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Current and future state of the management of Hirschsprung disease

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ABSTRACT

The enteric nervous system (ENS) consists of a network of neurons and glia that control numerous complex functions of the gastrointestinal tract. Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of ENS along variable lengths of distal intestine due to failure of neural crest-derived cells to colonize the distal intestine during embryonic development. A patient with HSCR usually presents with severe constipation in the neonatal period and is diagnosed by rectal suction biopsy, followed by pull-through procedure to surgically remove the affected segment and reconnect the proximal ganglionated intestine to the anus. Outcomes after pull-through surgery are suboptimal and many patients suffer from ongoing issues of dysmotility and bowel dysfunction, suggesting there is room for optimizing the management of this disease. This review focuses on discussing the recent advances to better understand HSCR and leverage them for more accurate and potentially less invasive diagnosis. We also discuss the potential future management of HSCR, particularly cell-based approaches for the treatment of HSCR.

BACKGROUND

A complex network of enteric neurons and enteric glial cells (EGCs) comprise the enteric nervous system (ENS) and are responsible for controlling numerous complex functions of the gastrointestinal tract including digestion, absorption, passage of food and waste, epithelial barrier function, maintenance of a healthy microbiome, and preservation of host immunity.[1] The ENS extends along the entire length of the gastrointestinal tract, is composed of 200–600 million neurons[2] and approximately seven times as many glial cells,[3] and is capable of autonomous function without signaling input from the brain or spinal cord.[4] The majority of enteric neurons and EGCs are arranged in two interconnected networks, the myenteric (between the longitudinal and circular muscle layers) and the submucosal (between the epithelium and circular muscle layer) plexuses.

Both neurons and EGCs arise from neural crest cells (NCCs) that migrate into and along the gut mesenchyme during embryologic development.[5] In human ENS development, NCCs emerge from the neural tube at postconception week 4 and complete their colonization of the hindgut by week 7.[6] This process of colonization is highly dependent on coordinated interactions between NCCs and their surrounding environment. The fate of NCCs is largely determined by the specific axial level of the neural tube (cranial, cardiac, vagal, trunk, and sacral) from which they arise.[5] The ENS is predominantly populated by vagal-derived NCCs. Sacral-derived NCCs contribute to a lesser extent, specifically to colorectal ENS development, and enter the distal hindgut only after the arrival of vagal NCCs.[7 8] An additional contribution to the distal intestinal ENS comes from Schwann cell precursors that migrate into the colon along extrinsic nerve fibers.[9 10] To form a functional ENS, NCCs must survive, migrate in the appropriate direction, proliferate, differentiate into neurons and glia in the correct ratio and at the appropriate time, and form a functional neuroglial network.[1] These processes are tightly regulated both spatially and temporally by multiple neurotrophic factors, signaling pathways, and transcription factors.[5 11]

Any disruption in enteric NCC migration, proliferation, or differentiation can lead to Hirschsprung disease (HSCR), a developmental disorder that occurs when enteric NCCs fail to complete their craniocaudal migration along the gut mesenchyme. This failure results in distal intestinal aganglionosis of a variable length and affects 1 in 5000 live births.[12] In the majority of cases (80%), only the rectosigmoid colon is affected, but the aganglionosis extends more proximally in the remainder, even rarely involving the entire intestinal tract.[12 13]

CURRENT MANAGEMENT OF HSCR

Most patients with HSCR present with classic symptoms characterized by failure to pass meconium within the first 48 hours of life. This prompts a water-soluble contrast enema, which has several characteristic findings in HSCR, including reversal of the rectosigmoid

ratio (where the diameter of the rectum is less than that of the sigmoid colon), a spiculated sawtooth pattern of the rectal mucosa, and a transition zone. These findings, however, are not always present. In one study, 75% of HSCR neonates had an abnormal rectosigmoid ratio, 85% had a transition zone, and 50% had a sawtooth pattern of the rectal mucosa.[14] Radiographic abnormalities may be absent in the neonatal period due to an insufficient amount of time for proximal bowel dilation to occur. Anorectal manometry can be used as an adjunctive test since all patients with HSCR have an absent rectoanal inhibitory reflex.[15] A definitive diagnosis is performed via rectal suction biopsy (RSB), and the tissue is evaluated by H&E and acetylcholinesterase (AChE) and/or calretinin staining. This is the current gold standard for diagnosis and has a 100% sensitivity and 99.1% specificity.[16]

The primary treatment for HSCR is surgery to remove the aganglionic segment, followed by a pull-through procedure to reconnect the remaining ganglionated intestine to the anus.[17 18] The pull-through operation can be performed transanally for short-segment aganglionosis, with an abdominal component that is done either laparoscopically or via open surgery to identify the level of aganglionosis prior to initiating the pull-through and also often to facilitate the dissection. The operation can also be performed as a single-stage procedure or after a diverting ostomy. There are three commonly performed pull-through procedures, as described by Swenson *et al*,[19] Yancey-Soave,[20] and Duhamel.[21] Outcomes and complication rates of the various procedures are similar and the specific procedure is typically chosen based on surgeon experience and preference. Timing of pull-through surgery varies across clinical practice. In a European review of pediatric surgical practice, pull-through procedures were performed at diagnosis in 33% of neonates and after 4 months of age, or when the patient was >5 kg, in 67% of patients.[22] While awaiting definitive surgery, 77% of patients underwent rectal irrigations, 22% underwent rectal dilatation/stimulation, and 33% had a diverting stoma.[22]

Unfortunately, there are several long-term complications following pull-through procedures and subsequent bowel dysfunction is relatively common. Complications include obstructive symptoms, fecal soiling, Hirschsprung-associated enterocolitis (HAEC), and psychosocial distress.[13 23–27] Constipation rates following pull-through surgery vary widely between studies, from 4%[28] to ~30%.[29] Many of these patients are unable to defecate without the assistance of anal dilation or rectal irrigation. There are five common causes of obstructive symptoms following pull-through surgery, including mechanical obstruction; retained aganglionosis, hypoganglionosis, or transition zone pull-through; internal sphincter achalasia; proximal bowel dysmotility; and functional constipation due to withholding behavior.[30] A thoughtful evaluation of the cause is essential to target the treatment effectively.

Some patients experience the opposite problem, with increased stooling frequency occurring in 44% of patients.[28] It is estimated that fecal soiling affects 35%–50% of patients with rectosigmoid disease and a higher proportion of those with total colonic aganglionosis.[29] A healthy rectum serves as a reservoir for stool, with its distension resulting in the urge to defecate. Pull-through procedures either completely (Swenson and Yancey-Soave) or partially (Duhamel) disrupt this reservoir and involve dissecting near the internal anal sphincter and its surrounding innervation, with varying degrees of fecal incontinence occurring in nearly half of cases. Unfortunately, fecal incontinence has a major impact on a child's quality of life,[31] and nearly 50% of children aged 5–15 years following pull-through surgery described a negative effect on their social life.

HAEC, a condition of intestinal inflammation characterized by fever, abdominal distension, diarrhea, and occasionally sepsis, occurs in approximately 28%–42% of patients post-operatively.[28 32 33] HAEC is the leading cause of death in HSCR[34] and needs to be taken very seriously. This complication can be recurrent, as up to 21% of patients can have ≥4 episodes of post-operative enterocolitis.[28] The cause of HAEC remains incompletely understood, and therefore treatment is supportive and includes antibiotics and rectal irrigations.

FUTURE MANAGEMENT OF HSCR

Non-invasive diagnosis

A definitive diagnosis of HSCR is made by detecting the absence of ganglion cells on rectal biopsy. RSB combined with H&E and AChE staining is the gold standard for the diagnosis of HSCR, although many recent studies have reported that calretinin staining can provide highly accurate diagnosis with 100% sensitivity and 99.1% specificity, even by inexperienced pathologists.[16] The suction biopsy is taken from above the dentate line and therefore does not cause the child pain, and must include the submucosa, which contains the submucosal plexus. This procedure is well tolerated, but RSBs are more likely to be non-diagnostic in older children due to the thickness of the rectal mucosa. In a review of RSBs, the overall complication rate was only 0.65% (which included persistent bleeding requiring blood transfusions, perforation, and pelvic sepsis), but the rate of insufficient specimens was approximately 10%.[35] Patients who have RSBs with insufficient tissue must undergo a transanal full-thickness rectal biopsy, which requires general anesthesia.

The first step of a pull-through is to determine the level of aganglionosis by taking intra-operative biopsies, allowing the surgeon to identify the proximal transection margin. These biopsies are typically full thickness and are evaluated via frozen section by an experienced pathologist. This takes up valuable time during the procedure, and non-invasive ways to visualize enteric ganglia would substantially streamline this process. Therefore, there is a strong impetus to identify non-invasive diagnostic

techniques. The myenteric plexus has been successfully visualized without tissue sampling using non-invasive imaging. Spectral imaging has been able to differentiate aganglionic bowel from normal intestine in mice with HSCR.[36] Full-field optical coherence microscopy, which is based on the broadly used technology of optical coherence tomography in medical imaging and endoscopy, can visualize the myenteric plexus in both humans and mice and has similarly been able to differentiate aganglionic from normal bowel in HSCR mice.[37 38] Using this technology, enteric ganglia and interganglionic fiber tracts can be identified, but individual neurons and glia are unable to be visualized.[39] An additional potential strategy includes using confocal laser endomicroscopy, but this requires endoscopic removal of the mucosa and administration of a neuronal dye to visualize the ENS, so it is not yet entirely non-invasive.[40] Another recent study used ultra-high frequency ultrasound on surgically resected specimens of patients with HSCR and was able to differentiate regions of aganglionosis from normal bowel, although this technology has not yet been successfully applied in vivo.[41] With continued advancements in imaging technologies, clinical application of non-invasive visualization of the enteric ganglia is likely around the corner.

Artificial intelligence to improve diagnostic accuracy

Diagnosing HSCR requires an experienced pathologist to perform immunohistochemical staining and manually review dozens of slides looking for the presence of enteric ganglion cells. The tissue is evaluated with H&E and AChE/calretinin staining and the diagnosis of HSCR is made based on the absence of ganglion cells and calretinin-positive neurites, and often supported by the presence of AChE-positive hypertrophic nerves. This process is very time-consuming and is dependent on the expertise of the available pathologist, and therefore standardization and automation of this process would be beneficial. In resource-poor settings or communities without easy access to healthcare, diagnostic automation could be particularly useful. There have been numerous studies comparing the performance of artificial intelligence (AI) algorithms with pathologists in the detection and classification of various disease processes.[42]

Pull-through specimens have been used to train algorithms that were 92.3% and 91.5% accurate at detecting ganglion cells.[43] A recent study used an AI-assisted approach to improve the efficiency and accuracy of diagnosing HSCR.[44] This approach used an algorithm to automatically detect enteric ganglion cells, and slides with ganglion cells were then manually reviewed by a pathologist. In normal colon, the algorithm detected enteric ganglion cells with 96% sensitivity and 99% specificity, and was also able to identify an HSCR case that was previously misdiagnosed. Importantly, the expert pathologist was correctly able to make all HSCR diagnoses based solely on the images suggested by the algorithm, with over 95% time saved.[44] Machine learning approaches have the potential to revolutionize diagnostic accuracy, although the application of AI in clinical medicine needs to overcome significant challenges, including algorithm transparency, data standardization, and interoperability across multiple platforms.

Genotype-phenotype correlation

Due to the complex inheritance of HSCR, including incomplete penetrance, variable expression, and multigenic inheritance, the genetic profile cannot currently predict disease phenotype. A better ability to predict disease course and severity would be very valuable in managing patients in the pre-operative and post-operative periods. HSCR has been shown to arise from three types of mutations in cell fate genes of neural crest-derived cells including: common non-coding variants, rare coding variants, and copy-number variants.[45] At least one of these pathological variants was found in 72% of patients with HSCR, and the odds ratios (OR) for HSCR increased by a factor of approximately 67 based on the combination of pathological variants, where the risk of HSCR increases from 1 in 18 100 with no pathological variants to 1 in 120 if all three types were present.

The relatively low risk of 1 in 120 despite having a high-risk genetic profile,[45] as well as the incomplete penetrance, indicates that other environmental and epigenetic factors likely contribute to the complex phenotype of this disease. Although significant progress has been made identifying genetic risk factors and new mutations that lead to HSCR, there remains a significant knowledge gap. It is unknown why the majority of patients with a high-risk genetic profile do not develop the disease, why some patients have short-segment disease while others have total colonic involvement, why some present within the first 48 hours of life while others develop symptoms later in childhood, or why some patients develop HAEC.

Epigenetic changes likely contribute to the incomplete penetrance observed in HSCR. Epigenetic regulation modifies gene expression and thus phenotype without altering the nucleotide sequence or genetic profile. Alterations in DNA methylation and histone modification can turn particular genes on or off. DNA hypermethylation silences genes while hypomethylation upregulates gene expression. Similarly, histone proteins have residues that can be altered via methylation/demethylation, acetylation/deacetylation, phosphorylation, ubiquitination, and sumoylation,[46] which subsequently affects the structure of chromatin and thereby regulates gene expression. *RET* is a tyrosine kinase transmembrane receptor expressed by enteric neural crest-derived cells, whose activation promotes NCC proliferation, survival, migration and differentiation into enteric neurons.[47 48] *RET* coding mutations account for 15%–35% of patients with sporadic HSCR and 50% of familial cases.[11 49] Additionally, non-coding *RET* mutations are common and account for approximately two-thirds of sporadic cases.[50] Epigenetic regulation may also play a role, as *RET* expression has been shown to be regulated by DNA methylation

in the peripheral white blood cells from patients with HSCR.[51] Furthermore, the protein complex HOXB5 alters chromatin configuration, which could result in decreased *RET* expression and HSCR.[52] Since there have been so many genes implicated in HSCR, epigenetic analysis is very complicated. However, emerging technologies in single-cell epigenetics, including CUT&Tag[53] and ATAC-seq,[54] may help elucidate critical epigenetic changes that contribute to disease phenotype.

Predictive potential may exist in the resection specimen from pull-through surgery, which includes the aganglionic portion, the transition zone, and a cuff of normal ganglionated bowel. There is increasing evidence that even the ganglionic normal colon in HSCR is not entirely normal, which may explain some of the ongoing bowel dysfunction experienced after surgery. In a Hirschsprung mouse model of short-segment HSCR, the normal duodenum had increased levels of excitatory motor neurons expressing calretinin as compared with normal controls.[55] Another study in Hirschsprung mice revealed distorted neurotransmitter expression in the proximal ganglionated colon, with increased expression of nitric oxide synthases (NOS) and vasoactive intestinal peptide (VIP) neurons, decreased cholinergic neurons, and decreased overall neuronal density.[56] By studying the normal ganglionic colonic margin included in the resection specimen, predictive models could be developed to identify which patients are at risk for post-operative issues with dysmotility and enterocolitis.

Cell therapy for HSCR

Given the high rate of bowel dysfunction following pull-through surgery, there exists strong interest in finding new ways to regenerate a functional ENS and preclude the need for surgery in HSCR. Both stem cell therapy, to replace the missing neurons and glia in the aganglionic segment, as well as potential treatments to activate the transdifferentiation of endogenous enteric glial cells into enteric neurons, are being actively studied.

Cell sources

A population of enteric neuronal stem cells (ENSCs) can be isolated from the gut wall from children[57–59] and adults,[60] from both the small intestine and the colon,[61] and from both mucosal and full-thickness biopsies.[62] These ENSCs are self-renewing, exhibit high rates of neurogenesis in vitro, and proliferate in culture to form clusters of concentrated neural stem cells called neurospheres.[59 60 63] These neurospheres can be delivered to the colon via laparotomy, through a peri-anal approach, or via endoscopic injection in mice[64] and swine.[65] Following transplantation, ENSCs engraft and differentiate into neurons and glial cells.[66]

Importantly, ENSCs have the ability to give rise to enteric neurons and appropriate neuronal subtypes.[67] Embryonic ENSCs effectively engrafted when transplanted into aganglionic embryonic explants and were able to regulate tissue contractility.[58] Furthermore, ENSCs isolated from the proximal ganglionic colon of postnatal HSCR mice were successfully transplanted into the aganglionic distal colon and differentiated into enteric neurons and glia.[68] More recently, autologous ENSCs were isolated from a segment of small bowel and used to replace missing neurons and glia in a model of focal aganglionosis induced using diphtheria toxin. The transplanted cells formed neo-ganglia and restored colonic contractile activity as shown by electrical field stimulation and optogenetics.[69] Another recent study used sacral and vagal neural crest-derived human pluripotent stem cells (hPSCs) to rescue a mouse model of total colonic aganglionosis.[70]

Optimization to maximize the efficacy of cell therapy

Although these findings are encouraging, most cell therapy studies in postnatal animals have been limited by relatively poor engraftment, migration, and proliferation of cells.[5 71 72] Current research therefore emphasizes the optimization of stem cell efficacy, including supplementing growth factors into culture conditions,[61] co-transplantation of cells with other molecules packaged in liposomal nanoparticles,[73 74] or direct modification of donor cells using viral vectors.[68] For example, when glial cell line-derived neurotrophic factor (GDNF) is supplemented into the culture media, neurospheres have increased neurogenic potential.[61 75] Additionally, in vivo co-administration of ENSCs with 5-Hydroxytryptamine type 4 (5-HT4) receptor agonist-loaded nanoparticles significantly enhanced neuronal density and proliferation.[73] Similarly, transduction of donor cells with a lentivirus that knocked down the heparan sulfate proteoglycan, agrin, enhanced the migration of stem cells following transplantation onto gut explants and in vivo.[76] Our lab is currently studying the utilization of novel isolation and expansion methods to increase the number and proportion of ENSCs that comprise our donor cells with the goal of enhancing the neurogenic potential of transplanted cells.

Attempts to circumvent immunological challenges

Nearly all studies on ENSC transplantation have been allogeneic, meaning that the donor cells are obtained from the same species but not from the exact same animal that is receiving the transplant. Autologous ENSC transplantation is feasible, but requires a piece of bowel to be removed to isolate and culture the donor cells.[69] Therefore, the identification and use of other sources of autologous cells that are easier to access is critical for clinical translation. Skin, dental pulp, adipose tissue, and bone marrow all contain neural progenitor cells with potential for use in regenerative therapy. Skin-derived precursors (SKPs) have been isolated from rodents,[77] swine,[78] and humans.[79] Rat SKPs were successfully transplanted into aganglionic rodent intestine[77] and human SKPs engrafted into swine colon 1 week following transplantation.[79] Similarly, dental pulp stem cells (DPSCs) have been isolated from humans,[80 81] and although they have yet to

be transplanted into the gut, they hold promise as they were successfully transplanted into a rat model of spinal cord injury.[82] Neural stem cells have also been isolated from the subcutaneous adipose tissue of both mice and humans, and transplantation of these cells into mouse models of gastroparesis and HSCR improved gastrointestinal function.[83] Furthermore, neural stem cells have been isolated from a neurovascular niche in the bone marrow and form neural networks following transplantation.[84] Pluripotent stem cells have been used,[85] but these are burdened with their own risks, primarily the risk of cancer development.

Use of cell therapy as an adjunct treatment for HSCR

Until this point, we have been discussing the role of cell therapy in regenerating the ENS in the aganglionic segment. Another possibility is to use cell therapy as an adjunct, rather than a replacement, for surgery. For example, the proximal ganglionic colon in HSCR is often not entirely normal, containing either relative hypoganglionosis or an imbalance of neuronal subtypes. Although this has yet to be investigated, delivering ENSCs to the proximal colon at the time of pull-through surgery could theoretically be beneficial in restoring an ENS that more closely approximates that of someone without HSCR. Additionally, in HSCR there is an excess of extrinsic excitatory cholinergic signaling and a lack of inhibitory nitrergic signaling in the internal anal sphincter (IAS),[86] leading to an elevated resting pressure. All patients with HSCR have internal sphincter achalasia with an absent rectoanal inhibitory reflex, referred to as IAS achalasia.[15] This does not recover after pull-through surgery, but most children are able to overcome the tonic contraction to defecate normally. However, for some children, IAS achalasia will contribute to multiple complications including obstructive symptoms, overflow fecal incontinence, and fecal stasis associated with increased risk of HAEC. For these patients, Botox injection into the IAS is very effective at facilitating stooling and decreasing hospitalization.[87] For patients for whom Botox injection is helpful, they may undergo repeat injections as frequently as every 3–6 months. Stem cell therapy is being pursued as a treatment for IAS incontinence, and therefore may be a possible option both for primary IAS achalasia and for post-operative issues due to IAS injury or dysfunction.[88 89] Patients that benefit from botulinum injection following pull-through procedure may therefore be ideal candidates for regenerative therapy.

Potential of enteric glial cells as neural progenitors

While some groups are pursuing stem cell therapy as a potential treatment for neurointestinal disease, others are attempting to activate the endogenous neural progenitor cells that are still present in the gut. Postnatal enteric neurogenesis arises from the transdifferentiation of a subset of EGCs.[90–95] Adult enteric neurogenesis does not occur under normal, steady-state conditions, but rather only after specific perturbations and/or injury to the intestine. In one study, a subset of EGCs were found to differentiate into enteric neurons after the direct chemical ablation of the myenteric plexus,[90] whereas another group found that chemical colitis triggered robust neurogenesis by Plp1+ and Sox2+ EGCs.[92] It is now understood that certain EGCs can become neurons under the appropriate conditions; however, the exact subtype of EGCs capable of this response and the specific conditions required to trigger this response are not well understood. Prior in silico work revealed that Gfap+ intraganglionic EGCs retain open chromatin at loci associated with neuronal differentiation and therefore remain epigenetically poised to become neurons, suggesting that this subtype of EGCs can be a source of new neurons.[94]

EGCs are widely distributed throughout all the layers of the gut wall, including within the submucosal and myenteric ganglia, and in close association with the nerve fibers that extend into the mucosa and intramuscular space.[96 97] EGCs have been categorized into four types based on their morphology and location: type I EGCs have astrocyte-like morphology and reside within the ganglia, type II EGCs have a fibrous morphology and reside within interganglionic connectives, type III EGCs are located outside the ganglia in close association with neuronal fibers at the level of the myenteric plexus, and type IV EGCs have a bipolar morphology and reside within the smooth muscle layers directly adjacent to nerve fibers.[96] Glial cells, in the form of Schwann cells, also reside along the extrinsic nerve fibers, which are hypertrophied in the aganglionic region of HSCR.[98] While multiple studies have shown glial cells residing within these hypertrophic nerve bundles,[99 100] there has only been one report of a population of EGCs inhabiting the intramuscular space in HSCR.[97] Demonstrating the activation and transdifferentiation of endogenous EGCs, investigators showed that rectal administration of GDNF via enema induced the proliferation of Schwann cells from the extrinsic nerve fibers and led to the generation of new neurons.[99] We are currently studying the neurogenic potential of the endogenous glia that reside within the intramuscular space in HSCR, which, if able to be activated to undergo neurogenesis, could represent a potential new therapy for HSCR.

CONCLUSION

Hirschsprung disease is the most common congenital neurointestinal disease. Since its description in 1886, the medical community has made significant advances in understanding the pathophysiology of the disease, making the diagnosis quickly and accurately, and managing the disease in the pre-operative, operative, and post-operative periods. However, long-term outcomes remain suboptimal and there is substantial room for improvement. We anticipate future changes in the management of HSCR to include the optimization of diagnostics using non-invasive imaging techniques and the aid of AI to review pathology slides, improvements in our understanding of the correlation between genotype and disease phenotype, as well as the predictive potential of long-term bowel function from the surgical resection specimen. Finally, we expect that regenerative cell therapy could become a novel strategy for treating the disease by replacing

the missing enteric neurons and potentially eliminating the need for pull-through surgery.

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