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Glutamine Restores Tight Junction Protein Claudin-1 Expression in Colonic Mucosa of Patients With Diarrhea-Predominant Irritable Bowel Syndrome

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Abstract

*Background:*Recent studies showed that patients with diarrhea-predominant irritable bowel syndrome (IBS-D) had an increased intestinal permeability as well as a decreased expression of tight junctions. Glutamine, the major substrate of rapidly dividing cells, is able to modulate intestinal permeability and tight junction expression in other diseases. We aimed to evaluate, ex vivo, glutamine effects on tight junction proteins, claudin-1 and occludin, in the colonic mucosa of patients with IBS-D. *Materials and Methods:* Twelve patients with IBS-D, diagnosed with the Rome III criteria, were included (8 women/4 men, aged 40.7 ± 6.9 years). Colonic biopsy specimens were collected and immediately incubated for 18 hours in culture media with increasing concentrations of glutamine from 0.6–10 mmol/L. Claudin-1 and occludin expression was then measured by immunoblot, and concentrations of cytokines were assessed by multiplex technology. Claudin-1 expression was affected by glutamine ($P < .05$, analysis of variance). In particularly, 10 mmol/L glutamine increased claudin-1 expression compared with 0.6 mmol/L glutamine (0.47 ± 0.04 vs 0.33 ± 0.03, $P < .05$). In contrast, occludin expression was not significantly modified by glutamine. Interestingly, glutamine effect was negatively correlated to claudin-1 (Pearson $r = -0.83$, $P < .001$) or occludin basal expression (Pearson $r = -0.84$, $P < .001$), suggesting that glutamine had more marked effects when tight junction protein expression was altered. Cytokine concentrations in culture media were not modified by glutamine treatment. *Conclusion:* Glutamine increased claudin-1 expression in the colonic mucosa of patients with IBS-D. In addition, glutamine effect seems to be dependent on basal expression of tight junction proteins. (*JPEN J Parenter Enteral Nutr*. XXXX;xx:xx-xx)

Keywords

irritable bowel syndrome; diarrhea; glutamine; tight junctions; claudin; occludin

Clinical Relevancy Statement

Intestinal permeability frequently occurs in patients with diarrhea-predominant irritable bowel syndrome (IBS-D) and has been proposed to be involved in IBS pathophysiology. In our pilot study, glutamine was able to restore tight junction protein, claudin-1, in patients with IBS-D that should be confirmed in further clinical studies.

Introduction

Irritable bowel syndrome (IBS) is a common functional bowel disorder characterized by abdominal pain or discomfort associated with altered bowel habits and/or abdominal bloating and/or defecation disorders.[1,2] The pathophysiology of IBS is multifactorial, and several factors, including gut microbiota, genetic polymorphism, and nutrition and psychosocial factors, have been reported.[3] Recent studies reported an alteration of intestinal permeability[4,5] or of factors regulating intestinal permeability.[6–8] Intestinal permeability was correlated with visceral hypersensitivity in some studies.[5,8] The paracellular permeability of the intestinal barrier is regulated in part by tight junctions (TJs) between epithelial cells. Among the protein complex composing the TJs, 3 proteins have been mainly studied in IBS[6,8]: occludin

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and claudin-1, which are transmembrane proteins, and zonula occludens-1 (ZO-1), a cytosolic protein. We previously reported that occludin and claudin-1 expression were altered in patients with IBS with predominant diarrhea (IBS-D), while ZO-1 remained not significantly affected.[6] However, a reduction of ZO-1 was observed by other research groups.[8,9]

Interestingly, Zhou et al[4] showed that glutamine synthetase expression was lower in the small bowel and colonic mucosa of patients with IBS-D who had an increased intestinal permeability compared with controls. These data suggest that glutamine mucosal content may be decreased in these patients as previously reported in patients with inflammatory bowel diseases.[10] Glutamine, the preferential substrate for rapidly dividing cells, has beneficial effects in intensive care patients by limiting infectious complications and intestinal permeability.[11] In vitro studies revealed that glutamine deprivation was associated with an increase of paracellular permeability in intestinal epithelial cells.[12,13] In this condition, TJ proteins were altered.[14] In contrast, glutamine supplementation restored paracellular permeability[12,13,15] and TJ protein expression and localization[14,15] in these in vitro studies. Glutamine also regulates intestinal permeability in injured conditions both in animals[16] and in humans.[17,18] However, the effects of glutamine on TJ proteins during IBS remain unknown, as recently underlined.[19]

In the present study, we aimed to evaluate, in a pilot ex vivo study, the effects of glutamine on TJ protein expression in the colonic mucosa of patients with IBS-D.

Materials and Methods

Patients

Twelve patients, who met the Rome III criteria for IBS with predominant diarrhea[1] and seen in the Department of Gastroenterology of the Rouen University Hospital, were consecutively included between December 2011 and July 2012. Patients with IBS with the following criteria were excluded: (1) patients with IBS with predominant constipation or alternating constipation and diarrhea, (2) blood dyscrasia, (3) personal history of autoimmune disease, (4) personal history of inflammatory bowel disease, (5) personal history of digestive cancer, or (6) macroscopic (except for diverticulosis, hyperplasic polyp, and angiodysplasia) and histological abnormality of the colonic mucosa. A complete colonoscopy was performed under general anesthesia after large bowel cleansing with 4 L of a macrogol solution. To our knowledge, it remains unknown whether bowel-cleaning preparations can affect intestinal permeability or TJ protein expression. Biopsies were taken in the descending colon in all cases. Two biopsy specimens for each patient were analyzed by a pathologist to exclude any histologic abnormality. The protocol was approved by the local committee for ethics, and a written consent from patients to participate in the study was obtained in all cases.

Table 1. Lactate Dehydrogenase (LDH) Concentrations in Culture Media.[a]

	Glutamine, mmol/L			
Concentration	0.6	2	5	10
LDH, UI/L	100.4 ± 21.2	84.5 ± 14.6	93.0 ± 17.5	99.3 ± 20.8

[a]Data from 12 patients with diarrhea-predominant irritable bowel syndrome are shown as mean ± SEM.

Biopsy Culture

Eight colonic biopsy specimens (6.5 ± 0.2 mg, mean ± SEM) were obtained in each patient and immediately transferred in tubes containing cooled glutamine-free culture medium (Dulbecco's modified Eagle's medium [DMEM]; Eurobio, Les Ulis, France) supplemented with glucose (4.5 g/L), penicillin (0.1 U/L), streptomycin (10^{-4} U/L), 10% of fetal calf serum, and 0.6 mmol/L glutamine. Biopsy specimens were then processed within an hour as previously described.[20] Briefly, each biopsy specimen was bathed in 1 mL glutamine-free DMEM supplemented with glucose (4.5 g/L), penicillin (0.1 U/L), streptomycin (10^{-4} U/L), and 10% of fetal calf serum in a plastic 24-well culture plate. In addition, increasing concentrations of glutamine were used as follows: (1) 0.6 mmol/L, (2) 2 mmol/L, (3) 5 mmol/L, and (4) 10 mmol/L. For each glutamine condition, 2 biopsy specimens were used. Culture plates were incubated at 37°C during 18 hours in a chamber equilibrated with 5% CO_2. Lactate dehydrogenase (LDH) release, a marker of cell viability, was not affected by treatments (Table 1). Culture media and biopsy specimens were then transferred into vials, frozen in liquid nitrogen, and stored at -80°C until analysis.

Immunoblotting

Cultured biopsy specimens were homogenized in ice-cold lysis buffer containing 50 mmol/L Hepes (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L β-glycerophosphate, 100 mmol/L NaF, 2 mmol/L sodium orthovanadate, 0.1% of protease inhibitors, and 0.1% of phosphatase inhibitors. Immunoblots were performed as previously described.[7] Briefly, proteins (30 µg) were separated on 4%-12% Tris-Glycine resolving gels (Invitrogen, Cergy-Pontoise, France) and transferred to a nitrocellulose membrane (GE Healthcare, Orsay, France). After blocking and washes, overnight incubations at 4°C were done with mouse anti–claudin-1, rabbit antioccludin (1:1000; Zymed Laboratories, Fisher Scientific, Illkirch, France), rabbit anti–glutamine synthetase (1:500; Pierce Biotechnology, Rockford, IL), or mouse anti–β-actin (1:1000; Sigma-Aldrich, Saint Quentin Fallavier, France) antibodies. After additional washes, a 1-hour incubation with peroxidase-conjugated goat anti–rabbit or anti–mouse IgG (1:5000; Santa Cruz Biotechnology, Tebu-bio, Le Perray en Yvelines, France) was performed. Immunocomplexes were revealed by using the

ECL detection system (GE Healthcare). Protein bands were quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare).

Cytokine Immunoassays

Culture media were assessed for concentrations of interleukin (IL)–1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, interferon γ (IFNγ), and tumor necrosis factor α (TNFα) using a Fluorokine MAP kit (R&D Systems, Abingdon, UK) as previously described.[21] Results were expressed as pg/mg of proteins assessed in biopsy specimens by Bradford assay.[22]

Statistical Analysis

To evaluate the effects of increasing doses of glutamine, results were compared using nonparametric 1-way analysis of variance (ANOVA) for paired data (Friedman test) followed by Dunn's post hoc tests. In other cases, data were compared using a nonparametric Mann-Whitney test. Nonparametric Spearman correlations were also used. For all tests, $P < .05$ was considered significant.

Results

We included 12 patients with IBS-D: 8 women and 4 men, aged 40.7 ± 6.9 years.

Glutamine and TJ Proteins

Claudin-1 expression was modified by glutamine increasing concentrations ($P < .05$, Friedman test), while occludin was not significantly affected (Figure 1). Indeed, the incubation of biopsy specimens with 10 mmol/L glutamine increased claudin-1 expression (×1.4-fold change) compared with 0.6 mmol/L glutamine ($P < .05$, Dunn's posttest). We did not analyze ZO-1 expression in the present study, as we previously observed that its expression was not altered in the colonic mucosa of patients with IBS-D.[6] As claudin-1 expression was affected by glutamine, we also studied other claudins (ie, claudin-4 and claudin-7) but only in 11 patients with IBS-D because of tissue unavailability in 1 patient. Claudin-4 was not affected by glutamine, while claudin-7 expression was increased with 2, 5, and 10 mM glutamine compared with 0.6 mM glutamine (Suppl. Figure S1).

Interestingly, we observed a negative correlation ($r = -0.78$, $P = .0024$; Figure 2) between the basal expression of claudin-1 observed at 0.6 mmol/L glutamine and the effects of glutamine on claudin-1, expressed as the ratio of expression at 10 mmol/L glutamine on expression at 0.6 mmol/L glutamine. Even if we did not observe an effect of increasing concentrations of glutamine on occludin expression, a similar negative correlation was observed ($r = -0.88$, $P = .0002$; Figure 2). These results suggest that glutamine may affect claudin-1 and occludin expression according to their basal level. We thus analyzed the effects of glutamine on the expression of claudin-1 and occludin in 6 patients displaying a low basal expression of both claudin-1 and occludin (Figure 2). In these patients, glutamine at 10 mmol/L markedly increased expression of both claudin-1 (×1.9-fold change, $P < .01$) and occludin (×2.4-fold change, $P < .01$). To know whether low expression of TJ proteins may be related to a low expression of glutamine synthetase, we analyzed glutamine synthetase expression in the colonic mucosa of included patients with IBS-D. Unfortunately, we were not able to detect glutamine synthetase expression in our samples, while we observed a marked expression in a positive control (human liver sample, data not shown).

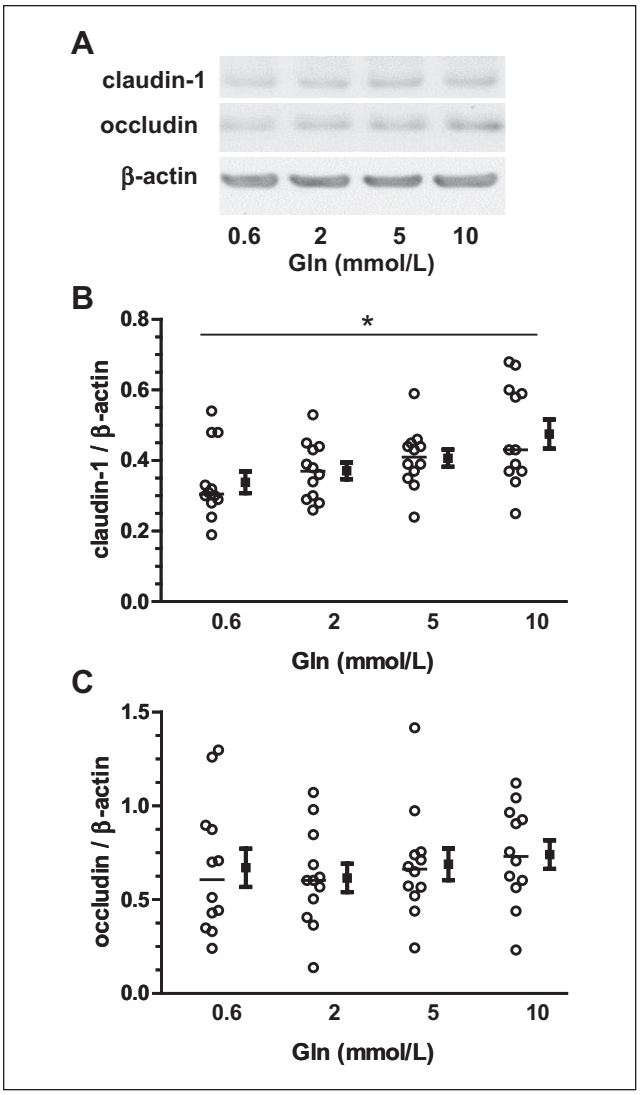

Figure 1. Representative immunoblots (A) and densitometric analyses of claudin-1 (B) and occludin (C) in colonic mucosa of patients with irritable bowel syndrome with predominant diarrhea (IBS-D, n = 12) after incubation with 0.6, 2, 5, or 10 mmol/L of glutamine during 18 hours. Values are shown as individual data and medians (left) or as mean ± SEM (right); Friedman test *P* values: B, $P = .0416$; C, not significant. *$P < .05$ using Dunn's post-tests.

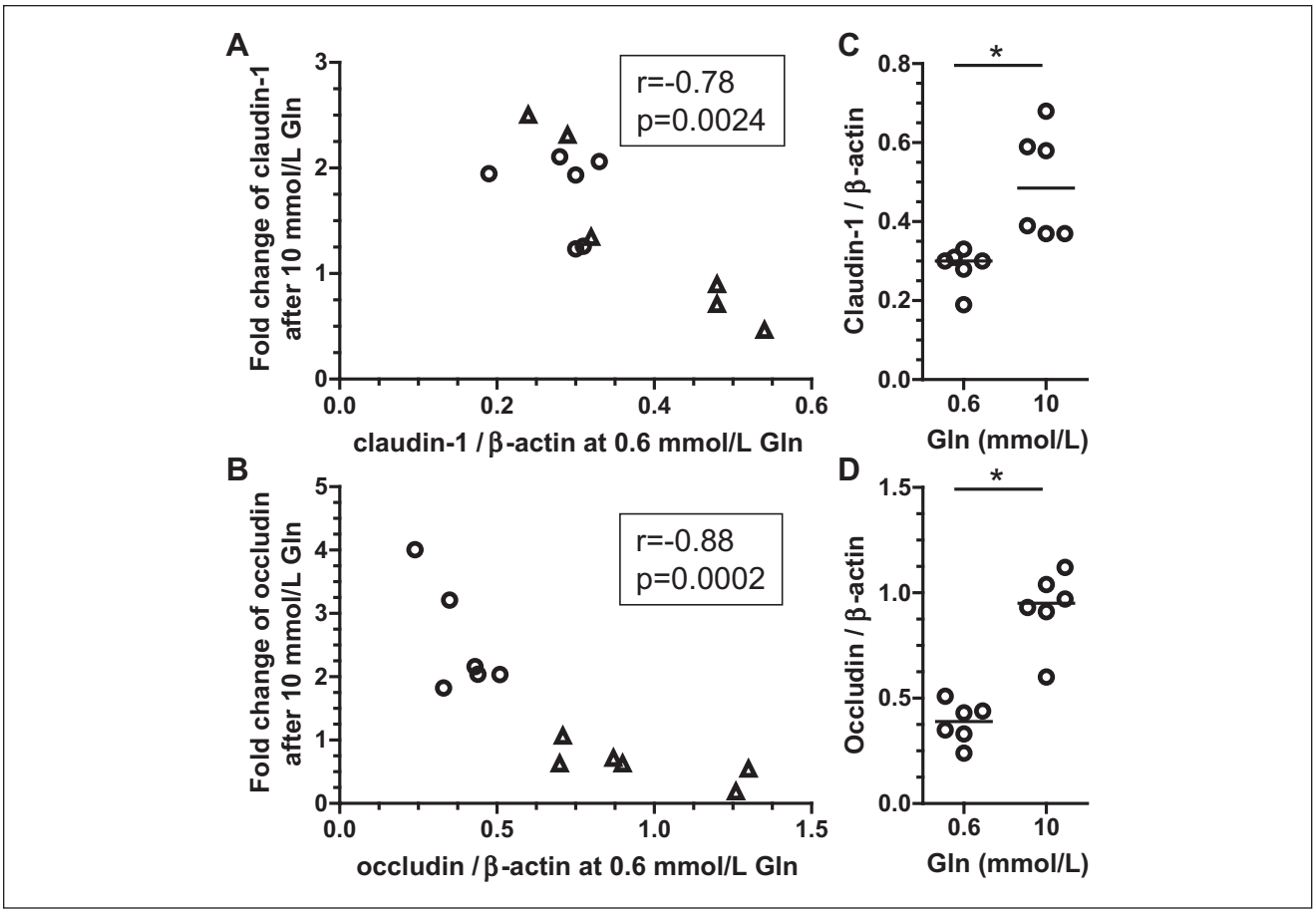

Figure 2. Effects of glutamine according to the basal expression of tight junction proteins. Correlation between the expression of claudin-1 (A) or occludin (B) in colonic mucosa of patients with irritable bowel syndrome with predominant diarrhea (IBS-D, n = 12) after 0.6 mmol/L glutamine and the effects of glutamine (ratio of expression at 10 mmol/L glutamine on expression at 0.6 mmol/L glutamine). (C, D) Effect of glutamine on claudin-1 (C) and occludin (D) expression in 6 patients showing a low basal expression. *$P < .05$ using Dunn's tests. Circles and triangles represent patients classified with low or high tight junction protein expression, respectively.

Glutamine and Cytokine Production

Glutamine did not significantly affect cytokine concentration in the culture media (Table 2). We observed only a trend for an increase of IL-10 production by increasing concentrations of glutamine, but the difference did not reach significance ($P = .0682$). To better understand the difference of TJ proteins in patients with IBS-D, we compared cytokine production according to the level of TJ proteins as described above. Interestingly, we observed that IL-2 production was significantly lower in patients showing a low expression of TJ proteins ($P < .05$; Figure 3).

Discussion

In this ex vivo study, our results suggest that glutamine is able to restore TJ proteins, claudin-1 and occludin, in the colonic mucosa of patients with IBS-D showing a low basal expression of these proteins.

An altered intestinal permeability has been reported in several studies during diarrhea-predominant IBS.[5,23] In addition, we recently observed that TJ proteins (ie, claudin-1 and occludin) were mainly affected in the colonic mucosa of patients with IBS-D compared with other IBS subtypes, with both a decrease of protein expression and an altered cell localization.[6] Interestingly, Martinez et al[9] also showed disruption of TJs in the small bowel mucosa in patients with IBS-D. The pathophysiological role of increased intestinal permeability remains debated, but correlations between intestinal permeability and visceral hypersensitivity[5,8] or between TJ protein expression and visceral hypersensitivity[6,8] have been reported.

Zhou et al[4] underlined that glutamine synthetase expression was reduced in the small bowel and colonic mucosa of patients with IBS-D who had an increased intestinal permeability. This decrease could be related to microRNA-29a expression that appeared enhanced in these patients.[4] Interestingly, in a recent

Table 2. Cytokine Concentrations in Culture Media.[a]

	Glutamine, mmol/L			
Cytokine, pg/mL	0.6	2	5	10
IL-1β	2.360 [0.039–37.61] 6.604 ± 3.334	4.021 [0.116–22.40] 5.895 ± 2.020	1.099 [0.000–60.75] 6.756 ± 4.979	1.527 [0.054–10.32] 2.936 ± 0.9737
IL-2	0.204 [0.000–0.815] 0.307 ± 0.089	0.226 [0.000–1.655] 0.312 ± 0.130	0.038 [0.000–1.036] 0.178 ± 0.088	0.114 [0.000–1.310] 0.253 ± 0.106
IL-4	0.442 [0.000–1.586] 0.539 ± 0.172	0.300 [0.000–2.771] 0.722 ± 0.263	0.215 [0.000–1.120] 0.352 ± 0.116	0.572 [0.000–1.682] 0.634 ± 0.145
IL-5	0.102 [0.000–0.171] 0.102 ± 0.016	0.117 [0.049–0.249] 0.123 ± 0.014	0.092 [0.000–0.406] 0.099 ± 0.033	0.106 [0.000–0.170] 0.098 ± 0.015
IL-6	50.4 [0.02–310.7] 78.1 ± 25.4	33.2 [17.7–214.6] 72.5 ± 19.2	32.5 [0.02–710.1] 94.9 ± 57.2	71.0 [0.11–259.4] 83.8 ± 22.9
IL-8	681 [5–1558] 609 ± 130	420 [98–2120] 620 ± 173	338 [5–1191] 412 ± 120	531 [7.5–1360] 536 ± 120
IL-10[b]	0.079 [0.000–1.544] 0.338 ± 0.150	0.085 [0.000–1.249] 0.297 ± 0.124	0.033 [0.000–0.805] 0.130 ± 0.066	0.189 [0.000–1.290] 0.291 ± 0.109
IL-12	0.473 [0.000–1.486] 0.584 ± 0.122	0.452 [0.148–0.860] 0.507 ± 0.075	0.403 [0.046–0.815] 0.411 ± 0.070	0.456 [0.203–0.752] 0.479 ± 0.051
IFNγ	0.053 [0.000–0.186] 0.050 ± 0.015	0.039 [0.000–0.221] 0.055 ± 0.017	0.026 [0.000–0.071] 0.029 ± 0.024	0.036 [0.010–0.162] 0.045 ± 0.043
TNFα	1.245 [0.006–6.280] 1.661 ± 0.521	0.974 [0.209–4.216] 1.554 ± 0.376	0.625 [0.000–4.951] 1.251 ± 0.497	0.801 [0.000–5.519] 1.244 ± 0.446

IFNγ, interferon γ; IL, interleukin; TNFα, tumor necrosis factor α.
[a]Data from 12 patients with irritable bowel syndrome with predominant diarrhea are shown both as median [range] and as mean ± SEM. Values are expressed in pg/mg proteins.
[b]$P < .1$ (analysis of variance).

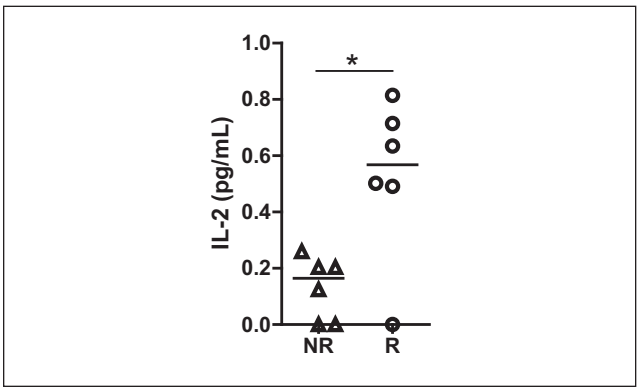

Figure 3. Concentration of interleukin-2 (IL-2) in culture media. Comparison of IL-2 concentrations between responders (R, n = 6) and nonresponders (NR, n = 6) to glutamine. *$P < .05$ using Mann-Whitney test. Circles and triangles represent patients classified with low or high tight junction protein expression, respectively.

study, Zhou et al[24] also showed that increased microRNA-29a altered claudin-1 messenger RNA (mRNA), leading to an alteration of intestinal permeability, and suggested that decreased glutamine synthetase may also affect claudin-1 level. In intestinal epithelial cell lines, glutamine deprivation has been associated with increased intestinal permeability.[12–14] In the present pilot study, we demonstrated that high doses of glutamine applied on the colonic mucosa of patients with IBS-D increased claudin-1 expression. By using a rectal route or targeted delivery of glutamine, high concentrations of glutamine can be easily achieved in the colonic lumen. Even if luminal amino acids are not considered to be able to enter the colonocytes,[25,26] previous data reported reduced colonic inflammatory response after a rectal supply of glutamine in both animals[27,28] and humans.[29] More interestingly, we observed that the effects of glutamine depended on the basal expression of claudin-1, suggesting that the lower the basal claudin-1 expression, the greater the glutamine effects. A similar correlation was observed for occludin. We also provided evidence that claudin-7 expression can be affected by glutamine. However, to our knowledge, claudin-7 expression has not been yet reported in the colonic mucosa of patients with IBS. In intestinal epithelial cell lines, glutamine also restored TJ protein expression after glutamine deprivation[14] or in injured conditions.[15] Unfortunately, we were not able to detect glutamine synthetase expression in all samples from patients with IBS-D in our study. Thus, we cannot provide data on the correlation between the expression of TJ proteins and the expression of glutamine synthetase. However, our study suggests, in accordance with the study by Zhou et al,[4] that some

patients with IBS-D, but not all, displayed low expression of TJ proteins and that glutamine may exert beneficial effects only in these patients. Further studies should evaluate in a larger population of patients with IBS first the relationship between glutamine mucosal content and intestinal permeability and, second, the effects of glutamine supplementation on in vivo intestinal permeability.

In the present study, we were not able to provide data on the mechanisms of action of glutamine due to the limited availability of tissue. Glutamine has been previously reported to affect many intestinal cellular functions such as cell survival, protein metabolism, inflammatory response, or stress response.[30] Previous studies reported that colonic epithelial cells were able to use not only butyrate but also glucose, glutamate, and glutamine.[26,31] We[15] and others[32] reported that glutamine must be converted in glutamate to affect TJ proteins in intestinal epithelial cell lines. In contrast, Seth et al[33] showed that inhibitor of glutaminase did not blunt glutamine effects. Unfortunately, in the present study, we were not able to provide data on glutamine utilization. Glutamine may also affect signaling pathways as previously described.[15,16] Further studies should evaluate the involved mechanisms of action, particularly glutamine metabolism.

During IBS, a low-grade inflammatory response has been proposed and may contribute to regulate visceral hypersensitivity[34] or gut barrier function.[7,21] We thus analyzed cytokine production by colonic mucosa. Glutamine had no effect on cytokine production in the present work whatever the studied cytokine. However, we observed that patients with IBS displaying low expression of claudin-1 and occludin had higher production of IL-2 than did other patients with IBS. To our knowledge, IL-2 colonic mucosal expression has not been studied in patients with IBS. For IL-2, only the (–330, +160) GT haplotype was significantly increased in patients with IBS compared with controls.[35] Experimental studies suggested that IL-2 alone does not affect intestinal permeability[36] but decreases intestinal permeability when coincubated with other cytokines.[37] Further studies should evaluate IL-2 intestinal production in patients with IBS according to intestinal permeability alteration.

In conclusion, in this pilot study, we suggest that topical glutamine is able to increase claudin-1 expression in patients with IBS-D and to restore TJ protein expression according to their basal expression. Further studies should be done (1) to better identify patients with altered intestinal permeability and (2) to evaluate the effect of glutamine delivered at the colonic level in vivo either by the rectal route or by using targeting tools.

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Statement of Authorship

J. Bertrand, P. Déchelotte, and M. Coëffier contributed to the conception/design of the research; J. Bertrand, I. Ghouzali, C. Guérin, C. Bôle-Feysot, M. Gouteux, P. Déchelotte, P. Ducrotté, and M. Coëffier contributed to the acquisition, analysis, or interpretation of the data; J. Bertrand and M. Coëffier drafted the manuscript. All authors critically revised the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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