Human Enterovirus Species B in Ileocecal Crohn’s Disease

Niklas Nyström, MD1, Tove Berg, PhD2, Elin Lundin2, Oskar Skog, PhD3, Inga Hansson, PhD3, Gun Frisk, PhD3, Ivana Juko-Pecirep3, Mats Nilsson, PhD3, Ulf Gylensten, PhD3, Yigael Finkel, MD, PhD4, Jonas Fuxe, PhD2,5 and Alkwin Wanders, MD, PhD3,5

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 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 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.

Clinical and Translational Gastroenterology (2013) 4, e38; doi:10.1038/ctg.2013.7; published online 27 June 2013
Subject Category: Inflammatory Bowel Disease

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) viruses11–15 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.15 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 phenotype21,22 we hypothesized that...
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 Coxackie 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 included highly inflamed regions in the intestine of five cases, no treatment has been given at the time point of endoscopy; only one patient received an anti-inflammatory or 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 uninjected 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 cytoplasmatic staining was defined as +/−, moderate staining as ++, and intense staining was defined as ++++. Regarding the virus staining within the myenteric ganglia, a distinct perineurial granular staining in any of the neuronal cells was judged as positive staining (+). In contrast, a strong cytoplasmatic staining in most of the neuronal cells within ganglia was regarded as intense staining (+++). A weak-to-moderate cytoplasmatic staining in most of the neuronal cells or a strong cytoplasmatic 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 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 strictureing ICD.** A cohort of nine patients between 8 and 25 years of age with strictureing 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 mutation (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 cytoplasmatic immunostaining for CBV was detected in crypt epithelial cells of the mucosa, and in both 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...
before treatment with various immunosuppressive drugs. Using an antibody (VP1) that recognizes a large range of HEV species 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 CVB was negative in all volvulus patients. Staining for Echovirus was positive in one out of six volvulus patients by IHC, but not by CISH.
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 confirmed 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 entire 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. In comparison, it was reported that NOD2 mutations are rare in Swedish children with CD (8.6%), and that they are exclusively heterozygous. 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.

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. 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 CBV 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 CBV

| Patients | IHC | CISH |
|----------|-----|------|
| 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 | 0 | 0 | 1,2 |

| Volvulus patients | | |
|-------------------|-----|------|
| 1 | Absent | 0 | 1 | 0 | 0 | 1 | 0 |
| 2 | Absent | 0 | 0 | 2 | 1,2 | 0 |
| 3 | Absent | 0 | 0,1 | 0 | 0,1 |
| 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, 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 (e) negative staining for microtubule-associated protein 2 (MAP2), a marker for nerve cell ganglia, in crypt epithelial cells (G) of the mucosa (e represents merged image of a and d). 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...
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, and in the central nervous system and peripheral nerves during mouse development. 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. Furthermore, HEV-B may establish latent infections in human myocytes and persist as dsRNA. Moreover, they have been shown to be able to initiate a chronic state of inflammation in the nervous system. 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. 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. 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.

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. 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.

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. 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.

**Financial support:** These studies were funded by research grants from the Uppsala County Council (ALF grant) to A.W., and from the Swedish Research Council, the Swedish Childhood Cancer Society and the Swedish Childhood Cancer Foundation to J.F.

**Potential competing interests:** None.

**Acknowledgements.** We thank Lars Holmquist at Uppsala University for valuable help in identifying study patients through medical registers. These studies were funded by research grants from the Uppsala County Council (ALF grant) to A.W., and from the Swedish Research Council, the Swedish Cancer Society, the Swedish Childhood Cancer Foundation and the Strategic Cancer Research Program (StratCan) at Karolinska Institutet to J.F.
Study Highlights

WHAT IS CURRENT KNOWLEDGE
- Ileocecal Crohn’s disease (ICD) is associated with myenteric plexitis and polymorphisms in NOD2.
- The 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.

1. Silverberg MS, Satsangi J, Ahmad T et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 2005; 19(Suppl A): 5–36.
2. Levine A, Griffiths A, Markowitz J et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. Inflamm Bowel Dis 2011; 17: 1314–1321.
3. Sartini A, Castellani L, Buonfiglioli F et al. Update on Crohn’s disease: a polymorphic entity. Minerva Gastroenterol Dietol 2011; 57: 89–96.
4. Chilli R, Dodero M, Cella G. On the histology and histopathology of the jejunal mucosa obtained by biopsy–observations and critical analysis. Gastroenterologia 1964; 102: 111–124.
5. Ferrante M, de Hertogh G, Havary T et al. The value of myenteric plexitis to predict early postoperative Crohn’s disease recurrence. Gastroenterology 2006; 130: 1595–1606.
6. Ng SC, Lied GA, Kamm MA et al. Predictive value and clinical significance of myenteric plexitis in Crohn’s disease. Inflamm Bowel Dis 2009; 15: 1499–1507.
7. Sokol H, Polin V, Lavergne-Slove A et al. Plexitis as a predictive factor of early postoperative clinical recurrence in Crohn’s disease. Gut 2009; 58: 1218–1225.
8. Hubbard VM, Arceu K. Viruses, autophagy genes, and Crohn’s disease. Viruses 2011; 3: 1281–1311.
9. Nasar SA, Arce M, Khaja A et al. Role of ATG16L1, NOD2 and L23R in Crohn’s disease pathogenesis. World J Gastroenterol 2012; 18: 412–424.
10. Simmons A, Crohn’s disease: genes, viruses and microbes. Nature 2010; 466: 699–700.
11. Petermann I, Huebner C, Browning BL et al. Interactions among genes influencing bacterial recognition increase IBD risk in a population-based New Zealand cohort. Hum Immunol 2009; 70: 440–446.
12. Sabbah A, Chang TH, Hamack R et al. Activation of innate immune antiviral responses by Nod2. Nat Immunol 2009; 10: 1073–1080.
13. Cadwell K, Patel KK, Matoney NS et al. Virus-plus-susceptibility gene interaction determines Crohn’s disease gene Atg16l1 phenotypes in intestine. Cell 2010; 141: 1135–1145.
14. Ahmad T, Satsangi J, McGovern D et al. Review article: the genetics of inflammatory bowel disease. Aliment Pharmacol Ther 2001; 15: 731–748.
15. Mannon PJ. Environmental triggers, genetic background, and Crohn’s disease. Gastroenterology 2011; 140: 363–365.
16. Alirezaei M, Flynn CT, Wood MR et al. Pancreatic acinar cell-specific autophagy disruption reduces coxsackievirus replication and pathogenesis in vivo. Cell Host Microbe 2012; 11: 298–305.
17. Murray PJ. Beyond peptidoglycan for Nod2. Nat Immunol 2009; 10: 1053–1054.
18. Cadwell K, Liu YY, Brown SL et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008; 456: 259–263.
19. Kohlo KL, Klimola P, Simonen-Tikka ML et al. Enteric viral pathogens in children with inflammatory bowel disease. J Med Virol 2012; 84: 345–347.
20. Masclee AM, Penders J, Pierik M et al. Enteropathogenic viruses: triggers for exacerbation in IBD? A prospective cohort study using real-time quantitative polymerase chain reaction. Inflamm Bowel Dis 2013; 19: 124–131.
21. Cuffari C. The genetics of inflammatory bowel disease: diagnostic and therapeutic implications. World J Pediatr 2010; 6: 203–209.
22. Economou M, Trikalinos TA, Loizou KT et al. Differential effects of NOD2 variants on Crohn’s disease risk and phenotype in diverse populations: a metaanalysis. Am J Gastroenterol 2004; 99: 2393–2404.
23. Rhoades RE, Tabor-Godwin JM, Tsung G et al. Enteroviruses infections of the central nervous system. Virolology 2011; 411: 288–305.
24. Li Y, Pan Z, Ji Y et al. Enterovirus replication in valvular tissue from patients with chronic rheumatic heart disease. Eur Heart J 2002; 23: 567–573.
25. Miao LY, Pioz C, Gray-Johnson J et al. Monoclonal antibodies to VP1 recognize a broad range of enteroviruses. J Clin Microbiol 2009; 47: 3108–3113.
26. Yazdanyar S, Kamstrup PR, Tybjaerg-Hansen A et al. Penetration of NOD2/CARD15 genetic variants in the general population. CMAJ 2010; 182: 661–665.
27. Kodroiu M, Rubio C, Granath F et al. CARD15 mutations are rare in Swedish pediatric Crohn disease. J Pediatr Gastroenterol Nutr 2005; 40: 456–460.
28. Arme DK, Mack DR, Morgan K et al. Autophagy gene ATG16L1 but not IRGM is associated with Crohn’s disease in Canadian children. Inflamm Bowel Dis 2009; 15: 501–507.
29. Rasschaert P, Thysberg J, Pettersson S et al. The coxsackie- and adenovirus receptor (CAR) is an in vivo marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. Exp Cell Res 2006; 312: 1556–1560.
30. Tömöki RP, Johansson CB, Totrov M et al. Expression of the adenovirus receptor and its interaction with the fiber knob. Exp Cell Res 2000; 255: 47–55.
31. Kringel K, Hohenadi C, Canu A et al. Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: quantitative analysis of virus replication, tissue damage, and inflammation. Proc Natl Acad Sci USA 1992; 89: 314–318.
32. Feuer R, Mena I, Pagarigan RR et al. Coxsackievirus replication and the cell cycle: a potential regulatory mechanism for viral persistence/latency. Med Microbiol Immunol 2004; 193: 85–92.
33. Overman NM, Kim KS. Persistent coxsackievirus infection: enterovirus persistence in chronic myocarditis and dilated cardiomyopathy. Curr Top Microbiol Immunol 2008; 323: 275–292.
34. Tam PE, Messner RP. Molecular mechanisms of coxsackievirus persistence in chronic inflammatory myopathy: viral RNA persists through formation of a double-stranded coxsackievirus DNA without associated genomic mutations or evolution. J Virol 1999; 73: 10113–10121.
35. Feuer R, Ruller CM, An N et al. Viral persistence and chronic inflammatory polymyopathy in the adult central nervous system following Coxsackievirus infection during the neonatal period. J Virol 2009; 83: 9395–9399.
36. Hoflandter D. Intestinal permeability in patients with Crohn’s disease and their relatives. Dig Liver Dis 2001; 33: 649–651.
37. Edelblum KL, Turner JR. The tight junction in inflammatory disease: communication breakdown. Curr Opin Pharmacol 2009; 9: 715–720.
38. Schultke JD, Pioz C, Amasheh M et al. Epithelial tight junctions in intestinal inflammation. Ann NY Acad Sci 2009; 1165: 294–300.
39. Martino TA, Petric M, Weingartl H et al. The coxsackie-adenovirus receptor (CAR) is used by reference strains and clinical isolates representing all six serotypes of coxsackievirus group B and by swine vesicular disease virus. Virology 2000; 271: 99–108.
40. Coyne CS, Bergelson JM. CAR: a virus receptor within the tight junction. Adv Drug Deliv Rev 2005; 57: 869–882.
41. Walters RW, Freimuth P, Moninger TO et al. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 2002; 110: 789–799.
42. Kellow JE. Myenteric plexitis in enteric dysmotility: what are the implications for clinical practice? Gut 2009; 58: 1042–1043.
43. Collins SM. The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. Gastroenterology 1996; 111: 1683–1699.

Clinical and Translational Gastroenterology is an open-access journal published by Nature Publishing Group. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/