > 0.05$, respectively). In addition, IL6 rs10499563 C and IL10 rs1800872 A were associated with supratherapeutic trough infliximab levels; IL10 rs3024505 T was associated with infratherapeutic levels ($p < 0.05$).

Conclusion: Genotyping of the variants identified in the genes encoding TLR2, TNFRSF1B, IL6 and IL10 reported herein represent a promising tool for the identification and selection of those patients who will benefit most from infliximab.

1. Introduction

Crohn's disease (CD) is a chronic inflammatory autoimmune disease of pathogenesis not fully understood that arises from the interaction between environmental and genetic factors [1,2]. CD significantly impacts patients' lives and represents a substantial cost to health systems [3]. Since CD is a chronic illness and although therapeutic options are increasing, they remain limited due to a high failure rate. Therefore, selecting treatment options able to provide responses in the long term is of major importance. Biological therapy is used when immunomodulators and corticosteroids are not effective for inducing or maintaining remission, with several monoclonal antibodies directed against various targets being effective in inflammatory bowel disease (IBD) [4].

Tumor necrosis factor-alpha (TNFα) is a key proinflammatory cytokine involved in the immune pathogenesis of IBD [5]. Anti-TNFα

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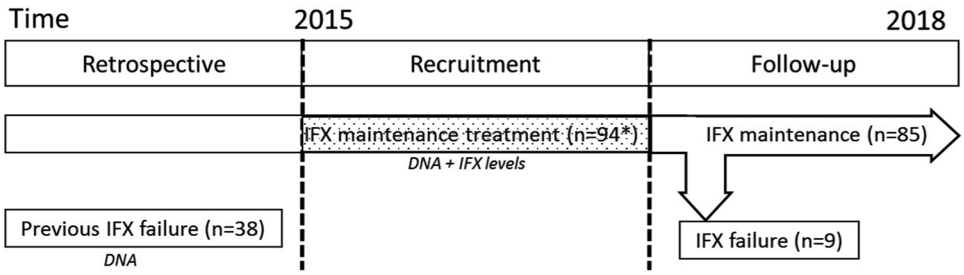

Fig. 1. Sampling timeline. **Retrospective phase:** patients who failed to IFX prior recruitment (n = 38). Only DNA was analyzed. **At recruitment:** patients actively treated with IFX as maintenance therapy (n = 94). DNA and serum were collected. **Prospective phase:** Patients were followed until failure to IFX or until the end of the follow-up. All 132 patients were included to identify polymorphisms associated with long-term response: 85 patients who remained on IFX maintenance treatment after follow-up and 47 patients who failed IFX before recruiting (n = 38) and after follow-up (n = 9). The dotted area represents those patients included to identify DNA variants associated with IFX trough levels. *The analysis was performed with 93 patients because one of them had no IFX levels data.

drugs, such as infliximab (IFX) and adalimumab, were the first antibody-based therapy for IBD patients and are generally used as a first-line biological option in CD. Anti-TNFα drugs have proven to be effective and safe, although approximately 13–40% of patients are primary non-responders and 23–46% will lose response over time (secondary non-responders) [6]. Currently, there are not reliable predictors of clinical response before anti-TNF therapy is started; therefore, and also taking into account the high cost and potentially severe side effects of anti-TNF biological drugs, the identification of underlying factors involved in the individual responses to them is mandatory.

Mucosal healing 3 months after starting therapy is the most useful predictor of long-term response to anti-TNFα therapy [7]. However, assessment is based mainly on colonoscopy, which is limited because of its invasiveness, high cost and resource utilization. As a result, useful but inconclusive non-invasive biomarkers such as serum C-reactive protein and fecal calprotectin, both markers of inflammation, are used to monitor response in the clinical setting [8,9]. The level of anti-TNF just before the next administration, known as trough level, is increasing its use as a non-invasive biomarker. In addition, anti-TNFs drugs are antibodies against TNF that can provoke immunogenicity, a response of the immune system, generating anti-drug antibodies (ADAs). These ADAs bind to the anti-TNF drug reducing the free functional drug, neutralizing the therapeutic effect and resulting in a loss of response [10–13]. In fact, measuring trough serum levels of anti-TNFα drugs, together with their corresponding neutralizing antibodies, in order to prolong the time these drugs are effective, has been the focus of research in recent years and its use in clinical practice has been normalized [14,15]. A study with 955 active CD enrolled patients demonstrates that suboptimal week 14 anti-TNF concentration predicts loss of response at week 54 and immunogenicity, and the development of these ADAs predicts low drug concentrations due to their neutralizing effect [16]. However, all of these studies are focused in measure response at times shorter than one year.

Aside from anti-TNFα drugs, several biologic agents with different targets were subsequently approved to treat CD patients, including ustekinumab, an interleukin blocker, and vedolizumab, a biological anti-adhesion drug. It will soon be possible to choose the most appropriate first-line target for each patient to maintain effectiveness in the long term [17]. Therefore, accurate identification of patients likely to have a long-term response to anti-TNFα therapy may be extremely useful in clinical practice.

Pharmacogenetic tests are easy, cheap, minimally invasive, blood-based options that can be used instead of serum drug level monitoring to help to predict response to drugs [18]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) plays a critical role in the pathogenesis of CD and is positively correlated with the score of CD activity [19]. Variations in genes related to the NFkB and TNFα pathways were recently associated with an early response to anti-TNFα drugs in IBD [20–24]. These include genes involved in the NFkB-mediated inflammatory response, TNFα-signaling and cytokines regulated by NFkB. However, none of these have yet been investigated with the aim of identifying patients who will respond to anti-TNFα drugs in the long term. Moreover, most pharmacogenetic studies provided inconclusive results and need to be validated in multiple cohorts. Given that trough serum anti-TNFα levels are directly associated with the response to this therapy, searching for genetic polymorphisms related to trough IFX levels might identify potential biomarkers to predict the response to IFX and thus enable us to identify the best candidates for this therapy or select patients who would benefit the most from alternative drugs acting against different targets. To our knowledge, no study has assessed whether these polymorphisms affect trough serum IFX levels.

The aims of this study were, on the one hand, to identify polymorphisms able to predict a long-term response to IFX in patients with CD, on the other hand, to identify variants associated with trough serum IFX levels in an homogeneous population of patients, those who were in clinical remission and actively treated with IFX at standard doses.

2. Material and methods

2.1. Patients and study design

An observational, longitudinal, ambispective study was conducted. Inclusion criteria were: patients over 18 years old diagnosed with CD and treated at any time with infliximab. Patients being treated with intensified infliximab doses were excluded (5 mg/kg every 4 weeks or 10 mg/kg every 8 weeks). The recruitment period was 2015–2018 (Fig. 1). In the retrospective phase, 38 patients who previously failed to IFX prior to study starting point were recruited. At recruiting, 94 patients who were actively treated with IFX as maintenance therapy at stable doses (5 mg/kg every 6 or 8 weeks) were included. A prospective phase was conducted until the failure to IFX or until the end of the follow-up (2018). During follow-up, 9 out of 94 patients failed to IFX therapy and at the end of the study 85 remained as responders. Patients were recruited in the IBD Units of 2 Spanish hospitals (*Hospital General Universitario Gregorio Marañón* and *Hospital General de Tomelloso*).

The first aim was to identify polymorphisms associated with long-term response to IFX in patients with CD. For this purpose, all recruited patients were included. The primary endpoint was failure to IFX treatment by genotypes. Failure was defined as withdrawal of IFX, and/or switching to another anti-TNF due to loss of effectiveness according to clinical, biochemical and endoscopic data or the need for abdominal surgery related to CD progression. Time-to-failure was defined as the time from the initiation of induction therapy to the date of failure. A blood sample was collected from all patients in a tube containing EDTA for genetic analyses. The main study variables of this aim were genotypes and time-to-failure to IFX.

The second goal was to identify DNA variants associated with IFX trough levels, a strong predictor of response. For this reason, we include a homogeneous cohort of patients, those who were actively treated with standard doses of IFX at recruitment. A single additional blood sample was collected in a tube with silica particles immediately before the administration of a scheduled IFX dose to measure trough serum IFX

levels. Due to a limitation of the ELISA technique, ADA levels (UA/mL), another strong indicator of failure to treatment, were measured only in those patients with drug levels below 1 μg/mL. Samples were preserved frozen at −20 °C to −40 °C until analysis. The main study variables for this second aim were genotypes and trough serum IFX levels at recruitment.

The following clinical and demographic variables were collected for all patients: age, sex, date of CD diagnosis, date of initiation of IFX treatment, hospital, line of anti-TNF for IFX treatment (first or second anti-TNF administered), smoking status, surgery at any time, C-reactive protein, and date of failure to IFX or date of end of follow-up. For patients receiving IFX at recruitment, the dose, frequency of administration, and trough serum IFX level were recorded.

2.2. Sample size

Sample size was calculated accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 105 subjects in the reference genotype group with 34 events (failure to IFX treatment) and 26 subjects in the non-reference genotype group with 8 events are required, assuming proportion survivals of 0.73 and 0.5 in the reference and non-reference group, respectively. Depending on the single nucleotide polymorphism (SNP), the reference group will be the homozygous wild type alone or combined with the heterozygous. It has been anticipated a drop-out rate of 0%.

2.3. Single nucleotide polymorphisms selection

SNPs, in genes involved in the NFkB-mediated inflammatory response (Toll-like receptors, *TLR2, TLR4,* and *TLR9;* Lymphocyte antigen 96 *LY96; CD14;* Mitogen-activated protein kinase 14, *MAP3K14*), TNFα-signaling (*TNF,* TNF receptor superfamily members, *TNFRSF1A* and *TNFRS1B; FASLG;* TNF alpha-induced protein3, *TNFAIP3*) and cytokines regulated by NFkB (Interleukins, *IL1B, IL10, IL6,* and *IL17A*) previously reported associated with anti-TNF response, but not long-term response, were selected (Table 1). [21–24]. For this study, the minor allele frequencies (MAF) were higher than 0.05 (5%) in all SNPs. MAF is the frequency at which the second most common allele occurs in a given population, it is widely used in population genetics studies because it provides information to differentiate between common and rare variants in the population.

2.4. DNA isolation and genotyping

Genomic DNA was isolated from 200 μL of whole blood using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany). The DNA concentration was measured using a Q5000 spectrophotometer (Quawell Technology Inc, San Jose, CA, USA). Polymorphisms were genotyped using TaqMan probes in a StepOnePlus Real-Time PCR System, according manufacturer's instructions (Life Technologies, Carlsbad, CA, USA) and analyzed using StepOnePlus v2.3. A successful of 100% in genotyping was obtained for all SNPs except a 99.24% for rs1816702, rs3804099, rs5030728, rs352139, rs11465996, rs4149570, rs6927172, and rs1800872). Hardy-Weinberg equilibrium was analyzed to detect deviations in genotype frequency.

2.5. Trough IFX levels

Trough serum IFX levels were measured using ELISA (Promonitor®IFXv2 kit, Progenika Biopharma, Derio, Spain) in a Triturus automation system (Grifols, Barcelona, Spain), according to manufacturer's instructions. The therapeutic range was defined as trough IFX levels ranging from 3 to 7 μg/mL. Levels above or below this range were considered, respectively, supratherapeutic or infratherapeutic.

2.6. Statistical analysis

For the clinical and demographic variables of study population, continuous variables are expressed as the mean and standard deviation (SD) or as the median and interquartile range (IQR); qualitative variables are presented as absolute and relative frequencies. The chi-squared test (Fisher exact-test, where appropriate) or *t* test was used to compare qualitative and quantitative variables, respectively.

The association of genotypes with long-term response to IFX was analyzed using Kaplan-Meier curves (time-to-failure curves). The adjusted hazard ratios (HR) and *p* values were calculated using sex and line of biological treatment as covariates, with 95% confidence interval (CI).

A linear-by-linear association chi-squared test was used to investigate the univariate associations between polymorphisms and supra- and infratherapeutic trough serum IFX levels. Then, binary logistic regression using sex and line of treatment as covariates were also

Table 1
Single nucleotide polymorphisms tested for pharmacogenetic analysis.

Gene	ID	Molecular consequences	Transcript ID	Nucleotide change	Amino acid change	MAF	Pathway
TLR2	rs1816702	Intron variant	NM_001318789.1	c.−17 + 324T > C	NA	0.142	A
	rs3804099	synonymous variant	NM_001318789.1	c.597C > T	Asn199Asn	0.451	A
TLR4	rs5030728	Intron variant	NM_138554.3	c.261–385G > A	NA	0.305	A
TLR9	rs352139	Intron variant	NM_017442.3	c.4–44G > A	NA	0.482	A
LY96	rs11465996	Promoter	NA	−1716C > G	NA	0.356	A
CD14	rs2569190	Promoter	NA	−260G > A	NA	0.474	A
MAP3K14	rs7222094	Intron variant	NM_003954.4	c.256 + 203C > T	NA	0.475	A
TNFRSF1A	rs4149570	Promoter	NA	−610G > T	NA	0.336	B
	rs767455	synonymous variant	NM_001065.3	c.36 T > C	Pro12Pro	0.492	B
TNFRSF1B	rs1061622	missense variant	NM_001066.2	c.587 T > G	Met196Arg	0.239	B
	rs1061624	3' UTR variant	NM_001066.2	c.*188A > G	NA	0.477	B
	rs3397	3' UTR variant	NM_001066.2	c.*215T > C	NA	0.626	B
TNF	rs1800629	Promoter	NA	−488G > A	NA	0.173	B
	rs361525	Promoter	NA	−418G > A	NA	0.074	B
TNFAIP3	rs6927172	Promoter	NA	−190190G > T	NA	0.175	B
FASLG	rs763110	Promoter	NA	−844T > C	NA	0.164	B
IL10	rs1800872	Promoter	NA	−627C > A	NA	0.208	C
	rs3024505	Downstream variant	NA	c.*2077C > T	NA	0.181	C
IL1B	rs4848306	Promoter	NA	−4301G > A	NA	0.464	C
IL6	rs10499563	Promoter	NA	−6394T > C	NA	0.195	C
IL17A	rs2275913	Promoter	NA	−197A > G	NA	0.354	C

Data from HAPMAP for Caucasian population; Genome Reference Consortium Human Build 38, Organism: Homo sapiens (human) GRCh.38; A, NFkB-mediated inflammatory response; B, TNF signaling; C, cytokines regulated by NFkB. MAF, minor allele frequency; ID, dbSNP Identification number; NA, non-applicable.

analyzed. P values < 0.05 was considered statistically significant. All data were analyzed using the Statistical Package for the Social Sciences v.21 (SPSS Inc, Chicago, IL, USA).

2.7. Ethical considerations

The study was conducted in accordance with the World Medical Association Declaration of Helsinki and Spanish regulations. It was approved by the ethics committees of Hospital General Universitario Gregorio Marañón and Hospital General de Tomelloso. All patients provided their written informed consent to participate.

3. Results

3.1. Patients' characteristics

The whole study population comprised 132 patients diagnosed with CD who were used to identify biomarkers to predict long-term response to IFX in patients with CD (Fig. 1). The study population included: 85 responder patients who remained on IFX maintenance treatment until end of follow-up, and 47 patients who failed to IFX before recruitment (n = 38) or during follow-up (n = 9) (Fig. 1). The median age at diagnosis was 27.4 years (IQR 16.7; range 10.8–76.7). The IFX treatment started at a median of 63.5 months after diagnosis (IQR 108.4; range 0.0–383.4), with no differences in age or sex. In one hospital, the percentage of patients who did not respond to IFX was lower than in the other, with no differences in demographic data. IFX was the first biological drug used in 121 patients (91.7%), and 11 patients (8.3%) received IFX after switching from adalimumab. Remission was recorded in 85 patients (64.4% of total), with a median follow-up of 71.0 months (IQR 52.9 months; range 7.0–120.0) from the onset of IFX. The median time-to-failure in the 47 patients (35.6%) who experienced clinical activity of CD during IFX treatment was 29.5 months (IQR 40.3; range 2.0–93.6) (Table 2).

For the second purpose of identifying variants associated with IFX levels, the study population included 94 patients who were actively treated with standard IFX doses during the recruitment period, finally the analysis was performed with 93 patients because serum was not available for one of them and trough IFX levels were obtained at recruitment (Fig. 1). The median trough IFX level for these patients was 3.0 µg/mL (IQR 3.3; range 0–11.4). Trough serum IFX therapeutic levels (3–7 µg/mL) was observed in 38 out of 93 (Table 3).

Table 3
Patients characteristics for the study of genotypes associated with trough serum IFX levels.

Characteristic	Overall (n = 93)
IFX Dose/schedule	
5 mg/kg/8 weeks	83 (62.9%)
5 mg/kg/6 weeks	10 (7.6%)
Drug levels, median, (IQR, range) (µg/mL)	3 (3.25, 0-11.4)
Trough serum Infliximab level	
< 3 µg/mL; n (%)	46 (49.5%)
3-7 µg/mL; n (%)	38 (40.8%)
> 7 µg/mL; n (%)	9 (9.7%)
ADAs	10/21 (10.75%)

IQR, interquartile range; IFX, infliximab; ADAs, antidrug antibodies.

3.2. DNA variants associated with long-term response to IFX

Three SNPs were significantly associated with long-term response to IFX in the univariate analysis (Fig. 2). After multivariate Cox regression analysis, all values remained significant. Kaplan-Meier curves for the analysis of the associations between the rest of DNA variants and long-term response to IFX are shown in Supplemental Fig. 1. The number of subjects by genotype can be found into the figures of the Kaplan-Meier curves.

After Cox regression the variant rs1816702 C in homozygosis in the *TLR2* gene was predictive of a longer time-to-failure of (or better response to) IFX than the TT genotype (HR, 0.128; 95%CI, 0.02-0.99; p = 0.049). However, only 1 patient was homozygous for the T allele. A second variant in *TLR2* (rs3804099) was also associated with an improved response to IFX; patients with TT genotype maintained their response to IFX over 10 years of follow-up, as compared with patients with the CC genotype (HR, 0.039; 95%CI, 0.18-0.88; p = 0.023). In the multivariate model for *TNFRSF1B* (rs1061624), the AA and GA genotypes were significantly more predictive of long-term response to IFX than the GG genotype (HR, 0.041; 95%CI, 0.18-0.92; p = 0.030).

3.3. DNA variants associated with trough serum IFX level

Two SNPs in *IL6* and *IL10* were significantly associated with trough

Table 2
Characteristics of the patients for the study of genotypes associated with long-term response to IFX.

Characteristic	Overall (n = 132)	Non-Responders (n = 47)	Responders (n = 85)	*p* value
Age (years)				
At diagnosis, median, (IQR, range)	27.4 (16.2, 10.76-76.7)	25.7 (11.3, 11.7–58.7)	28.6 (18.7, 10.76–76.7)	0.091
At start of treatment, median, (IQR, range)	36.6 (18.6, 12.5-81.4)	35.2 (18.3, 20.6–64.5)	36.9 (19.3, 12.5–81.4)	0.429
Months from diagnosis to onset of therapy, median, (IQR, range)	63.5 (108.4, 0–383.4)	81.3 (126.1, 0.5–383.4)	50.1 (100.5, 0–321.1)	0.129
Sex				
Male; n (%)	67 (50.8%)	22 (46.8%)	45 (52.9%)	0.586
Female; n (%)	65 (49.2%)	25 (53.2%)	40 (47.1%)	
Recruiting centre				
Hospital Universitario Gregorio Marañón; n (%)	105 (79.5%)	44 (93.6%)	61 (71.8%)	0.003
Hospital General de Tomelloso; n (%)	27 (20.5%)	3 (6.4%)	24 (28.2%)	
Line of Anti-TNFα				
1st; n (%)	121 (91.7%)	41 (87.2%)	80 (94.1%)	0.201
2nd; n (%)	11 (8.3%)	6 (12.8%)	5 (5.9%)	
Smoking status				
Former smoker; n (%)	28 (21.2%)	6 (12.8%)	22 (25.9%)	0.118
Current smoker; n (%)	28 (21.2%)	12 (25.5%)	16 (18.8%)	0.382
Never smoker; n (%)	76 (57.6%)	29 (61.7%)	47 (55.3%)	0.582
Previous Surgery	60 (45.5%)	24 (51.1%)	36 (42.2%)	0.190
C-reactive protein CRP, median, (IQR, range) (n = 91)	2 (3, 0–46)	2.5 (6, 1–46)	2 (3, 0–41)	0.05
Time to failure (months), median (IQR, range)		29.5 (40.3, 2–93.6)		
Follow-up time (months); median (IQR, range)			71 (53, 7–120)	

IQR, interquartile range; IFX, infliximab; Non-Responders, patients who failed to IFX therapy; Responders, patient who respond to IFX therapy until the end of follow-up;1st, patients for whom infliximab is their first anti-TNF; 2nd, patients for whom infliximab is their second anti-TNF.

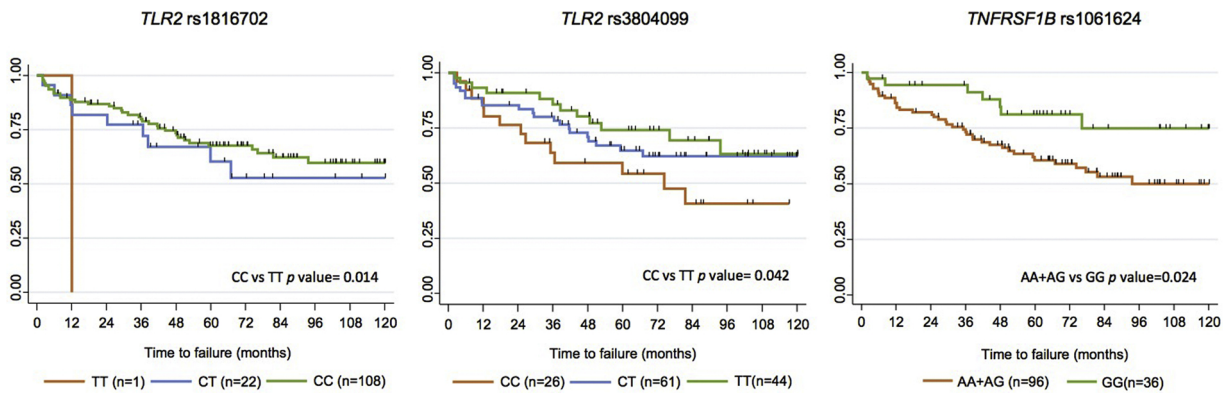

Fig. 2. SNPs associated with long-term response to infliximab. Kaplan-Meier curves for the three SNPs associated with response are represented. Sample sizes for each SNP and genotype are showed. Genotype comparisons and significant *p* values for univariate analysis are inserted into the Kaplan-Meier curves.

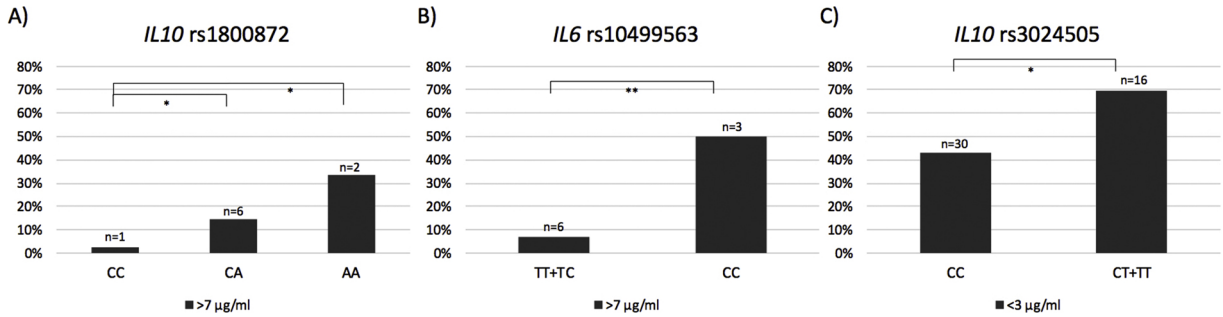

Fig. 3. Polymorphisms associated with supratherapeutic (A, B) or infratherapeutic trough serum infliximab level (C). IFX level > 7 were considered as supratherapeutic. IFX level < 3 µg/mL were considered as infratherapeutic. **p* value < 0.05; ** *p* value < 0.01.

serum IFX levels over the therapeutic range in both the univariate and the multivariate analyses. A SNP in *IL10* was associated with infratherapeutic trough IFX levels (Fig. 3).

Trough IFX levels > 7 µg/mL were found in 33% of patients with CD carrying the AA genotype for *IL10* (rs1800872), in 14.3% of heterozygous CA genotype patients, and in only 2.2% of patients with the CC genotype (CC *vs.* CA, OR, 9.48 [95%CI, 1.05–85.71], p = 0.045; CC *vs.* AA, OR, 20.84 [95%CI, 1.41–307.79], p = 0.027) (Fig. 3A).

On the other hand, 50% of patients with the CC genotype in *IL6* (rs10499563) presented trough IFX levels > 7 µg/mL, compared with only 6.9% of those with the CT or TT genotypes (OR, 21.43; 95%CI, 2.34–196.43; p = 0.007) (Fig. 3B).

The T allele of rs3024505 in *IL10* was associated with an increased probability of infratherapeutic trough IFX levels < 3 µg/mL (Fig. 3C). Thus, 69.6% of CT or TT genotype patients presented infratherapeutic levels compared to 42.9% of CC genotyped patients (OR, 0.33; 95%CI, 0.12–0.92; p = 0.033).

No additional SNPs were correlated with supra- or infratherapeutic trough IFX levels.

4. Discussion

Several parameters, including DNA variants, affect response to anti-TNF therapy in patients with CD, although current knowledge is limited and new biomarkers are necessary to more accurately predict individual responses and to select the drugs that best target the disease [24]. Trough serum IFX level has proven to be a useful parameter for predicting a sustained response to therapy, and guidelines have been provided to optimize results [25]. In the present study, we identified associations between DNA variants and both long-term response to IFX and trough serum IFX levels in a large series of patients with CD.

Previous research on patients with IBD or other autoimmune diseases has identified several polymorphisms that constitute potential biomarkers able to identify patients who respond to IFX [26,27]. However, the results were often contradictory and mostly focused on identifying an early response to therapy. Additional inconsistencies in the studies that have prevented more advances in this area include the heterogeneous criteria chosen by authors to define response or lack of response (clinical indices based on reported symptoms, endoscopic indices based on endoscopic findings, or concentrations of biomarkers of inflammation such as C-reactive protein or fecal calprotectin). Moreover, wide variability was recorded in the duration of IFX therapy to define whether a patient responded or not (from only 2 weeks to 1 year).

Our research is novel in that Kaplan-Meier time-to-failure curves were used to identify gene polymorphisms associated with long-term response to IFX. These have the advantage of incorporating a solid endpoint with strong clinical relevance, since we were able to include patients with a very low risk for failure with IFX after a maximum of 10 years taking the drug. This strategy was successfully used to compare long-term outcomes with IFX in populations with different genetic backgrounds [28].

Three polymorphisms were found to be associated with long-term response to IFX, namely, 2 of them in the *TLR2* gene (rs1816702, rs3804099), the third one in the *TNFRSF1B* gene (rs1061624).

Concerning *TLR2* polymorphisms, the first SNP is in an intron located at 17 nucleotides from a splice site of the *TLR2* gene which could affect to its right splicing and generate a transcript variant. The clinical significance of this change is unknown, but this SNP has been associated with immunology parameters or diseases, such as risk of CD, monocyte activation or vaccine response [21,29,30]. The second SNP rs3804099 in *TLR2* is a synonymous variant, but it has been related to increased cholesterol level, risk of pulmonary tuberculosis, legionella infection or hepatitis activity in HBV infected patients [31–34]. In contrast to our results, both SNPs in *TLR2* have not be related to anti-TNF response after 22 weeks of treatment [35], although they were

initially related [21]. These studies are not comparable due to follow-up time. Nevertheless, we analyzed our data at 22 weeks after starting IFX treatment and no significant results were observed for these 2 SNPs in *TLR2* (data not shown). In addition, the low number of subjects with a TT genotype for rs1816702 in our cohort is a limitation. More studies are required to clarify the role of these *TLR2* variants in the response to IFX.

TNFRSF1B is a single transmembrane glycoprotein which can induce cell apoptosis and survival [36]. Several DNA variants in *TNFRSF1B* have been associated with a response to anti-TNFα drugs, notably rs1061622 [37]. In our study, the SNP rs1061624 was associated with maintenance of long-term response to IFX. There are no data on the involvement of this SNP, which is located in the 3′UTR region, in the regulation of expression of *TNFRSF1B*. Patients carrying the A allele had a higher chance of failure with IFX. Even though this SNP was shown not to be associated with response in Japanese patients with CD treated with IFX, the diplotype A-T for rs1061624 + rs3397 was strongly associated with nonresponse [38]. Allele A was also associated with nonresponse to other drugs, such as 5-fluorouracil/cisplatin–based chemotherapy, and diseases such as cancer [39]. Since these findings point to the protective effect of the G variant against failure with IFX therapy, it suggests that it could represent a good candidate biomarker for long-term response to IFX in CD.

While these three SNPs have been associated with IFX outcomes, this is the first time they have been shown to be biomarkers of long-term response to this drug that are able to identify a group of patients with a low risk of treatment failure after mean of 71 months.

We also identified 3 SNPs that were associated with trough serum IFX levels. The optimal trough IFX level in IBD has been defined as 3–7 μg/mL [11] or even higher when mucosal healing is the objective [40]. Therefore, supra- and infratherapeutic levels of IFX should be associated, respectively, with improved and poor response to the drug. Genotype A in *IL10* rs1800872 and genotype C in *IL6* rs10499563 were associated with an increased probability of presenting serum IFX levels > 7 μg/mL. In contrast, the T allele of *IL10* rs3024505 was a risk factor for trough serum IFX levels < 3 μg/mL.

IL10 rs1800872 is located in the promoter region and, although it does not appear to have an effect on *IL10* expression [41], it has been related to immune system alterations that lead to susceptibility to infections [42]. *IL10* rs3024505 is located in immediately downstream of the end of transcription and no effect on gene expression has been reported. These 2 SNPs in *IL10* were associated with susceptibility to and severity of CD [43,44], although the findings were not validated by other authors [45,46].

Finally, the C allele of rs10499563, located -6331 nucleotides upstream from the start of *IL6* translation, has been associated with a decreased expression in serum and a lower risk of gastric cancer [47,48]. *IL6* rs10499563 was also associated with response to anti-TNFα treatment in IBD patients after 22 weeks of treatment suggesting that genetically determined high IL6-driven inflammatory response was associated with non-response [21,35]. While no significant association has been found between any of these SNPs and long-term outcome in this work, a nonsignificant trend towards a better response was observed for the C genotype in *IL6* rs10499563 (Supplementary Fig. 1L). For the first time, SNPs in *IL6* and *IL10* have been shown to be associated with trough serum IFX levels. More studies are required to elucidate the effect of this association on the clinical outcomes of CD patients receiving anti-TNF treatment.

The major limitation of the study is the sample size, which prevents obtaining conclusive results in those very rare polymorphisms. Sample size is also a limitation for analyses with small subgroups, such as in the case of patients who lose response to IFX after recruitment and the impact of trough serum IFX level. Another limitation was that trough serum IFX levels were only measured once and patients with a closed level to a cut-off point could change therapeutic category.

5. Conclusion

In conclusion, pharmacogenetic analysis allows us to identify genetic variants associated with trough serum IFX levels and long-term response to therapy in patients with CD. Cytokines closely related to IFX levels and variants in *TLR* were significantly associated with long-term responses. Current research to identify long-term responders to anti-TNF therapy may advance our ability to tailor treatment of CD.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2019.104478.

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