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Human Enterovirus Species B in Ileocecal Crohn's Disease

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OBJECTIVES: Advanced ileocecal Crohn's disease (ICD) is characterized by strictures, inflammation in the enteric nervous system (myenteric plexitis), and a high frequency of *NOD2* mutations. Recent findings implicate a role of *NOD2* and another CD susceptibility gene, *ATG16L1*, in the host response against single-stranded RNA (ssRNA) viruses. However, the role of viruses in CD is unknown. We hypothesized that human enterovirus species B (HEV-B), which are ssRNA viruses with dual tropism both for the intestinal epithelium and the nervous system, could play a role in ICD.

METHODS: We used immunohistochemistry and *in situ* hybridization to study the general presence of HEV-B and the presence of the two HEV-B subspecies, Coxsackie B virus (CBV) and Echovirus, in ileocecal resections from 9 children with advanced, stricturing ICD and 6 patients with volvulus, and in intestinal biopsies from 15 CD patients at the time of diagnosis.

RESULTS: All patients with ICD had disease-associated polymorphisms in *NOD2* or *ATG16L1*. Positive staining for HEV-B was detected both in the mucosa and in myenteric nerve ganglia in all ICD patients, but in none of the volvulus patients. Expression of the cellular receptor for CBV, CAR, was detected in nerve cell ganglia.

CONCLUSIONS: The common presence of HEV-B in the mucosa and enteric nervous system of ICD patients in this small cohort is a novel finding that warrants further investigation to analyze whether HEV-B has a role in disease onset or progress. The presence of CAR in myenteric nerve cell ganglia provides a possible route of entry for CBV into the enteric nervous system.

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by segmental, transmural inflammation, and fissuring abscesses. On the basis of clinical features, such as age of diagnosis, location of disease, and stricturing/penetrating behavior, CD is subclassified in several clinical phenotypes.[1,2]

Ileocecal CD (ICD) is a distinctive phenotype, which is characterized by its localization in the ileocecal region of the intestine, and by the fact that it more often than other CD phenotypes leads to strictures, stenosis, perforation, and surgical resection. Another common feature of ICD is lesions in the enteric nervous system.[3,4] Myenteric plexitis is an inflammatory reaction of the enteric nervous system, which is characterized by infiltration of lymphocytes, plasma cells, eosinophilic granulocytes, and mast cells, as well as granulomas.[5] Its widespread presence provides a bad prognostic sign for recurrence of CD after surgery.[5–7] Inflammation in the enteric nervous system can also be detected in areas of the bowel with a noninflamed mucosa, indicating that these lesions may precede mucosal inflammation and provide a neuronal pathway for the spreading of inflammation.[5]

The etiology of CD is unknown but it is considered a polygenic disease, which develops in a complex interplay between environmental factors and aberrant immune responses in a genetically susceptible host. In support of this, a majority of CD susceptibility genes that have been identified, including *NOD2* and *ATG16L1*, have known immune cell functions.[8–10] Recently, these genes were shown to have important roles in the immune response against single-stranded RNA (ssRNA) viruses[11–15] and the ATG16L1 pathway, also a prerequisite for Coxsackie virus replication.[16] NOD2 was shown to act as an intracellular pattern recognizing receptor for ssRNA viruses.[12] Loss of function of both genes may lead to defective autophagy and less effective clearance of ssRNA viruses.[17,18]

These findings suggest that enteropathogenic viruses could have a role in CD.[3] However, recent reports have failed to detect several types of enteropathogenic viruses in fecal samples from patients with CD.[19,20]

On the basis of the specific characteristics of ICD, the recent data showing important roles of *NOD2* and *ATG16L1* in immune responses against ssRNA viruses, and the fact that disease-associated polymorphisms in *NOD2* are particularly common in the ICD phenotype,[21,22] we hypothesized that

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ssRNA viruses with tropism both for the intestinal epithelium and the nervous system may have a role in ICD. In particular, we were interested to study the presence of human enterovirus species B (HEV-B), which belong to the Picornavirus family, in ICD. HEV-B, which consist of several subspecies, including Coxsackie B virus (CBV) and most of the Echoviruses, are ssRNA viruses that can cause mild gastroenteritis and also penetrate into the nervous system causing encephalitis.[23] We therefore investigated the presence of HEV-B in ileocecal resections from pediatric patients with advanced ICD.

METHODS

Patient material. All patients with pediatric onset stricturing ICD, residing in Uppsala County, Sweden, who had undergone ileocecal resection at the Uppsala University hospital between 1997 and 2010 were identified through medical registers ($n=9$). All nine patients were retrospectively included after informed consent, five boys/men and four girls/women, age at diagnosis 11.8 (8.5–15.5) years, age at surgery 17.0 (9–24.8) years, and duration from diagnosis to surgery 3.9 (0–9.3) years. In addition, the resection margins from surgical specimen from patients who had undergone surgery owing to intestinal volvulus were included ($n=6$), two men and four women, age at surgery 66.3 (43–74) years. All patients, but one (patient 7), were under immunosuppressive therapy at the time point of surgery (Supplementary Table S3).

The surgically removed specimen were fixed in 4% buffered formaldehyde, paraffin-embedded, sectioned (3 mm), and stained with hematoxylin and eosin according to standard procedures. All sections were re-evaluated by a gastrointestinal pathologist. Several full thickness blocks from each formalin-fixed specimen were chosen for further investigation. At least two of the blocks included highly inflamed regions in the intestine of CD patients and one block from the proximal intestinal resection margin represented a lower inflamed region of each patient.

Lower endoscopies were performed under general anesthesia by the use of endoscopes with 2.0 or 2.8 mm work channels sized for pediatric or adult usage, respectively. In this study, biopsies were collected from terminal ileum or colon cecum and, in addition, three different locations of the colon (right, transverse, and left colon). Tissue samples obtained from biopsies were fixed in formaldehyde, paraffin-embedded, sectioned (3 μm), and stained with hematoxylin and eosin according to standard procedures.

Endoscopical biopsies from the time point of diagnosis from six of these nine patients were available and retrospectively included. In five cases, no treatment has been given at the time point of endoscopy; only one patient received an anti-inflammatory treatment. In addition, biopsies from nine further patients without any anti-inflammatory or immunosuppressive treatment were included retrospectively after informed consent, five boys/men and four girls/women, age at endoscopy 14.0 (9–16) years (Supplementary Table S3).

Immunohistochemistry and immunofluorescence staining. We deparaffinized tissue sections from the biopsies in xylene and rehydrated them in graded alcohols according to standard procedures. Sections were heated in Tris EDTA buffer, pH 9 (DAKO, Glostrup, Denmark, S2367) in a microwave oven at 750 W for 10 min, followed by 350 W for 15 min for antigen retrieval. The slides were allowed to cool for 20 min and washed in distilled water. The tissue sections were blocked and stained by immunohistochemistry (IHC) or immunofluorescence techniques with antibodies against the following human viruses: enterovirus, CBV, and Echovirus, and the virus receptor CAR (Supplementary Table S1). We used human pancreatic islets infected *in vitro* with an enterovirus as a positive control and uninfected pancreatic islets as a negative control, primary antibodies were omitted, and, in addition, an irrelevant primary polyclonal antibody against *Helicobacter pylori*, a nonhuman protein, was used.

IHC was visualized by the use of Dako REAL Envision Peroxidase/DAB detection system (DAKO) and hematoxylin counterstaining. For immunofluorescence staining, sections were blocked in 5% goat serum and 2% bovine serum albumin in phosphate buffered saline 0.2% Tween, followed by incubation with primary antibodies overnight at 4 °C and secondary antibodies (1:200) for 1 h at room temperature. The slides were mounted in mounting solution with DAPI (4',6-diamidino-2-phenylindole, Vectorshield; Vector Laboratories, Burlingame, CA) to counterstain cell nuclei. Imaging was performed with confocal laser scanning microscopy (LSM 700; Zeiss AB, Stockholm, Sweden).

Semiquantification of the presence of HEV-B in surgical specimens. The IHC sections were assessed in a semiquantitative manner. Regarding viruses within the epithelium of intestinal crypts, weak or negative cytoplasmic staining was defined as $+/-$, moderate staining as $++$, and intense staining was defined as $+++$. Regarding the virus staining within the myenteric ganglia, a distinct perinuclear granular staining in any of the neuronal cells was judged as positive staining ($+$). In contrast, a strong cytoplasmic staining in most of the neuronal cells within ganglia was regarded as intense staining ($+++$). A weak-to-moderate cytoplasmic staining in most of the neuronal cells or a strong cytoplasmic staining in only a few neuronal cells was judged as moderate positivity ($++$). Representative images of enteric nerve ganglia stained for HEV-B and graded according to the procedure described above are included in Supplementary Figure S2.

Chromogenic *in situ* hybridization. The tissue sections were deparaffinized in xylene and rehydrated in graded ethanol according to standard procedures. The *in situ* hybridization were performed according to IIIB (ISH protocol for detecting mRNA with digoxigenin-labeled oligonucleotide probes) in chapter 5 in 'Non-radioactive *In Situ* Hybridization Applications Manual' provided by Roche Applied Science. Pretreatment of the tissue sections included treatment with 100 mM glycine in phosphate buffered saline, permeabilization with 10 μg/ml of Proteinase K at 37 °C for 30 min, post-fixation in 3.7% paraformaldehyde before acetylation using 0.25% acetic acid in triethanolamine buffer. A digoxigenin-labeled probe (Integrated DNA Technology, Coralville, IA, USA) concentration of 0.25 μM in the hybridization buffer was

used for the tissues and they were incubated overnight in 37 °C. A cocktail of six different enterovirus-specific digoxigenin-labeled probes[24] were used for the virus detection and human β-actin (ACTB) was used as a positive control (Supplementary Table S2). The immunological detection was carried out with the kit 'DIG Nucleic Acid Detection Kit' (Roche Applied Science, Penzberg Germany) according to the included protocol, with a 5-h color development step.

Genetic analyses. Three polymorphisms in *NOD2* (rs2066844, rs2066845, and rs5743292) and one in *ATG16L1* (rs2241880) were genotyped using TaqMan genotyping assays (Applied Biosystems, Foster City, CA) and the 7900 HT Fast Real Time PCR system (Applied Biosystems) using Absolute QPCR ROX mix (ABGene, Epsom, UK) and 10 ng of DNA per reaction. Thermal cycling consisted of an initial step at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR. Genotypes were assigned using the SDS Software v2.3 (Applied Biosystems).

Ethical considerations. All studies were approved by the regional medical Ethics Board in Uppsala. Written informed consent was given by patients older than 18 years and parents of children, and children older than 12 years of age at the time of the study.

RESULTS

Myenteric plexitis and presence of HEV-B in patients with stricturing ICD. A cohort of nine patients between 8 and 25 years of age with stricturing ICD was included in the study. Patients were diagnosed with ICD at the age of 8–15 years and all underwent ileocecal resection, in one case (patient 7) at the time of diagnosis and in the other cases between 6 months and 9 years after diagnosis (demographic data are summarized in Supplementary Table S3). All patients received medication in terms of various combinations of antibiotics and/or immunosuppressive drugs (Supplementary Table S3).

Genetic analysis showed that three out of nine ICD patients (patients 2, 3, and 4) were heterozygous, whereas one (patient 1) was homozygous for disease-associated missense (rs2066844/rs2066845) or frameshift (rs5743293) mutations in *NOD2* (Table 1). Four out of the nine patients (patients 1, 3, 5, and 8) were homozygous and four patients were heterozygous (patients 2, 4, 7, and 9) for a disease-associated missense mutation (rs2241880) in *ATG16L1.* One patient (patient 6) had no disease-associated mutations in *NOD2* or *ATG16L1*. Analysis of the surgical specimens revealed the presence of mucosal inflammation and myenteric plexitis (Figures 1a and b). In comparison, no signs of mucosal inflammation or myenteric plexitis were observed in resection margins from the ileocecal region of patients who had undergone surgery due to intestinal volvulus (Figure 1c). To study the presence of HEV-B we stained these surgical specimens by IHC using antibodies specific for CBV and Echovirus. Positive cytoplasmic immunostaining for CBV was detected in crypt epithelial cells of the mucosa, and in both

Table 1 Data from genetic analysis of CD-associated polymorphisms in *NOD2* and *ATG16L1* genes in nine patients with advanced ICD

Patient	*ATG16L1*	*NOD2*		
	rs2241880 (A/G) (missense mutation)	rs5743293 (– /C) (frameshift mutation)	rs2066844 (C/T) (missense mutation)	rs2066845 (C/G) (missense mutation)
1	GG	–	TT	GG
2	AG	–	CC	CG
3	GG	– /C	CC	GG
4	AG	–	CT	GG
5	GG	–	CC	GG
6	AA	–	CC	GG
7	AG	–	CC	GG
8	GG	–	CC	GG
9	AG	–	CC	GG

CD, Crohn's disease; ICD, ileocecal Crohn's disease.

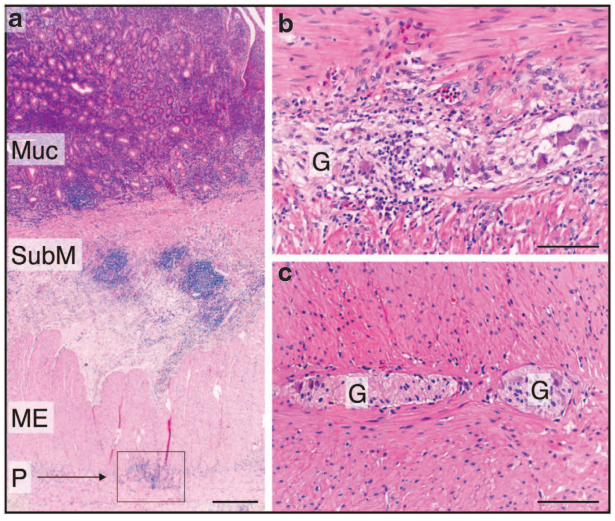

Figure 1 Myenteric plexitis in patients with ileocecal Crohn's disease (ICD). (**a**) Representative image of an hematoxylin and eosin-stained transmural section from the ileocecal region of a patient with ICD, showing inflammation in both the mucosa (Muc) and in the enteric nervous system (myenteric plexitis (P)) within the muscularis externa (ME) layer of the submucosa (SubM). (**b**) Representative high-magnification image showing plexitis in a myenteric ganglia (G) in the ileocecal region of a patient with ICD. (**c**) Representative high-magnification image showing no signs of P in a volvulus patient. Bars = (**a**) 500 μm; (**b**, **c**) 100 μm.

neurons and glial cells of myenteric ganglia in patients with ICD (Figures 2a and b; Supplementary Figures S1a–c). No positive staining for CBV was detected in surgical specimens from volvulus patients (Figures 2c and f). High-magnification images revealed a granular, perinuclear staining pattern of CBV in the nerve cell ganglia in ileocecal resections from patients with ICD (Figure 2d). A similar type of staining was detected for Echovirus in crypt epithelial cells of the mucosa (data not shown) and in myenteric ganglia (Figure 2e) in patients with ICD, but not in volvulus patients (Figure 2g).

Next we were curious to see whether HEV-B could be detected in the intestinal tract of ICD patients not only in advanced disease but also at the time of diagnosis, and thus

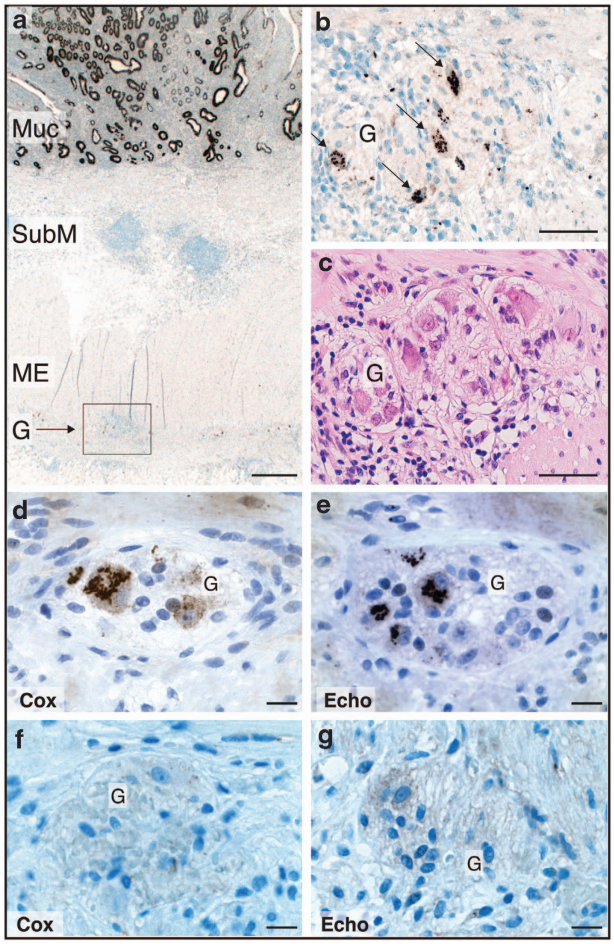

Figure 2 Detection of human enterovirus species B (HEV-B) in patients with ileocecal Crohn's disease (ICD) by immunohistochemistry (IHC). (**a**) Representative image of a transmural section of the ileocecal region of a patient with ICD, stained by IHC with an antibody specific for Coxsackie B virus (CBV). Positive staining for CBV was detected both in the crypt epithelium of the mucosa (Muc) and in myenteric ganglia (G) within the muscularis externa (ME) layer of the submucosa (SubM). Representative images showing (**b**) positive, perinuclear staining of CBV in G of a patient with ICD and (**c**) myenteric plexitis on an hematoxylin and eosin-stained section. Representative high-magnification images showing positive, perinuclear staining of (**d**) CBV and (**e**) Echovirus in G of a patient with (**d**, **e**) ICD and (**f**, **g**) negative staining in a patient with volvulus. Bars = (**a**) 500 μm; (**b**, **c**) 100 μm; (**d**–**g**) 20 μm. Cox, coxsackie B Virus; Echo, Echovirus.

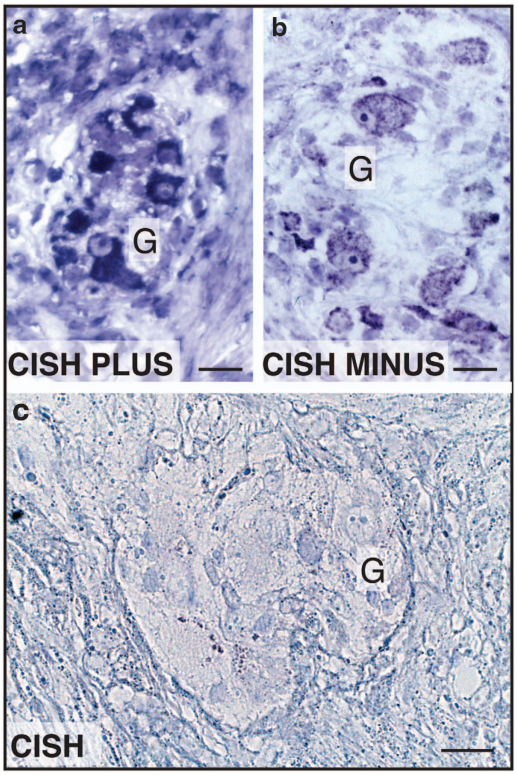

Figure 3 Detection of human enterovirus species B (HEV-B) in patients with ileocecal Crohn's disease (ICD) by chromogen *in situ* hybridization (CISH). (**a**–**c**) Representative high-magnification images showing (**a**, CISH PLUS) positive, perinuclear CISH staining for HEV-B virus template, (**b**, CISH MINUS) weaker staining for the replication template, and (**c**) negative CISH staining in a patient with volvulus. Bar = 20 μm.

before treatment with various immunosuppressive drugs. Using an antibody (VP1) that recognizes a large range of HEV species[25] we found positive staining for HEV-B in intestinal epithelial cells of colon biopsies from those six ICD patients that were included in these analyses (Supplementary Figure S3; Supplementary Table S4). In addition, positive staining for HEV was detected in intestinal epithelial cells of colon biopsies from another nine CD patients at the time of diagnosis (patients 10–18; Supplementary Table S4). Positive staining for CVB and Echovirus was detected in 4 of 15 CD patients at the time of diagnosis. One of these patients (patient 2) had been pretreated with Mesalazine.

Replication of HEV-B in myenteric ganglia in patients with ICD. To verify the positive staining for CBV and Echovirus in patients with ICD, we performed chromogen *in situ* hybridization (CISH) using probes recognizing a broad range of HEV-B. Positive staining for HEV-B (positive-stranded RNA) was detected in myenteric ganglia (Figure 3a), which further supported the presence of HEV-B in the enteric nervous system in patients with ICD. The replication template (negative-stranded RNA) was also positive in ICD patients, but was weaker than the virus template (Figure 3b), suggesting that virus replication occurred at a slow rate. These findings were further supported by positive immunostaining for double-stranded RNA which forms during viral replication, and the interferon-α-induced enzymes oligoadenylate synthetase and protein kinase R, both induced during virus infection (Supplementary Figure S1d–f). No positive signal for HEV-B by CISH was detected in ileocecal resections from patients with volvulus (Figure 3c).

Summarized data from IHC and CISH analysis revealed the presence of myenteric plexitis in all patients with ICD, but in none of the volvulus patients (Table 2). Positive stainings for CBV and Echovirus in both crypt epithelial cells and myenteric ganglia were detected in seven out of nine ICD patients by IHC and in all ICD patients by CISH. Staining for CBV was negative in all volvulus patients. Staining for Echovirus was positive in one out of six volvulus patients by IHC, but not by CISH.

Table 2 Summarized data from IHC and CISH analyses of the presence of myenteric plexitis and HEV-B in nine patients with advanced ICD and six patients with volvulus

Patients		IHC				CISH
	Plexitis	CBV, c	CBV, g	Echo, c	Echo, g	HEV-B
ICD patients						
1	Present	3	2_3	3	2	2_3
2	Present	3	3	3	3	2
3	Present	3	3	2_3	3	2
4	Present	1_2	3	1_2	3	2_3
5	Present	3	2_3	2_3	2	1_2
6	Present	3	3	2_3	2_3	2
7	Present	3	1_2	3	1_2	2
8	Present	0	0	0	0_1	2_3
9	Present	0	0_1	0	0_1	1_2
Volvulus patients						
1	Absent	0	1	0	0_1	0_1
2	Absent	0	0	2	1_2	0
3	Absent	0	0_1	0	0_1	0
4	Absent	0	0	0*	0	0_1
5	Absent	0	0_1	0	0	0_1
6	Absent	0	1	0	0_1	0

c, crypt epithelium; CBV, Coxsackie B virus; CD, Crohn's disease; CISH, chromogen *in situ* hybridization; g, ganglia; ICD, ileocecal Crohn's disease; IHC, immunohistochemistry.

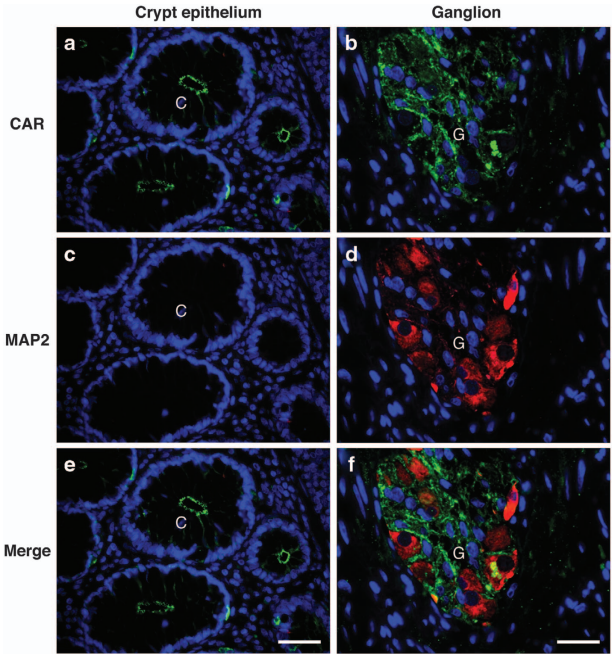

Figure 4 Immunofluorescence analysis of the expression of the Coxsackie- and adenovirus receptor (CAR) in the mucosa and submucosa of the ileocecal region in patients with ileocecal Crohn's disease (ICD). Representative high-magnification images showing positive staining for CAR in (**a**) tight junctions and (**c**) negative staining for microtubule-associated protein 2 (MAP2), a marker for nerve cell ganglia, in crypt epithelial cells (C) of the mucosa (**e** represents merged image of **a** and **c**). Representative high-magnification images showing positive membrane staining for (**b**) CAR and (**d**) positive cytoplasmic staining for MAP2, in myenteric ganglia (G) of the submucosa (**f** represents merged image of **b** and **d**).

Presence of the Coxsackie- and adenovirus receptor (CAR) in myenteric ganglia. Because of the presence of CBV in myenteric ganglia we were curious to find out whether the receptor for CBV, CAR, is expressed in myenteric ganglia. Immunofluorescence staining of ileocecal resections and subsequent confocal microscopy revealed positive staining for CAR in both crypt epithelial cells and in myenteric ganglia (Figure 4). The staining pattern of CAR in ganglia was membranous and was observed in both neurons, which was verified by costaining for the neuron-associated microtubule-associated protein 2, and in glial cells. These results provide a mechanism by which CBV could enter into the enteric nervous system.

DISCUSSION

In this study we investigated the presence of HEV-B in ileocecal resections from a cohort of nine patients with characteristic features of advanced ICD, including strictures and inflammation, both in the mucosa and in the enteric nervous system (myenteric plexitis). We found that all but one ICD patient had disease-associated polymorphisms in *NOD2* or *ATG16L1*, or in both these genes. Three patients had heterozygous mutations in *NOD2* and one patient carried a homozygous mutation. Heterozygous mutations in these specific single-nucleotide polymorphisms of *NOD2* are associated with an increased risk of developing CD, which is even higher in patients with homozygous or compound heterozygous mutations.[22,26] In comparison, it was reported that *NOD2* mutations are rare in Swedish children with CD (8.6%), and that they are exclusively heterozygous.[27] The high frequency of NOD2 mutations observed in our study group may reflect that patients with ICD were specifically selected and that NOD2 mutations are most strongly associated with this CD variant.[21,22] Four patients were homozygous (GG) for the risk allele in *ATG16L1*, which is associated with a more than threefold elevated risk of developing CD compared with heterozygous mutations.[28]

In agreement with our hypothesis we found significant presence of CBV and Echovirus, two species of HEV-B, in ileocecal resections from all patients with ICD. The presence of virus was detected by IHC using antibodies recognizing various subspecies of CBV and Echovirus, respectively, and validated by the positivity for viral footprints. In addition, the presence of virus was detected at the RNA level through *in situ* hybridization. The two patients (patients 8 and 9) that were almost negative for virus detection, at least by IHC analysis, were, similar to most other patients, treated with antibiotics and immunosuppressive drugs before surgery. On the other hand, the presence of HEV-B was readily detected in one patient (patient 7) who underwent surgery at the time of diagnosis and, therefore, had not been pretreated with any immunosuppressive drugs.

Regarding the endoscopical biopsies that were taken at the time of diagnosis, all patients displayed a clear positivity for HEV, whereas specific positivity for CVB and Echovirus could only be seen in four of these 15 patients. These results demonstrated the presence of various subtypes of HEV in the intestinal tract of CD patients already at the time of diagnosis, indicating that virus presence was not primarily an effect of immunosuppressive treatment. However, it should be noted that one of the ICD patients in which positive staining for CVB

and Echovirus was detected in biopsies had, similar to most patients at time of surgery, been pretreated with immunosuppressive drugs (patient 2). Thus, it is possible that immunosuppressive treatment could exaggerate or promote virus infection or persistency in ICD patients with a given genetic background. Further studies are warranted to elucidate which subspecies of HEV are present in the intestinal tract of CD patients, to what extent virus presence is different from children not suffering from CD, and to what extent immunosuppressive treatment affects virus persistence.

We consider the fact that we were able to detect HEV-B with a combination of different methods a main strength of the study. The fact that no positive staining for HEV-B was detected in patients suffering from volvulus further strengthened the specificity of the positive results in the ICD group. On the other hand, we are aware that volvulus patients may not represent an optimal choice of control group. However, because of ethical reasons it was not possible to study surgical specimens from healthy children of the same age group. This, together with the fact that we studied a rather low number of ICD cases, represents weaknesses of this study.

HEV-B was detected in both epithelial cells and in nerve cell ganglia, which is in line with the tropism of HEV-B for both these cellular compartments. We also found that the cellular receptor for CBV, CAR, is expressed both in enteric epithelial cells and in nerve cells within myenteric ganglia. These data provide a possible mechanism of CBV entry into the enteric nervous system and are in line with published data showing that CAR is expressed in intestinal epithelial cells, where it localizes to tight junctions,[29] and in the central nervous system and peripheral nerves during mouse development.[30] It will be of interest for the future to perform more detailed studies to elucidate which subspecies of HEV-B are most abundant in patients with inflammatory bowel disease and also which receptors that can mediate virus entry into the enteric nervous system.

Our findings support the idea that impaired function of *NOD2* or *ATG16L1* leads to impaired clearance of ssRNA viruses. However, in some of the patients we did not observe a direct correlation between the presence of HEV-B and genotype. This may reflect that other factors, both genetic and environmental, affect virus infection and persistence. More large-scale studies are needed to determine whether polymorphisms in *NOD2* and/or *ATG16L1* are specifically linked to impaired clearance and persistence of HEV-B in patients with ICD. Our findings showing perinuclear distribution of HEV-B in nerve cells and positive staining for the negative RNA virus strand indicate that viruses entering into the enteric nervous system may be able to replicate there. In relation to this, it has been reported that HEV-B can replicate, although at a slow rate, in nondividing cells, such as neurons.[31,32] Furthermore, HEV-B may establish latent infections in human myocytes[33] and persist as dsRNA.[34] Moreover, they have been shown to be able to initiate a chronic state of inflammation in the nervous system.[35] Together with our data, this opens the possibility that HEV-B infections may persist in the enteric nervous system and be reactivated under certain conditions. This could explain relapses in CD, a characteristic phenomenon of the disease. On the other hand, it cannot be ruled out that our findings reflect *de novo* secondary HEV-B infections of the mucosa and the enteric nervous system in ICD patients with a malfunctioning immune response against ssRNA viruses.

It is possible that persistent or relapsing infections with HEV-B in the gastrointestinal tract might contribute to a dysfunctional intestinal barrier. Impaired mucosal barrier function is linked to loss or altered expression of tight junction proteins together with an increased paracellular permeability—a hallmark of CD.[36–38] A mechanism by which CVB infections could promote increased paracellular permeability is through their use of CAR as a virus receptor for attachment and entry into the host.[39,40] CAR is a tight junction-associated transmembrane protein, and upon virus infection CAR is internalized and depleted from tight junctions, which leads to increased paracellular permeability.[41]

Persistent infections with HEV-B may also have consequences for the neuromuscular function of the enteric nervous system. Myenteric plexitis has been shown to correlate with enteric dysmotility.[42] It is tempting to speculate that virus-infected neurons and glial cells can lead or contribute to an impaired neuromuscular motility as observed in CD.[43]

In conclusion, the results from this study show, for the first time, significant presence of HEV-B in the mucosa and enteric nervous system of patients with ICD. Our data should be put in perspective of what others have found and discussed in terms of a role of interplay between mutations in genes encoding proteins involved in innate viral immunity and autophagy, and the presence of microorganisms, both viruses and bacteria, as a triggering factor of CD.[8] Further studies are warranted to elucidate the presence of HEV-B in larger cohorts of ICD patients, and whether HEV-B has a role in the etiology of ICD. Optimally, such studies should be of larger scale and include control groups matched for age and treatment with immunosuppressive drugs. Further, it will be of importance to evaluate whether HEV-B is associated with ICD, not only in advanced disease but also at disease onset.

CONFLICT OF INTEREST

Guarantor of the article: Alkwin Wanders, MD, PhD.
Specific author contributions: Study concept and design: N.N., G.F., U.G., Y.F., J.F., A.W.; acquisition of data: N.N., T.B., E.L., O.S., I.H., I.J.-P., M.N., U.G., J.F., A.W.; analysis of data and drafting of the manuscript: N.N., G.F., U.G., Y.F., J.F., A.W.; funding: A.W. and J.F.; study supervision: A.W.; review of draft: all authors.
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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- Ileocecal Crohn's disease (ICD) is associated with myenteric plexitis and polymorphisms in *NOD2*.
- The gene product of NOD2 is implicated in clearance of single-stranded RNA (ssRNA) viruses.
- Human enterovirus species B (HEV-B) are ssRNA viruses with tropism for both epithelial and neuronal cells.
- The role of HEV-B in ICD is unknown.

WHAT IS NEW HERE

- HEV-B were commonly detected in patients with advanced ICD and at the time point of diagnosis.
- HEV-B were present in the enteric nervous system.
- The CAR receptor was present in the enteric nervous system providing a route of virus entry.

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