Received: 19 October 2018 | First decision: 4 November 2018 | Accepted: 13 December 2018

DOI: 10.1111/apt.15126

WILEY AP&T Alimentary Pharmacology & Therapeutics

Mucosal IL13RA2 expression predicts nonresponse to anti-TNF therapy in Crohn's disease

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Funding information
B Verstockt is a doctoral fellow and G Van Assche, S Vermeire and M Ferrante are Senior Clinical Investigators of the Research Foundation Flanders (FWO), Belgium. B Verstockt has also received research grants by the Belgium Week of Gastroenterology, the Belgian IBD Research and Development (BIRD), the European Crohn's and Colitis Organization (ECCO) and the IBD Patient's Association Flanders (CCV VZW). C Breynaert is supported by the Clinical Research Fund KOF (University Hospitals Leuven, Leuven, Belgium). This work was partially supported by an Advanced European Research Council (ERC) Grant (ERC-2015-AdG).

Summary

Background: Ileocolonic expression of *IL13RA2* has been identified as a predictive marker for nonresponsiveness to infliximab (IFX) in patients with Crohn's disease (CD).

Aim: To validate the *IL13RA2* biomarker, study its anti-TNF specificity and get a better understanding of the underlying biology driving its expression.

Methods: *IL13RA2* mucosal expression was studied in a cohort of adalimumab and vedolizumab treated patients. To identify the upstream regulators of anti-TNF nonresponsiveness, weighted gene co-expression network analysis was applied on publicly available microarray data of IFX-treated patients. Selected serum proteins, including TNF, were measured prior to first IFX exposure and compared between healers and nonhealers.

Results: Increased mucosal *IL13RA2* expression prior to start of biological therapy was predictive for anti-TNF nonresponsiveness specifically (AUROC, area under the curve = 0.90, $P < 0.001$ in anti-TNF vs AUROC = 0.63, $P = 0.30$ in vedolizumab treated patients). In baseline biopsies, TNF-driven pathways were significantly enriched in future anti-TNF nonhealers ($P = 5.0 \times 10^{-34}$). We found an increased baseline mucosal TNF burden in nonhealers ($P = 0.02$), and *TNF* mRNA correlated significantly with *IL13RA2* expression ($\rho = 0.55$, $P = 0.02$). Baseline serum TNF levels were significantly lower in nonhealers ($P = 0.04$), and correlated inversely with IFX serum induction levels ($r = -0.45$, $P = 0.002$ at week 6).

Conclusions: Increased mucosal IL13RA2 expression is associated with an increased mucosal TNF burden in CD patients. In view of its specificity for prediction of anti-TNF therapy resistance, mucosal *IL13RA2* expression is a potential biomarker for therapy selection and/or for the need of increased anti-TNF drug dosing.

The Handling Editor for this article was Dr Nicholas Kennedy, and it was accepted for publication after full peer-review.

1 | INTRODUCTION

The advent of anti-TNF agents has dramatically improved the treatment of inflammatory bowel disease (IBD) in the past two decades.[1] Despite an overall good response to anti-TNF therapy, up to 30% of patients are primary nonresponders.[2] Why patients fail a particular drug is still poorly understood and, more importantly, accurate and clinically validated biomarkers predicting nonresponse are currently lacking. Using microarray experiments, our group previously identified the expression of *IL13RA2* messenger ribonucleic acid (mRNA) in mucosal biopsies of IBD patients as a predictive marker for primary nonresponsiveness to infliximab (IFX) therapy, with a higher baseline expression in future nonresponders.[3,4] Recent data suggest that IL13RA2 on epithelial cells contributes to IBD by negatively influencing goblet cell recovery, goblet cell function and epithelial restoration after injury.[5] Why *IL13RA2*, which under physiological conditions is not significantly expressed in normal tissue except in testes,[6,7] interferes with the effect of anti-TNF therapy in IBD is currently unknown.

The present study aimed to identify if the enhanced *IL13RA2* signal is anti-TNF specific and if so, why *IL13RA2* expression is a predictive marker of efficacy of anti-TNF therapy. First, we studied the *IL13RA2* mRNA signal in a new cohort of adalimumab-treated CD patients, and questioned the specificity of its predictive value through RNA-sequencing of inflamed mucosal biopsies of vedolizumab treated patients. We then applied weighted gene co-expression network analysis (WGCNA)—a data mining method designed to study biological networks based on pairwise correlations between variables—on publicly available colonic microarray data of IFX-treated patients, in order to identify the upstream regulators of anti-TNF nonresponse in CD. Finally, predicted mucosal key drivers were quantified on the protein level in serum, prior to first IFX exposure, compared between healers and nonhealers and correlated with serum IFX induction levels.

2 | MATERIALS AND METHODS

2.1 | Patient selection

This study was conducted at the tertiary IBD referral centre of the University Hospitals Leuven (Leuven, Belgium). Inflamed mucosal biopsies of CD patients initiating therapy with IFX, adalimumab or vedolizumab, were taken prior to the first administration, stored in RNALater buffer (Ambion, Austin, TX, USA) and preserved at −80°C. Similarly, serum of CD patients with active endoscopic disease was taken prior to the first IFX infusion, centrifuged and stored at −20°C. The primary endpoint, response to therapy, was defined as the complete absence of ulcerations.[8] Baseline characteristics of the different therapy cohorts are summarized in Table 1.

All patients included in the analysis had given written consent to participate in the Institutional Review Board approved IBD Biobank (B322201213950/S53684), collecting serum, biopsies and clinical characteristics among other items.

2.2 | Gene expression studies in mucosal biopsies of CD patients

Gene expression profiles at baseline and 4-6 weeks after IFX initiation were obtained from a previously published cohort by our group (GEO GSE16879) using Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, USA) (Cohort A, 17 colonic and 12 ileal biopsies).[4] In a new cohort of adalimumab and vedolizumab treated patients (Cohort B: 8 colonic, 14 ileal biopsies; Cohort C: 11 colonic, 13 ileal biopsies), RNA was extracted from inflamed biopsies using the AllPrep DNA/RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity and quantity of total RNA were assessed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Next-generation single-end sequencing was performed using the Illumina HiSeq 4000NGS, after library preparation using the TruSeq Stranded mRNA protocol (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Raw RNA-sequencing data were aligned to the reference genome using TopHat version 2.1.1,[9] absolute counts generated using HTSeq,[10] whereafter counts were normalized and differential gene expression was assessed using the DESeq2 package.[11]

Microarray data of inflamed colonic biopsies (Cohort A) were used for WGCNA and for quantification of mRNA expression of particular genes of interest. Raw data were preprocessed (normalization (robust multichip average (RMA) method[12]) and quality-control evaluation (arrayQualityMetrics[13]) is done using Bioconductor (http://www.bioconductor.org) in R version 3.5.0 (R Development Core Team, Vienna, Austria). Only annotated genes were included in the downstream analysis. Additional, nonspecific filtering was applied on the normalized log 2 expression values to obtain only those probe sets hybridizing above background levels, that is only those probe sets with an intensity >log 2(100) in 5% of samples. The probe sets that passed this filtering (n = 22 316) were used for further analysis. Instead of differential gene expression, a WGCNA was performed.[14] A gene co-expression network was generated by calculating a gene pairwise Pearson correlation matrix. A soft threshold power value of 16 corresponding to 80.3% scale-free topology fit to generate an unsigned scale-free network in order to construct a topological overlap matrix. Gene probe sets were subsequently assigned to modules by average linkage hierarchical clustering. Minimal module size was set to 30 gene probe sets at a deepSplit = 3. Each module was tested for association with the distinct traits. A false discovery rate (FDR) correction was applied on the module-trait association testing, with a cut-off of 0.01. Module Eigengenes were defined as the first principal component, which summarizes the expression patterns of all gene probe sets into a single characteristic expression profile within a given module. Genes showing high correlation with the module eigengene were referred to as intramodular hub genes. Bio-functional analysis of significantly correlated modules and probe sets were performed with Ingenuity Pathway analysis

TABLE 1 Baseline characteristics of all included patients

	IFX biopsy cohort (n = 29, Cohort A)	ADM biopsy cohort (n = 22, Cohort B)	VDZ biopsy cohort (n = 24, Cohort C)	IFX serum cohort (n = 41, Cohort D)
Colonic biopsies, n (%)	17 (58.6)	8 (36.4)	11 (45.8)	NA
Ileal biopsies, n (%)	12 (41.4)	14 (63.6)	13 (54.2)	
Sex, women, n (%)	14 (48.3)	12 (54.5)	13 (54.2)	23 (56.1)
Age at initiation of therapy (y), median (IQR)	41.4 (28.4-51.3)	26.1 (21.9-48.9)	35.2 (25.2-47.6)	38.4 (26.3-46.0)
Disease duration (y), median (IQR)	15.8 (2.1-24.9)	3.9 (1.0-17.9)	5.1 (3.5-18.3)	8.9 (0.9-19.1)
Disease behaviour, n (%)				
Inflammatory (B1)	17 (58.6)	9 (40.9)	10 (41.7)	19 (46.3)
Stricturing (B2)	8 (27.6)	6 (27.3)	8 (33.3)	17 (41.5)
Penetrating (B3)	4 (13.8)	7 (31.8)	6 (25.0)	5 (12.2)
Perianal (p)	6 (20.7)	4 (18.2)	13 (54.2)	17 (41.4)
Disease location, n (%)				
Ileal disease (L1)	6 (20.7)	8 (36.4)	5 (20.8)	4 (9.8)
Colonic disease (L2)	12 (41.3)	3 (13.6)	3 (12.5)	12 (29.2)
Ileocolonic disease (L3)	11 (37.9)	11 (50.0)	16 (66.7)	25 (61.0)
Upper GI disease (L4)	0 (0.0)	0 (0.0)	2 (8.3)	0 (0.0)
C-reactive protein (mg/L), median (IQR)	8.2 (4.0-20.6)	5.3 (3.3-16.0)	3.3 (1.2-11.5)	9.7 (4.4-31.6)
Concomitant therapy during induction, n (%)	17 (58.6)	0 (0.0)	4 (16.7)	20 (48.8)
Immunomodulators	17 (58.6)	0 (0.0)	4 (16.7)	20 (48.8)
Topical and/or systemic steroids	4 (13.8)	4 (18.2)	17 (70.8)	7 (17.1)

ADM, adalimumab; IFX, infliximab; IQR, interquartile range; NA, not available; VDZ, vedolizumab; n, number; y, years.

(IPA, Aarhus, Denmark) and Gene Set Enrichment Analysis (GSEA, Broad Institute, Massachusetts Institute of Technology, and Regents of the University of California, USA).[15–17]

2.3 | Quantification of serum proteins and IFX serum levels

Based on the results of the microarray data, tumour necrosis factor (TNF), chitinase-3-like-protein 1 (CHI3L1) and interleukin 13 (IL-13) were measured in baseline serum samples using the MesoScale Discovery electrochemiluminescence technology (MSD, Rockville, MD, USA). Soluble IL-13Rα2 was quantified using an Enzyme-Linked Immuno Sorbent Assay (ELISA) kit (ab46112) from Abcam (Cambridge, UK). Triggering-receptor expressed on myeloid cells 1 (TREM1) was measured in duplicate using the Human sTREM-1 ELISA kit (HK348, Hycult Biotech, Uden, the Netherlands). IFX serum levels were measured using an ELISA (apDia, Turnhout, Belgium) on an automated ELISA processing system.

2.4 | Statistical analysis

All analyses were carried out using R version 3.5.0 (R Development Core Team, Vienna, Austria). Continuous variables are expressed as median and interquartile range. Unpaired data were compared using the Mann-Whitney *U*-test for continuous variables, and with Fisher's exact test for categorical variables. Correlations were assessed using the Pearson *r* correlation coefficient. Diagnostic performance was assessed with receiver operating characteristics (ROC) curve analysis. A $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Increased *IL13RA2* expression is unique to anti-TNF nonhealers

In cohort A (n = 29, originating from a previously published cohort[4]), mucosal *IL13RA2* mRNA expression prior to the start of IFX therapy was significantly upregulated in future nonhealers ($P = 7.7 \times 10^{-5}$, fold change [FC] = 2.7). After IFX exposure, *IL13RA2* mRNA dropped significantly in nonhealers ($P = 7.0 \times 10^{-3}$), but less significant than in healers ($P = 2.0 \times 10^{-5}$) (Figure 1A). Even after IFX exposure, nonhealers did not reach the lower baseline *IL13RA2* expression levels of healers.

In a new cohort of CD patients initiating adalimumab therapy (Cohort B, n = 22), endoscopically assessed after 6 months for healing,[18] baseline *IL13RA2* expression was 13.0-fold upregulated in future nonhealers ($P = 1.1 \times 10^{-4}$) (Figure 1B). In the third cohort of vedolizumab starters (n = 24), also endoscopically assessed after 6 months,[18] baseline *IL13RA2* mRNA expression was not significantly different between healers and nonhealers ($P = 0.3$, FC = 1.7) (Figure 1C) (Cohort C, n = 24). Additionally, *CHI3L1* mRNA, a recently discovered second ligand of IL13RA2,[19] was also differentially expressed at baseline between healers and nonhealers in both the

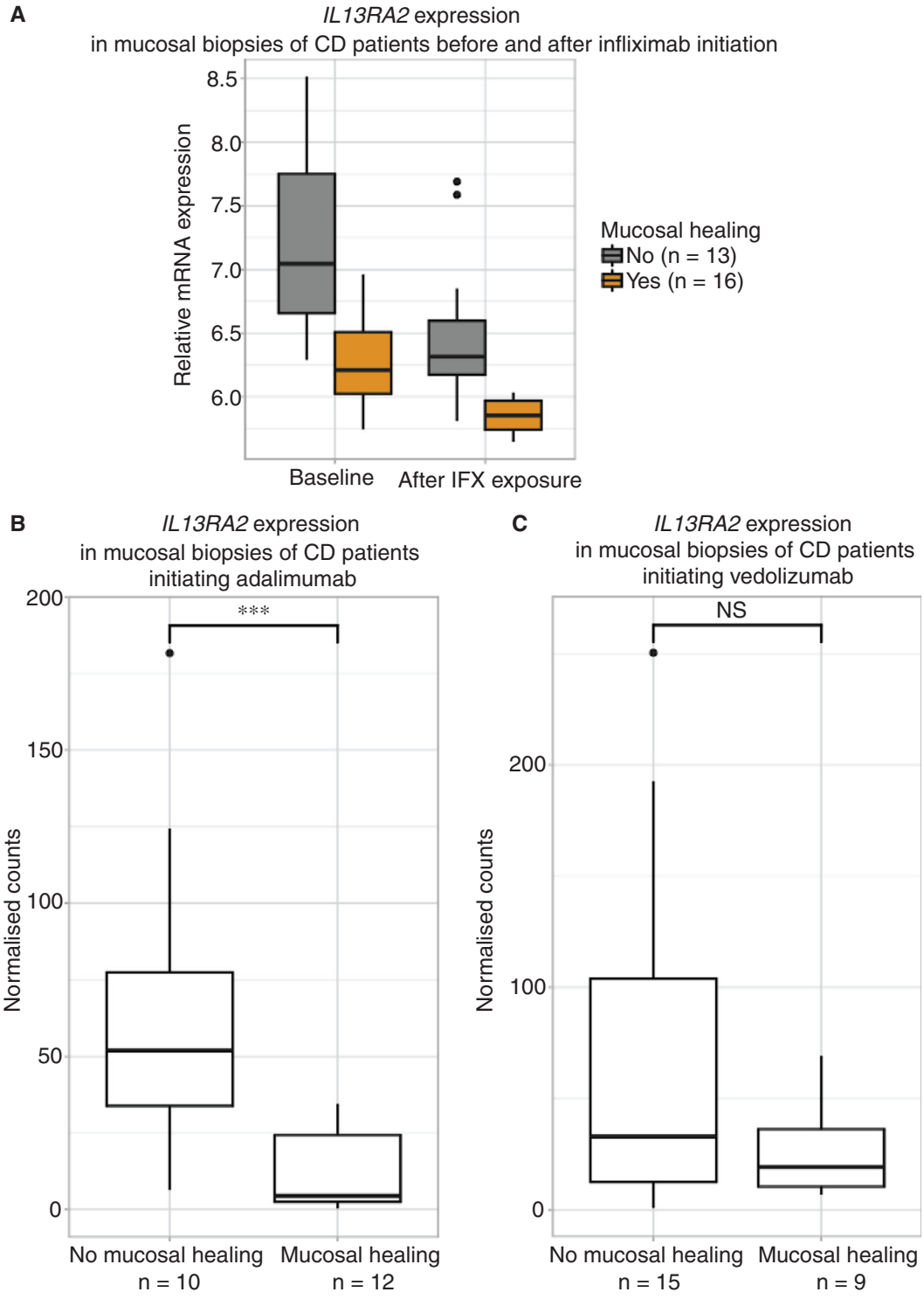

FIGURE 1 Mucosal *IL13RA2* mRNA in healers and nonhealers on different biological therapies. Relative mRNA expression (log2 transformed, microarray) of *IL13RA2* in inflamed colonic biopsies of patients with Crohn's disease (CD) prior to and 4-6 wk after (A) initiating infliximab therapy) (Cohort A). Normalised mRNA *IL13RA2* counts (RNA-sequencing) in inflamed colonic biopsies of CD patients prior to initiating adalimumab (Cohort B) (B) or vedolizumab (Cohort C) (C). ***$P < 0.001$. NS = not significant

IFX- and the adalimumab-treated patients (Figure 2A,B) ($P = 0.03$, $P = 0.003$ respectively), but not in the vedolizumab treated patients ($P = 0.8$) (Figure 2C). Overall, the predictive power of *IL13RA2* expression for anti-TNF nonresponsiveness in mucosal biopsies could be established in both the IFX and adalimumab-treated cohort (area under the curve, AUROC, 0.90 and 0.94 respectively, $P < 0.001$ in both groups), which was but not the case in vedolizumab treated patients (AUROC 0.63, $P = 0.3$).

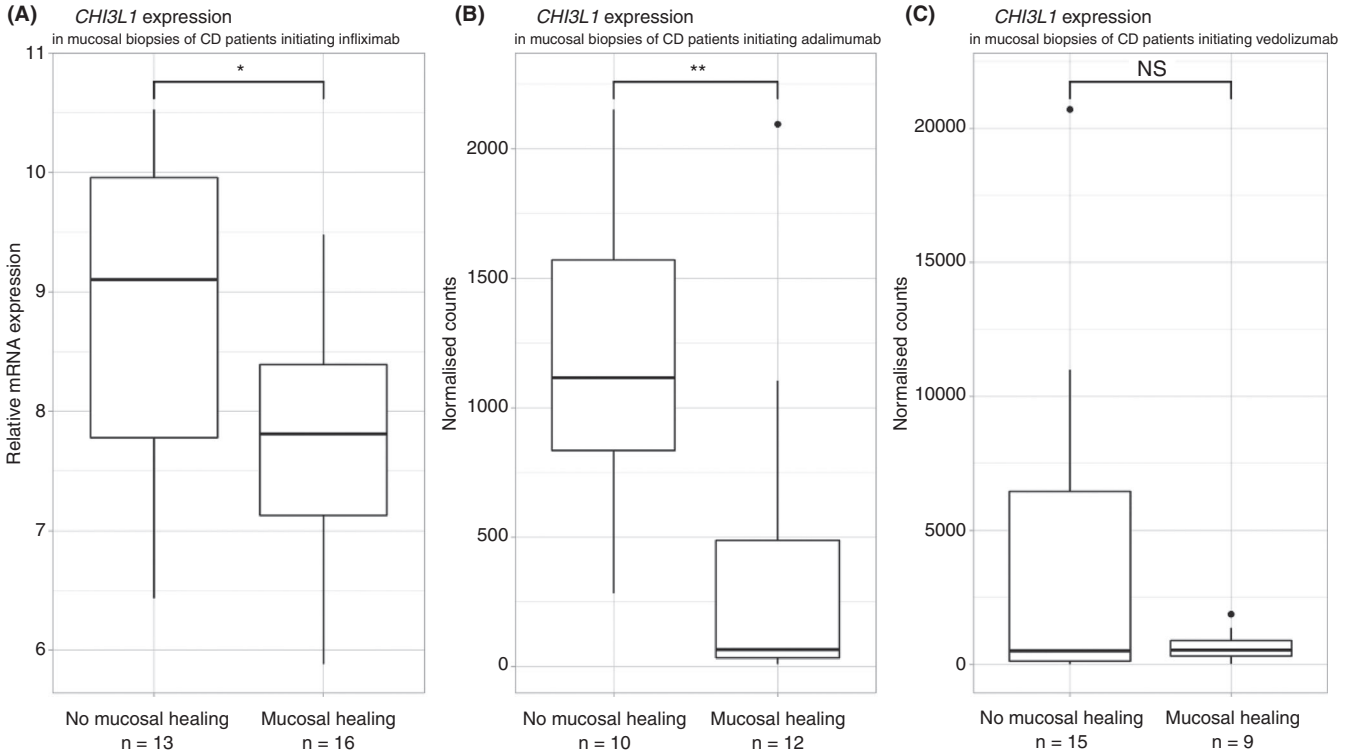

FIGURE 2 Mucosal *CHI3L1* mRNA in healers and nonhealers. Relative mRNA expression (log2 transformed, microarray) of *CHI3L1* in inflamed colonic biopsies of patients with Crohn's disease (CD) prior to initiating infliximab therapy (Cohort A) (A). Normalised mRNA *CHI3L1* counts (RNA-sequencing) in inflamed colonic biopsies of CD patients prior to initiating adalimumab (Cohort B) (B) or vedolizumab (Cohort C) (C). $^{*}P < 0.05$; $^{**}P < 0.01$. NS, not significant

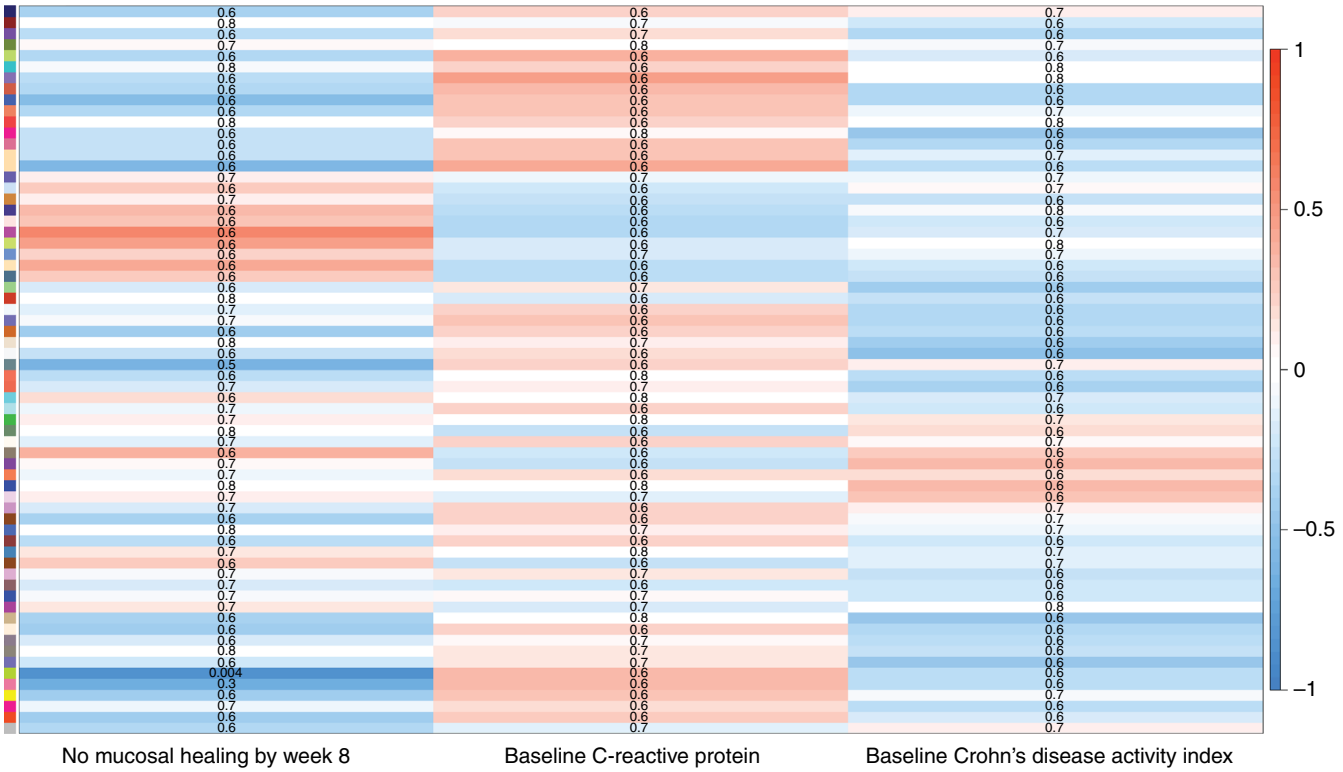

FIGURE 3 Co-expression network analysis: Module-trait relationships. Weighted gene co-expression network analysis on colonic mucosal biopsies of active Crohn's disease (CD) patients (Cohort A) prior to initiation of infliximab therapy. Heatmap represents the different modules detected (*Y*-axis) and their correlation with the different traits of interest (*X*-axis). Numbers represent the adjusted *P*-values, whereas the colours represent the strength of the association (blue negative and red positive correlation)

3.2 | Tissue TNF as a key driver in anti-TNF nonresponsiveness

In order to identify the clusters of genes driving anti-TNF nonresponsiveness, we performed WGCNA analysis on publicly available microarray data (Cohort A). Sixty-six modules of correlated gene probe sets were identified, of which only one (module "greenyellow") was significantly associated with mucosal healing to IFX therapy (FDR corrected $P = 0.004$). Module-trait analysis found identified no significant associations with either baseline C-reactive protein (CRP) or baseline Crohn's disease activity index (Figure 3). As expected, consensus clustering using the expression of all probe sets represented within the greenyellow module significantly discriminated responders from nonresponders ($P = 0.009$; Figure S1). More importantly, it separated samples on a biological basis, allowing further in-depth pathway analysis.

Pathway analysis on the 2013 probe sets represented in the greenyellow module associated with endoscopic response revealed the enrichment of TREM1 signalling in anti-TNF nonresponders ($P = 3.8 \times 10^{-10}$) as the most crucial immune pathway after more general granulocyte and agranulocyte adhesion and diapedesis pathways ($P = 6.7 \times 10^{-17}$ and 5.3×10^{-13} respectively). Predicted upstream analysis (using curated datasets in IPA) highlighted TNF ($P = 5.0 \times 10^{-34}$), TGFβ ($P = 8.3 \times 10^{-27}$) and IL-13 ($P = 3.3 \times 10^{-23}$) as the most likely upstream regulators of all pathways enriched in anti-TNF nonresponsive patients prior to treatment initiation. Indeed, *TNF* mRNA levels correlated significantly with *IL13RA2* expression ($\rho = 0.55$, $P = 0.02$), as well as with *TNFR2* expression ($\rho = 0.61$, $P = 0.009$) but not with *TNFR1* ($\rho = 0.21$, $P = 0.41$). Additionally, the *TNF/TNFR2* ratio was significantly higher in future responders ($P = 0.002$), which was not the case for the TNF/TNFR1 ratio ($P = 0.67$).

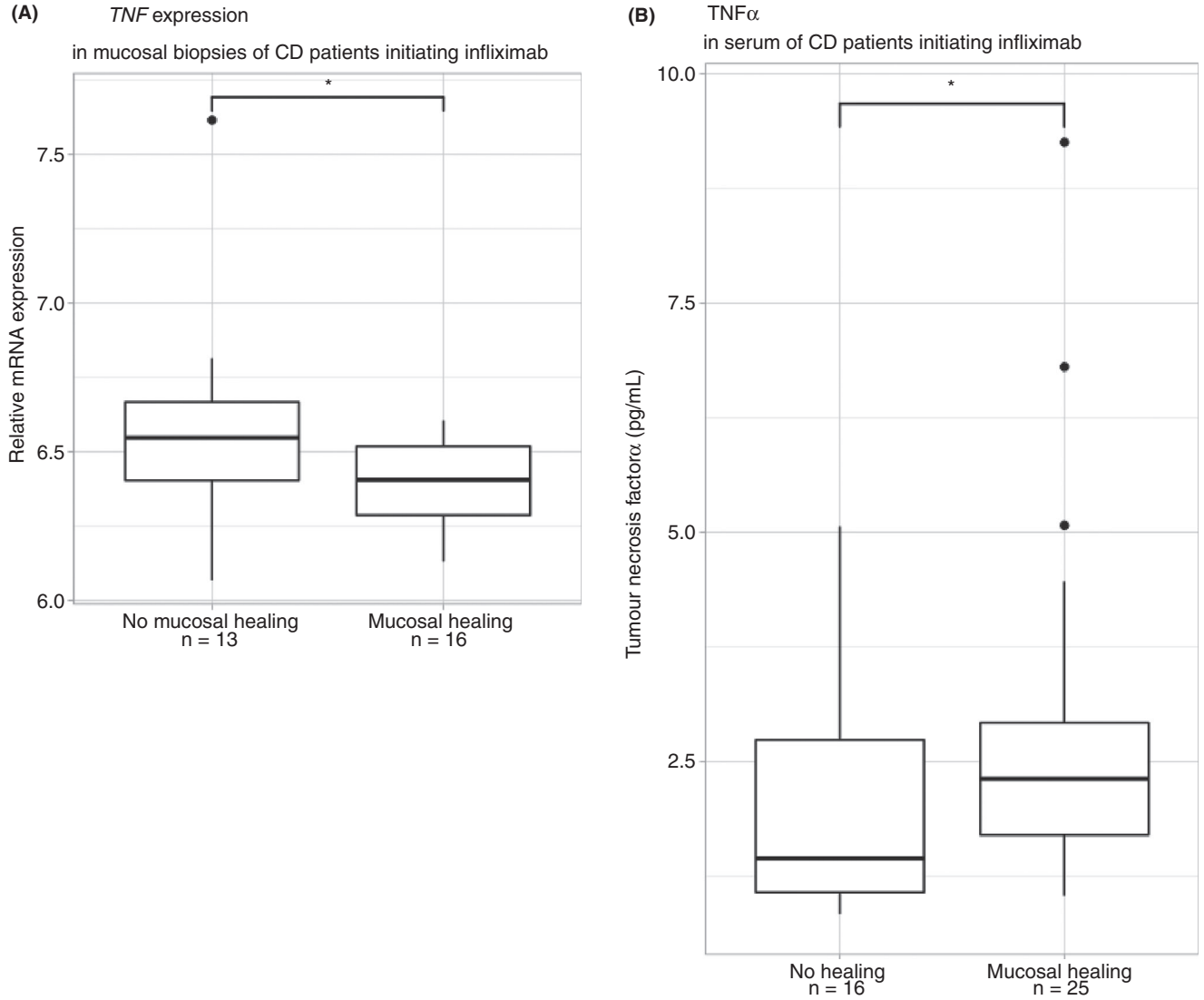

FIGURE 4 TNF levels at baseline in healers and nonhealers on infliximab therapy. Mucosal mRNA *TNF* (Cohort A) (A) and serum levels of TNF (Cohort D) (B) in patients with active Crohn's disease (CD) prior to initiating infliximab therapy. Patients are grouped according to the endoscopic response to infliximab therapy. *$P < 0.05$. NS = not significant

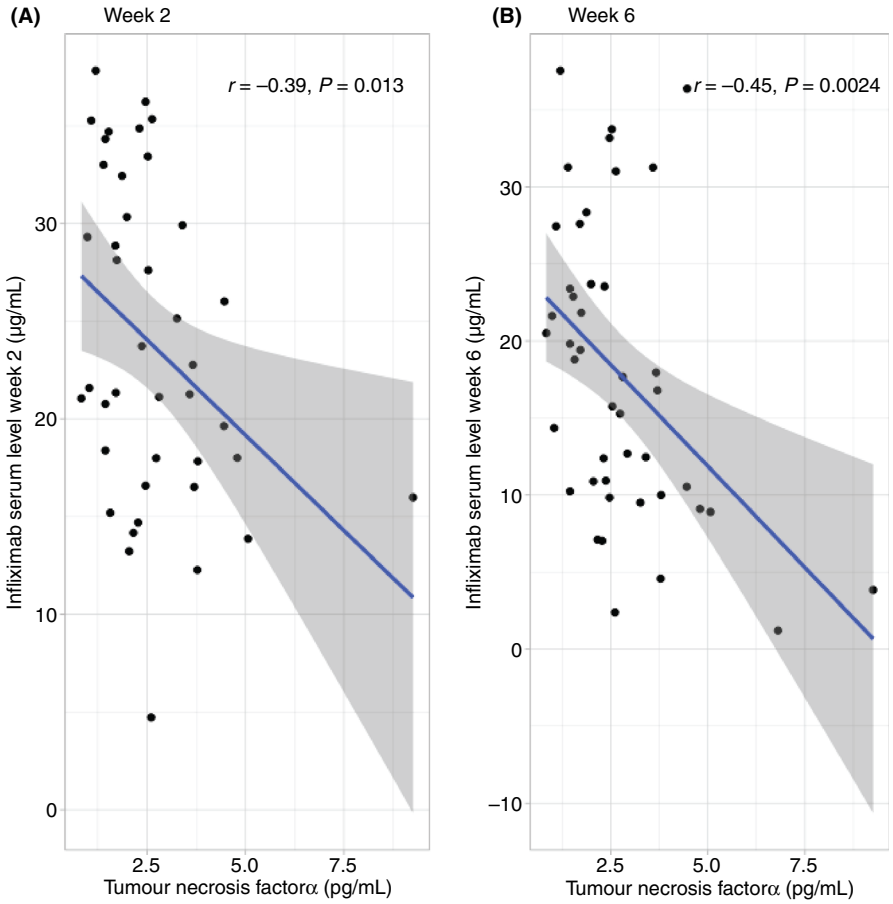

FIGURE 5 Correlation between serum TNF and infliximab induction serum levels. Pearson correlation between serum TNF prior to first infliximab infusion and infliximab serum levels at week 2 (A) and week 6 (B) (Cohort D)

3.3 | Lower systemic TNF burden in future nonresponders

In contrast to the elevated baseline mucosal *TNF* mRNA levels in IFX nonresponders (P = 0.02, FC = 1.2) (Figure 4A), baseline serum TNF levels were significantly lower in future nonhealers than in healers (P = 0.04, FC = 0.43) (Figure 4B) (Cohort D) and inversely correlated with IFX serum levels at weeks 2 and 6 (ρ = –0.39, P = 0.01; ρ = –0.45, P = 0.002 respectively) (Figure 5A,B).

Despite clear epithelial expression, soluble IL13RA2 (sIL13RA2) was not detectable in serum, which is in agreement with reports showing that IL13RA2 is only expressed on the membrane of epithelial cells, and is not shedded or expressed as a soluble molecule, due to the lack of alternative splicing in humans.[20,21] Serum CHI3L1 and IL-13 were not significantly different between healers and nonhealers at baseline (P = 0.1, P = 0.3 respectively) (Figure S2A,B), whereas a significant difference in sTREM1 could be observed (P = 0.02) as described earlier (Figure S2C).[22]

4 | DISCUSSION

IL13RA2 mRNA in intestinal mucosal tissues has been identified as a predictive biomarker for anti-TNF responsiveness in IBD.[3,4,23,24] In this study, we further elaborated on these previous findings. First, we could show that high *IL13RA2* mRNA expression is specific for prediction of resistance to anti-TNF induced mucosal healing, as we did not find similar accuracy in vedolizumab treated subjects. We further studied how expression of this receptor can be related to the effects of TNF neutralization. Instead of purely focusing on top differentially expressed genes, we therefore applied a co-expression network approach on publicly available transcriptomic data of IFX-treated CD patients, providing novel insights in anti-TNF nonresponsiveness. Subtle but biologically relevant differences at the top of a pathophysiological cascade can have important amplified downstream consequences which are not always picked up using differential gene expression analysis, but need more detailed network analysis. In accordance with some recent observations,[22,25] TREM1 signalling was one of the pivotal pathways upregulated in nonresponders. However, on an upstream level, TNF seemed the most dominant director, influencing several downstream pathways. TNF, the key upstream regulator driving the biological processes associated with anti-TNF nonresponsiveness in this network analysis, has previously been documented to influence *IL13RA2* expression.[26,27] The increased mucosal *IL13RA2* mRNA expression in anti-TNF nonresponders can therefore be seen at least partly as a downstream effect of the increased mucosal TNF burden. The significant IFX-induced decrease in *IL13RA2* expression in healers is another indirect proof suggesting that TNF is largely responsible for *IL13RA2* mRNA levels.

One could consider these findings on increased *TNF* burden in future nonresponders as being in conflict to previous findings demonstrating a significant enrichment of mucosal membrane-bound TNF (mTNF)-positive immune cells in future responders.[28] However, due to the design of the current study, we could not discriminate between mTNF and soluble TNF (sTNF) at the mRNA level, and only report the overall TNF burden. An increased mucosal mTNF with decreased sTNF expression in future responders could therefore not be excluded. Furthermore, the increased mucosal *TNF* mRNA expression in future nonresponders is in line with previous conclusions from the ATLAS-study, which demonstrated that in moderate to severely inflamed tissue, tissue TNF is not sufficiently neutralized by normal doses of anti-TNF agents, and high mucosal levels of TNF serve as a sink for anti-TNF.[29] The lower serum TNF levels in future nonresponders and its significant correlation with CRP do not come as a surprise, as elevated baseline CRP levels are known to be a predictor of anti-TNF responsiveness.[30–33] Because of the clear correlation between CRP and serum TNF, the inverse correlation observed between serum TNF and IFX serum levels is similar to the inverse correlation between serum levels and CRP documented earlier.[29,34] The higher mucosal TNF burden in future nonhealers is perhaps not sufficient to consider these patients true anti-TNF failures, as tissue anti-TNF concentrations may be insufficient for these patients to neutralize all tissue TNF. Because of the known mismatch between serum and tissue anti-TNF levels, especially in patients with active disease,[29] we hypothesize that targeting a higher serum trough level above the 7.0 μg/mL upper limit of the therapeutic window might better neutralize tissue TNF and hence increase mucosal healing rates, especially in patients with a high mucosal *IL13RA2* expression. Retrospective data already suggested that higher trough levels are safe and result in better endoscopic outcomes.[35–38] Similarly, recent prospective data from the UK PANTS study also demonstrated the beneficial impact of higher post-induction serum levels on 1-year outcome in anti-TNF-treated patients.[39] Future studies should therefore randomize patients with high mucosal expression of *IL13RA2* or high mucosal TNF burden between normal or increased doses of IFX, and evaluate if response rates can be improved by increased exposure, especially in those patients with a high baseline *IL13RA2* expression.

In conclusion, we confirmed the potential use of mucosal *IL13RA2* expression as an anti-TNF specific predictive marker for mucosal healing. Based on our network approach of transcriptomic data and the current literature, we propose that a high expression of *IL13RA2* mRNA to a large extent (if not exclusively) reflects higher TNF production in the tissue. It remains to be explored if higher anti-TNF exposure in patients with increased baseline *IL13RA2* expression can neutralize this increased TNF burden and hence then results in successful healing. For regulatory purposes, clinical trials investigate only a limit number of different dosages of a particular drug, in order to achieve the best outcome for the majority of patients. However, this "one-size fits all" principle does not take into consideration pharmacokinetic variability, which has already been proven to be of high importance in anti-TNF therapy.[40] Future research, studying higher IFX exposure based on personalised pharmacokinetic models, including biomarkers as for instance mucosal *IL13RA2* and whole blood *TREM1* expression, are therefore eagerly awaited.

ACKNOWLEDGEMENTS

The authors thank Vera Ballet and Eline Vandeput, Sophie Organe, Nooshin Ardeshir Davani, Helene Blevi, Tamara Coopmans and Willem-Jan Wollants for an excellent job in maintaining the Biobank database; and Vanessa Brys, Jens Van Bouwel, Wim Meert, Alvaro Cortes Calabuig, Celine Helsmoortel and Wouter Bossuyt the technical assistance with the RNA-sequencing library preparation and data generation.

Declaration of personal interests: B Verstockt received financial support for research from Pfizer; lecture fees from Abbvie, Ferring, Takeda Pharmaceuticals, Janssen and R Biopharm; consultancy fees from Janssen. G Van Assche received financial support for research from Abbott and Ferring Pharmaceuticals; lecture fees from Janssen, MSD and Abbott; consultancy fees from PDL BioPharma, UCB Pharma, Sanofi-Aventis, Abbott, Abbvie, Ferring, Novartis, Biogen Idec, Janssen Biologics, NovoNordisk, Zealand Pharma A/S, Millenium/Takeda, Shire, Novartis and Bristol Mayer Squibb. A Gils received financial support for research from Pfizer, MSD & Takeda; lecture fees from MSD, Janssen Biologicals, Pfizer, Takeda and Abbvie; consultancy fees from Takeda; holds a license agreement with R-biopharm, apDia and Merck. S Vermeire received financial support for research from MSD, Abbvie, Janssen and UCB Pharma; lecture fees from Abbott, Abbvie, Merck Sharpe & Dohme, Ferring Pharmaceuticals and UCB Pharma; consultancy fees from Pfizer, Ferring Pharmaceuticals, Shire Pharmaceuticals Group, Merck Sharpe & Dohme, and AstraZeneca Pharmaceuticals. M Ferrante received financial support for research from Takeda and Janssen; lecture fees from Ferring, Boehringer- Ingelheim, Chiesi, Merck Sharpe & Dohme, Tillotts, Janssen Biologics, AbbvieTakeda, Mitsubishi Tanabe, Zeria; consultancy fees from Abbvie, Boehringer-Ingelheim, Ferring, Merck Sharpe & Dohme, and Janssen Biologics.

SaV, BC, ST, JC, CB declare no conflicts of interest.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclose.

AUTHORSHIP

Guarantor of the article: Christine Breynaert.

Author contributions: BV contributed to the study concept and design, acquisition data, analysis and interpretation of data, drafting of the manuscript, statistical analysis and technical support. SaV: technical assistance, analysis and interpretation of data, critical revision of the manuscript. BC: interpretation of data, critical revision of the manuscript. ST: technical assistance with measuring of the serum

levels. GVA: critical revision of the manuscript. AG: material support and critical revision of the manuscript. JLC: design of the study and data interpretation, critical revision of the manuscript for important intellectual content. SV, MF and CB: study concept and design, acquisition of data, analysis and interpretation of data, material support, obtained funding and study supervision. All authors agreed with the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section at the end of the article.

How to cite this article: Verstockt B, Verstockt S, Creyns B, et al. Mucosal IL13RA2 expression predicts nonresponse to anti-TNF therapy in Crohn's disease. *Aliment Pharmacol Ther*. 2019;49:572–581. https://doi.org/10.1111/apt.15126