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Genetic and Transcriptomic Basis of Intestinal Epithelial Barrier Dysfunction in Inflammatory Bowel Disease

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Abstract

Background—Intestinal barrier defects are common in patients with inflammatory bowel disease (IBD). To identify which components could underlie these changes, we performed an in-depth analysis of epithelial barrier genes in IBD.

Methods—A set of 128 intestinal barrier genes was selected. Polygenic risk scores were generated based on selected barrier gene variants that were associated with Crohn's disease (CD) or ulcerative colitis (UC) in our study. Gene expression was analysed using microarray and quantitative reverse transcription PCR. Influence of barrier gene variants on expression was studied by cis-expression quantitative trait loci mapping and comparing patients with low and high risk scores.

Results—Barrier risk scores were significantly higher in IBD patients than controls. At single-gene level, the associated barrier SNPs were most significantly enriched in *PTGER4* for CD, and *HNF4A* for UC. As a group, the regulating proteins were most enriched for CD and UC. Expression analysis showed that many epithelial barrier genes were significantly dysregulated in active CD and UC, with overrepresentation of mucus layer genes. In uninflamed CD ileum and IBD colon, most barrier gene levels restored to normal, except for *MUC1* and *MUC4* that remained persistently increased compared to controls. Expression levels did not depend on cis-regulatory variants, nor combined genetic risk.

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Conclusions—We found genetic and transcriptomic dysregulations of key epithelial barrier genes and components in IBD. Of these, we believe mucus genes, in particular *MUC1* and *MUC4*, play an essential role in the pathogenesis of IBD, and could represent interesting targets for treatment.

Keywords

intestinal barrier; genetic analysis; inflammatory bowel disease; mucosal gene expression

Introduction

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are a group of chronic, relapsing inflammatory disorders of the gut that affect an increasing number of individuals around the world (1). The current hypothesis on the pathogenesis of IBD is that the disease results from complex interactions between the host genome, exposome, gut microbiome and mucosal immune system (2, 3). In this regard, also a dysfunctional intestinal barrier has long been recognized as a key pathogenic factor in IBD (4). The intestinal barrier is located at the interface between the external luminal environment and the internal immune system, and has the complex task to defend against potentially harmful molecules and microorganisms, while being permeable to essential nutrients and solutes (5). It is thought that intestinal barrier defects in IBD patients result in an increased uptake of luminal antigens across the intestinal epithelium, which in turn would trigger the immune system and the development of mucosal inflammation. However, whether mucosal barrier alterations represent a primary dysfunction in the aetiology of IBD, or develop as consequence of ongoing inflammatory processes in IBD patients, is not entirely clear (6). Observations of increased intestinal permeability in a proportion of healthy first-degree relatives of IBD patients suggest that intestinal barrier dysfunction might be genetically determined, and not only due to the impact of inflammatory mediators (7–15). Genome-wide association studies (GWAS) have also implicated the intestinal epithelial barrier as one of the key pathways in the pathogenesis of IBD (16–18).

The general structure of the intestinal barrier is based on several components contributing to its function as a physical barrier between the luminal and internal environment, together with elements from the mucosal immune system that create an immunological defence barrier (6, 19). The mucus layer provides the most apical line of defence against the luminal environment, and forms a sieve-like gel structure that prevents large particles and bacteria from contacting the underlying intestinal epithelium (20). Besides the predominant enterocytes, the epithelium is composed of other specialised cell types with a wide array of functions, including goblet cells that produce the gel-like mucus; paneth cells that secrete antimicrobial peptides reinforcing the immune barrier; and microfold cells that support transport of large luminal antigens and microbiota to immune cells in the lamina propria (5, 21). The intestinal epithelial cells themselves constitute by far the strongest determinants of the physical intestinal barrier through the establishment of an almost impermeable polarised monolayer along the gut wall in the absence of specific transporters. The intercellular space is furthermore sealed by junctional protein complexes, of which the tight junctions are located at the most apical pole of the epithelial cells. Tight junctions are the main

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gatekeepers of the paracellular space and can mediate permeability of ions and small molecules up to 20 kDa. Adherens junctions and desmosomes, in contrast, form strong adhesive bonds and are primarily responsible of maintaining tissue cohesion and integrity (22, 23). Both tight junctions and adherens junctions are dependent on scaffolding proteins for their formation, and may interact with the cytoskeleton and a broad range of signalling molecules for their regulation (24). At the basal side of the epithelium, hemidesmosomes take care of the firm attachment of the cells to the basement membrane and the extracellular matrix, which in turn also control intestinal functions (25). Given the complex organisation and regulation of the intestinal mucosal barrier, there is a need to identify which elements are most critical for the pathophysiology of IBD.

In the present study, we performed an in-depth characterisation of intestinal epithelial barrier genes in IBD patients, and combined genetic and transcriptomic approaches to get a better view on disease-relevant genes and components of the intestinal epithelial barrier. We first evaluated genetic risk scores based on variants in barrier genes, and searched for genes and barrier components that were most enriched at genetic level. Second, we investigated expression levels of barrier genes using intestinal mucosal tissue from IBD patients. Finally, we also analysed whether the barrier gene variants regulated the mucosal gene expression levels in our study cohort.

Materials and Methods

Ethical statement

Subjects were recruited at the outpatient IBD clinic of the University Hospitals Leuven, Belgium. The study was approved by the ethics committee of the UZ/KU Leuven (S53684/B322201213950), with written informed consent from all individuals prior to sample collection.

Selection of intestinal barrier genes

A literature search was performed in PubMed to select genes involved in intestinal epithelial barrier function. Different combinations of the following search terms were used: “inflammatory bowel disease”, “Crohn’s disease”, “ulcerative colitis”, “intestinal barrier function”, “intestinal integrity”, “intestinal epithelium”, “gut barrier”, “mucosal permeability” and “barrier genes”. Importantly, also genes without previous evidence for their significance in IBD, but essential for the structure of the intestinal barrier were included. For gene selection, we focused on the intestinal epithelium as physical barrier, and excluded genes involved in immunological barrier function. Subdivision of the genes into barrier components/categories was performed at the end of the selection.

Genetic risk study

Genotyping of 1696 CD patients, 884 UC patients and 849 unrelated controls from our center was performed before via Immunochip (Table 1) (17, 18). For this study, we first extracted all SNPs in the selected barrier genes, including markers located within 50 kb up- or downstream of the transcription start/end site of the genes (n=3220). All these SNPs passed quality control according to the criteria as described before (17, 18). Highly

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correlated SNPs (SNPs in high linkage disequilibrium, r^2>0.7) were subsequently excluded, leaving 1317 barrier SNPs for association. Comparative analysis between cases (CD or UC) and controls was performed using logistic regression in PLINK (v1.07). A CD or UC polygenic barrier risk score was defined for each individual by counting the total number of risk alleles for the nominally significant disease-associated SNPs (defined as uncorrected $p<0.05$) in the CD or UC versus controls analyses respectively. Comparison of the combined barrier risk scores between cases (CD or UC) and controls was done using Mann-Whitney U tests. Quartile analysis was done using the Chi-squared test in R 3.2.5.

To evaluate if specific genes or barrier components were enriched in independently associated SNPs, we compared the number of (non-)associated variants in a given gene to the number of (non-)associated variants in the other genes for gene-level enrichment; and the number of (non-)significant genes (significant defined as having at least one associated SNP) in a given barrier component with those in the other components for component-level enrichment. Comparisons were done using the Fisher Exact test in R 3.2.5 for 2x2 contingency tables, taking into account the total number of variants in each gene or barrier component. $P<0.05$ was considered as enriched.

Mucosal gene expression study

Patients and biopsies—Endoscopic mucosal biopsies were obtained from 198 IBD patients and 22 controls for microarray and/or quantitative reverse transcription PCR (qRT-PCR) analysis. The biopsy specimens included colon from 97 UC patients (74 with active disease, 23 with inactive disease), 34 CD patients (eight with active colonic disease, 26 with inactive disease), and 11 controls; and terminal ileum from 67 CD patients (51 with active ileal disease, 16 with inactive disease) and 11 controls (Table1). The uninflamed colon biopsies from CD patients were solely used for qRT-PCR analysis. Baseline characteristics from the individuals are presented in Supplementary Table 1. All biopsies were taken from different patients (no paired samples). Disease activity of the patients was based on endoscopic findings, with active disease defined as Mayo endoscopic subscore 2 for UC, and the presence of ulcers for CD patients. The control group, who underwent endoscopy for polyp screening, had normal mucosa at endoscopic level. The biopsies were immediately snap-frozen in liquid nitrogen upon extraction, and stored at -80°C until RNA isolation.

RNA isolation and microarray analysis—Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). Assessment of RNA integrity and quantity was performed by the 2100 Bioanalyzer (Agilent, Waldbronn, Germany) and the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, US), respectively. The isolated RNA was analysed with Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) (GSE75214), and as previously described by Vanhove *et al.* (26). Comparative analyses between the studied groups were performed using the R/Bioconductor LIMMA (linear models for microarray data) package (27). Differential expression was calculated based on moderated t-statistics with correction for multiple testing according to the Benjamini-Hochberg false discovery rate (FDR) (28). Because the main focus of this study was to evaluate the intestinal epithelial barrier, we filtered the results from the genome-wide comparative analyses for the gene probe sets representing the

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selected barrier genes. Gene probe sets with a >2-fold change (FC) and FDR<0.05, showing multiple testing correction for the entire array, were considered biologically significant. The gene probe set of *IL8* (inflammatory marker) was included to evaluate inflammation. Enrichment of genes in the specified categories of the intestinal barrier was evaluated using the Fisher-Exact test in R 3.2.5. Correlations with *IL8* were studied with the Spearman's Rank Correlation test in IBM SPSS statistics 22. The microarray log2 expression values were used for the correlation analyses, and the colon (n=116) and ileum (n=78) samples were studied separately. P<0.05 was considered significant.

In order to evaluate the relation between the barrier gene expression levels and genetic barrier risk, pairwise comparisons in LIMMA (as above) were performed for patients with low and high genetic barrier risk. Low and high risk was defined as a genetic barrier risk score below the 25th percentile value (quartile (Q) 1 = Q1), or above the 75th percentile value (Q4) respectively (Table 1).

Quantitative reverse transcription PCR—Based on the significance levels in the comparisons and/or their relevance for both tissue types (colon and ileum), the following genes were selected for validation by qRT-PCR: *MUC1*, *MUC4*, *TFF1*, *CLDN1*, *CLDN8*, *OCLN*, *DSG3* and *MAGI1*. Beta-actin was used as endogenous reference gene. The primer and probe sequences (Sigma-Aldrich, Diegem, Belgium) for the genes were custom-designed using OligoAnalyzer 3.1 software (see Supplementary Table 2). The RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) was used to synthesize cDNA from 0.5 µg total RNA, according to the manufacturer's protocol. Five samples were excluded, because insufficient cDNA was available. The qRT-PCR experiments were performed in duplicate using the SensiFast Probe No-ROX Kit (GC Biotech, Alphen aan den Rijn, The Netherlands) in a final reaction volume of 20 µl, on a Rotor-Gene 3000 instrument (Corbett Research, Mortlake, Australia). Cycle threshold values for each gene were determined by the Rotor-Gene 6 software package. The relative mRNA expression values of the barrier genes were calculated as ratio relative to the endogenous reference gene beta-actin (Pfaffl method) (29). Statistical analysis of the results was performed using two-tailed Mann-Whitney U tests for unpaired samples (IBM SPSS statistics 22), and a significance level of 0.05 was used.

Expression quantitative trait loci (eQTL) mapping

The genotype profiles and gene expression data were combined by performing cis-eQTL mapping on the available set of samples in our cohort with both genetic marker and microarray expression information: inflamed (n=56) and normal (n=20) colon from UC patients, inflamed colon (n=3) from CD patients, and inflamed (n=34) and normal (n=12) ileum from CD patients (Table 1). The maximal distance between each gene and SNP was limited to one mega-base (Mb). Only SNPs with a minor allele frequency (MAF)>0.05, and low linkage disequilibrium (r^2<0.1) were selected for analysis (n=17.108, of which 2329 in cis of the barrier genes). Direct pairwise regressions were performed using the Matrix eQTL package in R 3.2.5 (30). Each location (colon, ileum) and disease activity status (active, inactive) was analysed separately, because the microarray results pointed towards distinct profiles for these groups. Within each subgroup, we again filtered for MAF<0.05 during the

eQTL analysis to avoid false positive results. Correction for multiple testing was performed using the Benjamini-Hochberg procedure implemented in Matrix eQTL.

Results

Epithelial barrier gene selection and classification

We selected 128 genes related to physical intestinal barrier function. Of these, 25 were classified as part of the mucus layer, 34 as tight junctions, five as adherens junctions, 14 as desmosomes, four as hemidesmosomes, 17 as cytoskeleton, nine as extracellular matrix, and 20 as regulating proteins (see Supplementary Table 3).

Genetics of epithelial barrier genes

Eighty-two SNPs were nominally significant for association with CD, and 69 SNPs with UC (see Supplementary Table 4). None of these remained significant after correction for multiple testing ($<3.8 \times 10^{-5}$ for 1317 SNPs). When considering the total number of risk alleles for the nominally significant SNPs per individual, CD patients had significantly higher CD barrier risk scores compared to controls (median 82 [interquartile range (IQR): 77-87] versus 78 [IQR: 73-83], $p<2.2 \times 10^{-16}$) (see Supplementary Figure 1A for the distribution of the CD barrier risk scores). The median number of UC barrier risk alleles also was significantly higher in UC patients than in controls (68 [IQR: 64-73] versus 64 [IQR: 60-69], $p<2.2 \times 10^{-16}$) (see Supplementary Figure 1B for the distribution of the UC barrier risk scores). Quartile analysis of the barrier risk scores showed that a higher proportion of CD patients had CD barrier risk scores in Q4 versus controls (32.8% versus 17.1%), with proportionally less patients in Q1 versus controls (16.5% versus 32.4%) ($p<2.2 \times 10^{-16}$) (Figure 1A). Similar findings were seen for the UC barrier risk scores: more UC patients in Q4 (40.2% versus 19.8%), while less patients were found in Q1 compared to controls (15.4% versus 31.3%) ($p<2.2 \times 10^{-16}$) (Figure 1B).

In addition to the combined risk of the genetic barrier variants, we evaluated if the nominally associated SNPs were overrepresented in specific genes or components of the intestinal barrier. Comparison of the numbers of associated variants in the selected barrier genes showed enrichment in *MUC19*, *MUC22* and *TFF1* [mucus layer], and *PTGER4* [regulating proteins] for CD ($p=4.30 \times 10^{-2}$, 9.41×10^{-3}, 1.12×10^{-2} and 8.94×10^{-4}, respectively), whereas for UC most enrichment was seen in *MUC21* and *MUC22* [mucus layer], and *GNA12* and *HNF4A* [regulating proteins] ($p=4.87 \times 10^{-2}$, 2.47×10^{-2}, 7.85×10^{-3} and 5.67×10^{-3}, respectively) (see Supplementary Table 5). The barrier component with most genes associated with CD and UC was the group of regulating proteins, although enrichment of this component was only significant for UC ($p=2.18 \times 10^{-3}$) (see Supplementary Table 6 and Figure 2).

Mucosal barrier gene expression

Microarray analysis—Of the 128 selected genes, 125 were represented on the Human Gene 1.0 ST arrays by 132 different gene probe sets. To correlate the barrier gene mRNA expression levels with inflammation, we included the expression profile of *IL8*, represented by one extra gene probe set. In agreement with endoscopic disease activity, *IL8* expression

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was significantly higher in active IBD (UC and/or CD) compared to controls, whereas no differences were detected for *IL8* in uninflamed biopsies of IBD patients versus controls. Results of all comparisons are given in Supplementary Table 7. A heat map of the colonic and ileal expression values per gene probe set and individual is provided as Supplementary Figure 2 and 3.

Colonic expression of the epithelial barrier genes did not differ between UC and CD patients with active disease. As compared to controls, however, the expression of many barrier genes was dysregulated in the colon of active IBD (UC and/or CD) patients. The mRNA expression levels of *MUC1*, *MUC5B*, *EMCN*, *MCAM* and *TFF1* [mucus layer], *CLDN1* and *JAM2* [tight junctions], *DSG3* [desmosomes], *LAMA4* and *LAMC1* [extracellular matrix], and *TCF4* and *F2RL2* [regulating proteins] were >2-fold significantly upregulated in the inflamed colon of IBD patients, while the mRNA expression levels of *RETNLB* [mucus layer], *CLDN8* and *OCLN* [tight junctions], and *MAGI1* and *MEP1A* [regulating proteins] were >2-fold significantly downregulated in active IBD patients when compared to the colon of controls (Table 2). Of the different barrier components, the mucus layer was most enriched in differentially expressed genes ($p=4.97 \times 10^{-2}$) (see Supplementary Table 8). None of the barrier genes remained significantly dysregulated in the colon of UC patients with inactive disease as compared to their expression levels in controls (Table 2). All colonic dysregulated genes showed a highly significant correlation with *IL8*, confirming the direct impact of inflammation on epithelial barrier gene expression (see Supplementary Table 9 and Supplementary Figure 4 for the highest correlated ones).

In addition to the colonic mRNA expression levels, differences in barrier gene expression in the terminal ileum of CD patients with active and inactive disease, and controls were evaluated. Eight genes (*MUC1*, *MUC4*, *MUC5B*, *MUC6* and *TFF1* [mucus layer], *CLDN1* and *CLDN18* [tight junctions] and *F2RL2* [regulating proteins]) showed a >2-fold significantly increased expression in the inflamed ileal mucosa of CD patients compared to uninflamed tissue of controls, while the expression of *CLDN8* [tight junctions] was significantly downregulated (Table 3). The barrier component most enriched in genes with differential expression in the ileum of CD patients with active disease versus controls also was the mucus layer ($p=8.54 \times 10^{-3}$) (see Supplementary Table 8). Interestingly, the ileal expression of *MUC1*, *MUC4* [mucus layer] and *CLDN8* [tight junctions] remained dysregulated in the ileum of CD patients with inactive disease in comparison to controls. The mRNA expression of *MUC1* and *MUC4* was >2-fold significantly upregulated in inactive CD patients, while *CLDN8* was >2-fold significantly downregulated in patients compared to controls (Table 3). Again, significant correlations were found between the ileal mRNA levels of the dysregulated genes and *IL8* (see Supplementary Table 9 and Supplementary Figure 4 for the highest correlated ones).

Validation by qRT-PCR—The differential barrier gene expression levels of *MUC1*, *MUC4* and *TFF1* [mucus layer], *CLDN1, CLDN8* and *OCLN* [tight junctions], *DSG3* [desmosomes], and *MAGI1* [regulating proteins] from the microarray analysis were confirmed by qRT-PCR (Figure 3).

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As compared to the normal colon of controls, we found that the mRNA levels of *MUC1*, *TFF1*, *CLDN1* and *DSG3* were significantly upregulated in the inflamed colon of UC and/or CD patients, while the colonic expression levels of *CLDN8*, *OCLN* and *MAGI1* were significantly decreased in active IBD patients compared to controls. The more sensitive qRT-PCR results also showed increased mRNA expression levels of *MUC4* for these comparisons, while *OCLN* and *MAGI1* levels were significantly decreased in active CD patients. In addition, while no significant alterations were previously found for the colonic expression of the barrier genes between UC and CD patients with active disease, qRT-PCR analysis did identify significantly different levels of *CLDN8*, *DSG3*, *TFF1* and *MAGI1* in the colon of active UC patients when compared to active CD patients. Finally, qRT-PCR showed significantly increased expression levels of *MUC1*, *MUC4* and *DSG3* in the colon of UC patients with inactive disease versus controls. Evaluation of the genes in an additional cohort of 26 inactive CD patients demonstrated that *MUC1* and *MUC4* also were significantly upregulated in uninflamed colon samples from CD patients compared to healthy controls (p=0.043 and 0.009 respectively) (Figure 3).

In the ileum, we confirmed the differential expression of *MUC1*, *MUC4*, *TFF1*, *CLDN1* and *CLDN8* in active CD patients when compared to controls. The ileal expression of *MUC1*, *MUC4* and *CLDN8* also remained dysregulated in CD patients with inactive disease as seen in the microarray analysis. Additional differences were observed for *OCLN* and *MAGI1*, having significantly decreased levels in the inflamed ileum of CD patients when compared to the ileum of controls.

Influence of genetics on mucosal barrier gene expression

In order to evaluate if there were any cis-acting genetic variants affecting the barrier gene expression levels, we performed cis-eQTL mapping in each of the patient sample groups (inflamed colon, normal colon, inflamed ileum, normal ileum). No significant cis-eQTL signals were found after correction for multiple testing in any of the groups. We also did not find significant differences in the barrier gene expression levels between CD and UC patients with the lowest (<75 for CD, <62 for UC) and highest genetic barrier risk scores (>86 for CD, >70 for UC).

Discussion

This study represents a comprehensive report in which the different components of the intestinal epithelial barrier were analysed at genetic and transcriptomic level in the context of IBD, taking into account disease type (CD and UC), biopsy location (colon and ileum) and activity status (inflamed and uninflamed).

We found that the total number of risk alleles in epithelial barrier genes was significantly higher in CD and UC patients compared to controls, validating the known impact of the intestinal barrier for the pathogenesis of IBD. Further analysis of barrier gene variants highlighted the potential role of *MUC19*, *MUC22*, *TFF1* and *PTGER4* for CD, and *MUC21*, *MUC22*, *GNA12* and *HNF4A* for UC. At component-level, genes with associated variants were most enriched in the group of regulating proteins for both CD and UC. The mucosal gene expression study showed that the mRNA expression of many epithelial barrier genes

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was dysregulated in the inflamed colon and ileum of IBD patients, with a significant over-representation of mucus layer genes in both. During inactive disease, the expression of *MUC1* and *MUC4* remained commonly disturbed in intestinal samples of CD and UC patients, suggesting that these genes act as crucial players in IBD. In CD ileum, *CLDN8* also remained significantly lower expressed compared to controls as evaluated by both microarray and qRT-PCR. Analysis of the link between the genetic variants in the barrier genes and their expression alterations, however, did not show significant findings, which might indicate that both levels are not necessarily directly related and influenced by many other disease-specific factors. A schematic overview of the most interesting results is shown in Figure 4.

GWAS have previously identified multiple individual SNPs that are associated with the risk to IBD. Although the functional relevance of many of these SNPs is not known yet, the observed higher genetic barrier risk scores in IBD patients compared to controls suggest that patients also more commonly have a combination of disease-associated variants in intestinal barrier genes which could cause an intensification of the small effects from the individual risk variants. Part of the IBD patients may thus have a distinct genetic predisposition to have intestinal barrier defects, and respond differently – most likely in combination with other predisposing factors - to common environmental stimuli triggering disease onset or relapse. Enrichment analysis with associated barrier SNPs for CD showed that the most significantly enriched gene in this study was *PTGER4*. The *PTGER4* locus has already been identified in several other studies as associated with CD (17, 31). The gene encodes the prostaglandin receptor EP4, of which activation has been suggested to result in redistribution of junctional proteins and the cytoskeleton, with an increase in epithelial barrier disruption (32). The most significant enriched gene for UC was *HNF4A*, a transcription factor known for its essential role in the development and regulation of intestinal epithelial cells, and previously associated in a number of GWAS with UC (17, 33, 34). Ahn *et al.* showed that mice with a conditional knock-out of *Hnf4a* in intestinal epithelial cells had a markedly increased intestinal permeability and susceptibility to acute DSS colitis (35). Amongst the other enriched genes, *MUC19* (secreted gel-forming mucin) and *GNA12* (TJ regulator) are also extensively described based on their association in large GWAS and meta-analyses, whereas reports on genetic evidence for *MUC21*, *MUC22* and *TFF1* in IBD are rather limited (17, 18, 34). *MUC21* encodes a recently identified transmembrane mucin protein, in which one particular SNP has been associated to UC by Achkar *et al.* who looked into the major histocompatibility complex on chromosome 6p (36). In the context of lung diseases, both *MUC21* and *MUC22*, another membrane-bound mucin at epithelial surfaces, have been proposed as candidates for association with asthma, although it could not be excluded that other genes in close proximity including HLA regions are responsible for these signals (37). Changes in the integrity of the bronchial epithelium are thought to play a central role in the sensitisation to allergens and the development of asthma, a chronic inflammatory disease of the airways which represent a similar defence barrier as in the gut (38). The family of trefoil factors, including TFF1, has received considerable attention in a number of animal and intestinal expression studies, but has so far not been associated with the risk to IBD or other immune-related disorders. While its precise physiological function and regulation in the gut

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is not clear, TFF1 is thought to act in mucosal repair and reinforcement of the mucus layer by interaction with mucin molecules (39).

In addition to the enrichment analysis at single gene-level, which searched for multiple risk signals within the same genomic location, a component-level analysis was performed where we evaluated which barrier components had the highest number of genes with at least one associated SNP. We showed that the regulating proteins were most overrepresented, with multiple significant genes for both disease types, although only significant at $p<0.05$ for UC. We could assume that IBD barrier defects partly originate from effects of variants within different regulating barrier genes, together with some strong signals from individual genes of other barrier components like mucus layer factors that showed high enrichment at single-gene level. Of note, the group of regulating proteins involved a broad mixture of scaffolding proteins, transcription factors and previously associated regulatory genes, possibly creating a selection bias towards association. We also are aware that the genetic analysis had limited power to detect genome-wide significant findings. Given our current sample size and a significance level of $3.8x10^{-5}$, we only had 57% and 40% power to identify variants with an effect size of 1.5 and allele frequency of 0.1 for CD and UC respectively. Still, some of the most significant signals that we found, were already described in larger studies, as were the genes enriched in independent significant variants (e.g. *MUC19*, *HNF4A*).

The results from the gene expression study showed that IBD patients with active disease had major gene expression changes at different levels of the intestinal epithelial barrier validating many previous reports. Interestingly, there was a considerable overlap between genes dysregulated in the colon and ileum of both CD and UC patients during active disease (e.g. *MUC1*, *MUC5B*, *TFF1*, *CLDN1*, *CLDN8* and *F2RL2*). This suggests that these barrier molecules are affected in a similar way and represent largely the same barrier defects at both tissue sites under the influence of inflammatory mediators. The most aberrant changes during inflammation were found for *MUC1* in the ileum of CD patients, and *CLDN8* in the colon of UC and CD patients. MUC1 is synthesized by goblet and absorptive cells from the intestinal epithelium, and is expressed as a membrane-bound glycoprotein in the mucus layer (40). Consistent with our results, different studies have previously implicated increased *MUC1* gene and protein expression during inflammation (41–44). It was suggested by Kadayakkara *et al.* that an increase in *MUC1* gene expression may initially serve to protect the gut epithelium by strengthening the function of the mucus layer, while repetitive cycles of inflammation can induce an increased expression of an abnormal hypo-glycosylated protein form of MUC1 which attracts innate inflammatory cells and promotes the development of chronic inflammation and oncogenesis (45). *CLDN8* was the most downregulated gene in active IBD patients, as also frequently described in previous studies (46–49). CLDN8 belongs to the “sealing” proteins of the claudin family which restrict paracellular flux and decrease intestinal permeability, in contrast to pore-forming claudins such as CLDN2 which increase permeability of the intestinal barrier (50). In a study of Zeissig and colleagues, downregulation of CLDN8 was accompanied by CLDN2 upregulation at the tight junctions, intensely enhancing tight junction permeability in active CD patients (47). In our study, *CLDN2* gene expression was increased in the colon of active IBD patients compared to controls, but not more than 2-fold different. When comparing the number of differentially expressed genes for IBD patients with active disease versus

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controls, the mucus layer genes were most enriched. Taken these results together with the findings from the genetic study, we suggest a key role for the mucus layer component in the pathogenesis of IBD. Future studies should look at protein levels of mucus layer genes to dissect their biological working mechanism and functional relevance for IBD.

Remarkably, the common barrier genes that remained dysregulated during inactive disease were *MUC1* and *MUC4* in CD and UC colon (qRT-PCR) and CD ileum (microarray and qRT-PCR). In inactive ileum of CD patients, also *CLDN8* expression remained strongly dysregulated according to both microarray and qRT-PCR analysis. Like MUC1, MUC4 is a membrane-bound mucin protein at the apical side of the intestinal epithelial cells, and forms the glycocalyx which is situated just below the gel-like mucus layer (51). As opposed to studies of barrier gene expression changes during inflammation, less information is available on these barrier gene levels in quiescent disease (52, 53). A recent study of Peloquin *et al.* investigated a selection of 678 genes within previously identified IBD risk loci, and found that uninflamed samples of CD patients exhibited perturbed expression levels of particular genes with increased variances compared to healthy controls. They suggested that these genes are normally held under tight regulatory control, which is lost in the setting of CD (54). It could thus be that *MUC1* and *MUC4* are in a continuously, dysregulated state (primary or due to subclinical molecular inflammation) which can trigger disease onset and relapse in predisposed patients – and worsen with active inflammation. We should then suppose that high *MUC1* and *MUC4* levels have a detrimental effect on the intestinal barrier by expression of an aberrant form as suggested earlier for MUC1, or by causing a general imbalance in mucins which affects the mucus composition and function. An alternative hypothesis on persistent increases in *MUC1* and *MUC4* expression during inactive disease could be that they represent a secondary defence or repair mechanism to protect the gut and account for the damage of previous inflammation. Since *CLDN8* encodes a pore-sealing protein, it is acceptable that its expression has not returned to normal levels in controls when secondary to inflammation, and thus is less dynamic than other barrier genes, again promoting chronic reactivation of the disease. Although not significant in our genetic study, Franke *et al.* showed that the *MUC1* locus was genetically associated with CD, which would be in favour for the hypothesis of a primary role for this gene (55). For *MUC4* and *CLDN8*, no association reports are available in current literature.

In previous studies, our group has evaluated the effect of infliximab therapy on the mucosal expression of several genes involved in IBD (56–58). Albeit the primary goals of these studies were different, the majority of the dysregulated genes during active disease in the current study were also significantly dysregulated in the inflamed mucosa of IBD patients before their first infusion of infliximab. Strikingly, *MUC1* and *MUC4* also remained significantly upregulated in the colon of CD responders after infliximab treatment compared to controls, and the same was found for *MUC1* in the ileum of CD responders versus controls (see Supplementary Methods for a description of this cohort, and Supplementary Table 10 for all comparisons before and after infliximab treatment). Both genes did not show significantly altered levels in UC responders compared to controls, which is similar to our microarray findings in uninflamed colon from UC patients. Analysis with qRT-PCR, in contrast, did show persistent higher levels in the latter samples in our study, which might be

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explained by the higher sensitivity of qRT-PCR as opposed to microarray, or a more pronounced effect in the phenotype of CD versus UC.

When combining the results from the genetic and mucosal gene expression study, there was no direct link between variants in the epithelial barrier genes and differences in their expression. Neither single cis-acting variants in the barrier genes, nor the combined barrier risk scores were associated with the expression of the barrier genes at an FDR level of 5%. These results could imply that other mechanisms are primarily involved for the genetic barrier risk and expression changes seen in IBD patients in this cohort. For the *MUC19* risk locus, for example, it has been suggested that associated SNPs in the gene region probably exert their effect by inducing changes in mRNA conformation, translational efficiency or subcellular localisation rather than gene expression (59). Gene expression alterations of the barrier genes could also be regulated by SNPs further away from the genes, but because of the limited sample size of the overlapping cohort, we only examined eQTLs acting in cis (including a strict window of 1 Mb). Trans-eQTLs (>1 Mb from the barrier gene start/end sites) were not described here as it was shown that small effects of trans-variants are harder to detect and much more sensitive to statistical power (60). Unmistakeably, interesting signals could be missed in that way, indicating the need for larger sample sizes. Some recent studies have investigated genome-wide eQTLs in primary tissue cell types for IBD, and their overlap with the known IBD susceptibility loci (61–65). None of the top signals from these studies correspond with one of our selected barrier genes, confirming that we should search for other regulating mechanisms in these regions.

Taken together, the data in this study allowed us to get a better view on which genes and components from the intestinal epithelial barrier pathway are most critical for IBD, based on their genetic and transcriptomic significance. Identification of the most critical molecules could be necessary to enhance the development of novel barrier-restoring therapeutics. Today, several agents that modify intestinal barrier integrity have been proposed, but their clinical application is still limited - mostly due to shortcomings in the mechanistic and functional understanding of the intestinal barrier. One of the most promising agents for UC currently includes phosphatidylcholine, a major class of phospholipids in the colonic mucus layer. The delayed release of phosphatidylcholine in the gut is thought to reinforce the mucus layer. Our data also support the intestinal mucus layer as a key therapeutic target within the intestinal barrier. The compound has been shown to be an effective and safe therapeutic option for UC patients in phase II clinical trials, but more research is needed to understand its exact working mechanism, and its lack of efficacy for CD (66–68).

In conclusion, we provided an in-depth view on the genetic and transcriptomic basis of intestinal epithelial barrier defects in IBD. By using three different approaches, we identified a selection of barrier genes (e.g. *MUC1*, *MUC4, MUC22*) and components (e.g. mucus layer, regulating proteins) that may be plausible candidates for the onset or perpetuation of chronic gastrointestinal inflammation in IBD (Figure 4). Future studies focussing on the functional working mechanism of these genes and categories are required to uncover their precise role in the disease pathogenesis, and therapeutic potential.

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Supplementary Material

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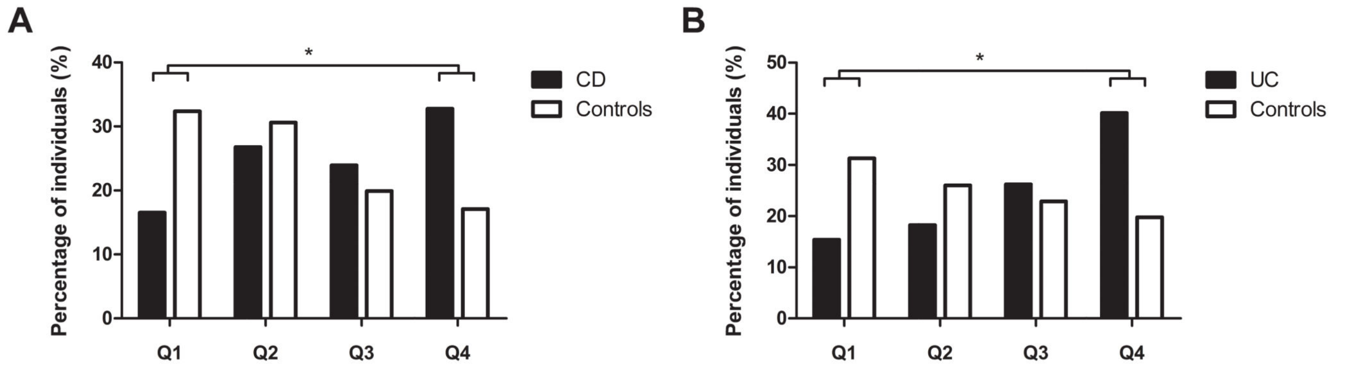

Figure 1. Quartile analysis of the barrier risk scores in patients and controls

The percentage of individuals in the quartiles (Q1-Q4) of the CD barrier risk scores (A) and UC barrier risk scores (B). Comparisons of the number of individuals in Q1 and Q4 was done with Chi-squared testing. *Statistically significant ($p<2.2x10^{-16}$ for A and B). CD, Crohn's disease; UC, ulcerative colitis; Q, quartile.

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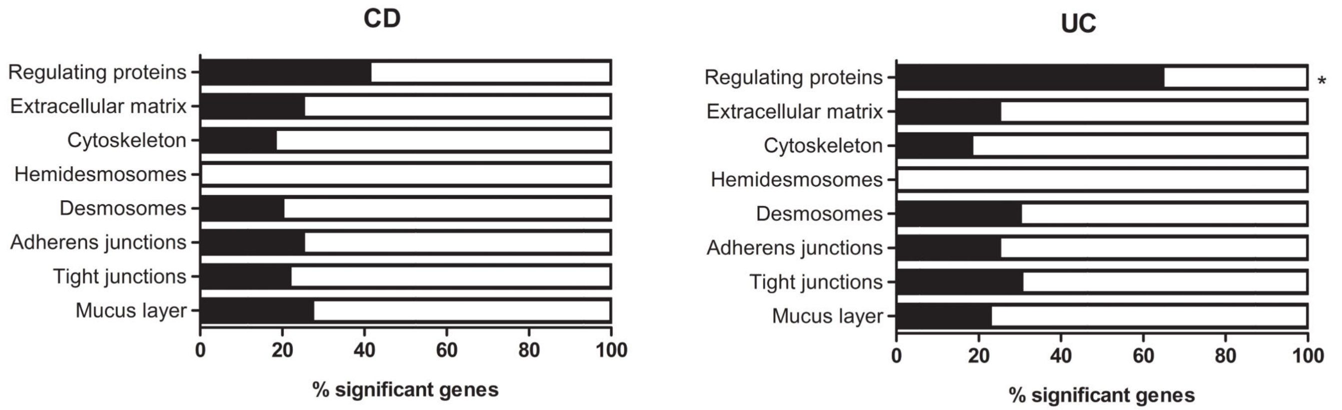

Figure 2. Enrichment analysis of associated barrier genes (1 SNP) per component

Bar plots representing the percentage of significant genes in each of the barrier components for CD (left) and UC (right). Only the set of regulating proteins was significantly enriched for UC in associated barrier genes compared to the other barrier components using Fisher Exact testing (*p<0.05).

CD, Crohn's disease; UC, ulcerative colitis.

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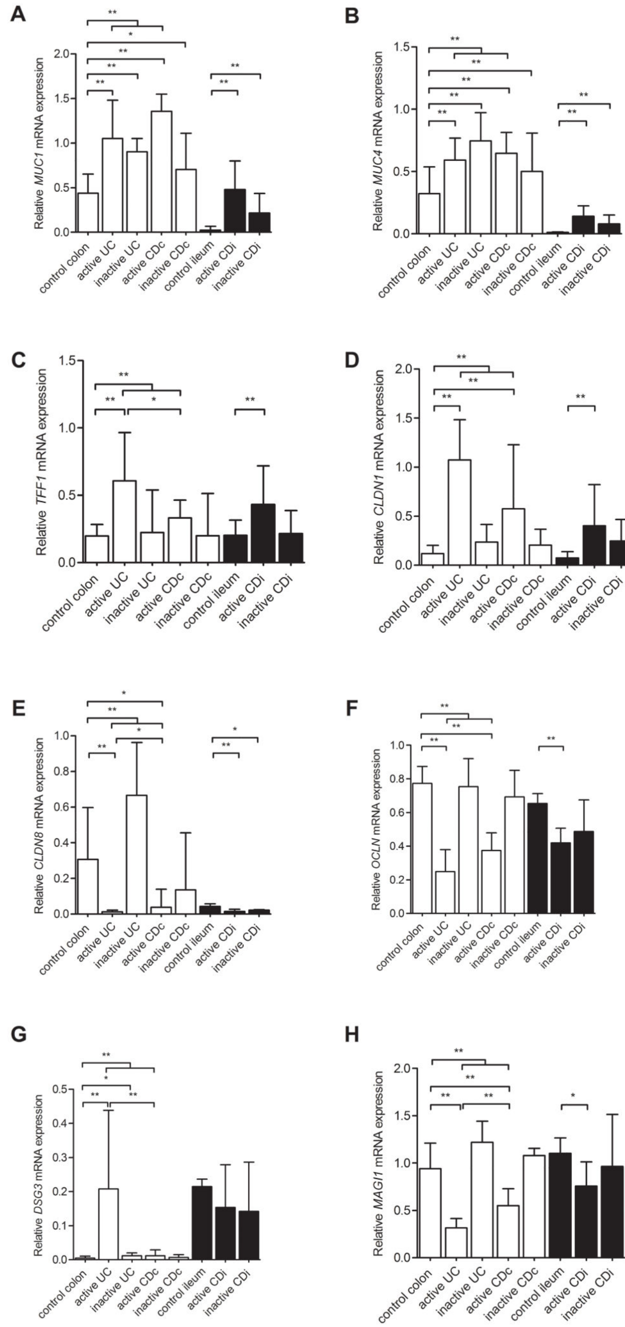

Figure 3. Relative expression levels of eight barrier genes with quantitative reverse transcription PCR

Bar plots representing the relative expression levels of eight barrier genes (A-H) measured by qRT-PCR in colon (white bars) from controls (n=11), active UC (n=72), inactive UC (n=22), active CD patients (n=8) and inactive CD patients (n=26); and ileum (black bars) from controls (n=11), active CD (n=51) and inactive CD patients (n=14). The expression levels are normalised to beta-actin. Data are expressed as medians with interquartile range. Comparisons between the subgroups were performed with Mann-Whitney U testing. Significant differences as described in the main text are indicated (*p<0.05, **p<0.01).

UC, ulcerative colitis; CDc, colon of Crohn's disease patients; CDi, ileum of CD patients.

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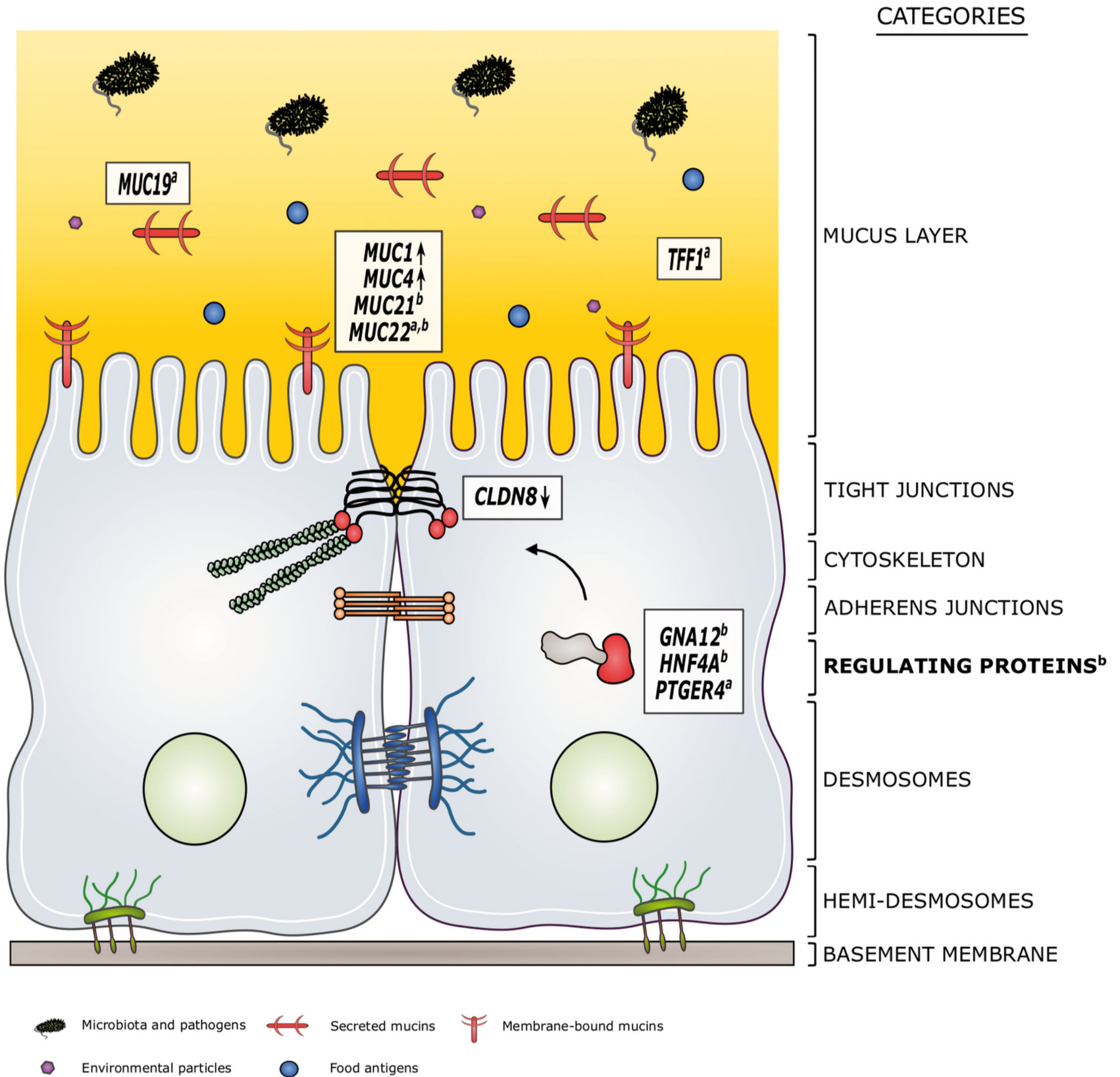

Figure 4. Schematic overview of the main results

The genetic and transcriptomic approaches in this study identified the potential role of particular epithelial barrier genes and components in the context of IBD.

We found that disease-associated variants were significantly enriched in *MUC19* (secreted mucin), *MUC22* (membrane-bound mucin), *TFF1* (stabilizing mucus layer protein) and *PTGER4* (regulating protein) for CD, and *MUC21* (membrane-bound mucin), *MUC22* (membrane-bound mucin), *GNA12* (regulating protein) and *HNF4A* (regulating protein) for UC. The most enriched barrier component was the set of regulating proteins for both CD and UC. At mRNA level, persistent changes in ileal and colonic *MUC1* (membrane-bound

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mucin) and *MUC4* (membrane-bound mucin) expression were found during inactive disease for CD and UC, pinpointing to a possible central role of these genes in IBD onset/relapse. In uninflamed CD ileum, also *CLDN8* expression remained strongly dysregulated.

Genetic predispositions and expression changes may together induce barrier dysfunction of the intestinal epithelium in IBD patients, and result in an enhanced uptake of harmful luminal antigens and initiation of inflammation.

Symbols within the mucus layer are explained below the figure. Structures within the epithelial cells are defined by the category names. Arrows indicate persistent up- or downregulation of the genes during inactive disease. [a]significant enrichment of variants associated with CD; [b]significant enrichment of variants associated with UC.

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Table 1

Overview of the number of samples

	Active UC		Inactive UC		Active CD		Inactive CD		Controls	
	Colon	Ileum	Colon	Ileum	Colon	Ileum	Colon	Ileum	Colon	Ileum
Genetic risk	884				1696				849	
Microarray	74	-	23	-	8	51	-	16	11	11
qRT-PCR	72	-	22	-	8	51	26	14	11	11
eQTL *	56	-	20	-	3	34	-	12	0	0
Q1/Q4-expression[a]*	32	-	12	-	2	15	-	4	0	0

UC, ulcerative colitis; CD, Crohn's disease; qRT-PCR, quantitative reverse transcription PCR; eQTL, expression quantitative trait loci; Q1/Q4-expression, expression analysis of the lowest genetic risk scores (quartile 1, Q1) and highest genetic risk scores (quartile 4, Q4).

*eQTL and risk comparisons were performed for the individuals with both genetic and microarray data.

[a]Number of samples within Q1 and Q4 of the CD or UC genetic risk scores.

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Table 2

Significant barrier genes in the colon of UC and CD patients versus controls

Gene symbol	Gene probeset ID	Colonic expression							
		Active UC vs controls		Inactive UC vs controls		Active CD vs controls		Active IBD vs controls	
		FC	P	FC	P	FC	P	FC	P
MUC1	7920642	**2.30**	9.39E-06	1.65*	4.17E-03	**2.52**	6.05E-05	**2.32**	2.83E-06
MUC5B	7937612	**2.66**	1.41E-05	1.84*	2.62E-02	**2.53**	6.76E-03	**2.65**	1.13E-05
EMCN	8101957	**2.08**	4.83E-04	1.29	2.70E-01	1.69*	2.49E-02	**2.04**	5.27E-04
MCAM	7952205	**2.94**	2.87E-08	1.48*	3.59E-02	**2.66**	2.92E-04	**2.91**	2.64E-08
TFF1	8070579	**3.23**	1.04E-07	1.31	4.54E-01	1.95*	2.20E-02	**3.08**	3.01E-07
RETNLB	8089394	**<u>0.40</u>**	8.10E-03	1.73	6.48E-02	1.19	7.01E-01	**<u>0.45</u>**	2.31E-02
CLDN1	8092726	**4.69**	8.74E-12	1.31	4.29E-01	**2.82**	1.52E-03	**4.46**	5.45E-11
CLDN8	8069795	**<u>0.07</u>**	2.95E-15	1.25	8.17E-01	**<u>0.15</u>**	2.42E-02	**<u>0.07</u>**	1.82E-13
OCLN	8105908	**<u>0.45</u>**	4.67E-04	0.74*	3.73E-03	0.55*	4.48E-05	**<u>0.46</u>**	3.46E-04
JAM2	8068024	**2.10**	5.56E-06	1.78*	3.11E-03	**2.08**	1.70E-03	**2.10**	2.96E-06
DSG3	8020762	**4.74**	1.04E-05	1.09	8.35E-01	1.17	6.48E-01	**4.13**	1.25E-04
LAMA4	8128991	**2.37**	8.08E-07	1.47	1.29E-01	**2.26**	5.18E-03	**2.36**	1.07E-06
LAMC1	7908041	**2.94**	1.12E-09	1.51*	4.17E-02	**2.50**	1.70E-03	**2.89**	1.96E-09
MAGI1	8088602	**<u>0.48</u>**	5.78E-18	0.78*	9.60E-04	0.56*	5.03E-05	**<u>0.49</u>**	2.59E-17
HNF4A	8062823	**<u>0.49</u>**	1.00E-05	0.93	3.69E-01	0.64*	1.46E-03	0.51*	1.43E-05
TCF4	8023415	**3.05**	1.79E-08	1.59	1.13E-01	**2.36**	1.70E-02	**2.98**	3.59E-08
MEP1A	8120088	**<u>0.21</u>**	6.11E-07	0.58*	1.89E-04	**<u>0.37</u>**	1.65E-04	**<u>0.22</u>**	8.80E-07
F2RL2	8112731	**3.18**	9.99E-10	1.31	2.49E-01	1.99*	6.63E-03	**3.04**	7.46E-09

Fold changes and FDR-corrected p for the barrier genes that were significantly upregulated (**bold**) or downregulated (**bold and underlined**) in the colon of IBD patients versus controls. Fold changes indicated with an asterisk represent genes with significant p, but less than 2-fold differential expression.

UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; FC, fold change; vs, versus.

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Table 3

Significant barrier genes in the ileum of CD patients versus controls

Gene symbol	Gene probeset ID	Ileal expression			
		Active CD vs controls		Inactive CD vs controls	
		FC	P	FC	P
MUC1	7920642	**8.47**	8.19E-11	**4.17**	2.42E-03
MUC4	8092978	**4.64**	3.02E-06	**2.81**	2.35E-02
MUC5B	7937612	**2.53**	4.45E-02	1.13	8.52E-01
MUC6	7945595	**3.88**	1.88E-02	1.50	3.12E-01
TFF1	8070579	**2.54**	9.86E-04	1.57	9.68E-02
CLDN1	8092726	**2.95**	8.78E-05	1.68	1.02E-01
CLDN8	8069795	**<u>0.39</u>**	2.84E-06	**<u>0.47</u>**	3.44E-02
CLDN18	8082928	**3.15**	2.27E-03	1.83*	5.68E-03
F2RL2	8112731	**2.06**	6.61E-03	1.23	3.62E-01

Fold changes and FDR-corrected p for the barrier genes that were significantly upregulated (**bold**) or downregulated (**<u>bold and underlined</u>**) in the ileum of CD patients versus controls. Fold changes indicated with an asterisk represent genes with significant p, but less than 2-fold differential expression.

UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; FC, fold change; vs, versus.