TITLE

Expression of immune-related genes in rectum and colon *descendens* of Irritable Bowel Syndrome patients is unrelated to clinical symptoms

RUNNING TITLE

Mucosal gene expression in IBS patients

AUTHORS

Javier Aguilera-Lizarraga¹, Morgane V Florens¹, Thomas Van Brussel², Egbert Clevers^{3, 4}, Lukas Van Oudenhove³, Diether Lambrechts², Mira M. Wouters¹, Guy E. Boeckxstaens¹

AFFILIATIONS

¹KU Leuven Department of Chronic Diseases, Metabolism and Ageing; Translational Research Center for Gastrointestinal Disorders, Laboratory for Intestinal Neuroimmune Interactions, Leuven, Belgium.

²Laboratory for Translational Genetics, Department of Oncology, KU Leuven, Leuven, Belgium; Laboratory for Translational Genetics, Vesalius Research Center, VIB, Leuven, Belgium

³KU Leuven Department of Chronic Diseases, Metabolism and Ageing; Translational Research Center for Gastrointestinal Disorders, Laboratory for Brain-Gut Axis Studies, Leuven, Belgium.

⁴Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.

CORRESPONDING AUTHOR

Prof. Dr. Guy E. Boeckxstaens

KU Leuven Department of Chronic Diseases, Metabolism and Ageing
Herestraat 49, box 701, 3000 Leuven, Belgium

Telephone: +32-16-330237

E-mail address: guy.boeckxstaens@kuleuven.be

ABSTRACT

Background: Mucosal-immune activation has been postulated to play an important role in the pathogenesis of irritable bowel syndrome (IBS). However, data are conflicting and often based on small patient cohorts. Here, we aimed to evaluate the gene expression of a large set of immune-related genes in mucosal biopsies from IBS patients and healthy volunteers (HV).

Methods: A total of 171 IBS patients and 127 HV were included in the study. Rectum biopsies were collected from a cohort of 70 HV and 77 IBS patients (Rome III) and colon *descendens* biopsies from another cohort of 57 HV and 94 IBS patients (Rome II). Gene expression was assessed using OpenArray technology and validated questionnaires were used to evaluate clinical characteristics (GI symptoms, somatization, anxiety and depression).

Key results: A subset of IBS patients (33%) with increased immune activation in the colon *descendens* was identified using multivariate analysis and displayed increased gene expression of *IL1B* (3-fold change), prostaglandin synthase *PTGS2* (2.1-fold change) and the G protein coupled receptor *MRGPRX2* (10.7-fold change). Clinical characteristics in this subgroup were however similar to the rest of the patient cohort. Analysis of rectal biopsies failed to identify such subgroup of “immuno-active” IBS patients in the other patient cohort.

Conclusion: A subset of IBS patients reveals evidence of immune activation in the colon *descendens*, but not in the rectum, however gene expression is unrelated to clinical symptoms. To what extent this subgroup might however respond to anti-inflammatory therapy remains to be investigated.

KEY POINTS

- The role of low-grade inflammation and mucosal immune activation in irritable bowel syndrome (IBS) is controversial and remains poorly understood.
- Multivariate analysis of the mucosal gene expression identified a subset of patients with evidence of increased immune activation in the colon *descendens*, but not in the rectum.
- The clinical characteristics of the immuno-active subgroup did not differ from the rest of the IBS patient cohort. Our results suggest no direct relationship between mucosal low-grade inflammation and symptoms in IBS.

KEYWORDS

IBS, immune activation, gene expression, colon *descendens*, rectum.

ABBREVIATIONS

IBS, irritable bowel syndrome; HV, healthy volunteer; PI-IBS, post-infectious irritable bowel syndrome; IL, interleukin; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; RNA-seq, ribonucleic acid sequencing; SD, standard deviation; PLS-DA, partial least squares – discriminant analysis; GI, gastrointestinal

INTRODUCTION

Irritable bowel syndrome (IBS) is a heterogeneous gastrointestinal (GI) disorder affecting up to 11.2% of the world-wide population¹. The symptom profile may vary among patients but typically includes chronic discomfort and abdominal pain associated with altered bowel habits in the absence of an organic cause. A variety of potential underlying mechanisms have been proposed, such as changes in mucosal intestinal permeability, altered expression and/or release of cytokines, aberrant immune response, food sensitivity, sensitization of sensory nerves and changes in gut microbiota. Numerous studies have reported evidence of microscopic or low-grade inflammation in intestinal biopsies of IBS²⁻⁴ and post-infectious (PI)-IBS patients⁴⁻¹⁰, as evidenced by augmented expression of pro-inflammatory markers or increased number of immune cells. In line, reduced anti-inflammatory IL10+ cells or downregulation of mucosal expression of *IL10* have been reported in patients with IBS^{11,12} and PI-IBS¹³.

However, data supporting the presence of chronic low-grade inflammation in IBS are conflicting. Indeed, some studies fail to detect increased cytokine mRNA expression¹⁴⁻¹⁹ or even report decreased expression of pro-inflammatory cytokines^{16,20,21}. Along the same line, inconsistent data have been reported on the role of mast cells in IBS. Barbara and colleagues showed increased number of mast cells and mediators released in colon *descendens* biopsies of IBS patients compared with healthy volunteers (HV)²². Of interest, a greater number of mast cell were found in close proximity to nerves in IBS patients, correlating with the frequency of abdominal pain and/or discomfort. Increased numbers of mast cells in IBS patients have frequently been reported^{3,23-28}. However, other studies fail to confirm such findings²⁹ or even showed lower numbers^{30,31}. Such discrepancies have been summarized in detail in a recent meta-analysis³², further underscoring the heterogeneity among IBS patients as potential explanation. Moreover, different cell count methodologies, small sample size of the studies and differences in the tissues evaluated (colon *ascendens*, colon *descendens*, rectosigmoid colon or rectum) may also account for such differences³². It should be emphasized though that previous studies evaluating mucosal gene expression in IBS only included a low number of IBS patients

and HV ($n < 50$) and evaluated a limited number of genes ($n = 2-7$)^{7,11,13,14}. Also studies including a greater sets of genes (91 genes by RT-PCR, microarray and RNA-seq analysis¹⁵⁻¹⁷) and using a more sophisticated pathway analysis, only studied a rather low number of patients ($n \leq 20$). Considering the heterogeneity of IBS, this is a major issue most likely contributing to the discrepancies reported so far. Of interest, using a more detailed analysis, a recent study including more than one hundred IBS patients suggested that mucosal immune activation can only be detected in a subset of patients³³. However, this study evaluated only 4 genes in mucosal biopsies and 7 cytokines in serum.

Based on the above, we aimed to compare the mucosal gene expression of an extensive list of immune markers ($n = 36$, including markers for inflammatory mediators, mast cell-related genes, proteases-related genes and opioid-precursors) between a large group of IBS patients and HV ($n = 171$ and $n = 127$, respectively) and studied the relationship between gene expression in biopsies (rectum, colon *descendens*) and clinical symptoms.

MATERIAL AND METHODS

Study population and material collection

Two independent cohorts of IBS patients and HV from Belgium and UK/USA/Canada were included in this study (**Table 1**). All participants were invited to undergo proctoscopy to collect mucosal biopsies. 70 HV, recruited by public advertisement, and 77 patients fulfilling ROME III criteria for IBS were recruited from the outpatient clinic of the Hospital UZ Leuven (Belgium) (participants recruited for several prospective studies: ref. S51973, S55485, S55484, S51440). These participants provided mucosal biopsies from the rectum. In addition, 57 HV and 94 patients meeting ROME II criteria for IBS were recruited in centers of UK/USA/Canada. Mucosal biopsies from the colon *descendens* were collected prospectively from these patients and were kindly provided by GlaxoSmithKline (GSK). HV were included if they had no abdominal symptoms or previous IBS diagnosis (based on physician assessment and negative Rome II or III criteria) and no former history of chronic gastrointestinal diseases or gastrointestinal surgery and were not on gastrointestinal medication. Local ethical

committees approved the study protocol. This study was performed in accordance with the ethical standards established in the Declaration of Helsinki. Ethical committee of GlaxoSmithKline, North Carolina, USA, and the ethical committee of the University Hospitals Leuven approved the protocols. Informed consent was obtained from all participants.

After collection, mucosal biopsies from rectum and colon *descendens* were immediately placed in RNA*later* (Qiagen) and retained overnight at 4°C. Next, RNA*later* was removed and samples were stored at -80°C until RNA extraction. Total RNA was extracted using RNeasy Mini kit (Qiagen) and transcribed into cDNA with using qScript cDNA SuperMix (Quanta).

Gene expression analysis

Mucosal gene expression was evaluated by OpenArray Real-Time (RT)-PCR technology using TaqMan Gene Expression Assays (ThermoFisher, **Supplementary Table 1**). QuantStudio™ 12K Flex Real Time PCR System (Thermo Scientific) was used according to the manufacturer's instructions. $C_t = 39$ was used as cutoff cycle for gene expression (OpenArray manufacturer's specifications). The expression of the following genes was evaluated: genes encoding for pro- or anti-inflammatory markers (*IL1B*, *IL4*, *IL6*, *CXCL8*, *IL10*, *IL12A*, *TNF*, *CCL2*, *TGFB1*, *IL17A*, *IL13*, *IL25*, *IL33*, *TSLP*, *IL17RB*, *ALOX5*, *PTGS2*), mast cell-related genes (*MRGPRX2*, *CMA1*, *TPSAB1*, *CPA3*, *KIT*, *FCER1A*, *FCGR2B*, *FCGR3B*), proteases-related genes (*PRSS3*, *SERPINB2*, *SERPINB5*, *SERPINB12*, *SERPINE1*, *CTSG*, *F2RL1*, *F2RL3*), opioid precursors (*POMC* and *PENK*) and histamine-related gene (*HDC*). *HPRT1* and *GAPDH* gene expressions were measured to be used as housekeeping genes.

For relative gene expression analysis, *HPRT1* was used as reference gene due to its lower variability among our population ($SD_{HPRT1} = 2.13$ vs. $SD_{GAPDH} = 3.08$). Thus, delta C_t (ΔC_t) was calculated between gene of interest and *HPRT1*, followed by delta delta C_t ($\Delta\Delta C_t$) calculation [ΔC_t (IBS) - ΔC_t (HV)]. Fold-change was calculated using $2^{-\Delta\Delta C_t}$ formula, normalized with the average expression of HV (set as 1).

Individual clinical assessment questionnaires

Participants completed questionnaires on demographics, psychological profile and gastrointestinal (GI) symptoms. In patients recruited in Belgium, anxiety and depression were assessed using the HADS anxiety and depression questionnaire³⁴ while the Kroenke Somatization Questionnaire (PHQ-15) was used to assess the severity of different somatic symptoms³⁵. In patients recruited in UK/USA/Canada, the functional bowel disorder severity index (FBDSI) was used to assess the severity of symptoms (only in IBS)³⁶, the Kendler DMS-IV Lifetime Depressive Symptoms questionnaire was used for the assessment of personality disorders³⁷, the Spielberger Trait Anxiety Scale was used to measure trait and state of anxiety³⁸ and the PHQ-15 was used to assess somatic symptoms.

Statistical analysis

Univariate analysis:

All univariate analyses were performed using Graphpad Prism (Prism 7, Graphpad Software, CA, USA). Relative gene expression data were analyzed using two-tailed Mann Whitney test (HV vs. IBS) or Kruskal-Wallis test (HV vs. IBS subgroups). To correct for multiple testing (36 genes), a p value <0.0014 was considered significant (HV vs. IBS). When IBS subgroups were included in the analysis, we corrected for multiple testing considering the number of subgroups: $p < 0.00069$ for 2 subgroups, $p < 0.00046$ for 3 subgroups and $p < 0.00035$ for 4 subgroups. Differences where the p value was >0.0014 (or corrected for multiple IBS subgroups) and <0.05 were described as tendencies. Frequency distributions were analyzed using Fisher's exact or χ^2 tests. Pearson correlation coefficient (r) was used to quantify the direction and magnitude of correlation between variables. Correlations were assessed between the relative gene expression and gastrointestinal (GI) symptom severity, somatization, anxiety and depression. Moreover, the difference able to be detected (effect size) with power = 80% and $\alpha = 0.05$ was quantified post-hoc, prior to any correction for multiple testing. This was calculated for HV and IBS patients (or subgroups) in both rectum and colon *descendens* biopsies. Briefly, we sampled two random lists of expression values 100,000 times and compared these using Mann-

Whitney U tests. We then identified the difference in gene expression at which 80% of p-values were below 0.05.

Multivariate analysis:

Multivariate analyses were performed to evaluate differences in the global immune-profile based on the relative expression of genes included in the study. We performed Partial Least Squares - Discriminant Analysis (PLS-DA) to evaluate whether the relative gene expression can discriminate HV from IBS patients using MetaboAnalyst 4.0³⁹. Gene expression data were power-transformed to optimize the normality of distribution. PLS-DA models are prone to over-fit data, therefore cross-validation is required. Results were validated using Leave-One-Out Cross-Validation (LOOCV), where prediction of observed scores was tested with Mann-Whitney U tests to confirm the accuracy of PLS-DA models. This analysis was carried out using R version 3.5.1.

RESULTS

Characterization of the patient cohorts

A total of 298 individuals were included in the present study, comprising 127 HV and 171 IBS patients from two different cohorts (**Table 1**). In Belgium, mucosal biopsies from the rectum were collected from 70 HV and 77 IBS patients (34 were defined as IBS-D, 11 as IBS-C, 15 as IBS-M and 17 as IBS-U). Moreover, 17 IBS patients reported the onset of their symptoms after a period of infectious gastroenteritis, i.e. PI-IBS (**Table 1**). In a second cohort consisting of IBS patients recruited in the UK, US, and Canada, mucosal biopsies from the colon *descendens* were collected from 57 HV and 94 IBS patients (46 identified as IBS-D, 32 as IBS-C and 16 as IBS-M) (**Table 1**). Symptoms and psychological factors were evaluated using structured questionnaires (**Table 2**).

Colon *descendens* shows increased immune activation in a subgroup of IBS patients unrelated to IBS subtype

In mucosal biopsies collected from the colon *descendens*, the overall relative expression of the genes evaluated was similar between HV and IBS. Of the 36 immune-related genes included, only the pro-inflammatory gene *IL1B* (2.1-fold change) and the serine protease inhibitor *SERPINE1* (1.7-fold change) were significantly upregulated in IBS compared to HV (**Figure 1a** and **Supplementary Table 2**). We quantified, post hoc, that differences of 1.54-fold change were able to be detected (power = 80% and $\alpha = 0.05$) between IBS patients and HV. The expression of the pro-inflammatory gene *CXCL8* and the MAS Related G-protein coupled receptor Family Member X2 gene *MRGPRX2*, known to be expressed in human mast cells and play a role in their degranulation⁴⁰, tended to be significantly increased in IBS patients, but statistical significance did not withstand Bonferroni-correction. As the underlying mechanisms may be different depending on the phenotype, we then compared the mRNA gene expression between IBS subgroups. Analysis of the mRNA gene expression among IBS patients classified by their predominant bowel habit showed a significant increase only in the expression of *IL1B* in each IBS subgroup (**Supplementary Table 3**).

Next, we evaluated whether the global immune-gene expression profile in the colon *descendens* was different between HV and IBS by using multivariate analysis. Partial Least Square – Discriminant Analysis (PLS-DA) is a supervised method used for dimension reduction of large amount of data and identification of features that drive group separation. PLS-DA of the relative expression of the 36 genes included in the study did not discriminate between HV and IBS. However, the distribution was more heterogeneous in IBS compared to that in HV. Of interest, 31 IBS patients scattered outside the 95%-confidence region of the HV population (**Figure 1b**), indicating that the immune-profile of this subgroup of IBS patients differs from that in HV and the rest of the IBS cohort. The Mas-related G-protein coupled receptor member X2 gene (*MRGPRX2*), receptor involved in IgE-independent activation of mast cells⁴⁰, and the pro-inflammatory genes *IL1B*, *CXCL8* and *IL12A* were identified as strongest variables driving the clustering (variable importance in the projection [VIP] scores >1.5) and, therefore, to be important in the defined profile of these patients. Thus, we referred to this subgroup

of patients as immuno-active IBS³³. Leave-one-out cross validation (LOOCV), used to evaluate the correct classification rate of PLS-DA model, confirmed the accuracy of the model ($p = 0.0003$).

We next evaluated if this subgroup of immuno-active IBS patients, was associated with a predominant bowel habit. PLS-DA showed that all IBS subgroups (IBS-D, IBS-C and IBS-M) were scattered proportionally within the different IBS subgroups (data not shown). Of the 31 IBS patients identified as immuno-active IBS, 14 were classified as IBS-D, 10 as IBS-C and 7 as IBS-M ($p = 0.6$, χ^2 test). Then, we used univariate analysis to evaluate whether the gene expression of the immuno-active IBS subgroup was different from the rest of the IBS patients (immuno-normal IBS) and HV. Of note, in addition to *IL1B* (3-fold change), the gene expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*) and *MRGPRX2* was significantly increased in immuno-active IBS patients compared to HV (2.1-fold change and 10.7-fold change, respectively, **figure 1c** and **Supplementary Table 4**). We quantified, post hoc, that differences of 1.84-fold change were able to be detected ($p < 0.05$, power = 80%) between immuno-normal and immuno-active IBS patients. Notably, the G protein coupled receptor *MRGPRX2* is of especial interest in view of its role in mast cell activation⁴⁰. It must be noted though, that the expression of *MRGPRX2* gene was low in terms of proportional expression. This gene was expressed only in 5% of the colon *descendens* biopsies of HV compared to 22% of the biopsies from IBS patients ($p = 0.0002$, Fisher's exact test). Of interest, *MRGPRX2* was more frequently expressed in samples from immuno-active IBS patients (39%) compared to immuno-normal IBS patients (14%) ($p = 0.016$, Fisher's exact test). No statistical difference was found between the proportional expression of *MRGPRX2* in HV and immuno-normal IBS patients ($p = 0.132$, Fisher's exact test). Moreover, the expression of *CXCL8*, *SERPINE1* and proenkephalin (*PENK*) tended to be increased in the immuno-active IBS subgroup compared to HV, however this difference did not hold true after Bonferroni-correction (**Figure 2c**).

No differences in mucosal gene expression in rectal biopsies between HV and IBS patients

Next, we evaluated if the findings obtained in the colon *descendens* can be confirmed in the rectum and a similar subset of immuno-active IBS patients can be identified. To this end, mRNA expression of rectal biopsies collected from another cohort was evaluated using univariate and multivariate analyses. As observed in colon *descendens* biopsies, univariate analysis showed that the overall relative gene expression was similar between HV and IBS (**Figure 2a** and **Supplementary Table 5**). Here, only *IL6* was statistically increased (after Bonferroni-correction) in IBS patients compared to HV. This increase was, however, rather small (1.4-fold change compared to HV). We quantified, post hoc, that differences of 1.46-fold change between IBS and controls were able to be identified (power = 80% and $\alpha = 0.05$). The pro-inflammatory gene *IL1B* and the serine protease inhibitor *SERPINB2* showed a trend towards an increase in IBS patients, although statistical significance was lost after Bonferroni-correction. Furthermore, no statistical differences were found between IBS patients classified by their predominant bowel habits and HV (**Supplementary Table 6**). In patients with PI-IBS (n = 17), only *IL6* was found to be upregulated (2.8-fold change) compared to HV and non-PI-IBS patients (**Figure 2b**). Notably, the gene expression of some pro-inflammatory cytokines (*PTGS2*, *IL1B*, *CXCL8* and *TNF*) tended to be higher in PI-IBS (**Supplementary Table 7**), but significance did not hold after Bonferroni-correction. The absence of statistical differences in these genes may be attributed to a very stringent level of significance used in the analyses.

In this cohort of patients, sex and gender ratio were different between HV and IBS. Thus, we evaluated if this had any influence in our analysis. We analyzed differences in gene expression in rectum biopsies from HV and IBS comparing male vs. female, and young vs. old individuals (individuals below or above the median age of each group, respectively). No differences were found when comparing male vs female or young vs old individuals, neither in HV nor IBS (data not shown). We therefore concluded that differences in age or gender ratio did not have a major impact on the gene expression in patients recruited in Belgium.

After confirming that the general population of IBS patients show a similar immune profile in rectal biopsies compared to HV, we next sought to determine if, similar to the findings in colon

descendens, a subset of patients displaying increased immune activation can be identified in the rectum. Multivariate PLS-DA showed no discrimination between HV and IBS patients. IBS patients scattered within the 95%-confidence region of the HV population (**Figure 2c**), indicating that all IBS patients and HV displayed a similar immune-profile in the rectum. Moreover, similar findings were obtained for the different IBS subtypes: PLS-DA analysis did not discriminate IBS subtypes or PI-IBS from non-PI-IBS and HV (data not shown).

Similar clinical parameters between immuno-normal and immuno-active IBS patients and lack of association with mucosal gene expression

Next, we assessed whether changes in the mucosal immune-profile displayed in immuno-active IBS patients in colon *descendens* biopsies could explain the symptoms characteristics of these patients. Of note, no significant differences were found when comparing GI symptoms severity, somatization, anxiety or depression scores between this subset with increased immune activation and immuno-normal IBS patients (**Supplementary table 8**). To evaluate whether the absence of statistical significance in our study was due to lack of power, we performed a two-tailed sensitivity power analysis comparing differences between two independent means. We determined that we were powered to detect an effect size of 0.62 (power = 80% and $\alpha = 0.05$) between immuno-normal and immuno-active IBS patients. The actual size effect for GI symptoms severity, somatization, anxiety or depression was however 0.11, 0.23, 0.38 and 0.10, respectively (**Supplementary table 8**). To evaluate if the absence of a statistically significant difference in GI symptoms was due to a type II error, we calculated the sample size required to acquire statistical significance (for instance, with effect size = 0.11, considering GI symptom severity as most important symptom amongst those evaluated [power = 80% and $\alpha = 0.05$]). Assuming the same ratio of immuno-active / immuno-normal (1:2) patients, this calculation indicated that the total sample size needed equaled 2942. These results illustrate that changes in clinical features in IBS patients based on the immuno active profile are small and most likely irrelevant. Moreover, it must be noted that the changes in symptoms observed in immuno-active IBS patients are

in the opposite direction of what one would expect, *i.e.* more symptoms in immune-active patients. While these patients showed upregulation in some genes, all clinical characteristics evaluated showed a trend towards a decrease compared to the immuno-normal patients (Supplementary Table 8). Based on these arguments, we believe our results indeed show true null findings.

Finally, to study potential underlying mechanisms leading to the reported symptoms, we evaluated if the relative gene expression was associated with clinical symptoms in immuno-active IBS patients. However, no statistically significant correlations were found (data not shown). These results suggest no direct relationship between mucosal low-grade inflammation and IBS symptoms.

DISCUSSION

The present study demonstrates that the expression profile of 36 genes involved in the immune response in the rectum and colon *descendens* of HV and IBS patients is largely similar. Univariate analysis revealed that IBS patients showed increased gene expression only of the pro-inflammatory markers *IL1B* (2.1-fold change) and the serine protease inhibitor *SERPINE1* (1.7-fold change) in the colon *descendens*, and IL-6 (1.4-fold) in the rectum compared to HV. By means of multivariate analysis (PLS-DA), we next identified a subset of IBS patients (33%), referred to as immuno-active IBS, with evidence of immune-activation in the colon *descendens*, a finding that could not be confirmed in rectal biopsies of another IBS cohort. Moreover, clinical symptoms did not correlate with the altered gene expression profile in immuno-active IBS patients, suggesting no direct relationship between mucosal low-grade inflammation, as defined by increased gene expression, and symptoms in IBS.

Low-grade inflammation is considered one of the underlying pathophysiological mechanisms of IBS, although the data supporting this hypothesis are rather conflicting^{2-4,15-18,20,21}. The sample size and number of cytokines considered in these studies are however limited, most likely explaining this controversy, especially given the heterogeneity of IBS. Here, we studied two large IBS cohorts and analyzed the gene expression of 14 cytokines, in addition to 22 extra genes encoding for immune

markers including opioid precursors, lipoxygenase and cyclooxygenase enzymes, immunoglobulin gamma Fc receptors, proteases, protease-inhibitors, protease-activated receptors and histidine decarboxylase enzyme. Univariate analysis revealed that only the inflammatory markers *IL1B* and *SERPINE1* were upregulated in the colon *descendens* and *IL6* in the rectum of IBS patients. However, the magnitude of such increase was rather small (≤ 2 -fold change). These data suggest that changes in gene expression are subtle and most likely limited to a subpopulation of patients³³.

To evaluate if immune activation may indeed be present in only a subgroup of patients, we used a more sophisticated analysis (*i.e.* multivariate analysis: Partial Least Square – discriminant analysis, PLS-DA). This approach identified a subset of IBS patients (33%), referred to as immuno-active IBS³³, with evidence of increased immune activation in the colon *descendens*. These results are in line with previous studies^{33,41} showing increased immune activation in a subset of Swedish IBS patients based on multivariate analysis of serum levels and mucosal expression of a small set of 5 and 9 cytokines, respectively. To further evaluate the potential role of immune activation in IBS, we compared the clinical characteristics of immuno-active versus immuno-normal IBS patients. In line with two previous Swedish studies^{33,41}, no differences in clinical symptoms could be identified between these two subgroups, suggesting no direct relationship between mucosal low-grade inflammation, assessed by gene expression analysis, and symptoms in IBS. However, previous clinical trials in IBS have reported clinical improvement with the mast cell stabilizer ketotifen⁴² and the histamine 1 receptor antagonist ebastine⁴³ while a subgroup of IBS patients seems to respond to treatment with mesalazine⁴⁴. These data strongly support that immune activation, and most likely mast cell activation, is involved in IBS, or at least in a subgroup.

In the immuno-active IBS subgroup, the genes encoding for the pro-inflammatory cytokine *IL1B* (3 fold), the prostaglandin-endoperoxide synthase 2 (*PTGS2*, 2.1-fold) and the G protein coupled receptor *MRGPRX2* (10.7 fold) were significantly increased. Notably, *MRGPRX2* drives IgE-independent activation of human mast cells mediated by neuropeptides, cysteine proteases, antimicrobial peptides and eosinophilic cationic proteins, and has been proposed to contribute to the pathogenesis of

asthma⁴⁵. *PTGS2*, one of the two cyclooxygenases involved in the conversion of arachidonic acid into prostaglandins, is upregulated upon IgE-activation of mast cells⁴⁶. IL-1 β on the other hand, is also released by mast cells⁴⁷ and, similar to histamine and serotonin^{43,48,49}, elevates basal firing and induces mechanical hypersensitivity of pelvic serosal afferents⁵⁰. Taken together, these data suggest that mast cell activation may drive the immuno-active profile of this subpopulation. It should be emphasized though that gene expression in intestinal biopsies does not allow identification of the activated immune cell population. Only careful assessment of individual cells will be able to detect possible changes in phenotype in the cell population involved. Moreover, accepting that sensitization of visceral afferents contributes to aberrant pain perception in IBS^{23,43}, intermittent and/or subtle changes in mast cell activation may be involved that remain undetected using RT-PCR of intestinal biopsies. Similarly, post-transcriptional modifications (i.e. phosphorylation, acetylation or glycosylation) leading to changes on functional activities of proteins cannot be detected using RT-PCR. These considerations imply that new techniques such as single-cell RNA-sequencing or mass cytometry of individual cells⁵¹ may be more appropriate to actually demonstrate changes in the activation status of immune cells and particularly to identify the cell populations involved in the pathogenesis of IBS.

In contrast to the findings in the colon *descendens*, we failed to find a subset of immuno-active IBS patients in rectal biopsies. This discrepancy may have several explanations. First and foremost, these are two different anatomical regions. Intestinal lymphocytes, macrophages, mast cells and other innate immune cells show a different distribution and functionality along the intestinal tract⁵² and the overall cellularity decreases along the large intestine in both HV and IBS patients⁵³. Of interest, IBS patients show an increased number of macrophages in the colon *descendens* compared to HV, but not in the rectum⁵³, potentially explaining the increased immune activation in the colon *descendens* of a subset of IBS patients, but not in the rectum. In addition, it should be emphasized that the Rome II criteria were used in the UK/US/Canada cohort while patients in Belgium were recruited based on the Rome III criteria. The Rome II criteria define a more severely affected population of IBS patients than

Rome III⁵⁴, which could contribute to a different immune profile of these patients. Finally, the signal of immune activation may be rather low and remain undetectable for the methodology used.

The finding that a subgroup of IBS patients displays a subtle degree of mucosal immune activation is of great interest. To date, we can only speculate on the underlying triggers or mechanisms. Differences in gut microbiota in IBS patients^{55,56} could explain, at least partially, differences in gene expression of immune markers without direct or major associations with clinical parameters. Another potential explanation may be related to the impact of psychological factors in IBS, as these have been linked with increased production of pro-inflammatory cytokines⁵⁷⁻⁶⁰. In our cohorts, in line with previous reports⁶¹⁻⁶³, IBS patients showed increased depression and anxiety scores compared to HV. Of note however, psychological factors did not differ between the immuno-active IBS subgroup and immuno-normal IBS patients.

It should be noted that our study has some limitations. Ideally, biopsies of the rectum and colon *descendens* should have been collected from the same individual to draw solid conclusions with respect to regional differences. Another weakness, as discussed above, is the fact that the selection criteria (Rome II vs Rome III) differed between the 2 cohorts studied. The strengths of our study though are the large sample size, the use of two different cohorts, the selection of a large set of genes potentially involved in the pathogenesis of IBS and the use of a multivariate analysis exploring the activation of physiological pathways rather than single genes.

In conclusion, our study demonstrates that the immune-profile, defined by gene expression, in the rectum and colon *descendens* in IBS patients is largely similar to HV and cannot be used to discriminate patients from healthy controls. Of interest, and in line with previous studies^{33,41}, we identified a subgroup of IBS patients with increased immune activation in the colon *descendens*, especially influenced by *IL1B*, *PTGS2* and *MRGPRX2*. This subgroup of patients showed similar GI symptoms, somatization, anxiety and depression scores than the rest of IBS patients, suggesting no direct relationship between mucosal low-grade inflammation, defined by gene expression, and symptoms in IBS. However, to what extent immuno-active IBS patients might nevertheless benefit

from treatment with anti-inflammatory agents cannot be excluded and requires further investigation. Moreover, high-throughput experimental methods like single-cell RNA-sequencing and high-dimensional mass cytometry are clearly required to further unravel the mechanisms underlying abnormal pain perception in IBS.

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DISCLOSURES:

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION:

JAL, MMW and GEB planned and designed the experiments. JAL performed RNA extraction from mucosal biopsies and performed data analysis. TVB performed the OpenArray RT-PCR experiments

assisted by DL. MVF acquired and analyzed clinical data. EC performed comparative analysis and assisted in data analysis together with LVO. JAL and GEB wrote and revised the manuscript. All authors corrected and approved the final version of the manuscript.

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TABLES

Table 1. Demographic of HV and IBS patients recruited in the study. Age data are represented as median $\pm$ IQR.

		HV	IBS	IBS-D	IBS-C	IBS-M	IBS-U	PI-IBS
Rectum biopsies	Total	70	77	34	11	15	17	17
	Age	44.5 (27-70)	32 (24-66)	30.5 (23-45)	31 (23-43)	29 (26-53)	36 (31-54)	31 (25-36)
	Gender (F/M)	23/27	58/19	27/7	8/3	12/3	11/6	14/3
Colon <i>descendens</i> biopsies	Total	57	94	46	32	16	-	-
	Age	50 (35-57)	53 (39-61)	54 (43-61)	48 (35-67)	57 (37-63)	-	-
	Gender (F/M)	46/11	72/22	34/12	25/7	13/3	-	-

Table 2. Data from questionnaires completed HV and IBS patients. Data presented as mean $\pm$ SD. Bolded and italic data show statistically significance ($p < 0.05$) relative to HV. †Anxiety and depression were assessed using the HADS anxiety and depression questionnaire and Kroenke Somatization Questionnaire (PHQ-15) was used to assess the severity of different somatic symptoms. ‡ Functional bowel disorder severity index (FBDSI) was used to assess the severity of symptoms (only in IBS), the Kendler DMS-IV Lifetime Depressive Symptoms questionnaire was used for the assessment of personality disorders, the Speilberger Trait Anxiety Scale was used to measure trait and state of anxiety and the PHQ-15 was used to assess somatic symptoms.

Cohort	Phenotype	IBS Symptoms severity	Depression score	Anxiety score	PHQ-15
Belgium† (rectum biopsies)	HV	-	1.48 $\pm$ 1.81	2.97 $\pm$ 1.96	3.11 $\pm$ 3.51
	IBS	-	4.41 $\pm$ 4.031	8.66 $\pm$ 4.53	8.94 $\pm$ 3.74
UK/USA/Canada‡ (colon <i>descendens</i> biopsies)	HV	-	4.35 $\pm$ 3.94	23.47 $\pm$ 3.87	8.72 $\pm$ 4.48
	IBS	60.4 $\pm$ 16.84	5.75 $\pm$ 4.00	25.04 $\pm$ 4.57	14.53 $\pm$ 4.88

FIGURE LEGENDS

Figure 1. Mucosal-immune gene expression in colon *descendens* biopsies. (a) Univariate analysis of the relative gene expression of 36 immune-related genes in HV (solid circles) and IBS patients (empty circles). (b) PLS-DA scatter plot showing the global mucosal immune-profile based on the 36 genes analyzed in HV (solid circles) and IBS (empty circles). Grey ellipse shows the 95%-confidence region of HV. (c) Univariate analysis of the relative gene expression of statistically significant genes (without testing for multiple comparison) in HV (solid circles), immuno-normal IBS patients (empty circles) and immuno-active IBS patients (empty squares). In (a) and (c) data are presented as mean with 95%

confidence interval. * $p < 0.0014$ (statistically significant after multiple correction, 36 genes); # $p < 0.05$ and > 0.0014 .

Figure 2. Mucosal-immune gene expression in rectal biopsies. (a) Univariate analysis of the relative gene expression of 36 immune-related genes in HV (solid circles) and IBS patients (empty circles). (b) Relative gene expression of IL6 cytokine in HV (solid circle), non-PI-IBS (empty circle) and PI-IBS (empty triangle). In (a) and (b) data are presented as mean with 95% confidence interval. * $p < 0.0014$ (statistically significant after multiple correction, 36 genes); # $p < 0.05$ and > 0.0014 . (c) PLS-DA scatter plot showing the global mucosal immune-profile based on the 36 genes analyzed in HV (solid circles) and IBS (empty circles). Grey ellipse shows the 95%-confidence region of HV.