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Research Article

Intestinal Epithelial Digestive, Transport, and Barrier Protein Expression Is Increased in Environmental Enteric Dysfunction

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ABSTRACT

Environmental enteric dysfunction (EED) is characterized by malabsorption and diarrhea that result in irreversible deficits in physical and intellectual growth. We sought to define the expression of transport and tight junction proteins by quantitative analysis of duodenal biopsies from patients with EED. Biopsies from Pakistani children with confirmed EED diagnoses were compared to those from age-matched North American healthy controls, patients with celiac disease, and patients with nonceliac disease with villous atrophy or intraepithelial lymphocytosis. Expression of brush border digestive and transport proteins and paracellular (tight junction) proteins was assessed by quantitative multiplex immunofluorescence microscopy. EED was characterized by partial villous atrophy and marked intraepithelial lymphocytosis. Epithelial proliferation and enteroendocrine, tuft, and Paneth cell numbers were unchanged, but there was significant goblet cell expansion in EED biopsies. Expression of proteins involved in nutrient and water absorption and that of the basolateral Cl^- transport protein NKCC1 were also increased in EED. Finally, the barrier–forming tight junction protein claudin-4 (CLDN4) was significantly upregulated in EED, particularly within villous enterocytes. In contrast, expression of CFTR, CLDN2, CLDN15, JAM-A, occludin, ZO-1, and E-cadherin was unchanged. Upregulation of a barrier–forming tight junction protein and brush border and basolateral membrane proteins that support nutrient and water transport in EED is paradoxical, as their increased expression would be expected to be correlated with increased intestinal barrier function and enhanced absorption, respectively. These data suggest that EED activates adaptive intestinal epithelial responses to enhance nutrient absorption but that these changes are insufficient to restore health.

Introduction

Environmental enteric dysfunction (EED) is an early childhood disease that is estimated to affect over 150 million children worldwide. Previously referred to as environmental enteropathy, EED is associated with severe malnutrition and irreversible physical and cognitive stunting. It can be particularly challenging to discriminate between severe acute malnutrition and EED, in part because there are no precise diagnostic criteria for EED. Response to hypercaloric supplementation in severe acute malnutrition, but not in EED, is one operational definition, but this can only be applied in retrospect. The pathophysiology that leads to EED remains poorly understood.

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EED is most common in underresourced regions of Asia, South America, and Africa, where the absence of clean drinking water and other hygienic challenges may contribute to its pathogenesis.[1,2] Consistent with this, small bowel bacterial overgrowth can occur. EED has also been associated with recurrent or persistent enteric infection, intestinal barrier loss, inappropriate immune cell activation, and an altered gut microbiome.[2-16] However, nonabsorbable antibiotics, efforts to improve sanitation, and hyperalimentation have all failed to reverse stunting or reduce EED prevalence.[1,17] This suggests that the pathophysiology of EED may include defects in nutrient absorption or metabolism.[4]

Early studies of histopathology compared EED to celiac disease. EED is not, however, responsive to gluten elimination. Recent transcriptomic analyses[4,8,18] have correlated alterations in transcription of genes associated with mucosal immune function, metabolism, and epithelial repair processes with EED,[4,18,19] but the specific antigens or pathogens that drive mucosal immune activation in EED have not been identified. Nevertheless, the reduced oral vaccine efficacy in regions where EED is endemic suggests generalized immune dysfunction.[9]

Here, we analyzed the expression of ion and nutrient transport and barrier proteins within duodenal biopsies from a well-characterized patient group. Unexpectedly, the expression of absorptive proteins and a barrier—enhancing tight junction protein was increased in EED. These results suggest that EED activates adaptive intestinal epithelial responses that are ultimately insufficient to restore homeostasis.

Materials and Methods

Subjects and Biopsies

Duodenal biopsies were obtained from 10 Pakistani children with confirmed EED diagnoses from Aga Khan University, Pakistan. Clinical features of these patients and morphological analyses of their biopsies have been reported previously.[20] Duodenal biopsies were collected from age- and sex-matched North American children, including healthy controls, patients with celiac disease, and patients with nonceliac disease along with villous atrophy or intraepithelial lymphocytosis (Table 1).

Immune Staining and Multiplex Immunofluorescence

Sections (5 μm) of formalin—fixed paraffin—embedded (FFPE) duodenal biopsies were baked overnight at 60 °C. TintoDeparaffinator Citrate or EDTA (BioSB) was used for heat—assisted deparaffinization, rehydration, and antigen retrieval in a pressure cooker (15 minutes). After rinsing with TintoDeparaffinator Hot Rinse (BioSB), sections were cooled in PBS. Tissue autofluorescence was minimized by 2 sequential incubations (1 hour each) in a bleaching buffer under broad-spectrum LED light. After incubation (10 minutes) with Serum Free Protein Block (Agilent), primary antibodies (Supplementary Table S1) diluted in Antibody Diluent (Agilent) were added and slides were incubated (8 hour) at room temperature. After washing with ImmunoDNA Washer solution (BioSB), sections were incubated with secondary antibodies and Hoechst 33342 (Life Technologies, 1 μg/mL), washed, and mounted using Vectashield Plus Antifade Medium (Vector Laboratories). Following imaging, coverslips were removed, fluorophores were inactivated, and slides were re-stained. After the first round of staining, only directly conjugated antibodies were used.

Table
Demographics of cases and controls

	EED	HC	CD	NCD
Age (months, mean ± SD)	16.0 ± 7.5	18.2 ± 5.6	16.7 ± 4.6	14.5 ± 6.0
Number	10	29	7	16
Gender (male%)	50%	58%	57%	56%

CD, celiac disease; EED, environmental enteric dysfunction; HC, healthy control; NCD, nonceliac disease.

Image Acquisition

Each biopsy was scanned using a DM4000 microscope with 20× NA 0.7 HC PLAN APO objective (Leica), motorized *xyz* stage and emission filter wheel (Ludl Electronic Products), multichannel dichroic and single-band emission filters (Semrock), Aura light engine (Lumencor), and ORCA-Flash 4.0 LT+ camera (Hamamatsu) controlled by Metamorph 7.8 (Molecular Devices) using custom journals.[21] Images of hematoxylin and eosin (H&E)—stained sections were acquired similarly using a transmitted white LED light source and MicroPublisher 5.0 camera (Q Imaging). Tiled images were stitched using Metamorph 7.8 and custom journals.

Morphometry

Villus height was measured manually using well-oriented regions within each biopsy. Quantitative immunostain analyses used CellProfiler (Broad Institute); villus and crypt regions were annotated manually. After using Hoechst—labeled nuclei to identify tissues, an epithelial mask was generated based on lateral membrane labeling, eg, E-cadherin (Supplementary Fig. S1). E-cadherin staining was also used to create masks, demarcating basolateral membranes for analyses of CLDN4, NKCC1, and Na^+/K^+-ATPase. NHE3, sucrase-isomaltase (SI), PEPT1, SGLT1, and CFTR were analyzed with apical membrane masks created using γ-actin staining (Supplementary Fig. S1). Tight junction masks generated based on ZO-1 labeling were used to analyze CLDN2, CLDN15, occludin (OCLN), and JAM-A expression. Preliminary validation analyses showed that E-cadherin and γ-actin staining was consistent across biopsies; E-cadherin was used to normalize signal intensity across biopsies (Supplementary Fig. S1). Intraepithelial lymphocytes were assessed as CD3+ cells detected as primary objects within the epithelial mask. Epithelial subpopulations were defined by POU2F3, CHGA MUC2, and LYZ expression and counted manually.

Statistical Analysis

Data are presented as mean ± SD. Normalized data are shown for immunostains, and absolute numbers are shown for villus height and cell numbers. Comparisons between groups were performed using the Kruskal-Wallis and Dunn's tests (Prism 9.2.0, GraphPad Software). $P < .05$ was considered significant.

Results

Since the first descriptions of growth stunting in extremely young children with repeated bouts of diarrhea, it has been clear

that malabsorption is a critical component of EED. Few studies have assessed EED histopathology, and none have examined transport protein expression within the involved mucosa. In part, this reflects hesitance to obtain intestinal biopsies from affected infants. Intestinal biopsies are now being obtained from infants with malnutrition and growth stunting with increasing frequency, but ethical and practical concerns have precluded endoscopy and biopsy of age-matched healthy control subjects from within the same environment. The latter is critical, as mucosal histology is well known to vary as a function of geography and season. For example, some changes associated with EED are present in well-nourished African children but not Western controls.[22] Although imperfect, we compared EED cases to age− and sex−matched North American healthy control subjects, patients with celiac disease, and individuals with nondiagnostic histologic abnormalities, ie, villous blunting or intraepithelial lymphocytosis, referred to as subjects with nonceliac disease.

Environmental Enteric Dysfunction Is Characterized by Villous Blunting and Intraepithelial Lymphocytosis

Consistent with previous reports,[8,23-25] we found that EED is associated with partial villous atrophy but noted that this differed from complete villous atrophy associated with pediatric celiac disease. Conversely, intraepithelial lymphocytosis in EED exceeded that of celiac disease. Moreover, despite villous blunting, epithelial proliferation, which was increased in celiac disease, was not altered in EED (Fig. 1). Epithelial apoptosis, measured as cleaved caspase-3−positive epithelial cells, was not different in any group. The combination of villous blunting without reduced epithelial proliferation suggests that epithelial turnover may be accelerated in EED.

Goblet Cell Numbers Are Increased in Environmental Enteric Dysfunction

We next evaluated whether epithelial differentiation was affected in EED. The numbers of POU2F3−positive tuft cells, chromogranin A−positive enteroendocrine cells, and lysozyme−positive Paneth cells were similar across all groups. However, MUC2−positive goblet cell numbers were significantly increased in EED, relative to healthy controls, and trended toward an increase in celiac disease (Fig. 2). The number of goblet cells in nonceliac disease biopsies extended over a broad range and was not significantly different than any other group, including EED. Subset analysis of the 2 histologies that comprised the nonceliac group showed that the numbers of MUC2−positive cells were significantly increased, relative to normal controls, in villous atrophy but not in lymphocytosis.

Na^+ and Nutrient Absorptive Processes Are Upregulated in Environmental Enteric Dysfunction

Although villous blunting in EED is incomplete relative to celiac disease, malnutrition is more severe in EED. We, therefore, evaluated whether nutrient malabsorption in EED reflected the downregulation of related proteins. First, we assessed the expression of SI, which is necessary for the absorption of complex carbohydrates and sucrose. SI expression in villous enterocytes was markedly upregulated in EED relative to healthy controls as well as nonceliac disease controls (Fig. 3).

Following breakdown by SI and other brush border digestive enzymes, monomeric nutrients, eg, glucose, are primarily absorbed via Na^+−coupled cotransporters. We, therefore, assessed the expression of SGLT1 (SLC5A1), the brush border Na^+-glucose cotransporter. Similar to that of SI, SGLT1 expression was significantly increased in EED relative to healthy control and celiac disease biopsies. SGLT1 expression in nonceliac controls clustered into 2 groups, but unlike goblet cell numbers, the groups did not correlate with villous atrophy or lymphocytosis.

Na^+-nutrient cotransport generates a transepithelial osmotic gradient that drives fluid absorption and activates the brush border Na^+-H^+ exchanger NHE3 (SLC9A3), which also contributes to this gradient.[26-28] The function and expression of NHE3 are reduced in acute and chronic diarrheal disorders.[29-32] In contrast, NHE3 expression was significantly upregulated in EED relative to healthy control and celiac disease biopsies (Fig. 3). The oligopeptide transporter PEPT1 (SLC15A1) expression was unchanged (Fig. 3).

Ultimately, apical Na^+−dependent transport, eg, by SGLT1 and NHE3, relies on the transmembrane Na^+ gradient created by the Na^+/K^+-ATPase. We therefore evaluated whether the increases in SGLT1 and NHE3 expression were accompanied by changes in Na^+/K^+-ATPase expression. In EED, Na^+/K^+-ATPase expression was significantly upregulated relative to healthy control and celiac disease biopsies (Fig. 3). Thus, both apical and basolateral membrane proteins required for Na^+, nutrient, and water absorption are upregulated in EED. Malabsorption in EED is, therefore, not due to insufficient transport protein expression.

Cl^- Transport Protein Expression Is Modified in Environmental Enteric Dysfunction

The data above indicate that proteins responsible for absorption are upregulated in EED. Nevertheless, patients with EED often suffer from diarrhea, suggesting either insufficient fluid absorption or increased fluid secretion. NHE3 upregulation would be expected to amplify fluid absorption. We, therefore , assessed whether the expression of CFTR (which mediates cAMP−dependent Cl^- secretion across the apical membrane) or NKCC1 (SLC12A2) (which transports Cl^- across the basolateral membrane) is altered in EED. CFTR expression was unchanged, but NKCC1 was upregulated in EED relative to healthy control and nonceliac biopsies (Fig. 4). Together with the activation of apical Cl^- channels, increased NKCC1 expression could potentially drive increased Cl^- and, consequently, fluid secretion. Functional analyses will be required to determine if this is a mechanism of diarrhea in EED.

Only Claudin-4 Expression Is Modified in Environmental Enteric Dysfunction

One biochemical marker of EED is an increase in the lactulose-mannitol fractional excretion ratio, a measure of intestinal permeability. We analyzed the expression of the tight junction proteins ZO-1, OCLN, JAM-A, CLDN2, CLDN4, and CLDN15 and the adherens junction protein E-cadherin. There were no changes in the subcellular distribution of tight junction proteins. Moreover, the funnel-like ZO-1 and OCLN profiles that are typical of epithelial shedding were not present.[33] Quantitatively, only CLDN4 expression was altered (Fig. 5). This increased expression was unexpected, as CLDN4 has been characterized as a barrier−forming CLDN.[34] Notably, similar to previous studies,[5] we

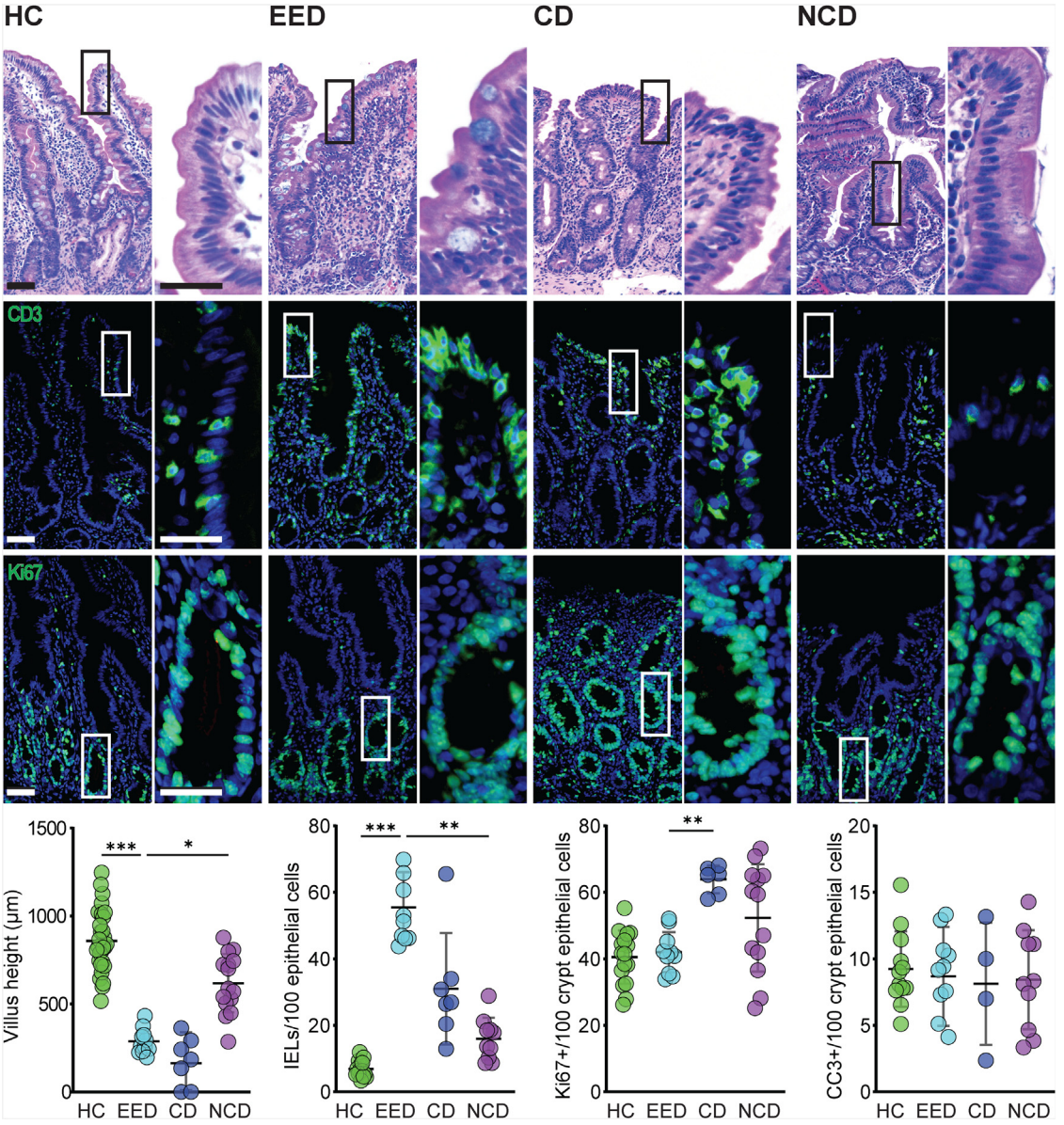

Figure 1.
Villus height was significantly reduced in children with EED or CD relative to HC and subjects with NCD. Villous atrophy in CD was significantly more severe than that in EED, but the number of $CD3^+$ IELs was more prominently increased in EED. Proliferation, assessed as the number of Ki67−positive cells, was increased in CD but unchanged in EED. The number of cleaved caspase 3−positive cells was similar in all groups. Bars = 50 and 20 μm. *$P < .05$; **$P < .01$; and ***$P < .001$. CD, celiac disease; EED, environmental enteric dysfunction; HC, healthy control; IEL, intraepithelial lymphocyte; NCD, nonceliac disease.

found that CLDN4 expression increased at villous tips. Sites of increased CLDN4 expression were not associated with epithelial damage.[5] In contrast, expression of OCLN, which is necessary for intraepithelial migration of some lymphocyte subpopulations,[35] was similar in EED and healthy controls but reduced, relative to healthy controls, in celiac disease and nonceliac disease biopsies.

Discussion

In recent years, numerous studies have assessed specimens from children with EED, including intestinal biopsies, feces, and urine. Some of these studies included quantitative analyses of histologic features visible with routine hematoxylin and eosin (H&E) stains, but in nearly all studies, histopathology and limited analyses of protein expression have been assessed subjectively. The data set analyzed here consists of 10 Pakistani children with EED defined, in part, as malnutrition, reduced weight-for-height *z* scores, and failure to respond to ready-to-use therapeutic food.[20] Due to the absence of biopsies from healthy Pakistani children, we used the following 3 distinct cohorts of age− and sex−matched North American children: normal, celiac disease, and nonceliac disease with either villous atrophy or increased numbers of intraepithelial lymphocytes. The last group was included because villous atrophy and intraepithelial lymphocytosis are recognized features of EED. Consistent with previous reports,[22] we found increased numbers of intraepithelial lymphocytes within small bowel biopsies from subjects with EED. Villous blunting in EED was less severe than that in celiac disease.[20] Our demonstration of enhanced expression of absorption−related transport and

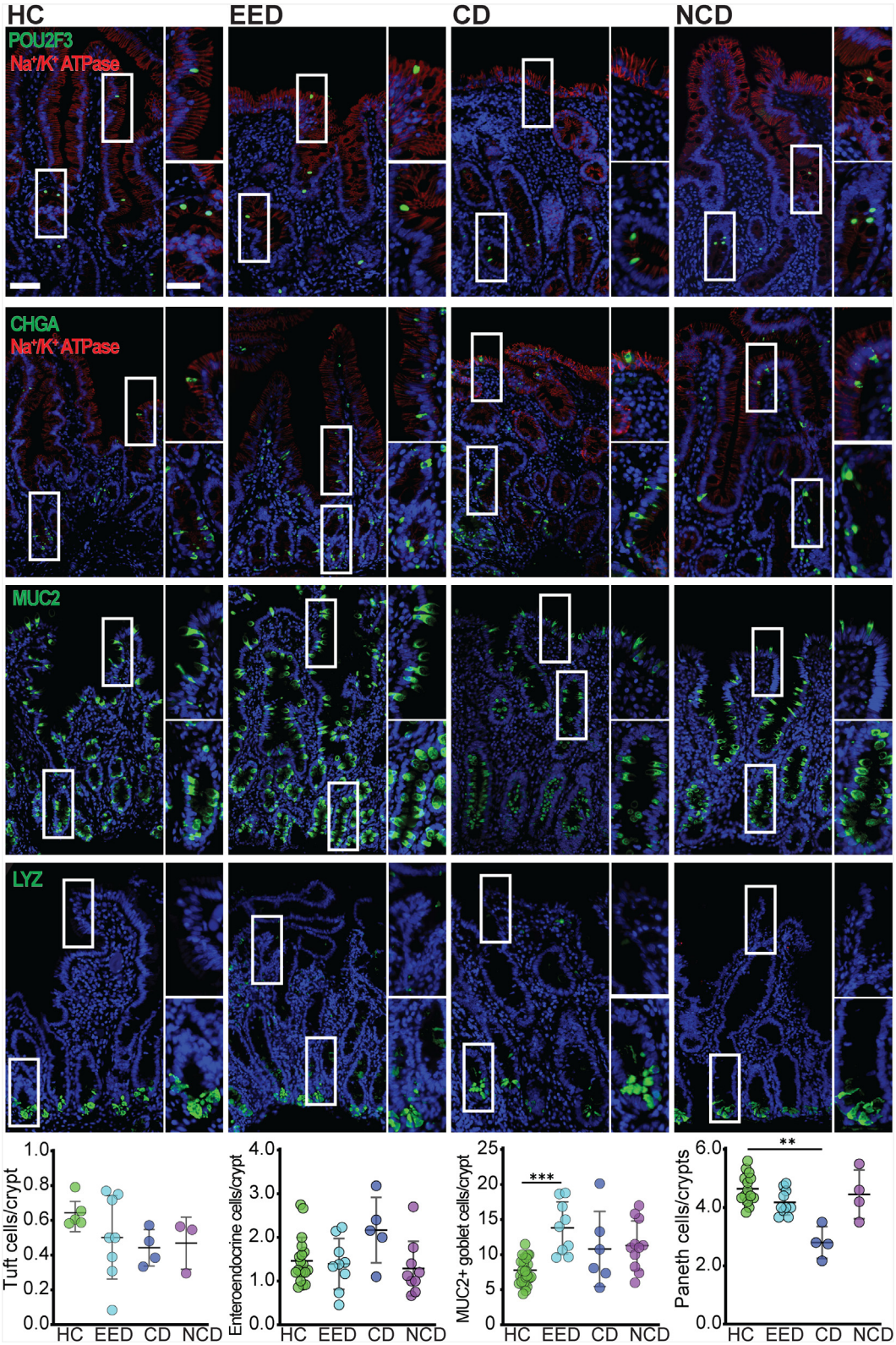

Figure 2.

Numbers of POU2F3−positive tuft cells and CHGA−positive enteroendocrine cells were similar in all groups. MUC2−positive goblet cell numbers were increased in EED relative to all other groups. Data shown are per crypt unit. When quantified per 100 epithelial cells, the numbers of MUC2−positive goblet cells were 14.7 ± 2.2, 19.8 ± 3.4, 16.5 ± 1.5, and 20.0 ± 4.3 for HC, EED, CD, and NCD groups, respectively. MUC2−positive goblet cell numbers in villous atrophy and intraepithelial lymphocytosis groups were 17.0 ± 3.8 and 23.1 ± 2.0, respectively. Goblet cell numbers were significantly increased in EED, NCD, and villous atrophy groups relative to HC. Loss of lysozyme−positive Paneth cells was evident in CD. Bars = 50 and 20 μm. *$P < .05$; **$P < .01$; ***$P < .001$.

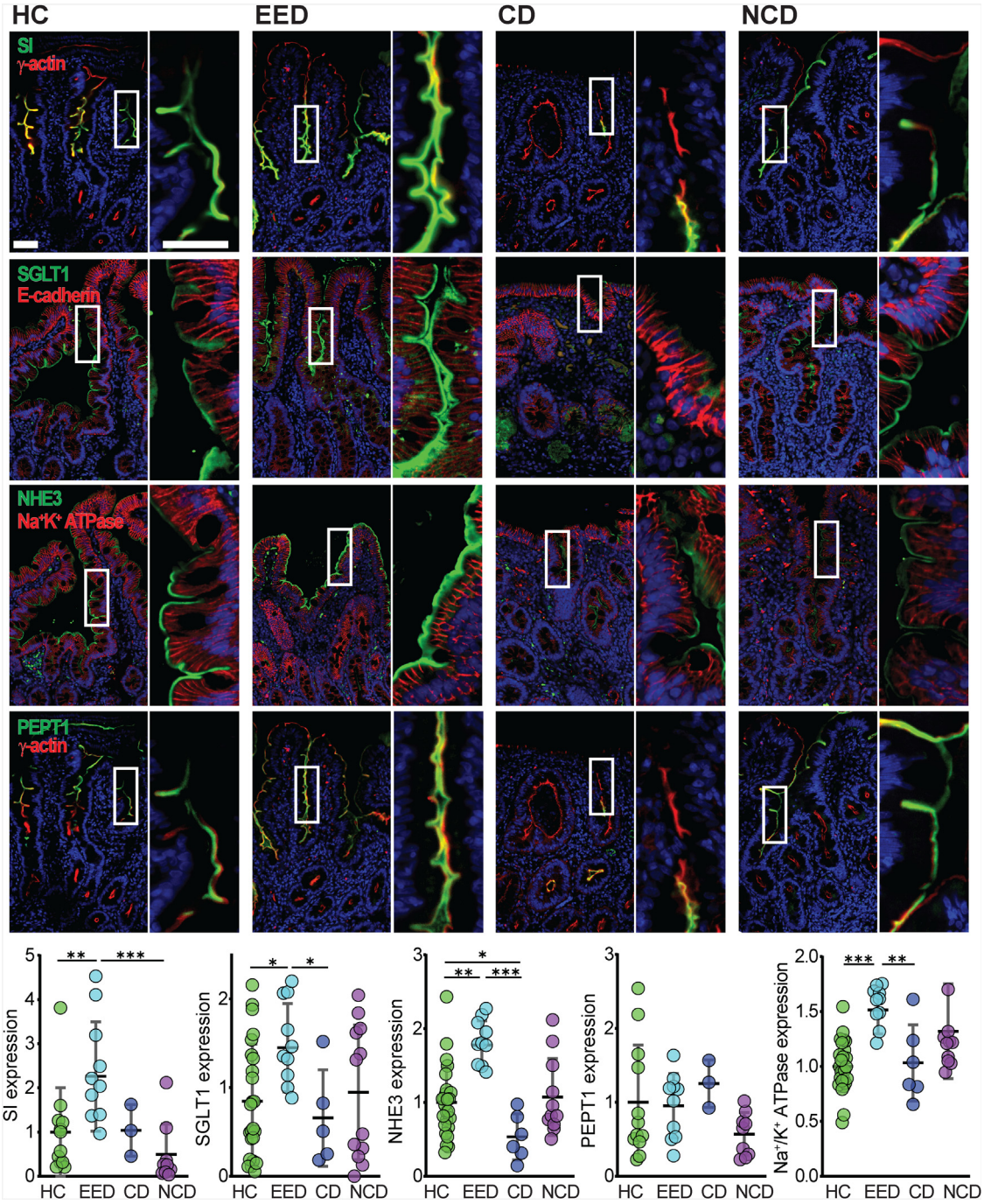

Figure 3.
Expression of the brush border proteins SI, Na^+-glucose cotransporter SGLT1, and Na^+/H^+ exchanger NHE3 (SLC9A3) was increased in EED, as was that of Na^+/K^+-ATPase. Conversely, NHE3 was reduced in CD. PEPT1 expression was not different in any group. Bars = 50 and 20 μm. *P < .05; **P < .01; and ***P < .001.

digestive proteins was unexpected, particularly in the context of malnutrition and villous blunting. In contrast, although the expression of absorptive proteins, except NHE3, per villus epithelial cell was not reduced in celiac disease, their overall expression was decreased due to near-total villous atrophy. The data suggest that, in EED, villous epithelial reprogramming leads to adaptive changes that would otherwise be expected to enhance nutrient absorption. These changes, however, fail to ameliorate malnutrition in EED.

In addition to specific proteins, we identified increased numbers of goblet cells in EED. This is consistent with a recent semiquantitative report of a new scoring system that detected increased number of goblet cells in Zambian subjects with EED[24] but another study using the same histologic scoring system failed to detect altered goblet cell numbers in Pakistani EED subjects. This inconsistency could reflect reliance on H&E morphology alone, which can miss goblet cells that have expelled their mucin vacuole, whereas we used immunohistochemistry. We did not

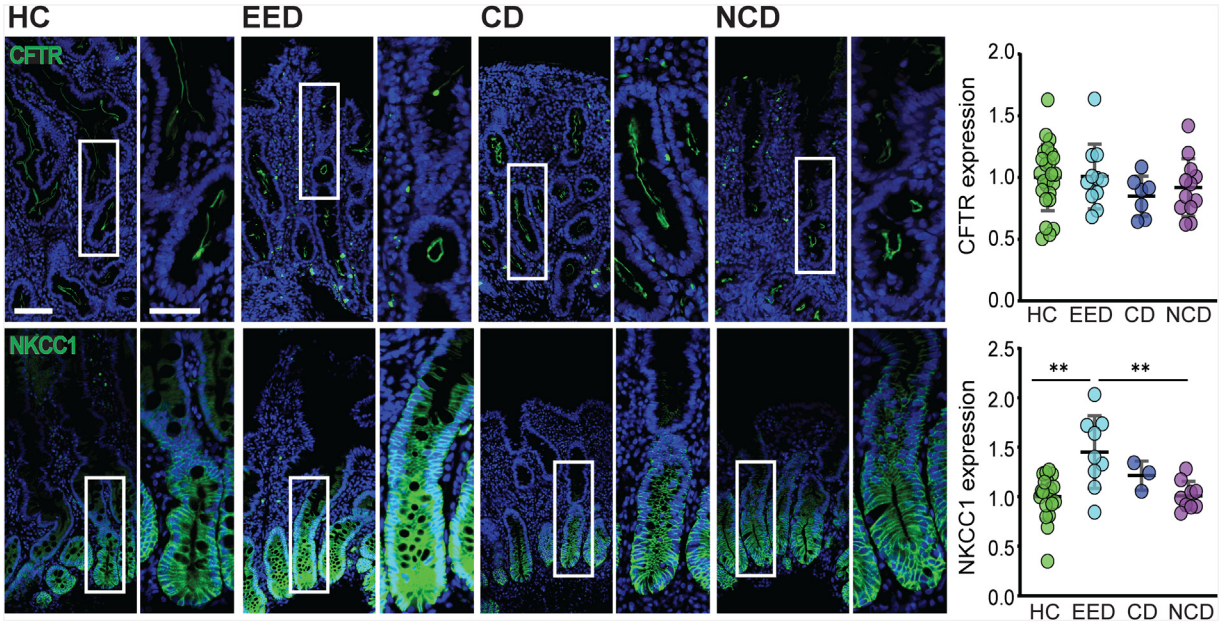

Figure 4.
CFTR expression was unchanged in EED. In contrast, expression of the basolateral Cl^- transporter NKCC1 was increased. Bars = 50 and 20 μm. *$P < .05$; **$P < .01$; and ***$P < .001$.

identify any changes in the number of tuft, enteroendocrine, or Paneth cells or in epithelial proliferation in EED. Previous studies have described either no change or reduced numbers of Paneth cells[25,36,37] but also relied on the histological presence of Paneth cell granules, which can be disrupted during processing.

Our data suggest that EED induces adaptive responses to counteract malabsorption and barrier dysfunction. It is, therefore, surprising that these changes did not restore nutrient absorption. Importantly, our data concur with prior work showing reduced SGLT1 and NHE3 expression in celiac disease. Although previous studies have not assessed Na^+/K^+-ATPase expression in either celiac disease or EED, it is notable that one study found that methylation of both *ATP1A1* and *ATP1B1*, which encode intestinal epithelial Na^+/K^+-ATPase, was altered in biopsies from EED subjects, relative to healthy North American controls; however, no transcriptional changes were detected.[4] Finally, our data demonstrate marked morphological and molecular differences between celiac disease and EED. It would be interesting for future studies to determine if the upregulation of SI, SGLT1, NHE3, and Na^+/K^+-ATPase can serve as a prognostic marker of responses to nutritional therapy.

All EED cases included in this study had a history of persistent diarrhea,[20] which could correlate with increased expression of NKCC1 and Na^+/K^+-ATPase, both of which are required for intestinal epithelial Cl^- secretion. We did not, however, detect increases in CFTR expression. While CFTR channel activation, without expression changes, could synergize with NKCC1 and Na^+/K^+-ATPase upregulation to drive net fluid secretion, it is also possible that other apical Cl^- transport pathways, eg, Ca^{2+}—activated Cl^- channels, are involved. Some studies have suggested that the Ca^{2+}—activated Cl^- channel may be TMEM16A/ANO1, but *TMEM16A* transcripts and TMEM16A protein are not present in intestinal epithelial cells.[38] We, therefore, cannot rule out changes in apical Ca^{2+}—activated Cl^- channel expression or activity in EED. Nevertheless, these data show that, despite inflammation, diarrhea in EED is not due to reduced NHE3 expression, as this was increased in EED. Our data do not refer to the underlying mechanism, but it is possible that this represents an adaptive response to net fluid secretion.

Because EED is characterized by epithelial barrier loss, we analyzed expression of intestinal epithelial tight junction proteins. Importantly, the expression of CLDN2, which is exquisitely responsive to inflammatory stimuli, was unaffected. Although *CLDN2* transcription in EED subjects has been reported to be increased relative to North American controls, the difference observed is more likely explained by the significantly younger age of EED cases, relative to controls, in that study, as intestinal epithelial CLDN2 expression is progressively downregulated after birth.[4,39] We did not detect changes in the expression of the other intestinal tight junction Na^+ channel—forming protein, CLDN15, despite reports of CLDN15 protein and *CLDN15* mRNA in the urine and feces of subjects with EED, respectively.[4,19]

In this set of Pakistani cases with EED, only CLDN4 expression was increased, consistent with a previous report on Zambian cases with EED.[5] CLDN4 has typically been thought of as a barrier—forming CLDN, and its upregulation may therefore represent a response to barrier loss in EED. However, this requires further study, as *Cldn4* knockout in mice and cell lines does not affect epithelial barrier function.[40-42] More importantly, increased CLDN4 expression suggests that increased tight junction permeability may not be the cause of barrier loss in EED. One alternative possibility is that EED—associated barrier loss is secondary to epithelial damage. However, the absence of increased epithelial proliferation, which is a sensitive marker of low-grade damage, does not support this hypothesis.

The major limitation of this study is the absence of healthy controls from the same environment as subjects with EED. This is common to all previous studies of EED, as practical and ethical considerations have prevented the biopsy of these children without a medical indication. As a result, previous studies have relied on biopsies from non—age-matched American or British children or adults or, in some cases, have not included any healthy controls.[4,18,19,22,24,43-45] Unfortunately, expression of transport and

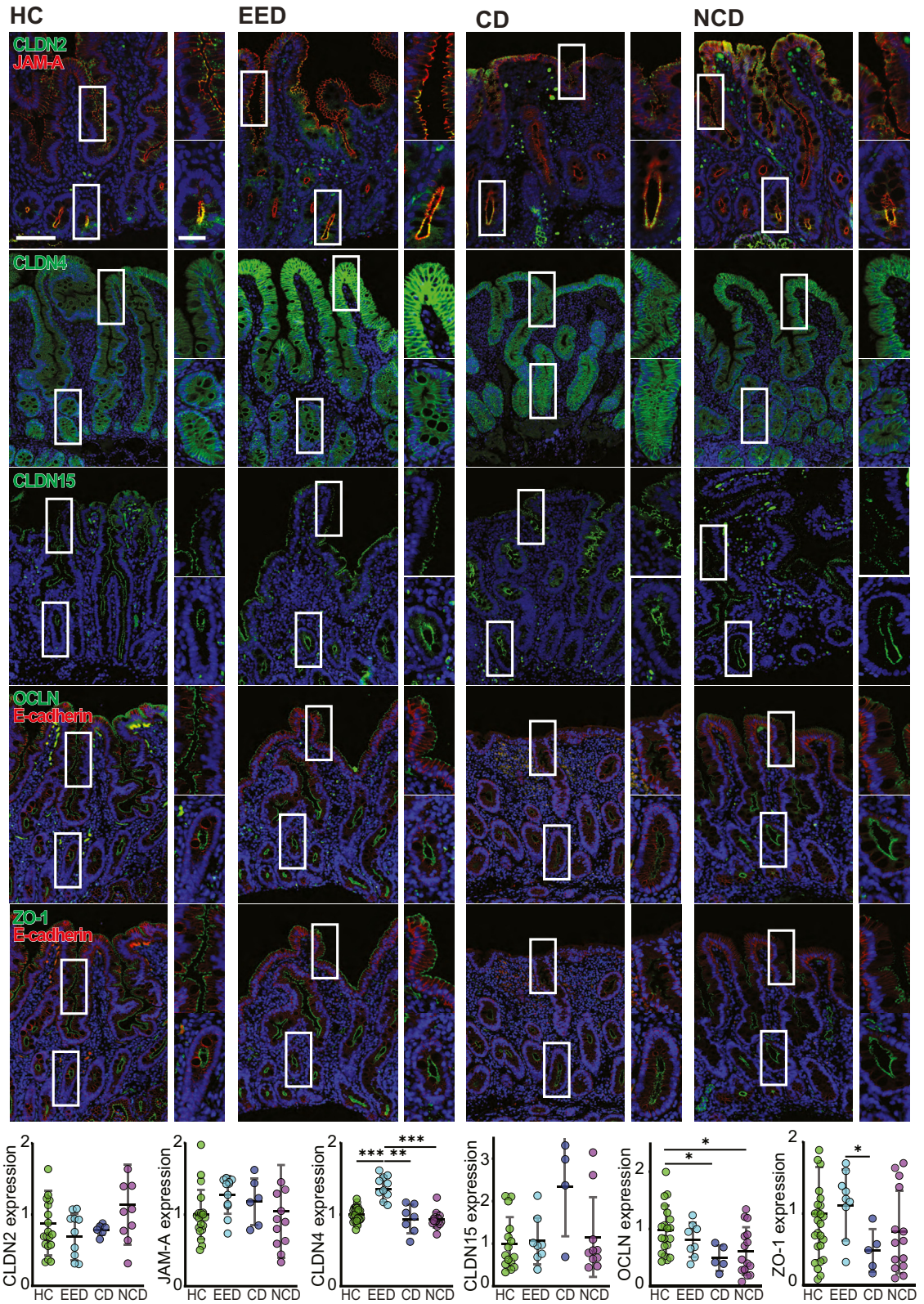

Figure 5.

Expression of barrier–forming CLDN4 increased in EED. Among the tight junction proteins examined, only CLDN4 displayed EED–associated expression changes. Increased CLDN4 expression was particularly evident in the upper half of villi. In contrast, expression of the pore–forming CLDN2 and CLDN15 and that of the leak pathway regulatory proteins OCLN, ZO-1, and JAM-A were unchanged. Similarly, E-cadherin expression was unchanged in EED. OCLN expression was reduced in CD. Bars = 50 and 20 μm. *$P < .05$; **$P < .01$; and ***$P < .001$.

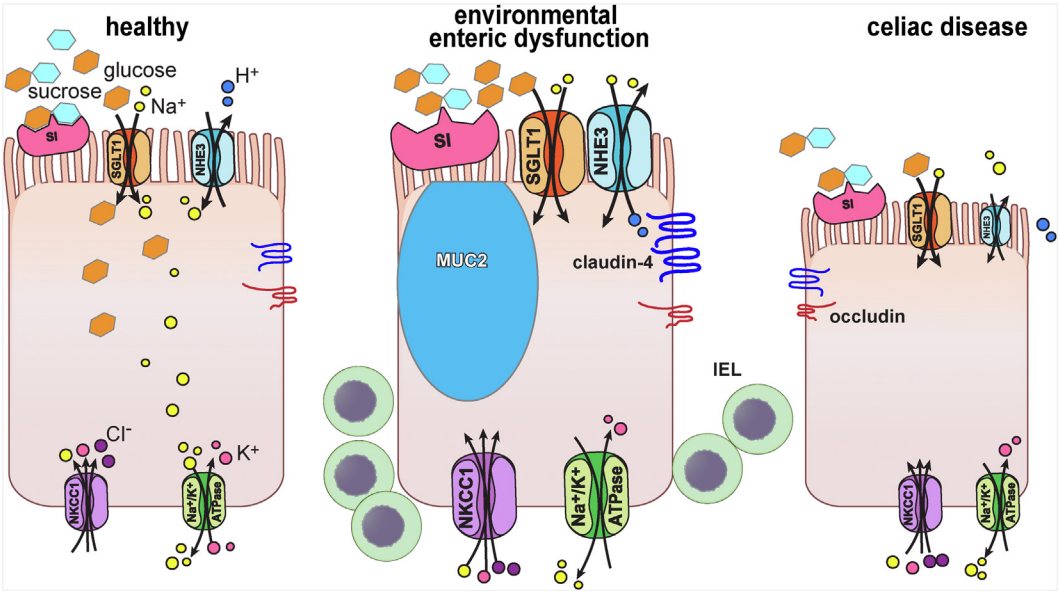

Figure 6.
Comparisons of protein expression in biopsies from healthy subjects and those with EED or CD. The cell on the left represents healthy physiology. SI breaks sucrose into glucose and fructose. Glucose is transported across the apical brush border by the Na^+-glucose cotransporter SGLT1 (SLC5A1). Glucose is then used as an energy source by the cell or exits the cell via basolateral GLUT2 (SLC2A2, not shown). Similarly, NHE3 (SLC9A3) takes advantage of the extracellular-to-intracellular Na^+ gradient to transport H^+ across the apical membrane from the cytoplasm to the lumen. In both cases, Na^+ is then pumped across the basolateral membrane by Na^+/K^+-ATPase. NKCC1 (SLC12A2), also located on the basolateral membrane, transports Na^+, K^+, and Cl^- into the cell, thereby providing the driving force for apical Cl^- secretion. SI, SGLT1, NHE3, NKCC1, and Na^+/K^+-ATPase are all upregulated in EED, as is the barrier–forming tight junction protein CLDN4. Increased numbers of MUC2–positive goblet cells and intraepithelial lymphocytes (IELs) are also present. Although, like EED, IELs are increased in CD, NHE3 and OCLN are downregulated on a per-cell basis. The loss of absorptive epithelial cells in CD due to near-total villous atrophy results in reduced overall absorptive protein expression.

barrier proteins, eg, SGLT1 and CLDN2, is regulated during development, in response to immune stimuli, and by diet and the microbiome.[18,39,46,47] Another limitation is that we only examined duodenal biopsies, and, therefore, cannot exclude contributions by lesions in the distal small intestine or colon. As a minor point, we noted that several study patients were infected with *Giardia*. Subgroup analysis showed that changes in the expression of the proteins studied were not associated with *Giardia* infection, but it is possible that other enteric or systemic infections may have had unrecognized effects.

As a whole, these quantitative analyses failed to explain the malabsorption and diarrhea that characterize EED. The expression data would, in contrast, be more consistent with increased of ions, nutrients, and water absorption (Fig. 6). From this perspective, intestinal epithelial cell upregulation of transport and barrier protein expression might be an appropriate compensatory response to malabsoprtion that is, nevertheless, inadequate to resolve malabsorption and diarrhea. The data further suggest that descriptive studies of gene and protein expression, including this work, will not elucidate the pathophysiology of EED and that functional analyses of intestinal transport and barrier functions will be critical to the mechanistic understanding of EED.

Author Contributions

S.A.A. and J.R.T. conceived the study and acquired funding for the study. S.A. and J.R.T. performed formal analysis. S.A., A.S., X.H., S.D.C.P., K.A., K.S., N.T.I., S.A.A., and J.R.T. performed the investigation. S.A., A.S., J.T.R., and J.R.T. designed the methodology. K.A., K.S., N.T.I., S.A.A., and J.R.T. procured the resources. S.A., A.S., J.T.R., X.H., S.D.C.P., K.A., K.S., N.T.I., and S.A.A. contributed to the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Data Availability

Primary data are available upon request.

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Declaration of Competing Interest

J.R.T. is a consultant for Entrinsic and Kallyope. All other authors declare no competing interests.

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.labinv.2022.100036

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