# Aquaporin Expression in Colonic Mucosal Biopsies From Irritable Bowel Syndrome With Diarrhea

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**INTRODUCTION:** **Aquaporin (AQP) channels are involved in regulating fluid homeostasis in the colon. Several AQP channels were detected in human colon epithelial cells. In a previous study, rats fed 1% (wt/wt) sodium cholate had increased AQP3, 7, and 8 levels, suggesting AQP involvement in bile acid diarrhea (BAD). Our aim was to compare AQP expressions in rectosigmoid mucosal (RSM) biopsies from patients with irritable bowel syndrome–diarrhea (IBS-D) (divided into those with normal or high fecal BA excretion) and in patients with IBS-constipation (IBS-C) compared with healthy controls.**

**METHODS:** **In RSM biopsies from 44 patients with IBS-D (with normal ($<$) or high ($>$2,337 $\mu$mol/48 hours (BAD)) fecal BA excretion), 10 patients with IBS-C, and 17 healthy controls, we measured expressions of AQP1, 3, 7, and 8, with RT-PCR (housekeeper gene *GAPDH*). We analyzed RNA for expression by RT$^2$-PCR assays, with expression calculated using $2^{-\Delta\Delta CT}$-based fold-change. Comparisons of IBS groups were corrected for false detection rate (Bonferroni correction for 12 comparisons; $P<0.0042$). AQP protein measurements on biopsies from 3 healthy controls, 3 patients with IBS-D, and 3 patients with BAD were performed by western blots (GAPDH housekeeping protein).**

**RESULTS:** **In RSM from patients with IBS-D (but not IBS-C), mRNA expression of AQP3 was decreased, and AQP7 and 8 were increased relative to controls. Fold differences were not different in IBS-D with or without BAD. Western blots confirmed increased expression of AQP7 and 8 and decreased AQP3 proteins in biopsies from patients with IBS-D compared with controls.**

**CONCLUSIONS:** **Increased AQP7 and 8 and decreased AQP3 expressions in RSM suggest that further studies on AQPs' potential role in the pathophysiology of diarrhea in IBS-D are warranted.**

## INTRODUCTION

Peripheral mechanisms are increasingly recognized in the etiology and manifestations of irritable bowel syndrome (IBS) (1). These include intestinal secretory mechanisms caused by peptides and amines produced by enteroendocrine cells or submucosal neurons, enterocyte secretory processes, and intraluminal factors (2). Among the secretory mechanisms in functional diarrheas, one of the few well-documented factors associated with the increase in intestinal secretion in patients with IBS-D is the increased sensitivity to bile acids (BAs) (3).

Aquaporin (AQP) water channels (4) are regulators of transcellular water flow; rapid water exchange across cells enables tissues and organs to secrete and/or absorb water as part of their physiological functions. Consistent with their expressions in most tissues, AQPs are associated with diverse physiological and pathophysiological processes. Transport through AQPs occurs by a common passive mechanism, but the regulation and cellular distribution of AQPs vary depending on the cell and tissue type. AQPs are involved in regulating cell volume, transcellular water flow and water homeostasis, surface expression of other membrane proteins and cell adhesion, and providing selective pores for the rapid movement of water across diverse cell membranes (4,5).

The literature on the gut distribution, selective permeability, and tissue localization of AQPs has been summarized previously (6). Several AQP channels were detected in human colon epithelial cells (AQP1, 3, 4, and 7–9): AQP1 in the apical plasma membrane in the bottom of crypts; AQP3 and AQP4 in the basolateral plasma membrane; AQP7 and 8 are expressed along the entire large intestine; AQP7 is located in colon superficial

epithelial cells, and AQP8 in the subapical colonic absorptive cells (6–11). They play a role in water trafficking from lumen to the interstitium by a transcellular route (12). AQP8 channels are selectively permeable to water. AQP3 appears to be a basolateral membrane and apical membrane channel with selective permeability for water, glycerol, and urea, although there are species differences in the rat and human intestines (11,13).

Rats fed sodium cholate for 72 hours had significantly increased fecal water content, significantly increased AQP3, 7, and 8 mRNA in proximal and distal colonic biopsies, and increased AQP7 and 8 protein, but reduced AQP3 protein levels in colonic mucosal biopsies (14). In fact, bile acid diarrhea (BAD) is increasingly recognized as a cause in up to 30% of patients with functional diarrhea (15,16), and the BA-fed rat studies suggest that it is important to understand the potential role of AQPs in mediating effects of BAs in patients with functional diarrhea or diarrhea-predominant IBS.

The cellular functions of AQPs are regulated by post-translational modifications, e.g., phosphorylation, ubiquitination, glycosylation, subcellular distribution, degradation, and protein interactions. Thus, glycosylation is essential for transport from the endoplasmic reticulum and for cell surface expression of the AQPs. AQP3 has 1 N-glycosylation site (17), AQP7 is O-glycosylated (2), and AQP8 is N-glycosylated (18). AQP8 allows flux of water, whereas AQP3, 7, and 9 also facilitate glycerol flux (19). Given the necessity of glycosylation for function, we examined the native proteins in whole-cell lysates. It is relevant to note that spontaneous deglycosylation has also been reported previously without any treatment or deglycosylation enzymes (20).

The expressions of these AQPs in patients with diarrhea-predominant IBS-D with high or normal fecal BA excretion are unknown. The aim of our study was to compare the expressions of AQPs in rectosigmoid mucosal (RSM) biopsies in patients with IBS-D with normal or high fecal BA excretion and in patients with constipation-predominant IBS (IBS-C) compared with healthy controls.

## MATERIALS AND METHODS
### Participants and rectosigmoid mucosal biopsies
We previously collected RSM biopsies (21) from patients with IBS-D (with normal or high (≥2,337 μmol/48 hr) fecal BA excretion) and IBS-C and healthy controls (Table 1). Patients consented to use biopsies in future research conducted by the investigators; data on the mucosal biopsies from 3 of the original cohort (21) were unsuitable for the mRNA expression studies (insufficient RNA integrity number to ensure quality of RT-PCR assay results). The research study was approved by the Mayo Clinic Institutional Review Board (IRB #17-009905). Among the patients with IBS-C, none were receiving bisacodyl, senna alkaloids, docusate, magnesium salts, secretagogues or morphine in the 48-hour period before the biopsies. However, a standard sodium phosphate enema (Fleets Enema) was administered immediately before undergoing the biopsy at unsedated flexible sigmoidoscopy.

### RT-PCR–based measurement of expressions of AQPs
We used RT-PCR–based measurement of expressions of AQP1, 3, 7, and 8 and housekeeper gene *GAPDH*. RNA was purified from RSM biopsies using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) including on-column DNase treatment to remove genomic

DNA. RNA quality was assessed on the Agilent Bioanalyzer. The resulting RNA (RNA integrity number, RIN > 7) was reverse transcribed by the use of the RT² First Strand Kit (Qiagen), and samples were analyzed for expression by RT²-PCR assays (AQP1, PPH59997A; AQP3, PPH14747A; AQP7, PPH22164B; AQP8, PPH13652A; GAPDH, PPH00150F, Qiagen).

### Selection of mucosal biopsy samples for protein studies using western blots
We conducted further studies of protein expression on RSM biopsies from 3 healthy controls, 3 patients with IBS-D with high BA excretion, and 3 patients with IBS-D with normal BA excretion. Biopsies selected were based on 2 criteria: protein content in mucosal biopsies available among participants and whose quantified mRNA expression was high in AQP7 and 8 mRNA expressions in the IBS-D group.

### Protein measurements by western blots
Whole-cell lysates were isolated from human RSM biopsies with the RIPA Lysis Buffer System (Santa Cruz Biotechnology, Dallas, TX). Briefly, tissue samples were homogenized in RIPA lysis buffer containing protease inhibitors. The homogenate was centrifuged at 720$g$ to remove nuclei and cellular debris. The supernatant was then centrifuged at 10,000$g$, and the resulting supernatant containing cytosolic and membrane fraction was used for the western blot analysis. Protein concentrations were determined by Bradford quantification (Sigma-Aldrich, St. Louis, MO). Proteins were separated by the use of 10% Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and blotted onto polyvinylidene difluoride (Millipore, Burlington, MA) membranes. The membranes were blocked with 5% bovine serum albumin in phosphate buffered saline/0.1% Tween, after which AQP primary antibodies (1:1,000; Abcam) were applied overnight at 4 °C.

GAPDH (1/3,000; Millipore) was used for normalization of protein loading for all the AQPs of interest. Membranes were washed with TBST (a mixture of tris-buffered saline and Polysorbate 20, also known as Tween 20) and incubated with horseradish peroxidase–conjugated secondary antibody (goat anti-rabbit, Abcam, or chicken anti-mouse, Santa Cruz Biotechnology) and were then visualized with chemiluminescence (Pierce, Rockford, IL) and autoradiography. Band densities were quantified with the Image J 1.50i (National Institutes of Health).

### Statistical analysis
Demographic information of IBS groups and healthy controls was compared using ANOVA on ranks, followed by Dunn multigroup comparison. For the direct comparisons between the patients with IBS-D with high or normal fecal BA excretion (based on 48-hour fecal BA 2337 μmol/48 hr), we used the Mann-Whitney rank-sum test because the variance among the 4 groups of participants was large and generally did not proceed beyond the initial ANOVA on ranks.

We compared mRNA fold expression relative to healthy subjects in the 3 groups: IBS-D with BAD, IBS-D without BAD, and IBS-C. This method uses $2^{-\Delta\Delta CT}$-based fold-change calculations and the Student $t$ test to calculate 2-tail, equal-variance $P$ values. $P$ values for comparisons of each IBS group vs the healthy control group were corrected for 12 comparisons (using

**Table 1** Demographics of IBS groups and healthy controls and fecal measurements of patients with IBS-D with high or normal fecal BA excretion (based on 48-hour fecal BA cutoff of 2337 μmol/48 hr)

|  | Healthy | IBS-D with high BA | IBS-D with normal BA | IBS-C | P value |
|---|---|---|---|---|---|
| N | 17 | 19 | 25 | 10 | |
| Age | 36 (23.2–52.8) | 39 (26.8–51.6) | 42 (28–66) | 46 (31–68.5) | 0.14 |
| F:M | 16:1 | 16:3 | 24:1 | 10:0 | |
| BMI, kg/m$^2$ | 26.9 (23.8–30.0) | 32.7 (28.7–36.7) | 28.2 (24.7–31.7) | 24.9 (20.7–37.6) | 0.038 |
| Fecal fat, g/d | | 10 (3–25) | 6 (3–15) | | 0.17 |
| Stool weight, g/48 hr | | 548 (246.4–1,039.8) | 333 (123–538) | | <0.001 |
| Fecal BA, μmol/48 hr | | 3,442 (2,566.3–10,311) | 1,065 (163–1942.6) | | <0.001 |

Values: median (10–90 percentiles).
The P value refers to comparison between IBS groups by ANOVA on ranks; the Mann-Whitney rank-sum test was used to compare the 2 IBS-D groups with high or normal fecal BA excretion.
BA, bile acids; F, females; IBS, irritable bowel syndrome; IBS-C, constipation-predominant irritable bowel syndrome; IBS-D, diarrhea-predominant irritable bowel syndrome; M, males.

Bonferroni correction) so that $P < 0.0042$ was statistically significant.

## RESULTS

We studied mRNA expression in RSM samples from 44 patients with IBS-D, 10 patients with IBS-C, and 17 healthy controls. Patient demographics at the time of RSM sample collection are shown in Table 1. Nineteen of the 44 patients with IBS-D had elevated fecal BAs. There were no differences in the sex or age of patients in the 4 groups. As previously noted (21), the only difference in demographics was in body mass index (BMI) across the 4 groups (ANOVA $P = 0.038$), with a numerically higher BMI in patients with IBS-D with high fecal BA excretion; although this did not reach statistical significance on Dunn multiple comparison test, the difference was significant ($P = 0.049$) by the direct Mann-Whitney rank-sum test between the 2 groups.

### AQP mRNA expression in the rectosigmoid mucosa

AQP expression data (expressed as fold difference relative to healthy controls) are shown in Figure 1 and Supplemental Table 1 (Supplementary Digital Content, http://links.lww.com/CTG/A21) and Supplemental Figure 1 (Supplementary Digital Content, http://links.lww.com/CTG/A21) in the Appendix. In RSM biopsies from patients with IBS-D with BAD or without BAD compared with healthy controls, mRNA expression of AQP3 was decreased, whereas AQP7 and 8 were increased relative to healthy controls (all $P < 0.0042$, correcting for false detection rate). Numerically, the fold differences were not different in patients with IBS-D with BAD or without BAD. The fold differences observed in patients with IBS-C did not differ from controls. There were numerically reduced mRNA expressions of AQP1 in both diarrhea groups; however, the P values (both $< 0.05$) were not significant after false detection rate correction ($P < 0.0042$).

### AQP protein expression

Data on the patients whose biopsies were included in the study of mucosal AQP protein expression are summarized in Table 2. We observed that compared with healthy controls, there was

increased expression of AQP8 ($P = 0.048$), nonsignificant increase in AQP7 ($P = 0.262$), and decreased expression of AQP3 ($P = 0.024$) in mucosal biopsies from patients with IBS-D (Figure 2). Moreover, there was borderline increased AQP7 protein expression in IBS-D without BAD compared with IBS-D with BAD ($P = 0.061$). AQP3 showed 2 bands on western blot; the quantification of the bands separately provided similar significant data ($P < 0.01$ for both, relative to healthy controls) to the quantification of both bands together (Figure 2). Unlike AQP7 and 8, AQP3 had 2 bands, which represent glycosylated (upper band) and deglycosylated AQP3 (lower band).

## DISCUSSION

Our study shows increased AQP8 mRNA and protein expressions and decreased AQP3 mRNA and protein expressions in the rectosigmoid mucosa of patients with IBS-D relative to healthy controls. In contrast, patients with IBS-C (disease control) had AQP mRNA expression that did not differ from controls. The reduced AQP3 expression may reduce absorption of water; conversely, increased AQP8 may result in fluid diffusion into the lumen in the presence of higher luminal osmolality, such as short chain fatty acids or microbial products in the colon. There was also statistically increased AQP7 mRNA expression in the rectosigmoid mucosa in both IBS-D groups and borderline increased protein expression of AQP7 in the IBS-D subgroup without BAD. In general, these findings concur with the observations in rats fed sodium cholate, except that the latter studies showed increased mRNA for AQP3, but (similar to our observations in human colon biopsies) reduced AQP3 protein expression. Thus, in the rats treated with sodium cholate, there were reductions in expression of AQP3, similar to observations in patients with IBS-D in our study.

Generally, there were no differences in AQP mRNA or protein expression in patients with IBS-D with overt BAD compared with those with normal total fecal BA excretion. Increased AQP7 and 8 protein expressions with reduced AQP3 protein expression in patients with IBS-D suggest another potential mechanism for the looser bowel movements in
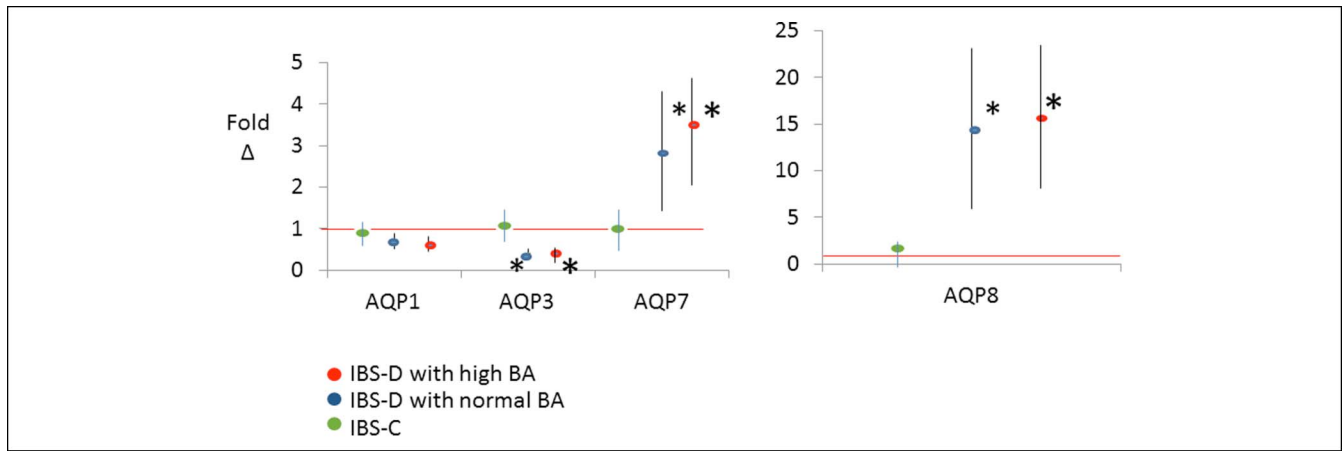
**Figure 1** mRNA expressions: decreased AQP3 and increased AQP7 and 8 in patients with diarrhea-predominant irritable bowel syndrome (IBS-D) with high or normal fecal bile acid (BA) excretion with no significant differences in mucosa from patients with constipation-predominant IBS (IBS-C) relative to healthy control mucosa. Note that the horizontal line at 1 represents expression in healthy controls. *Indicates a significant $P$ value <0.0042; mean (95% CI); AQP, aquaporin.

patients with IBS-D, that is, not restricted to patients with BAD; this adds to the information provided by rat studies after exposure to sodium cholate. The current report provides the first evidence in IBS-D of increased expression of AQP7 and 8 and decreased expression of AQP3 in colonic mucosa that may be potential mechanisms for increased fluid diffusion through colonocytes, resulting in the altered stool consistency in patients with IBS-D.

Whereas our patients with IBS-D had greater expressions of AQP7 and 8, and obesity has been associated with increased AQP expression in adipose tissue, facilitating glycerol release from the adipose tissue and distribution to various tissues and organs (22), there is no evidence in the literature that increased BMI results in altered AQP expression in any gastrointestinal tissues, such as the colonic epithelium. Therefore, it seems highly unlikely that the increased AQP expression observed in our study is related to the higher BMI in the patients with IBS-D.

Our results on AQP8 differ from those reported by Wang et al. (23) who observed reduced AQP8 mRNA expression in the ascending colon biopsies from patients with IBS-D compared with

controls. However, it is worth noting that in the same patients reported by Wang and Hou (23), there were no differences observed in descending colon biopsies approximately from the distal left colonic mucosal biopsies in our studies. Rat models of IBS-D have also been associated with decreased AQP8 (24); however, it is unclear whether this is relevant, given the differences in AQP8 molecules reflected in their molecular weight in rats and humans (14).

There is conflicting information about the function of AQP3 in previous literature. AQP3 channels were down-regulated in a model of slow transit constipation in rats (25) or increased in morphine-induced constipation in rats (26). Conversely, the secretory hormone, vasoactive intestinal polypeptide, increased AQP3 expression in colonic epithelial cell line (27). Commonly used medications such as osmotic laxatives including magnesium sulfate, and "stimulants" such as bisacodyl and sennosides change AQP3 expression to cause increased fluid secretion, whereas opioid agonists such as morphine increased fluid absorption through AQP3 channels in colonocytes (28). None of the participants in our studies were receiving these medications, although some did receive

**Table 2** Participants whose mucosal biopsies were tested for aquaporin 3, 7, and 8 protein expressions

|  | Healthy | IBS-D with high BA | IBS-D with normal BA | $P$ value |
|---|---|---|---|---|
| N | 3 | 3 | 3 | |
| Age, yr | 36 (31–40) | 48 (39–50) | 34 (32–42) | 0.10 |
| Females | 3 | 3 | 3 | |
| BMI, kg/m$^2$ | 25.1 (22.0–38.3) | 26.7 (24.5–45.5) | 31.2 (22.5–35.3) | 0.88 |
| Fecal fat, g/24 hr | | 7 (4–12) | 5 (2–27) | 0.68 |
| Stool weight, g/48 hr | | 548 (508–595) | 374 (58–492) | 0.14 |
| Fecal BA, $\mu$mol/48 hr | | 3,861 (3,442–11,327) | 576 (463–1,497) | 0.11 |

Values: median (10–90 percentiles).
The $P$ value refers to comparison between IBS groups by ANOVA on ranks.
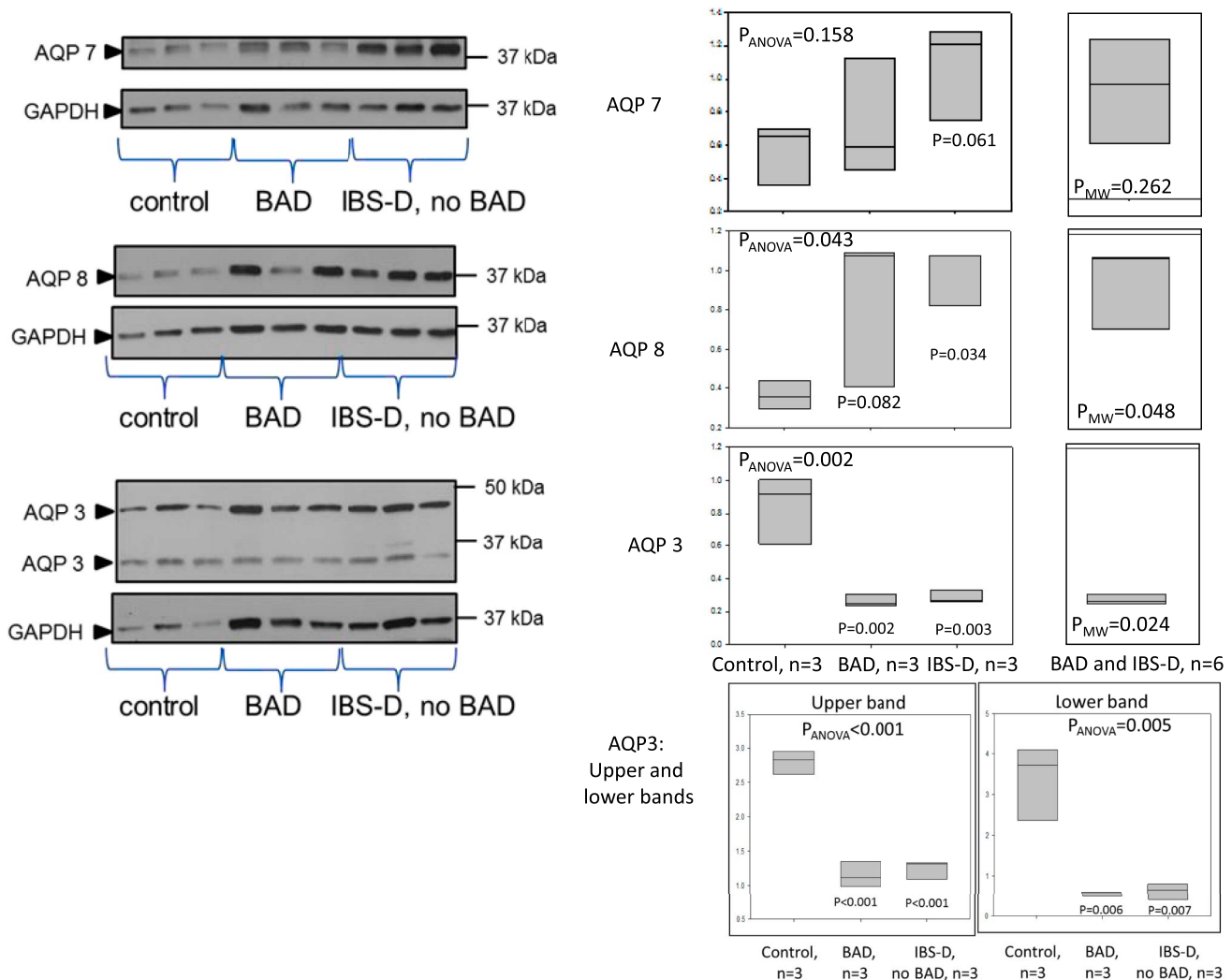BA, bile acids; IBS-D, diarrhea-predominant irritable bowel syndrome.

**Figure 2** Western blots showing increased AQP8 and borderline increased AQP7 and decreased AQP3 expressions in rectosigmoid mucosal biopsies in diarrhea-predominant irritable bowel syndrome (IBS-D) compared with controls (using GAPDH as housekeeping protein and correcting for the GAPDH expression in the analysis). Separate quantification of the upper and lower protein bands of AQP3 shows identical trends with reduced AQP3 in both bile acid diarrhea (BAD) and IBS-D groups compared with healthy controls. AQP, aquaporin.

a magnesium sulfate enema immediately before the biopsy. Importantly, magnesium sulfate increased expression of AQP3 in colonocytes (28). On the other hand, the laxatives, daio-kanzoto, and sennoside A decrease AQP3 expression in the colon to inhibit water transport from the luminal to the vascular side, leading to a laxative effect (29). We observed reduced expression of AQP3 in mucosal biopsies from patients with IBS-D, with no significant difference between patients with IBS-C and healthy controls.

Our studies point to the relevance of AQP3, 7, and 8 channels in the spectrum of the entire group of patients with IBS-D. We were unable to demonstrate differences in expressions of AQP7 and 8 in patients with BAD compared with IBS-D; however, we are conducting studies with larger sample sizes to investigate this further, and we are exploring expressions in both proximal and distal colon mucosal biopsies. This is relevant because the concentrations of BAs in the right colon may be up to 4 times higher than the concentrations in stool and distal colon. In fact, the study in rats

that were fed sodium cholate (1% by weight) (14) likely exposed the colonic mucosa to an 8-fold higher concentration of BAs compared with BA levels in the human colon of patients with IBS-D (see calculation in Appendix, Supplementary Digital Content, http://links.lww.com/CTG/A21). Further studies of BA loading into the colon or treatment with the ileal BA transport inhibitor, elobixibat (30), may provide further insights on the effects of BAs on AQP proteins in humans.

Potential limitations of our study include the relatively small number of biopsies subjected to protein expression studies. However, the protein studies did indeed confirm the findings on mRNA expression. The wide variation in expressions of AQP7 and 8 proteins in the BAD group may have led to a type II error compared with the less variable protein expression observed in the patients with IBS-D. We are currently pursuing further studies of terminal ileal, right and left colon mucosal biopsies to examine the expression of AQPs in different regions, and these studies may provide

further insights on potential differences between patients with high or normal BA loading to the right colon. This is relevant because passive absorption of BAs in the colon (which absorbs approximately 75% of the BAs entering the cecum) may reduce their potential impact on AQPs, which may be apparent in biopsies from the right colon, which was not investigated in our previous studies.

Another limitation is that our studies do not provide an explanation for the mechanism leading to the changes in mRNA or protein expression or the functional consequences of the changes in expression that are observed in the different patient cohorts. Our studies are intended to foster further research, and it is relevant to note that given the absence of fold differences in AQP expressions between patients with IBS-D with normal or high fecal BA excretion, it would be relevant initially to perform further research in IBS-D as a whole and then search for potential interactions with fecal BA excretion.

Other potential mechanisms for the reduced AQP3 expression might be mucosal immune activation or the effects of intraluminal proteases, which are factors described in patients with IBS-D (1). It is also conceivable that the increased AQP7 and 8 expressions represent compensatory changes to counterbalance other mechanisms that might be causing ion secretion, such as the increased mRNA expression of GUCA2B and PDZD3 documented in previous studies (21,31). However, these issues need to be examined in future studies.

It is also conceivable that unintended bias was introduced in the protein expression studies because these studies could only be conducted in samples with sufficient protein expression. Because PCR and western blot analyses were performed on mucosal biopsies, we also cannot be certain that changes observed are occurring in epithelial cells rather than in other mucosal cells, such as fibroblasts and immune cells.

In conclusion, increased AQP7 and 8 and decreased AQP3 expressions in RSM biopsies are identified in a study of 44 patients with IBS-D with high or normal fecal BAs, but not in 10 patients with IBS-C, suggesting a possible role of AQPs in the pathophysiology of diarrhea in a broader group of patients with IBS-D, not just patients with BAD. AQPs may be potential targets for treatment of diarrhea, although it remains to be seen whether these small, integral membrane proteins are actually amenable to modulation by medications (32).

**CONFLICTS OF INTEREST**

**ACKNOWLEDGEMENTS**

## Study Highlights

**WHAT IS KNOWN**

✓ Several AQP channels have been detected in human colon epithelial cells and seem to function in water flux across the colonic epithelium in health and in disease states.

✓ Rats fed sodium cholate had increased colonic mucosal expression of AQP3, 7, and 8, suggesting a role of AQPs in BAD.

**WHAT IS NEW HERE**

✓ mRNA and protein expressions of AQP7 and 8 in RSM biopsies are increased, and AQP3 is decreased in diarrhea-predominant IBS, but not in constipation-predominant IBS, compared with healthy control mucosa.

✓ mRNA expression of AQP3, 7, and 8 does not seem to be different among patients with IBS-D with normal or increased fecal BA excretion.

**TRANSLATIONAL IMPACT**

✓ Altered AQP expression may constitute a mechanism contributing to the development of diarrhea in IBS-D, and may in the future serve as a therapeutic target.

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FUNCTIONAL GI DISORDERS