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Mucosal gene signatures to predict response to infliximab in patients with ulcerative colitis

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Short title: Infliximab mucosal predictive response signature

Keywords: Ulcerative colitis, infliximab, gene expression profiling

Abbreviations: ACT, Active Ulcerative Colitis Trial; B-H: Benjamini and Hochberg; FC, fold change; FDR, false discovery rate; IL-11, interleukin-11; IL-13Ralpha2, interleukin-13 receptor alpha 2; IQR, interquartile range; LIMMA, linear models for microarray data; NR, non-responders; PAM, prediction analysis of microarrays; PTGS2, prostaglandin-endoperoxide synthase 2; qPCR, quantitative RT-PCR; R, responders; RMA, robust multichip average; STC1, stanniocalcin-1; TNF-alpha, tumor necrosis factor-alpha; TNFRSF11B, osteoprotegerin; UC: ulcerative colitis

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ABSTRACT

Background & Aims: Infliximab is an effective treatment for ulcerative colitis (UC) with over 60% of patients responding to treatment and up to 30% reaching remission. The mechanism of resistance to anti-TNF-alpha is unknown. This study used colonic mucosal gene expression to provide a predictive response signature for infliximab treatment in UC.

Methods: Two cohorts of patients who received their first treatment with infliximab for refractory UC were studied. Response to infliximab was defined as endoscopic and histologic healing. Total RNA from pre-treatment colonic mucosal biopsies was analyzed with Affymetrix Human Genome U133 Plus 2.0 Arrays. Quantitative RT-PCR was used to confirm microarray data.

Results: For predicting response to infliximab treatment, pre-treatment colonic mucosal expression profiles were compared for responders and non-responders. Comparative analysis identified 179 differentially expressed probe sets in cohort A and 361 in cohort B with an overlap of 74 probe sets, representing 53 known genes, between both analyses. Comparative analysis of both cohorts combined, yielded 212 differentially expressed probe sets. The top 5 differentially expressed genes in a combined analysis of both cohorts were osteoprotegerin, stanniocalcin-1, prostaglandin-endoperoxide synthase 2, interleukin-13 receptor alpha 2 and interleukin-11. All proteins encoded by these genes are involved in the adaptive immune response. These markers separated responders from non-responders with 95% sensitivity and 85% specificity.

Conclusion: Gene array studies of UC mucosal biopsies identified predictive panels of genes for (non-)response to infliximab. Further study of the pathways involved should allow a better understanding of the mechanisms of resistance to infliximab therapy in UC.

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease involving the mucosa of the colon distal to the anal verge. The pathogenesis of UC is believed to result from an interaction of genetic factors, the immune response to microbial dysbiosis and environmental factors. Cigarette smoking and appendectomy have both been associated with a decreased risk of developing UC(1). Five-aminosalicylates, corticosteroids, azathioprine/6-mercaptopurine are the current treatments for UC and patients who fail these treatments were until recently referred for colectomy. The ACT trials have shown that infliximab (Remicade; Centocor, Inc., Malvern, PA, USA), a mouse/human chimeric monoclonal IgG1 antibody to tumor necrosis factor alpha (TNF-alpha), is efficacious in the treatment of patients with refractory UC and may avoid colectomy(2). However, around 40% of patients treated do not respond to infliximab and predictors of response are currently lacking.

Microarray technology is a powerful tool that enables the measurement of the expression of thousands of genes simultaneously(3). This technology has been used to elucidate the pathogenic processes underlying different diseases and to identify predictive gene profiles(4;5).

The aim of the present study was to identify mucosal gene signatures predictive of response to infliximab in anti-TNF-alpha naive UC patients using high-density oligonucleotide arrays. We studied two independent cohorts of patients, one at the University Hospital Leuven (cohort A) and another cohort of patients who participated in the placebo-controlled ACT1 study(2) (cohort B).

METHODS

Patients

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Cohort A

Twenty-four patients with active UC, refractory to corticosteroids and/or immunosuppression, were studied in cohort A (Table 1a). The study was carried out at the University Hospital Leuven (tertiary referral centre) (ClinicalTrials.gov number, NCT00639821) and all patients were followed-up long-term. The ethics committee of the University Hospital approved the study and all individuals gave written informed consent.

Table 1a Baseline characteristics of the UC patients from cohort A

	Responders (n=8)	Non-responders (n=16)	P-value
Male/Female (%)	4/4 (50/50)	10/6 (62.5/37.5)	0.67
Median (IQR) age at first IFX (years)	28.4 (24.3-41.8)	45.8 (36.5-62.3)	0.03
Median (IQR) weight at first IFX (kg)	72 (57.8-78.5)	73.3 (68.5-80.3)	0.41
Median (IQR) duration of disease prior to first IFX (years)	10.3 (4.1-17.3)	7.3 (2.6-13.3)	0.67
Median (IQR) C-reactive protein at first IFX (mg/dL)	1.65 (1-9.6)	6.5 (2.9-19.1)	0.09
Median (IQR) Mayo score before first IFX	10 (8.8-10)	9.5 (8.8-10.3)	0.95
Concomitant medication at first IFX (%)			
5-Aminosalicylates	5 (62.5)	13 (81.3)	0.36
Corticosteroids	2 (25)	5 (31.3)	1
Azathioprine/6-Mercaptopurine	7 (87.5)	8 (50)	0.18
Corticosteroids + Immunosuppressants	2 (25)	1 (6.3)	0.25
Active smoking at first IFX (%)	1 (12.5)	1 (6.3)	1

IQR, interquartile range; IFX, infliximab

Consecutive patients with active UC who consented with the study were included. The patients had a total Mayo score between 6-12 and an endoscopic subscore of at least 2(2). The UC patients underwent colonoscopy with biopsies from diseased rectum within a week prior to the first intravenous infusion of 5 mg infliximab per kg body weight. The patients underwent a second flex sigmoidoscopy with rectal biopsies 4 weeks after the first infliximab infusion in case of a single infusion and at 6 weeks if they received a loading dose of infliximab at weeks 0, 2 and 6. The endoscopist was not blinded to treatment. Half of the biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C for RNA isolation. The residual biopsies were fixed in Carnoy's fixative for up to 5 hours and then dehydrated, cleared and paraffin-embedded for histologic examination. The features of chronic intestinal inflammation were scored in haematoxylin-eosin stained slides from the paraffin blocks of each patient using a previously reported scoring system for UC(6). The pathologists who scored the biopsies (KG and GDH) were blinded to treatment.

The response to infliximab was assessed 4 weeks after first infliximab treatment for patients who received a single infusion at induction and 6 weeks after first infliximab treatment for patients who received a loading regimen. Response was defined as a complete mucosal healing with a Mayo endoscopic subscore(2) of 0 or 1 and a grade 0 or 1 on the histological score for UC(6). Patients who did not achieve healing were considered non-responders although some of them presented endoscopic and/or histologic improvement.

Colonic biopsies were also obtained from 6 control subjects who underwent colonoscopy for screening for polyps. These subjects gave informed consent for the study.

Cohort B

Endoscopies were carried out and biopsies were collected during the ACT1 trial(2), a placebo controlled trial of infliximab therapy in refractory UC (ClinicalTrials.gov number,

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NCT00036439), at protocol-specified time points from a subset of randomized patients. Endoscopists and pathologists in this study were blinded to therapy. The institutional review board or ethics committee at each site approved the ACT1 protocol and all patients provided informed consent.

For the current study, 23 pre-treatment colonic mucosal biopsies from 22 active UC patients (2 colonic mucosal biopsies were obtained within two weeks from the same patient), who received a loading dose of infliximab (5 or 10 mg/kg) at weeks 0, 2 and 6 for refractory UC were analyzed in cohort B (Table 1b). The biopsies were collected 15 to 20 centimetres distal from the anal verge.

The response to infliximab was assessed 8 weeks after their first infliximab treatment. The definition of response was identical to cohort A.

Table 1b Baseline characteristics of the UC patients from cohort B

	Responders (n=12)	Non-responders (n=10)	P-value
Male/Female (%)	6/6 (50/50)	4/6 (40/60)	0.69
Median (IQR) age at first IFX (years)	39 (28.5-46.8)	51.5 (36-59.8)	0.34
Median (IQR) weight at first IFX (kg)	75 (70.5-87.3)	69 (56-78.5)	0.09
Median (IQR) duration of disease prior to first IFX (years)	12.3 (4.8-16.8)	6.8 (3.8-11)	0.35
Median (IQR) C-reactive protein at first IFX (mg/dL)	0.7 (0.5-1.4)	1.35 (0.7-2.2)	0.28
Median (IQR) Mayo score before first IFX	8.5 (7.8-9.3)	10 (9-10.8)	0.13
Concomitant medication at first IFX (%)			
5-Aminosalicylates	6 (50)	6 (60)	0.69
Corticosteroids	8 (66.7)	5 (50)	0.67
Azathioprine/6-Mercaptopurine	4 (33.3)	6 (60)	0.39
Corticosteroids + Immunosuppressants	2 (16.7)	3 (30)	0.62
Active smoking at first IFX (%)	1 (8.3)	0 (0)	1

IQR, interquartile range; IFX, infliximab

RNA isolation

For cohort A, total RNA was extracted from the biopsy specimens using the RNeasy Mini Kit (Qiagen, Benelux B.V.), according to the manufacturer's instructions. The integrity and quantity of total RNA were assessed with a 2100 Bioanalyzer (Agilent, Waldbronn, Germany) and Nanodrop ND-1000 spectrophotometer (Nanodrop technologies). The extracted RNA was used for microarray analysis and in some cases for quantitative RT-PCR (qPCR) analysis.

For cohort B, total RNA was isolated from the biopsies with RNeasy mini-kit according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA quality and quantity were analyzed with a 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA).

Oligonucleotide array hybridization

For samples from both cohorts, all steps were performed according to Affymetrix expression analysis technical manual 701021Rev.5 (Affymetrix, Santa Clara, CA, USA). Briefly, total RNA was reverse-transcribed into cDNA using the SuperScript Choice System (Invitrogen, Carlsbad, CA, USA). cDNA was *in vitro* transcribed to cRNA and biotin-labelled (Affymetrix). Biotinylated cRNA was purified and fragmented. The quality of labelled and fragmented cRNA was assessed with the Agilent 2100 Bioanalyzer. Fragmented cRNA (15 μg) was hybridized overnight to the Human Genome U133 Plus 2.0 Array (Affymetrix), which comprised of 54675 probe sets. The arrays were washed, stained with streptavidin-phycoerytrin and scanned on the Affymetrix 3000 GeneScanner. The resulting image files

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(.dat files) were analyzed using Affymetrix GCOS software, and intensity values for each probe cell (.cel file) were calculated. Quality evaluations of the microarrays were as expected. The data were deposited at Gene Expression Omnibus under the series accession numbers GSE14580 for cohort A and GSE12251 for cohort B.

Data analysis

The microarray data were analyzed using Bioconductor tools(7) in R (version 2.7.2, http://www.r-project.org/). The robust multichip average (RMA) method(8) was performed on the affymetrix raw data (.cel files) from both cohorts to obtain an expression value for each probe set. In cohort B, there were 2 pre-treatment colonic microarray profiles obtained from the same patient but only 1 of these was used for further data analysis. Because the severe correction for 54675 comparisons may have caused false negatives, we next performed the analysis with probe sets that hybridized above background levels to the patient samples. A non-specific filtering was applied on the log2 RMA normalized data from the pre-treatment UC samples from both cohorts to remove the probe sets with low overall intensity and variability that are unlikely to carry information about the phenotypes under investigation. Only probe sets with an intensity > log2(100) in at least 10% of the samples and an interquartile range (IQR) of log2 intensities across the samples > 0.5 were included, leaving 9183 probe sets for further data analysis (Supplementary table 1). Probe set annotations were obtained through the Affymetrix NetAffx website (http://www.affymetrix.com/analysis/index.affx) or the UCSC Genome Browser website (http://genome.ucsc.edu/). Linear models for microarray data (LIMMA)(9) and prediction analysis of microarrays (PAM)(10) were used for supervised data analyses. For comparative analysis, LIMMA was used to identify probe sets that are different between responders and non-responders, based on moderated *t*-statistics. To correct for multiple testing, the false discovery rate (FDR) was estimated from p-values derived from the moderated *t*-statistics using the method of Benjamini and Hochberg (B-H)(11). Probe sets with a > 2-fold change and a FDR < 0.05 were selected as differentially expressed. For class prediction, PAM with leave-one-out cross validation was carried out on the filtered dataset and on the top 20 and top 5 differentially expressed known genes that were identified by LIMMA analysis between responders and non-responders, to see if these subsets accurately predict response or non-response to infliximab and to identify the lowest misclassification error rate based on these subsets. Unsupervised average-linkage hierarchical clustering, using Euclidian distance as metric, was applied to data obtained from the data analysis to visualize gene/sample relationship. The results of the clustering were visualized as a 2-dimensional heatmap with 2 dendograms, one indicating the similarity between patients and the other indicating the similarity between genes. The Bio Functional Analysis tool in the Ingenuity Pathway Analysis program (Ingenuity Systems®, www.ingenuity.com) was used to identify biological functions and/or diseases that were most significant to the dataset of differentially expressed probe sets, identified by LIMMA analysis between responders and non-responders.

For both cohorts the baseline characteristics were compared between responders and non-responders (Table 1a and 1b). The comparisons were performed with the Mann-Whitney *U*-test for continuous variables and Fisher's exact test for categorical variables, using SPSS 16.0 software (SPSS, Chicago, IL). A p-value of < 0.05 was considered significant.

Quantitative RT-PCR

To validate the microarray data, qPCR was performed for osteoprotegerin (*TNFRSF11B*), stanniocalcin-1 (*STC1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), interleukin-13 receptor alpha 2 (*IL-13Ralpha2*), interleukin-11 (*IL-11*) and beta-actin. Beta-actin was used as

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the endogenous reference gene. cDNA was synthesized from 0.5 µg of total RNA from cohort A samples using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany), following the manufacturer's protocol. Primers and dual-labelled probes were designed using OligoAnalyzer 3.0 software (http://biotools.idtdna.com/analyzer/) and synthesized by Sigma-Genosys (Haverhill, UK). The oligonucleotide sequences are available upon request. qPCR was performed in a final reaction volume of 25 µl on a Rotor-Gene 3000 instrument (Corbett Research, Mortlake, Australia), using QuantiTect Multiplex PCR NoROX Kit (Qiagen, Venlo, NL), according to the manufacturer's instructions. Cycle threshold values were determined by Rotor-Gene 6.0.16 software. All samples were amplified in duplicate reactions. The relative expression of target mRNA levels were calculated as a ratio relative to the beta-actin reference mRNA(12). Results were analyzed using the Mann-Whitney *U*-test using SPSS 16.0 software. A p-value of < 0.05 was considered significant.

A glossary of terms used in methods is provided in Supplementary Appendix 1.

RESULTS

Response to infliximab

Two independent UC cohorts (cohort A and cohort B) were studied to identify mucosal gene signatures predictive of response to infliximab in UC. The response to infliximab was defined as complete endoscopic and histologic healing. Of the 24 UC patients in cohort A, 8 patients were responders and 16 patients were non-responders, while cohort B had 12 responders and 10 non-responders. No baseline characteristics were significantly different between responders and non-responders in both cohorts, except for age in cohort A which was significantly lower in responders than in non-responders (Table 1a and 1b).

Comparative analysis between responders and non-responders

For predicting response to infliximab treatment based on gene profiles, pre-treatment expression profiles were compared for responders and non-responders in each cohort and both cohorts combined, using LIMMA.

When all probe sets on the microarray (54675 probe sets) were included in the LIMMA analysis, the stringent correction for multiple testing resulted in only one differentially expressed probe set between responders and non-responders for UC in cohort A, namely *IL-13Ralpha2*. Therefore a non-specific filtering was first applied on the normalized data from the pre-treatment UC samples from both cohorts to eliminate non-relevant probe sets, leaving 9183 probe sets for further comparative analyses (Supplementary table 1).

In cohort A, LIMMA analysis identified a total of 179 probe sets that were decreased in responders compared with non-responders (Table 2 and Supplementary table 2). Since age in cohort A was significantly different between responders and non-responders, LIMMA analysis with age as confounder was performed. No probe sets were differentially expressed between responders and non-responders, when adjusted for age (lowest FDR 0.06). However, the top-ranked probe sets were similar as compared to the LIMMA analysis without age as confounding factor. A higher FDR can be explained by a reduction in power due to the decreased number of degrees of freedom by inclusion of age in the model. Moreover, no evidence was found for age-related differential gene expression (results not shown).

In cohort B, a total of 361 probe sets were differentially expressed in responders compared to non-responders, with 38 probe sets showing an increased signal and 323 probe sets a decreased signal in responders compared to non-responders (Table 2 and Supplementary table 2). For the two cohorts combined (cohort A and cohort B), a total of 212 differentially expressed probe sets were identified by LIMMA analysis, with 5 probe sets showing an

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increased signal and 207 probe sets a decreased signal in responders compared to non-responders (Table 2 and Supplementary table 2).

Table 2 Summary of the results from LIMMA analyses between the pre-treatment expression profiles of responders and non-responders in cohort A, cohort B and both cohorts combined

Comparative analysis	UC cohort A R(n=8)/NR(n=16)	UC cohort B R(n=12)/NR(n=10)	UC cohort A and B R(n=20)/NR(n=26)
Increased probe sets in R	0	38	5
Decreased probe sets in R	179	323	207
Total	179	361	212

NR, non-responders; R, responders

There was an overlap of 74 differentially expressed probe sets, representing 53 different known genes, between the LIMMA analyses in cohort A and B, and these common probe sets were all downregulated in responders compared to non-responders (Table 3 and Figure 1).

Table 3 Fold change of the 74 common differentially expressed probe sets between LIMMA analysis in cohort A and LIMMA analysis in cohort B

Probe Set ID*	Gene Symbol	Cohort A FC (R/NR)	Cohort B FC (R/NR)	Probe Set ID	Gene Symbol	Cohort A FC (R/NR)	Cohort B FC (R/NR)
202422_s_at	*ACSL4*	0.30	0.33	229947_at	*PI15*	0.14	0.09
226517_at	*BCAT1*	0.39	0.47	220014_at	*PRR16*	0.39	0.42
204103_at	*CCL4*	0.38	0.39	1554997_a_at	*PTGS2*	0.21	0.12
219947_at	*CLEC4A*	0.49	0.44	204748_at	*PTGS2*	0.22	0.13
231766_s_at	*COL12A1*	0.28	0.39	223809_at	*RGS18*	0.46	0.41
205159_at	*CSF2RB*	0.42	0.42	209071_s_at	*RGS5*	0.46	0.49
214974_x_at	*CXCL5*	0.23	0.10	203535_at	*S100A9*	0.39	0.20
206336_at	*CXCL6*	0.22	0.25	220330_s_at	*SAMSN1*	0.35	0.43
207610_s_at	*EMR2*	0.40	0.37	1555638_a_at	*SAMSN1*	0.38	0.39
217967_s_at	*FAM129A*	0.36	0.38	206211_at	*SELE*	0.27	0.17
217966_s_at	*FAM129A*	0.47	0.44	202627_s_at	*SERPINE1*	0.40	0.38
1554899_s_at	*FCER1G*	0.42	0.39	202498_s_at	*SLC2A3*	0.41	0.31
203561_at	*FCGR2A*	0.39	0.33	218404_at	*SNX10*	0.49	0.48
1554741_s_at	*FGF7*	0.33	0.38	227697_at	*SOCS3*	0.41	0.29
229435_at	*GLIS3*	0.41	0.43	201858_s_at	*SRGN*	0.40	0.36
211959_at	*IGFBP5*	0.28	0.43	204597_x_at	*STC1*	0.26	0.24
203424_s_at	*IGFBP5*	0.45	0.34	230746_s_at	*STC1*	0.19	0.21
206420_at	*IGSF6*	0.48	0.35	204595_s_at	*STC1*	0.35	0.28
206924_at	*IL11*	0.13	0.17	229723_at	*TAGAP*	0.30	0.24
206172_at	*IL13RA2*	0.22	0.20	210664_s_at	*TFPI*	0.50	0.45
205207_at	*IL6*	0.22	0.17	209278_s_at	*TFPI2*	0.16	0.15
205798_at	*IL7R*	0.45	0.49	210176_at	*TLR1*	0.41	0.32
210511_s_at	*INHBA*	0.23	0.22	201645_at	*TNC*	0.22	0.32
226001_at	*KLHL5*	0.39	0.49	204933_s_at	*TNFRSF11B*	0.32	0.23
205269_at	*LCP2*	0.40	0.42	204932_at	*TNFRSF11B*	0.28	0.24
229937_x_at	*LILRB1*	0.42	0.44	231227_at	*WNT5A*	0.25	0.39
210146_x_at	*LILRB2*	0.33	0.26	205990_s_at	*WNT5A*	0.28	0.32
206953_s_at	*LPHN2*	0.42	0.45	213425_at	*WNT5A*	0.31	0.32
206584_at	*LY96*	0.38	0.46	232297_at	N/A	0.44	0.49
220122_at	*MCTP1*	0.44	0.47	227140_at	N/A	0.16	0.17
203434_s_at	*MME*	0.33	0.39	209960_at	N/A	0.42	0.43
205828_at	*MMP3*	0.25	0.30	242388_x_at	N/A	0.42	0.37

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224940_s_at	*PAPPA*	0.39	0.39	229802_at	N/A	0.33	0.28
228128_x_at	*PAPPA*	0.35	0.38	226218_at	N/A	0.41	0.46
224941_at	*PAPPA*	0.39	0.43	226847_at	N/A	0.36	0.37
203708_at	*PDE4B*	0.41	0.31	226237_at	N/A	0.36	0.32
211302_s_at	*PDE4B*	0.44	0.29	209683_at	N/A	0.44	0.47

FC, fold change; N/A, not available; NR, non-responders; R, responders
*Full annotation for each probe set is given in Supplementary table 1

Ten overlapping genes came up in the top 20 differentially expressed known genes for cohort A and 3 overlapping genes belonged to the top 20 genes of cohort B (Figure 1). Figure 2 shows the top 10 most significant biological functions that were associated with the list of the common probe sets.
To identify which biological processes and/or diseases are associated with (non-)response to infliximab treatment, a Bio Functional Analysis was performed on the differentially expressed probe sets from each LIMMA analysis (Supplementary table 3). The Bio Functional analyses showed a common predominance of the biological functions: immune response, cellular movement, cellular growth and proliferation, haematological system development and function, cell-to-cell signalling and interaction, cell death and tissue morphology/development.

Class prediction analysis of responders and non-responders
PAM analysis was carried out on the top 20 and top 5 differentially expressed known genes that were identified by LIMMA analysis between responders and non-responders in cohort A, cohort B and both cohorts combined (Table 4). The top 20 and top 5 gene classifiers used for the PAM analyses in cohort A, cohort B and both cohorts combined are listed in Supplementary Table 4.
In cohort A, PAM analysis of the top 20 and top 5 genes allowed classification of samples as responder and non-responder with an overall accuracy of 91.7% (22/24) and 83.3% (20/24), respectively. The top 20 and top 5 gene classifiers of cohort A were used to predict the (non-)response of the samples from cohort B. Both cohort A classifiers predicted 3/12 responders (25% sensitivity) and 10/10 non-responders (100% specificity) in cohort B correctly. Hierarchical clustering of the log2 expression values from the top 20 (Figure 3a) and top 5 (Figure 3b) genes in cohort A resulted in 2 major clusters of responders versus non-responders, with two non-responders misclassified in the cluster of responders.
In cohort B, PAM analysis of the top 20 and top 5 genes revealed an overall accuracy of 86.4% (19/22) and 90.9% (20/22), respectively. The top 20 and top 5 gene classifiers of cohort B predicted the samples from cohort A with an overall accuracy of 66.7% and 70.8%, respectively. Cluster analysis of the log2 expression values from the top 20 and top 5 genes in cohort B are shown in figure 3c and figure 3d, respectively.
For the two cohorts combined, PAM analysis of the top 20 and top 5 genes from the LIMMA analysis for both cohorts combined allowed classification of responders and non-responders with an overall accuracy of 84.8% (39/46) and 89.1% (41/46), respectively. Hierarchical cluster analysis of the two cohorts combined, using the 20 and the 5 gene classifier, are shown in figure 3e and figure 3f, respectively. For the two cohorts combined, PAM analysis showed that a predictive signature of 20 gene probes was not better than a panel of 5 gene probes to differentiate responders from non-responders.

Table 4 Summary of the results from PAM analyses in cohort A, cohort B and both cohorts combined

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PAM analysis			
UC cohort A (R=8, NR=16)	**Sensitivity**	**Specificity**	**Overall accuracy**
Top 20 genes	100% (8/8)	87.5% (14/16)	91.7% (22/24)
Top 5 genes	75% (6/8)	87.5% (14/16)	83.3% (20/24)
The top-ranked gene classifiers of cohort A were used to classify samples in cohort B (R=12, NR=10)	**Sensitivity in cohort B**	**Specificity in cohort B**	**Overall accuracy in cohort B**
Cohort A top 20 genes	25% (3/12)	100% (10/10)	59.1% (13/22)
Cohort A top 5 genes	25% (3/12)	100% (10/10)	59.1% (13/22)
UC cohort B (R=12, NR=10)	**Sensitivity**	**Specificity**	**Overall accuracy**
Top 20 genes	91.7% (11/12)	80% (8/10)	86.4% (19/22)
Top 5 genes	91.7% (11/12)	90% (9/10)	90.9% (20/22)
The top-ranked gene classifiers of cohort B were used to classify samples in cohort A (R=8, NR=16)	**Sensitivity in cohort A**	**Specificity in cohort A**	**Overall accuracy in cohort A**
Cohort B top 20 genes	87.5% (7/8)	56.3% (9/16)	66.7% (16/24)
Cohort B top 5 genes	87.5% (7/8)	62.5% (10/16)	70.8% (17/24)
UC cohort A and B (R=20, NR=26)	**Sensitivity**	**Specificity**	**Overall accuracy**
Top 20 genes	95% (19/20)	76.9% (20/26)	84.8% (39/46)
Top 5 genes	95% (19/20)	84.6% (22/26)	89.1% (41/46)

NR, non-responders; R, responders

PAM analysis was also performed on the filtered dataset for every cohort separately and for both cohorts combined. The minimum misclassification error rate for cohort A was obtained by 13 probe sets, for cohort B by 5877 probe sets and for the pooled data by 15 probe sets. The genes referred by these probe sets overlapped with the top 20 and top 5 differentially expressed genes.

Validation of the microarray data by qPCR

To confirm the microarray data, qPCR was performed for the top 5 differentially expressed known genes of the LIMMA analysis of both cohorts combined (*TNFRSF11B* (Figure 4a), *STC1* (Figure 4b), *PTGS2* (Figure 4c), *IL-13Ralpha2* (Figure 4d) and *IL-11* (Figure 4e)). LIMMA analysis in cohort A showed also increased mRNA expression of these genes in untreated UC when compared to controls. qPCR confirmed the differential expression of these genes between the responders, non-responders and controls in cohort A.

DISCUSSION

There is a great need at defining molecular mechanisms that underlie (non-)response to anti-TNF-alpha therapy in UC. In this study we investigated endoscopic mucosal biopsy derived mRNA expression to identify a 'gene expression fingerprint' distinguishing primary non-responders from responders and to identify gene panels predictive of response to infliximab, a chimeric IgG1 monoclonal antibody to TNF-alpha.

Two independent cohorts of patients with refractory UC who received a first treatment with infliximab were studied. We classified patients as responders if they achieved complete healing both endoscopically and histologically. All other patients were considered non-responders although some of them showed clinical improvement or showed mucosal healing at a later time point. We used mucosal healing as an endpoint as it is less open to bias than a symptom score, and there is little chance that complete mucosal healing would occur only by spontaneous disease evolution.

Microarray analysis of pre-treatment mucosal biopsies for both cohorts A and B yielded 179 and 323 probe sets respectively that were downregulated in responders versus non-responders.

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Although the differentially expressed probe sets were not the same for the two cohorts, a comparative analysis of cohort A and B showed an overlap for 74 differentially expressed probe sets, representing 53 different known genes. These common probe sets were all downregulated at baseline in responders compared to non-responders. The top biological functions that were overrepresented within the lists of differentially expressed probe sets were cellular movement, haematological system development and function, immune response and cell death. It is not surprising that there is no perfect overlap for the signatures found in both cohorts. This is likely due to differences in patient populations, different environmental background and concomitant therapies.

For each cohort, the top differentially expressed genes were used for predicting response to infliximab in UC and the overall accuracy with the sensitivity and specificity were calculated. The overall accuracy of the top 20 and top 5 genes for cohort A were 92% and 83%, respectively. For cohort B, overall accuracy was 86% and 91% for the top 20 and top 5 genes, respectively. When using cohort B to validate the predictive signature obtained in cohort A, we found a 100% sensitivity but a low specificity. When using cohort A to validate the predictive signature of cohort B, we found an overall accuracy of around 70%.

We also carried out an analysis of the microarray data from both cohorts combined. For both cohorts combined, the number of gene probes used for prediction was reduced from an initial panel of 20 to 5 gene probes. The defined 5 gene signature panel encoded the following proteins: osteoprotegerin (TNFRSF11B), stanniocalcin-1 (STC1), prostaglandin-endoperoxide synthase 2 (PTGS2), interleukin-13 receptor alpha 2 (IL13Ralpha2) and interleukin-11 (IL11), and predicted the response to infliximab therapy with 89% accuracy. We confirmed the predictive value of each of the top 5 probes derived from the combined analysis by qPCR.

TNFRSF11B is a member of the TNF-receptor superfamily that functions as a soluble decoy receptor. It is binding to RANK ligand, thus preventing the interaction with RANK and thereby inhibiting the differentiation and activation of osteoclasts. Moreover, modulation of RANKL-RANK interaction with exogenous recombinant osteoprotegrin reverses skeletal abnormalities and reduces colitis by decreasing colonic dendritic cell numbers(13). It is also binding to TRAIL, an important regulator of cell survival(14;15).

STC1, a secreted, homodimeric glycoprotein that is expressed in a wide variety of tissues, maintains endothelial permeability in TNF-alpha treated endothelial cells through preservation of tight junction protein expression, suppression of superoxide anion production, and inhibition of the activation of NFkappaB and JNK, suggesting an important role for this protein in regulating endothelial functions(16).

PTGS2, also known as cyclooxygenase 2b, is the key inducible enzyme in biosynthesis of prostaglandins, prostacyclin and thromboxanes. Together with NO synthase, this enzyme is overexpressed in chronic inflammation and its overexpression occurs early in UC-associated neoplasia(17).

The importance of IL-13Ralpha2 in colitis has recently become apparent. Originally, it was believed that IL-13Ralpha2 acts as a decoy receptor(18). A study from Fichtner-Feigl however showed that IL-13 signalling through IL-13Ralpha2 is involved in the induction of TGF-beta1 production and fibrosis(19).

IL-11 has been used to treat inflammatory bowel disease because this cytokine decreases NFkappaB activity, but the studies were not successful. Recent studies have shown that IL-11 induces Th2 polarization of human CD4(+) T cells(20). Moreover, IL-11Ralpha, the main receptor for IL-11, plays a key role in the pathogenesis of IL-13-induced inflammation and remodelling by activation of TGF-beta1(21) and acts in concert with IL-13Ralpha2 in this respect.

We think that the markers identified in this study are specific for anti-TNF therapy and not for resistance therapy overall as all patients had already failed other therapies before being treated

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with infliximab. Moreover, the five genes identified, encode for proteins involved in signalling in the adaptive immune response, inflammation and TNF pathways. Further study of the genes and pathways identified in the present study should allow a better understanding of the molecular mechanisms of infliximab action and mechanisms of resistance to anti-TNF therapy. We plan to develop a predictive diagnostic chip with these 5 genes and to further prospectively validate this gene signature in UC patient candidates for anti-TNF treatment. In this manner optimal use of resources could be achieved and safety improved.
In conclusion we described gene expression profiles in mucosal biopsies from two cohorts of infliximab naive patients prior to infliximab therapy. Our studies demonstrate that a limited number of genes involved in the inflammatory cascade account for resistance of UC to respond to anti-TNF-alpha therapy.

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Conflicts of interest: PR, SV, GVA and KG report the following conflicts of interest: grant support, lecture fees and consulting fees from Centocor and Schering-Plough. GT, KL, CM, KM and FB are employees of Centocor Research and Development, Inc. XS is a former employee of Centocor Research and Development, Inc. and is currently an employee of Ethicon, Inc.

FIGURE LEGENDS

Figure 1. Venn diagram of the differentially expressed probe sets between responders and non-responders before treatment in UC cohort A and UC cohort B. An overlap of 74 differentially expressed probe sets, representing 53 different known genes, was observed between both cohorts. Ten (bold italic) and 3 (underline) overlapping genes were present in the top 20 significantly genes of cohort A and B, respectively. The full annotation for each gene is given in Supplementary table 1.
Figure 2. Bar chart representing the top 10 most significant biological functions that were associated with the list of overlapping probe sets of the LIMMA analysis in cohort A and in cohort B. The functional categories are displayed along the *x*-axis and the *y*-axis indicates the significance score.
Figure 3. Hierarchical cluster analysis of responders (R) and non-responders (NR), based on the log2 expression values of the top 20 and top 5 differentially expressed known genes**, identified by LIMMA analysis in cohort A **(a, b)**, cohort B **(c, d)**, the two cohorts combined **(e, f)**. Individual samples are shown in columns and genes in rows. The log2 expression values for individual genes are indicated by colour, as shown in the scale (colour key), with yellow indicating a high level of expression and blue a low level of expression. The responders (R) and non-responders (NR) are coloured red and blue, respectively. **For probe sets representing the same gene, the most significant probe set representing that gene was taken for the analysis.
Figure 4. Quantitative RT-PCR analysis of TNFRSF11B (**a**), STC1 (**b**), PTGS2 (**c**), IL-13Ralpha2 (**d**) and IL-11 (**e**) in controls and pre-treatment UC samples from cohort A. The small horizontal lines in the graphs indicate the average of each group. NR, non-responders; R, responders. *p-value < 0.05, **p-value < 0.01.

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SUPPLEMENTARY MATERIAL

Supplementary Appendix 1. A glossary of terms used in methods.

Supplementary Table 1. A list of annotations of the filtered probe sets, obtained by the Affymetrix NetAffx website (http://www.affymetrix.com/analysis/index.affx) or UCSC Genome Browser website (http://genome.ucsc.edu/). A non-specific filtering was applied on the log2 RMA normalized data from the pre-treatment UC samples from both cohorts to remove probe sets with low overall intensity and variability that are unlikely to carry information about the phenotypes under investigation. Only probe sets with an intensity > log2(100) in at least 10% of the samples and an interquartile range (IQR) of log2 intensities across the samples > 0.5 were included, leaving 9183 probe sets for further data analysis.

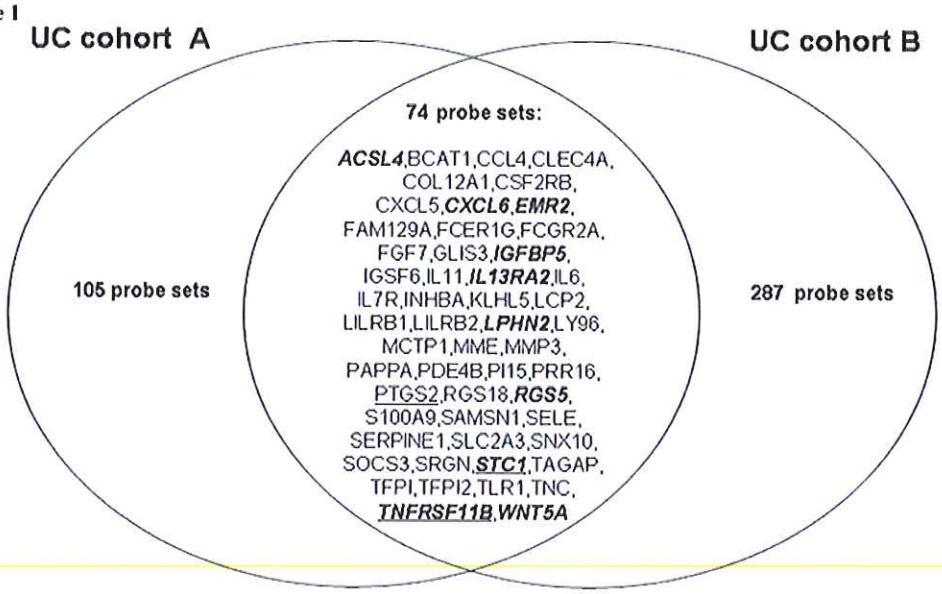
Supplementary Table 2. A list of the differentially expressed probe sets between responders (R) and non-responders (NR) before treatment in cohort A, cohort B and both cohorts combined, identified by LIMMA analysis (FDR < 5% and 2-fold cut off). The probe sets are ranked based on the increasing order of the false discovery rate (FDR). The top differentially expressed genes** used in cohort A, cohort B and both cohorts combined for PAM and hierarchical cluster analyses are coloured red. **For probe sets representing the same gene, the most significant probe set representing that gene was used for the PAM and hierarchical cluster analyses.

Supplementary Table 3. Bio Functional Analyses (B-H P-value < 0.001) of the differentially expressed probe sets between responders and non-responders from the LIMMA analyses in cohort A, cohort B and both cohorts combined. The biological processes and/or diseases are ranked based on the increasing order of the B-H P-value.

Supplementary Table 4. The subset of top 20 and top 5 differentially expressed genes** that were identified by LIMMA analysis in cohort A, cohort B and the two cohorts combined, and used for PAM analysis in cohort A, cohort B and the two cohorts combined. The scores indicate whether the gene is up- or downregulated in the two classes of samples. The genes are ranked based on the decreased predictive value. R: responders, NR: non-responders **For probe sets representing the same gene, the most significant probe set representing that gene was taken for the PAM analysis (see Supplementary table 2).

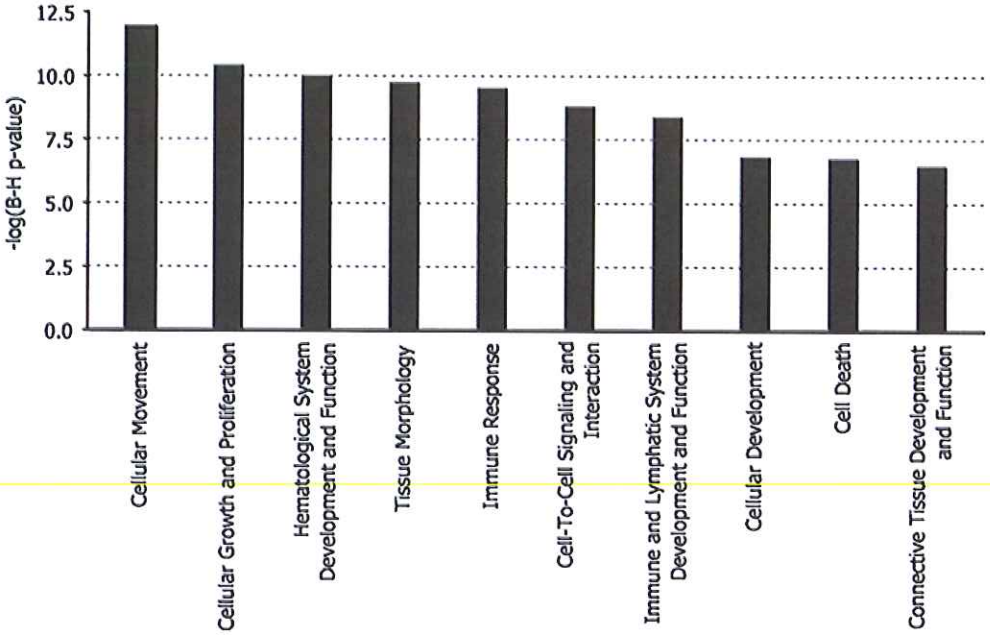
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Figure 1



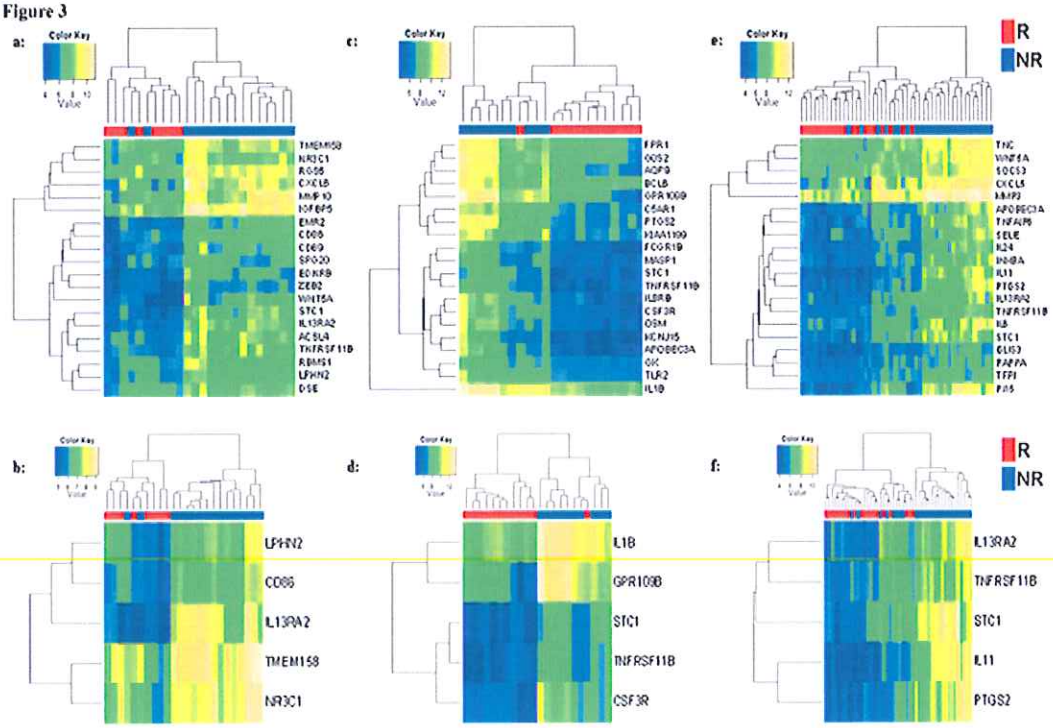
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Figure 2



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Figure 3



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Figure 4

