Hirschsprung-Associated Enterocolitis: Transformative Research from Bench to Bedside

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Abstract

Hirschsprung disease (HSCR) is a congenital disease that is characterized by the absence of intrinsic ganglion cells in the submucosal and myenteric plexuses of the distal colon and is the most common cause of congenital intestinal obstruction. Hirschsprung-associated enterocolitis (HAEC) is a life-threatening complication of HSCR, which can occur either before or after surgical resection of the aganglionic bowel. Even though HAEC is a leading cause of death in HSCR patients, its etiology and pathophysiology remain poorly understood. Various factors have been associated with HAEC, including the mucus barrier, microbiota, immune function, obstruction of the colon, and genetic variations. In this review, we examine our current mouse model of HAEC and how it informs our understanding of the disease. We also describe current emerging research that highlights the potential future of HAEC treatment.

Keywords

- Hirschsprung's disease
- Hirschsprung-associated enterocolitis
- Pathophysiology
- translational research

Introduction

Hirschsprung Disease

Hirschsprung disease (HSCR), first described by Harald Hirschsprung,[1] is defined as a congenital disease that is characterized by the absence of intrinsic ganglion cells in the submucosal and myenteric plexuses of the hindgut/distal colon.[2] The incidence of HSCR has been reported to be approximately 1/5,000 live births.[3] Clinical symptoms of HSCR in newborns include functional obstruction characterized by delayed passage of meconium within the first 24 to 48 hours of life, abdominal distension, vomiting, and constipation.[4]

There has been continued development in our understanding of the pathogenesis of the disease, and the development of a variety of surgical options. Medical research into HSCR has steadily grown over the last three decades (►Fig. 1), with continued interest in translational models which can help to identify the underlying pathogenesis and explore novel treatment. The current standard treatment for HSCR is surgical pull-through operation resecting the aganglionic bowel, and the normal ganglionic intestine is then anastomosed to the rectum or anus. However, the procedure requires part of the intestine to be removed leading to various post-operative complications. Fistula or stenosis of the anastomosis may appear as short-term complications, and long-term outcomes can include fecal incontinence or recurrent constipation.[5–8] Furthermore, 20 to 38% of HSCR patients may develop enterocolitis despite technically successful surgery, a condition referred as Hirschsprung-associated enterocolitis (HAEC). These associated complications can have long-term impacts on the quality of life of patients and necessitate a deeper understanding of both the disease's pathogenesis and its associated complications.

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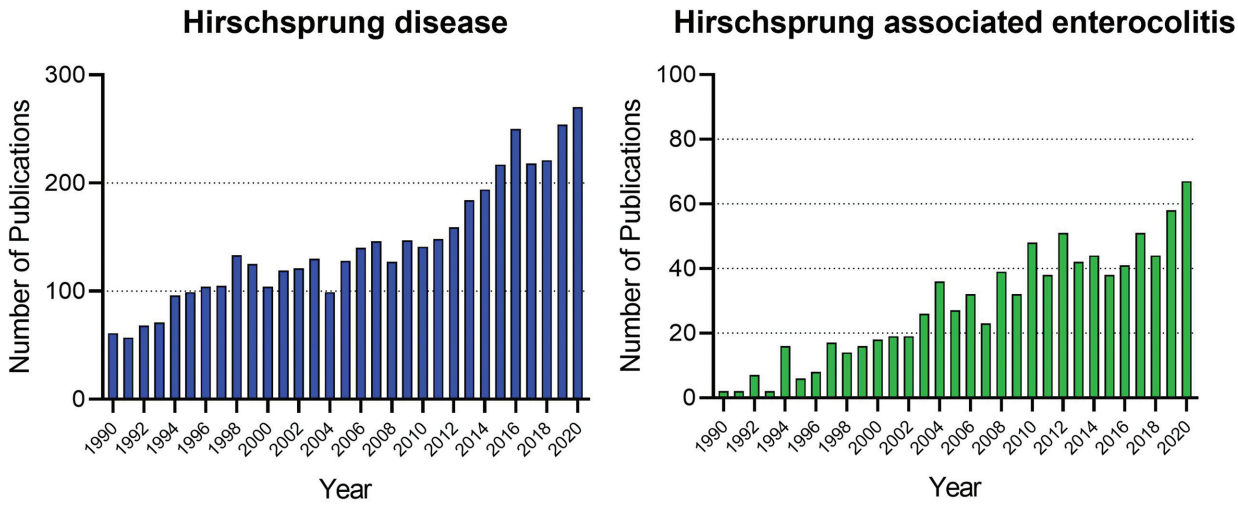

Fig. 1 Peer-reviewed publications for Hirschsprung disease (*blue*) and Hirschsprung-associated enterocolitis (*green*) on PubMed over the past 30 years. Search query of: "Hirschsprung" [Title/Abstract] OR "Hirschsprung's" [Title/Abstract] OR "HSCR" [Title/Abstract] for the Hirschsprung's disease manuscripts. Search query of: "Hirschsprung associated enterocolitis" [Title/Abstract] OR "HAEC" [Title/Abstract] for the Hirschsprung-associated enterocolitis manuscripts.

Hirschsprung-Associated Enterocolitis

HAEC is the most common and serious complication of HSCR, with the potential to develop into generalized sepsis, potentially leading to a high mortality rate. HAEC classically occurs in HSCR patients with abdominal distention, fever, and foul-smelling stools. It accounts for most of the morbidity and mortality of children with HSCR.[9,10] Although the surgical correction of HSCR is mostly successful, challenges remain as HAEC can still develop.[11,12] Additionally, the incidence of HAEC appears to be unchanged between the pre-operative and post-operative periods in both animal models and humans.[13–15]

The pathogenesis of HAEC appears complex and is not completely understood yet.[10] It usually presents with both gastrointestinal (GI) and generalized symptoms ranging from fever and diarrhea to bloodstained stools and septic shock, while in chronic cases, it affects growth and development.[16,17] Currently, treatments for HAEC are relatively nonspecific and can consist of a combination of antibiotics, rectal irrigations, and bowel rest.[18,19] These measurements are often directed toward treating the acute symptoms rather than targeting the factors that are thought to contribute to the disease. Recent research into the pathogenesis of the disease with animal models, and a better understanding of the underlying etiology may substantially improve the quality of life of the patients who developed HAEC.

Etiology of HAEC

Investigations focused on histopathologic changes in HAEC-affected bowel have suggested that the distal obstruction in HSCR is likely the leading cause of HAEC.[20,21] However, clinical observation of continued susceptibility to HAEC after pull-through procedure contradicts this conclusion.[13,15] The etiology of HAEC is poorly understood and controversial. The many proposed potential pathological processes of HAEC can be classified into three main abnormalities of the intestinal homeostasis, namely ones of (1) intestinal barrier dysfunction, (2) abnormal innate immune response, and (3) abnormal microbiota.

In the case of intestinal barrier dysfunction, viral and/or bacterial infectious pathogens may lead to the development of HAEC but the causative organisms remain elusive with conflicting data identified.[22–24] Studies have also demonstrated that there are decreased turnover and alterations in various subsets of mucins which play a fundamental role in the luminal barrier integrity of patients with HSCR.[25] Although there exists variation in the literature among what mucin receptors are suspected of being involved,[26,27] interestingly in most cases these findings seem to extend beyond the aganglionic region of the bowel.

Researchers have shown that there is a defect of adaptive immunity in HSCR. Cheng et al[28] found that splenic lymphopenia and a small-sized spleen existed in *Ednrb* knockout mice, which is characterized by a 5 to 20-fold reduction in the amount of $CD19^+$ mature B cells, $CD4^+$ T cells, and $CD8^+$ T cells. Other studies have also shown that in severe HAEC, thymic involution, splenic lymphopenia, and suppression of B-cell production were also present.[29] These results mimic the clinical manifestations seen in neonates who develop sepsis and highlight the importance of early management. Secretory IgA is also important for intestinal immunity, Moore et al identified an increase in IgM and IgG populations without a concomitant IgA increase in the aganglionic and transitional segments of the intestine in HSCR patients.[30] Of note, the reduction of B-cell-produced secretory IgA have been detected in *Ednrb* knockout mouse model and have shown that this may be due to a combination of both an intrinsic B-cell defect in antibody production and an extrinsic defect in IgA transport in HAEC.[31] In addition, the abnormalities of glial cells may lead to HAEC.[32,33]

The HAEC recurrence rate can have quite a varying range with as high as 50% recurrence in some cases.[34] This may be attributable to sustained histopathological alterations in the intestinal mucosa or the immunodeficiencies/dysfunction of

intestinal immunity mentioned above. Interestingly, the assessment of stool specimens from a patient using amplified ribosomal DNA restriction analysis has suggested that the recurrence may be associated with a specific distribution of intestinal flora, which is influenced by antibiotic use.[22] Using high throughput sequencing, Li et al showed that HSCR patients without HAEC had relatively distinct and more stable microbiota relative to their HAEC patient counterparts.[35] The microbiotas in HSCR patients were characterized by a higher prevalence of Bacteroidetes, while HAEC patients had a higher prevalence of Proteobacteria. Neuvonen et al also found a significantly increased abundance of Proteobacteria in HAEC patients and a decrease in enterobacteria and Bacilli.[36] In fact, in early 2020, Tang et al were able to identify a group of 21 microbiome signature operational taxonomic units that can predict postoperative HAEC with an 85% accuracy.[37] These studies among others suggest the potential of modulating the gut microbiome as a potential way to prevent the development or progression of HAEC in HSCR patients.

Animal model of HSCR

Animal models of HSCR play a critical role in our understanding of the important anatomical features, pathogenesis, and underlying molecular mechanisms of the disease. They are also vital in allowing us to model disease progression changes with novel treatments and in finding strategies to assess the effectiveness of treatment. Over the last few decades, several models of HSCR have been developed. These models have been used in numerous studies on a wide range of animals like mice, rats, rabbits, and pigs and have helped to effectively model human aganglionosis that is seen in HSCR. Additionally, the animal models have also allowed us to appropriately assess disease progression and find associated markers that have since opened the door to a wide spectrum of potential targets to reduce negative outcomes in patients. Animal models can generally be broken down into three main types of models, namely, chemical or teratogen-induced models, and genetic knockout models (►Table 1).

Chemical-Induced Models of HSCR

The benzalkonium chloride (BAC) rat model of HSCR was first described in 1978 and has become a well-established model since then to investigate the underlying mechanism of HSCR as it is highly similar in its progression to human HSCR.[38–40] BAC is a cationic surface-acting agent that attaches to cell membranes and through selective neuronal ablation leads to irreversible depolarization, cell damage, and death.[41] In the intestinal

Table 1 Animal models of Hirschsprung disease

Type of model	Model	Chemical used or target gene	Underlying mechanism
Chemical-induced models	Benzalkonium chloride (BAC) rat model	Benzalkonium chloride (BAC), a cationic surfactant	BAC ablates the myenteric neurons and glia of the intestine leading to the development of aganglionosis.[38–41]
	Retinoic acid	Several potential targets, two common ones are retinol-binding protein 4 and retinaldehyde dehydrogenase 2	Retinoic acid is required for lamellipodia formation and enteric neural crest cell migration.[43]
	Ibuprofen	Ibuprofen, a nonselective cyclooxygenase inhibitor	Ibuprofen reduces enteric neural crest cell migration, lamellipodia formation, and levels of motility genes like Rac1.[45]
	Mycophenolate	Mycophenolate inhibits inosine-5′-monophosphate dehydrogenase	Prevents de novo guanine nucleotide synthesis which leads to defects in multiple neural crest derivatives and intestinal aganglionosis.[46,47]
Genetic models	*Ret* gene	*Ret* gene knockdown or GDNF deletion/mutation or Ntn knockout	Ret gene encodes a receptor tyrosine kinase for GDNF, Ntn, Atm, and Psp.[48] Knockdown models or GDNF knockout produces aganglionosis and plays an important role of *Ret* gene and have been shown in human HSCR in up to 50% of familial cases.[51–54]
	Endothelin gene	*Ednrb* or *Edn3* mutation	Endothelin receptor b and Edn3 are required for proper ENCC migration and expression.[62,63] Mutations have been seen in human HSCR patients and *Ednrb* knockout is a commonly used animal model of HSCR.[66,67]
	Other models (Sox 10, Phox2b, Pax3, Shh, and Ihh)	Various mutation models that affect ENCCs	Mutation in various genes that affect ENCC development as they leave the neural tube, migrate to the intestine, or allow for ENCC differentiation can lead to aganglionosis that mimics HSCR.[68–71]

Abbreviations: ENCC, enteric neural crest cell; GDNF, glial cell line-derived neurotrophic factor; HSCR, Hirschsprung disease.

region where BAC is used, the myenteric neurons and glia are almost completely ablated leading to the development of aganglionosis which mimics human HSCR.[41] The submucosal neurons, however, are not affected by BAC treatment. This model has the advantage of having had many years of use and is relatively inexpensive with good long-term survival.

Another chemical model involves depleting retinoid signaling or vitamin A. Downregulation of retinol-binding protein 4 and retinaldehyde dehydrogenase 2 have both shown that the retinoic acid pathway, in which they are involved, plays a critical role in colonic aganglionosis confirming the importance of retinoic acid (active form of vitamin A) signaling in ganglionic development.[42] The underlying mechanism is believed to be the diminished retinoic acid activity which leads to impaired lamellipodia formation and reduces enteric neural crest cell (ENCC) migration in response to glial cell line-derived neurotrophic factor (GDNF).[43] These findings suggest that vitamin A deficiency may act in combination with potential genetic factors to increase disease penetrance and expression.[43,44]

Other chemical models have also been explored and shown to reduce ENCC migration. One such example is ibuprofen, a nonselective cyclooxygenase inhibitor, which has been shown to reduce ENCC migration and lamellipodia as well as levels of motility genes like Ras-related C3 botulinum toxin substrate 1 (Rac1).[45] Another example is mycophenolate, a potent, reversible, non-competitive inhibitor of inosine-5′-monophosphate dehydrogenase, which is required for de novo guanine nucleotide synthesis and has also been shown to reduce ENCC migration and lamellipodia.[46] Like other chemical models, this reduction in ENCC migration and lamellipodia leads to intestinal aganglionosis. However, just a delay in neural crest cell migration with the eventual development of a normal enteric nervous system (ENS) was founded in both studies. Additionally, inhibition of inosine-5′-monophosphate dehydrogenase 2 (Impdh2) causes a defect in multiple neural crest derivatives which leads to intestinal aganglionosis, craniofacial abnormalities, and malformations of major vessels.[47] The chemical-induced models seemed to represent the models of perturbed ENS development, the genetic models have been established to overcome these hurdles.

Genetic Models of HSCR

HSCR has a complex genetic etiology with several genes and loci being potentially associated with either isolated HSCR or syndromic forms. Implicated genes often play a critical role in regulating proper ENCC migration and/or appropriate enteric nervous system development. Disruptions in these genes lead to remarkably phenotypic expression in animal models similar to human HSCR. Two of the most well-studied genes that have produced useful HSCR animal models are the *Ret* gene and the endothelin (Edn) gene family.[48]

The *Ret* gene encodes a receptor tyrosinase kinase which has four ligands, GDNF, neurturin (Ntn), artemin (Atm), and persephin (Psp),[48] with another receptor (glycosylphosphatidylinositol-linked receptor) to form a complex that is important for molecular adhesion and neural crest migration.[49,50] *Ret* gene mutations have been shown to play a vital role in human HSCR, accounting for 15 to 20% of patients with sporadic HSCR and up to 50% in familial cases.[51–54] A total/global knockout of the *Ret* gene in mice causes complete intestinal aganglionosis; however, it also leads to kidney agenesis and death of the mouse pups at birth.[55–58] GDNF deletion has also been shown to produce a similar phenotype as it is a critical Ret activator. GDNF/Ret signaling has been shown to play a critical role in the formation of specific neuron subtypes and enteric nervous system development overall. Ntn has also been shown to play a critical role in the maintenance of enteric neurons and ganglia and in promotion of neuronal differentiation and the ENCC proliferation.[59,60] Ntn-deficient mice have reduced nerve fiber density with associated abnormalities in bowel motility.[59,61]

Endothelins are peptides with receptors that normally are responsible for constricting blood vessels and affecting blood pressure. The endothelins act via surface transmembrane receptors, endothelin receptor A (Ednra), and endothelin receptor B (Ednrb).[48] Ednrb and Edn3 are both expressed in the enteric neurons and gut mesenchymal cells of fetuses and are required for appropriate enteric neural migration and expression.[62,63] The Edn3–Ednrb pathway has been shown to play an important role in ENCC migration and in maintaining enteric progenitors.[64,65] Heterozygous *Ednrb* mutations have been identified in non-syndromic HSCR patients.[66] Homozygous mutations of *Ednrb* and *Edn3* genes by contrast have been reported in HSCR patients with the type-2 Waardenburg syndrome. Interestingly, *Ednrb* mutations have been associated with short-segment HSCR, while *Ret* mutations appear to be associated mainly with long-segment aganglionosis.[66] Neural crest cell-specific deletion of Ednrb has also been shown to lead to reduced neuronal density in the ENS of the colon and may account for the dysmotility seen in HSCR.[67] The *Ednrb* knockout/knockdown models provide both mechanistic and translational understanding of HSCR and its associated symptoms.

Other gene mutations have also been implicated in ENCC differentiation or migration. These include, Sox10, a member of the SRY-related family of transcription factors and expressed in vagal ENCCs as they leave the neural tube.[68] Paired-like homeobox2b, a transcription factor expressed in migrating ENCCs, enteric neurons, and glial cells.[69] *Pax3*, a member of the paired-box containing family of nuclear transcription factors that is expressed in neural cell precursors giving rise to an enhancer in the *Ret* gene,[70] and Sonic hedgehog, and Indian hedgehog genes also potentially affect the survival and differentiation of ENCCs.[71] Targeted deletion of these genes among others all are viable options and provide avenues in understanding what is potentially leading to aganglionosis, its underlying causes, and how to best target therapeutics to address it.

Animal Models of HAEC

Genetic Murine Models: Understanding the Pathogenesis of HAEC

HAEC is responsible for the most serious morbidity and mortality from HSCR. Animal models that can mimic the

Table 2 Hirschsprung disease models that have studied the associated enterocolitis (HAEC)

Type of model	Model	Overview
Genetic	Piebald-lethal mice[73,74]	Natural mutation that leads to a recessive phenotype identical to *Ednrb* knockout mice that disrupts enteric neural crest development.
	Ednrb knockout mice[62,72]	*Ednrb* mutation reliably models human HAEC, and knockout allows for targeted assessments.
	Ednrb-deficient rats[75–77]	Can be naturally occurring null mutation in *Ednrb* gene as in case of spotting lethal (sl) rats. Similar functional use to the knockout model but using larger animals (rats).
Chemical induced	Benzalkonium chloride piglet[88]	Chemical induction under general anesthesia of 5-day-old piglets leading to induced partial aganglionosis and HAEC-like development.
Ex vivo	Intestinal organoids[89]	Cultured organoids from intestinal stem cells and pluripotent stem cells of animals or humans can help mimic intestinal conditions and be utilized to understand the effect of chemical, physical, and environmental conditions which can lead to aganglionosis and HAEC development.

Abbreviation: HAEC, Hirschsprung-associated enterocolitis.

inflammatory process can help provide insight into the etiology of HAEC as well as provide an avenue to test potential treatment options. Three rodent models have been used to study HSCR and its associated entercolitis[15] (►Table 2). These include Ednrb null mice,[62,72] piebald-lethal mice,[73,74] and endothelin receptor B-deficient rats.[75–77] There are additional mutant rodent models that have been used to study HSCR in general and these include Endothelin-3 ligand-deficient mice,[63] Hoxb5 dominant-negative conditional (Cre-Lox) transgenic mice,[78] erbB2/nestin-Cre conditional mutant mice,[79] Dom spontaneous mutant mice,[80] conditional β-1 integrin knockout mice,[81] trisomy 16 mice,[82,83] and mice deficient in the c-ret proto-oncogene,[84] and the fmc/fmc (familial megacecum and colon) rat[85] which all have phenotypes that mimic HSCR.

By utilizing some of these models among other newly developed ones, several interesting findings have been identified with respect to HAEC etiology which may provide therapeutic targets. Chen et al found that in the *Ednb* knockout mouse model the intestinal cells of Cajal lost their C-KIT expression in the dilated portion of the colon resulting in damaged pacemaker function and intestinal motility.[86] They found that proinflammatory macrophage activation may act as a phenotypic switch to intestinal cells of Cajal and as a result represent a promising therapeutic target for HAEC. Another recent study using a glial cell line-derived neurotrophic factor receptor α-1 (GFRa1) hypomorphic mouse model demonstrated that a 70 to 80% reduction in GFRa1 results in HSCR and associated HAEC in mice and that this process proceeds from goblet cell dysplasia and mucin abnormalities to epithelial damage.[87] Won et al used endothelin receptor B-deficient rats as a model for long-segment HSCR and examined the myogenic mechanism involved in the intestinal obstruction of this model.[76] They found that there was both increased contractility of the smooth muscle and thickness of the intestinal muscular wall contributing to the intestinal obstruction in this model, offering a more functional finding. The diversity of models available and the variety of approaches being taken have greatly expanded our understanding of HAEC as a result and have helped to reduce its mortality over the last few decades.

Chemical-Induced Model: Toward Clinical Translation

In an attempt to create a model of HAEC closer to the clinical representation of the human disease, some investigators have employed in large animal such as piglets, whose GI tract shares ontogenetic similarities close to that of humans. Arnaud et al used a piglet model of iatrogenic rectosigmoid hypoganglionosis to study the impact of the enteric nervous system on gut barrier function and microbiota development.[88] This is an effective study in using a larger animal model to demonstrate the effects of hypoganglionosis especially in replicating the gut barrier issues and proinflammatory states seen in smaller animal models. Adequate animal models will provide the ability to assess medical options and especially surgical interventions. However, the use of larger animal models like this has some severe disadvantages that may make them of limited use. For instance, it is arduous to maintain a large enough number of piglets for adequate statistical significance. Additionally, unlike mice, knockout models and genetic markers are far more difficult to create, as transgenic lines are often unavailable or producing such lines may take a long time and be very costly.

Ex Vivo Model (Organoids): Emerging Future Directions

Intestinal organoids provide a potential translational bridge between our current animal models and the human studies. It has been shown that recent advances in culturing of intestinal and pluripotent stem cells have allowed us to develop an intestinal organoid model.[89] This model allows for the development of an in vitro model that can utilize either human or animal tissue to produce mini self-encapsulated systems that mimic GI tissue and by proxy allow us to both understand what is happening at the intestinal wall and test potential treatments. Furthermore, the transplantation of these intestinal organoids by enema has been shown to be able to rescue damaged colonic epithelium in mice with

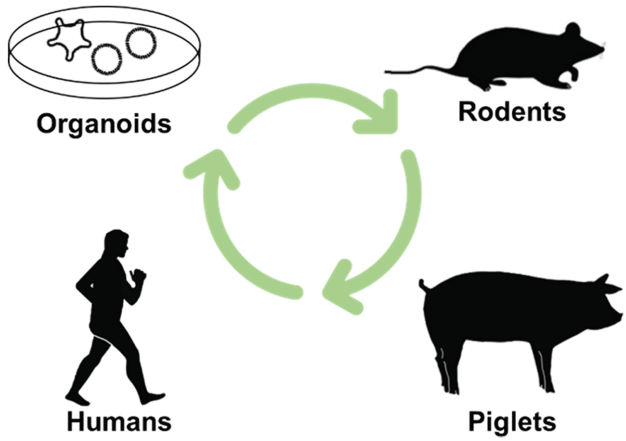

Fig. 2 Diverse models of Hirschsprung disease and its associated colitis allow for a greater potential of identifying possible viable treatments. Human clinical data and tissue, mouse models that mimic HAEC, a piglet model that reproduces the aganglionosis, and intestinal organoid models in combination that allow for a greater mechanistic understanding of the disease and more opportunity to try varying potential treatments.

dextran sulfate sodium-induced colitis.[90] These models may be expanded to study other GI diseases including HSCR and provide a promising avenue for future research. The combination of mouse/rat models, piglet model, intestinal organoids, and associated clinical data from human studies/samples has allowed for a multipronged approach to studying HSCR and HAEC while expanding our understanding of the disease and providing a platform to test varying potential treatment options (►Fig. 2).

Conclusion and Future Perspective

HAEC remains a challenging clinical condition with many unresolved problems. The pathophysiology of the disease and the mechanisms that lead to the intestinal inflammation and damage are yet to be fully characterized. Additionally, the clinical management and therapeutic options for patients are restricted, as demonstrated by the consistent mortality and morbidity rates of the patients affected. The usage of animal models has advanced our knowledge on HAEC and helped in providing better overall management for patients. These models are now well characterized and will continue to be of tremendous use in the development of future studies designed to better understand the disease and to translate the findings into clinical interventions. With the continued use of murine models of HAEC and the more recent use of larger animals and intestinal organoids derived from human tissue, there is great potential for translational therapeutics. However, currently, there is a lack of clinical trials available in the field, and thus, the management strategies remain quite limited. Greater effort should be placed on translating animal study findings into the clinical application and assessing the potential safety and efficacy of such treatment options as they may hold great promise for HAEC patients in the future.

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Conflict of Interest
None declared.

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