1

GENERAL INTRODUCTION
DEVELOPMENT OF THE ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) is the complex network of neurons and glial cells that occurs along the entire length of the gastrointestinal tract and is responsible for the peristaltic movements of the bowel, as well as regulation of gut metabolism. Although the ENS is partially controlled by the central nervous system (CNS), it largely functions autonomously. The neurons and glial cells of the ENS are organized in interconnected ganglia that are localized in two plexuses in the gut wall. The myenteric (Auerbach’s) plexus is located between the longitudinal and circular muscles of the gut wall and innervates these muscular layers. The submucosal (Meissner) plexus is localized within the submucosa and regulates gut homeostasis1,2. The ENS is entirely derived from the neural crest, a cell population that originates at the border of the non-neural ectoderm and the neural plate. Upon closure of the neural tube, neural crest cells delaminate and migrate throughout the body and differentiate into various cell types including melanocytes, craniofacial cartilage, adrenal medulla, dorsal root ganglia and the neurons and glia of the ENS3.

The majority of the ENS is derived from vagal neural crest cells that originate from the neural tube at the level of somites 1 to 74. Vagal neural crest cells invade the foregut at 4 weeks of gestation in human embryonic development5. These enteric neural crest cells (ENCCs) proliferate extensively. Vagal neural crest cells migrate in a rostral to caudal direction to colonize the foregut, midgut and hindgut, whereas sacral neural crest cells move in the opposite direction. Colonization of the entire length of the human gastrointestinal tract is completed by the 7th week of gestation5.

The development of ENS has been studied in detail in mice, where neural crest cells colonize the gut between embryonic day 9.5 (E9.5) and E13.56 (Figure 1). Time-lapse microscopy of fluorescently labeled ENCCs in mice has shown that ENCCs migrate along the gut in interconnected strands7. Individual cells break from these strands at the wavefront and assemble into new chains of migrating cells. As the gastrointestinal tract develops, the midgut and hindgut become juxtaposed in a hairpin loop at mouse embryonic day 11.5. A subpopulation of the migrating ENCCs use this spatial organization as a shortcut from the midgut to the hindgut via the mesentry (Figure 1). These trans-mesenteric ENCCs extensively contribute to the ENS in the murine hindgut8. Although the hairpin loop is also observed in human embryonic development, it is yet unclear whether ENCCs cross...
the mesentery in humans. Interestingly, advancing murine ENCCs pause their migration for approximately 12 hours when reaching the caecum, before continuing colonization of the hindgut and caecum. Whether this pause in migration occurs in humans is also as yet unknown.

The hindgut is partially colonized by sacral neural crest cells that originate at the level of somite 24 in mice. Sacral neural crest cells enter the hindgut late during ENS development and migrate in the opposite direction to the vagal neural crest cells (Figure 1). Studies using quail-chick chimeras showed that the contribution of the sacral neural crest to the ENS is limited, but contributes up to 17% of neurons in the chick hindgut. Although vagal and sacral neural crest cells have different migratory properties, they are morphologically indistinguishable after the gut is completely colonized.

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**Figure 1. Colonization of the developing gastrointestinal tract by ENCCs in the mouse.** Vagal neural crest cells delaminate from the neural tube (E9.5) and migrate in a rostro-caudal direction to colonize the gut (E10.5-E13.5). Sacral neural crest cells contribute to the colonization of the hindgut in opposite direction later during development. Adapted from Heanue & Pachnis (2007) and Sasselli et al. (2012).
In order to form a functional ENS, ENCCs have to differentiate into many neuronal subtypes and glia. Markers of early neuronal and glial differentiation are found in ENCCs that are actively migrating and proliferating. Early differentiating enteric neurons are found just behind the migratory wavefront, while glial precursors are found further away from the wavefront, indicating that enteric neuronal differentiation precedes glial differentiation. ENCCs are a heterogeneous cell population. Terminally differentiated neurons are observed when ENCCs are still mitotic and migratory. However, these represent different cell populations, as markers of terminally differentiated neurons are found exclusively in post-mitotic, non-migratory cells. Different neuronal subtypes arise at different developmental stages. Serotonergic (5-HT) and cholinergic (ChAT) neurons arise early, whereas neurons expressing ENK, NPY, VIP and CGRP appear later and neurons and glial cells continue to be appear postnatally.

**HIRSCHSPRUNG DISEASE**

**Clinical presentation**

Hirschsprung disease (HSCR) is a congenital disorder that is characterized by the absence of enteric ganglia in the distal region of the intestine and it is thought to be the result from incomplete colonization of the gut by ENCCs. The absence of enteric ganglia (aganglionosis) results in tonic contraction of the muscles in the affected segment of the bowel, leading to abdominal distension (megacolon) proximal to the affected segment (Figure 2A-C). HSCR patients commonly present with vomiting, chronic constipation, failure to pass meconium, and less frequently with enterocolitis. Aganglionosis generally affects the most distal region of the bowel and the length of the affected segment varies between patients. Aganglionosis from the anal sphincter up to the rectosigmoid or sigmoid colon is defined as short-segment HSCR and comprises 80% of the HSCR cases (Figure 2A). In long-segment HSCR (15% of the cases) the aganglionosis extends beyond the sigmoid colon and in total colonic aganglionosis (5% of the cases) there is a lack of enteric ganglia along the entire length of the colon. In rare cases, termed total intestinal aganglionosis, even the small intestine, stomach and esophagus can be affected.
**Diagnosis**

The majority of HSCR patients are diagnosed within the first 48 hours after birth. HSCR is sometimes diagnosed during childhood and in rare occasions during adult life. The absence of ganglia in a rectal biopsy confirms a HSCR diagnosis\(^\text{19}\). Aganglionosis can also be detected by increased immunoreactivity for acetylcholinesterase in hypertrophied extrinsic nerve fibers\(^\text{20}\). Additional diagnostic proof can be obtained using anorectal manometry\(^\text{21}\); measurement of the relaxation of the rectum in response to inflation of a rectal balloon. Barium enema radiography may be used to assess the length of obstructed colon and may show a dilated proximal segment.

![Image of normal and HSCR colon](image_url)

**Figure 2. Intestinal aganglionosis and clinical presentation of HSCR.** A) Schematic representation of a normal colon, displaying the different colonic segments, and a HSCR-affected colon. B) X-ray of a HSCR patient showing tonic contraction of the affected segment and dilatation of the proximal bowel. C) Abdominal distension in a newborn suffering from HSCR. Adapted from http://www.ShowMe.com and http://www.artikelkeperawatan.info.
Treatment

Left untreated, the mortality rate of HSCR is ~75%\(^22\). Treatment consists of surgical resection of the aganglionic segment and rejoining of the ganglionic gut with the anal sphincter. This so-called pull-through surgery was first performed by Dr. Orvar Swenson in 1948\(^23\) and several modifications to the original procedure have been described. These are known as the Soave, Duhamel and Boley procedures. From the 1990’s open surgical methods have started to be replaced by laparoscopic and transanal endorectal resection and pull-through surgeries\(^24,25\). These procedures require reduced operating and hospitalization times, and show improved clinical outcome\(^26-28\). Depending on the physical condition of the patient, a colostomy might be created proximally of the aganglionic segment to bypass the abdominal obstruction. In these cases a second surgical procedure is required to perform the pull-through surgery. Even though surgical treatment reduces the mortality rate of HSCR to as little as a few percent\(^29\), long-term follow-up studies showed that many patients suffer from impaired bowel function, constipation, fecal soiling, perianal dermatitis, enterocolitis and associated social problems\(^30,31\).

It has been hypothesized that replenishment of the aganglionic gut with neuronal progenitor cells (NPCs) may provide a future therapy for HSCR. NPCs have successfully been isolated from human embryonic and postnatal mucosal gut\(^32-35\). Moreover, these cells can be expanded \textit{in vitro} when cultured as neurosphere-like bodies (NLBs) and can be differentiated into neurons and glia. Metzger and co-workers have transplanted human NLBs into aganglionic gut segments derived from HSCR patients. Transplanted NPCs migrated away from the NLBs to colonize the aganglionic gut and differentiated into functional enteric neurons\(^35\). Importantly, these authors and others\(^33\) showed that NLBs can be generated from HSCR patient biopsies with similar efficiency as from non-HSCR gut. Although this is an important step towards the clinical use of autologous stem cells in transplantations, cell numbers are currently too low to replenish aganglionic gut segments of HSCR patients.

An alternative and unlimited source of neural crest cells for transplantation to HSCR patients could be provided by human induced pluripotent stem (iPS) cells. Human iPS cells can be differentiated \textit{in vitro} into neural crest cells, can be purified by flow cytometry, and can subsequently be differentiated into peripheral neurons and glial cells\(^36\). When transplanted into chick embryos or mice, human embryonic stem cell-derived neural crest cells are migratory and differentiate \textit{in vivo}\(^37\). \textit{In vitro} neural crest differentiation has been further
optimized to obtain neural crest cells of vagal origin\textsuperscript{38}. When transplanted into a transgenic mouse model for HSCR, these cells extensively migrate, up to the distal colon, and completely rescue disease-related mortality\textsuperscript{38}. Moreover, pepstatin A was found to rescue the impaired migratory capacity of neural crest cells in a genetically engineered HSCR model system, suggesting that pepstatin A could improve the outcome of therapeutic neural crest cell transplantations in HSCR patients\textsuperscript{38}. Despite their clinical promise, a current limitation of hiPS cell-derived neural crest cells for transplantations is their safety, as remaining undifferentiated hiPS cells in the graft can lead to tumor formation in the donor\textsuperscript{39}.

**Epidemiology**

The prevalence of HSCR is around 1:5,000 live births, but varies between ethnic groups. The disease is 2-fold and 3-fold more common among Asians than among Caucasians and Hispanics, respectively\textsuperscript{40}. In all ethnicities, the prevalence of HSCR is higher in males than in females, but it is unclear what causes this bias. This gender bias is most prominent in short-segment HSCR, with a 4:1 male:female ratio, compared to a 2:1 ratio in long-segment HSCR\textsuperscript{41}. Although HSCR mostly presents sporadically (non-familial), HSCR can present in a familial form (5-20% of cases). In general, the risk for siblings of HSCR patients to develop the disease as well (recurrence risk) is 4%, but ranges from 1 to 33% depending on the extent of aganglionosis and the gender of both the patient and sibling (Figure 3). Long-segment HSCR gives a high recurrence risk and has a dominant mode of inheritance with incomplete penetrance. Short-segment HSCR has a much lower recurrence risk and follows a multifactorial or recessive mode of inheritance\textsuperscript{41}. Most HSCR cases present as an isolated trait. However, 30% of the HSCR cases are associated with congenital syndromes, including Down syndrome (Trisomy 21) that accounts for 7% of all HSCR cases\textsuperscript{42}.

**GENES ASSOCIATED WITH HIRSCHSPRUNG DISEASE**

**RET signaling pathway**

Linkage analysis on familial HSCR patients found that the major gene for HSCR maps to chromosome band 10q11.2\textsuperscript{44,45} and *RET* was identified as the gene carrying mutations in this locus\textsuperscript{46,47} (see Table 1 for a list of all HSCR-associated genes). Mutations in the coding regions of *RET* have been found in ~50% of
familial HSCR cases and 15-35% of sporadic cases. Even though coding RET mutations have a dominant mode of inheritance, coding RET mutations have an incomplete penetrance in HSCR (72% in males and 51% in females), suggesting that other genetic aberrations are needed for disease development. In addition to HSCR, RET mutations are involved in Multiple Endocrine Neoplasia type 2 (MEN2) and Familial Medullary Thyroid Carcinoma (FMTC). RET mutations in MEN2 and FMTC are restricted to specific domains of RET and are activating, gain of function mutations. RET mutations in HSCR, on the other hand, are scattered throughout the coding sequence of RET. A wide variety of coding RET mutations has been identified in HSCR patients, including large and small deletions, missense, nonsense, and splice-site mutations. In HSCR, RET mutations generally result in a loss of RET function and lead to haploinsufficiency. However, activating mutations that are associated with MEN2 have been reported in HSCR patients as well.

Besides rare, coding mutations, also common, non-coding variants in RET have been associated with HSCR. Two independent haplotypes in the RET locus have been identified. The first haplotype spans 27 kb between 4 kb upstream of

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**Figure 3. Recurrence risk and incidence of HSCR by phenotype and contributing genetic variant.**

HSCR mostly affects a short segment of the bowel, is most common in males and predominantly occurs sporadically. The recurrence risk is higher for long-segment HSCR, females and familial cases. The more common forms of HSCR have a large contribution of common genetic variants with low penetrance, whereas the rare and more heritable forms of HSCR are caused by more penetrant, rare variants. Adapted from Emison et al. (2010).
the RET promoter, 2 SNPs in an evolutionary conserved enhancer in intron 1 and a synonymous SNP in exon 2. The two SNPs in the RET promoter sequence reduce the binding affinity of NKX2-1 (TTF1) to the RET promoter, but the contribution of these SNPs to RET promoter activity is limited\(^5^6\). Similarly, the synonymous SNP in exon 2 does not influence RET expression levels\(^5^7\). The two SNPs in the conserved enhancer in intron 1 are in strong linkage disequilibrium. Nevertheless, both SNPs influence the activity of the enhancer independently\(^5^8,5^9\). Mice expressing the LacZ reporter gene under control of the enhancer element containing both SNPs show that the temporospatial expression of LacZ is similar to that of RET, suggesting that the enhancer element is indeed regulating RET expression\(^6^0\). Interestingly, the penetrance of the intron 1 enhancer SNPs is higher in males than in females. Overall, these SNPs have a 10 to 20-fold higher contribution to disease risk than coding mutations\(^5^8\). The predisposing haplotype is present in ~60% of Caucasian patients, compared to 20% of controls. In Asians, the frequency of the predisposing haplotype is higher; 85% in patients and 40% in controls\(^6^1\). The high frequency of the predisposing haplotype might contribute to the higher incidence of HSCR among Asians. The second HSCR-associated RET haplotype contains a synonymous SNP in exon 14 and a SNP in the 3’UTR. The 3’UTR SNPs is protective against HSCR by increasing the stability of RET mRNA\(^6^2\). Again, the frequency of the haplotype in different ethnicities is concordant with the prevalence of HSCR, as the protective haplotype is present in 4-8% of Caucasians and is virtually absent in the Asian population\(^6^3\).

Both rare, coding mutations and common, non-coding variants in RET contribute to the risk of developing HSCR. However, their relative contributions depend on gender, length of the aganglionic segment and familiality\(^4^3\). The enhancer SNPs are more common in males, short-segment HSCR and sporadic cases. Rare, coding mutations on the other hand, are more frequent in females, long-segment HSCR and familial cases. The presence of rare and common genetic variants in RET, and possibly also in other genes, therefore contributes to the gender-, segment length- and familiality-dependent bias in recurrence risk and incidence of HSCR.

**RET signaling in ENS development**

RET is a receptor tyrosine kinase that is expressed by ENCCs throughout ENS development. RET can be activated by four different ligands, glial cell line-
Table 1. HSCR-associated genes and loci.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Phenotype</th>
<th>Frequency of coding mutations</th>
<th>Inheritance</th>
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<tr>
<td>RET</td>
<td>10q11.2</td>
<td>Non-syndromic HSCR / MEN2A</td>
<td>50% familial, 15–35% sporadic</td>
<td>Dominant, incomplete penetrance</td>
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<td>GDNF</td>
<td>5p13.1</td>
<td>Non-syndromic HSCR</td>
<td>Rare</td>
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<td>GFRα1</td>
<td>10q25.3</td>
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<td>1 case reported</td>
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<td>NRTN</td>
<td>19p13.3</td>
<td>Non-syndromic HSCR</td>
<td>Very rare</td>
<td>Non-Mendelian</td>
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<tr>
<td>PSPN</td>
<td>19p13.3</td>
<td>Non-syndromic HSCR</td>
<td>Very rare</td>
<td>Non-Mendelian</td>
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<td>EDNRB</td>
<td>13q22.3</td>
<td>Non-syndromic HSCR</td>
<td>3-7%</td>
<td>Dominant (de novo in 80%)</td>
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<td>EDN3</td>
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<td>ECE1</td>
<td>1p36.12</td>
<td>HSCR, craniofacial and cardiac defects</td>
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<td>Dominant</td>
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<td>6%</td>
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<td>PHOX2B</td>
<td>4p13</td>
<td>CCHS</td>
<td>&lt;5%</td>
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<td>ZFHX1B / ZEB2</td>
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<td>Mowat-Wilson syndrome</td>
<td>&lt;5%</td>
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derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN). These ligands bind to GDNF family receptor alpha (GFRα) 1-4, respectively, that act as co-receptors in RET signaling. Ligand binding to RET, and GFRα co-receptors result in RET dimerization and trans-phosphorylation of tyrosine residues in the intracellular domain of RET. The phosphorylated tyrosine
residues function as docking sites for a wide range of downstream signaling cascades, including the ERK, PI3K/AKT, p38-MAPK and JNK pathways, that are critical for proliferation, migration, differentiation and survival of ENCCs and their derivatives. Evidence that RET is required for the development of the ENS has been obtained in Ret knockout mice, as these mice fail to colonize the bowel beyond the stomach. However, the required RET gene dosage differs between humans and mice. Only homozygous Ret knockout mice are aganglionic, but heterozygous animals develop normally. In humans, on the other hand, mutations in RET are heterozygous and lead to a disease phenotype with reduced penetrance.

Like Ret-deficient mice, mice lacking the RET ligand GDNF, or its coreceptor GFRα1, also display total intestinal aganglionosis. GDNF acts as a chemoattractant to vagal ENCCs as they migrate along the developing gut. In addition, GDNF is required for the proliferation and survival of ENCCs, induces neuronal differentiation, and is a chemoattractant for outgrowing neurites. In humans, heterozygous mutations in GDNF have been found sporadically in isolated HSCR cases.

Of the remaining RET ligands, mutations in NRTN and PSPN have been identified in HSCR patients, but RET signaling by NRTN and PSPN is less critical for ENS development than signaling by GDNF. NRTN is involved in neuronal differentiation of ENCCs, as mice lacking the NRTN co-receptor GFRα2 exhibit a reduced density of cholinergic projections and decreased intestinal motility. Despite the indispensable role for the GFRα co-receptors in RET signaling, mutations in these genes have not yet been reported in HSCR patients, except for a GFRα1 deletion in a single patient.

**Endothelin signaling pathway**

In addition to RET, signaling through the endothelin receptor type B (EDNRB) is also critical for the development of the ENS. EDNRB is a G protein-coupled receptor that is expressed by ENCCs and activated by its ligands endothelin (EDN) 1-3 that are secreted by the gut mesenchyme. Mutations in the endothelin pathway account for ~5% of all HSCR cases. Most of these mutations were found in EDNRB, but mutations in EDN3 have been reported as well. In addition, a single mutation has been found in endothelin converting enzyme 1 (ECE1), an enzyme that processes endothelins in the gut mesenchyme before secretion into the lumen of the gut. Mutations in EDNRB were first identified in a Mennonite population with a high prevalence of HSCR. Both heterozygous and homozygous
ENDRB mutations occurred in this population. Several homozygous patients presented with additional symptoms, including deafness and hypopigmentation, a condition known as Shah-Waardenburg syndrome. The presence of heterozygous ENDRB mutations in isolated HSCR has later been confirmed. Likewise, homozygous mutations in the ligand EDN3 cause Shah-Waardenburg syndrome, whereas heterozygous mutations in EDN3 lead to isolated HSCR.

Disruption of Ednrb, Edn3 or Ece1 in mice leads to aganglionosis and abnormal pigmentation, resembling the Shah-Waardenburg phenotype. In the mouse gut, EDNRB signaling is required for the migration of ENCCs towards EDN3 between developmental stage E10 and E12.5. Additionally, activation of EDNRB by EDN3 induces ENCCs to proliferate, maintains their precursor state and prevents premature differentiation.

Neuregulin signaling
A genome-wide association study (GWAS) performed on HSCR patients revealed a strong association of SNPs in intron 1 of neuregulin 1 (NRG1) in a Chinese population. Subsequent fine-mapping of the NRG1 locus showed that the four most highly associated SNPs map to the NRG1 promoter region and these variants reduce NRG1 expression levels in gut tissue. The association between common variants in NRG1 and HSCR has been confirmed in independent Thai and Indonesian populations, but no significant association between NRG1 and HSCR was found in patients of European ancestry. Sequencing analysis of the NRG1 coding region revealed that, in addition to common variants, also rare, coding variants are found in HSCR patients. These were found in both Chinese and Spanish patient cohorts, implicating NRG1 in the development of HSCR not only in Asians, but also in Caucasians.

Following up on the initial GWAS performed on Chinese HSCR patients, these data were re-purposed to assess the role of copy number variants (CNVs) in HSCR. In this study, CNVs in NRG3, a paralog of NRG1, were found to be associated to HSCR. Additional evidence implicating NRG3 in HSCR was obtained in an exome sequencing study on a Chinese HSCR family that identified NRG3 as the top candidate gene in this family.

Neuregulins are a family of proteins that are involved in synaptic plasticity in neurons and differentiation into glial cells. The different neuregulins (NRG1-4) have many splice variants that are either membrane bound or secreted. Secreted neuregulins bind to various different heterodimers of ErbB receptors 1-4.
The importance of Nrg-ErbB signaling in the murine ENS has been shown by conditional knockout of ErbB2 in Nestin-expressing cells. These mice displayed a loss of enteric neurons and glia and distension of the colon, reminiscent of HSCR.

**Semaphorin class 3 signaling**

A GWAS study on HSCR patients of European ancestry detected an association to the semaphorin class 3 locus, that contains seven SEMA3 genes (SEMA3A through SEMA3G). These authors found that SEMA3A, SEMA3C and SEMA3D were expressed in the mouse ENS, and therefore performed targeted sequencing of these genes in HSCR cases. Damaging variants were found in SEMA3A, SEMA3C and SEMA3D and knockdown of sema3c and sema3d in zebrafish embryos resulted in HSCR-like phenotype. The strongest association within the semaphorin class 3 locus maps in between SEMA3A and SEMA3D. This inspired a mutational screening of SEMA3A and SEMA3D that identified several coding variants in these two genes.

**HSCR-associated transcription factors**

Mutations in transcription factors have mostly been implicated in the genetic etiology of syndromic forms of HSCR, such as Shah-Waardenburg syndrome, congenital central hypoventilation syndrome (CCHS), Mowat-Wilson syndrome and Pitt-Hopkins syndrome.

Heterozygous mutations in SOX10 cause Shah-Waardenburg syndrome. This is opposed to the Endothelin pathway in Shah-Waardenburg, where homozygous mutations are involved. SOX10 mutations were originally restricted to syndromic HSCR, however a single case was reported of a truncating mutation in a non-syndromic HSCR patient. SOX10 is expressed by ENCCs as they invade the foregut and is critical for maintenance of their progenitor state. SOX10 mutations are also found in another neurocristopathy, namely Kallmann syndrome.

CCHS is a rare respiratory disorder that is associated with HSCR and is primarily caused by mutations in PHOX2B. CCHS results from loss of autonomic neurons that fail to develop in the absence of PHOX2B.

Mowat-Wilson syndrome, which is characterized by mental retardation, facial abnormalities, epilepsy and HSCR, is caused by mutations in ZFHX1B (official name ZEB2, sometimes referred to as SIP1). ZFHX1B is required for the formation of vagal neural crest cells.
Pitt-Hopkins syndrome is a rare genetic disorder that is characterized by mental retardation, wide mouth and distinctive facial features, and intermittent hyperventilation followed by apnea. Pitt-Hopkins syndrome is caused by mutations in TCF4\textsuperscript{119,120}. A single patient has been reported that, in addition to the characteristic features of Pitt-Hopkins syndrome, presented with HSCR\textsuperscript{121}.

As mentioned earlier, two SNPs in the promoter region of RET overlap with a binding site of the transcription factor NKX2-1. Sequence analysis of the coding regions of NKX2-1 identified one heterozygous mutation in the DNA binding domain of the gene\textsuperscript{56}. Functional analysis showed that the mutant NKX2-1 caused a reduction in RET promoter activity, but only in combination with the predisposing SNPs in the RET promoter. In contrast to the other transcription factors involved in HSCR (SOX10, PHOX2B, ZFHX1B and TCF4), which are associated with syndromic forms of the disease, the NKX2-1 mutation was found in a patient with non-syndromic HSCR. However, NKX2-1 mutations are also associated with congenital hypothyroidism, a condition that can present in syndromic HSCR\textsuperscript{122,123}.

Other genes in HSCR
Mutations in cell adhesion molecule L1CAM have been reported in patients suffering from the rare comorbidity of X-linked hydrocephalus and HSCR\textsuperscript{124–127}. The phenotypic spectrum of L1CAM mutations is extensive however, and one case was reported of two siblings who presented with HSCR and a hypoplastic corpus callosum, but without hydrocephalus\textsuperscript{128}. Another cell adhesion molecule, DSCAM, was found to be associated with HSCR through a chromosome 21 scan in Down syndrome patients with HSCR\textsuperscript{129}. Although the gene was identified in patients with Down syndrome and HSCR, a replication study on non-syndromic HSCR patients confirmed the association between DSCAM and HSCR\textsuperscript{129}.

Homozygosity mapping in a family presenting with Goldberg-Shprintzen syndrome identified homozygous nonsense mutations in KBP (KIAA1279, official name KIF1BP) as a cause for this syndromic form of HSCR\textsuperscript{130}. Recently, the role of KBP in Goldberg-Shprintzen syndrome was confirmed by two reports who found homozygous nonsense mutations and deletions in KBP, respectively\textsuperscript{131,132}. KBP interacts with microtubule-associated proteins and is required for neuronal differentiation and neurite outgrowth\textsuperscript{133}.

Due to the multipotent character of ENCCs, a study was performed to determine the expression levels of stem cell-expressed genes in ENCCs from gut biopsies. The authors found a low expression level of DNMT3B in ENCCs isolated
from HSCR patients compared to control individuals. Subsequent genetic analysis of a large patient cohort found three potential damaging variants in *DNMT3B*\(^{134}\). DNMT3B regulates the expression of neural crest specifier genes and loss of DNMT3B prolongs the duration of neural crest production and emigration from the neural tube\(^{135,136}\).

Through pathway-based epistasis analysis of GWAS data on Chinese patients, *PTCH1* and *DLL3* were identified as susceptibility genes for HSCR\(^{137}\). The association between *PTCH1* and HSCR has later been confirmed in an independent patient cohort\(^{138}\). *PTCH1* acts as a receptor in hedgehog signaling and *DLL3* encodes a ligand for Notch. Both hedgehog and Notch signaling are involved in gliogenesis, underlining the critical balance between neuronal and glial differentiation in ENS development.

**HSCR susceptibility regions**

Linkage analysis on HSCR families has been a widely adopted methodology to identify HSCR-associated loci and led to the identification of 10q11, the locus that contains *RET*\(^{44,45}\). A study by Bolk et al. showed that in fact the majority of HSCR families have linkage to the *RET* locus, although only half of these families carry coding *RET* variants\(^{139}\). Interestingly, this study identified 9q31 as a second susceptibility region exclusively in patients without coding *RET* variants. Fine mapping of the 9q31 locus in Dutch families detected association to *SVEP1* in the absence of *RET* variants, but could not be replicated in Chinese samples. The Chinese population did show association to *IKBKAP*. However, in contrast to the linkage analysis by Bolk et al., this association was most significant in patients carrying coding *RET* variants\(^{140}\). *Ikbkap* is important for ENS development in zebrafish, as knockdown of the gene causes aganglionosis\(^{141}\). Several other loci have been found by linkage analysis and for some of these loci, no causal gene has yet been identified. In the Mennonite population where *EDNRB* was discovered, there was not only linkage to the *EDNRB* locus, but this study also found that the 21q22 locus is a risk modifier of HSCR\(^{142}\). Other studies reported linkage to 3p21 and 19q12 in addition to the *RET* locus\(^{143}\) and linkage to 16q23 in addition to the *RET* and *EDNRB* loci\(^{144}\). Finally, the 4q31-q32 locus was identified in a HSCR family that showed no linkage to any of the known HSCR genes, yet the 4q31-q32 locus was not sufficient to cause the disease\(^{145}\).
Interactions between HSCR pathways

The genetics of HSCR are complex and genetic variants in the above-mentioned genes contribute to HSCR, but are not sufficient to cause the disease. It is a combination of predisposing factors that leads to the development of HSCR. For example, a HSCR patient was reported to have mutations in both RET and EDNRB, each inherited from a healthy parent\textsuperscript{146}. Furthermore, the joint transmission of RET and EDNRB haplotypes was demonstrated in a Mennonite population\textsuperscript{82,144}. These genetic interactions were corroborated by the functional interaction between Ret and Ednrb in mouse models of aganglionosis\textsuperscript{144,147,148}. Association studies of RET and NRG1 in HSCR have shown that the combination of SNPs in these genes gives a higher disease risk than SNPs in RET or NRG1 alone\textsuperscript{98,99,149}. The balance between RET and NRG signaling is important for ENS development, as it was shown that NRG1 inhibits GDNF-induced neuronal differentiation of ENCCs, while GDNF downregulates the expression of the NRG1 receptor ErbB2\textsuperscript{149}. Epistasis between RET and SEMA3 was demonstrated and the risk of developing HSCR correlates with the number of risk alleles in these genes\textsuperscript{100}. Indeed, knockdown of sema3c in zebrafish increases the extent of aganglionosis in conjunction with knockdown of ret\textsuperscript{101}. In several congenital syndromes that are associated with HSCR, the predisposing RET allele in intron 1 is overrepresented in patients that present with HSCR, suggesting that RET is a modifier in syndromic HSCR\textsuperscript{150}.

ENS DEVELOPMENT GENES NOT ASSOCIATED WITH HSCR

As described here, a large number of genes have been associated with HSCR and these genes have functional roles in the development of the ENS. In addition to these HSCR-associated genes, many other genes play a role during ENS development and are therefore candidate HSCR genes. For example, loss of Indian hedgehog (Ihh) causes segmental aganglionosis in mice and loss of Sonic hedgehog (Shh) results in abnormal innervations in the gut\textsuperscript{151}. Total colonic aganglionosis is observed in homozygous Pax3 mutant mice, or in Pax3/Tcof1 double heterozygotes\textsuperscript{152,153}. Other mouse models of aganglionosis include Sall4 and β1-integrin knockout mice that fail to colonize the distal bowel and Mash1 knockout mice display aganglionosis of the esophagus\textsuperscript{154–156}. Furthermore, mice lacking Dcc
fail to develop submucosal ganglia and loss of TrkC, its ligand Nt3 or Hlx1 results in reduced numbers of enteric ganglia\textsuperscript{157,158}.

A forward genetic screen for loci affecting ENS development found partially penetrant aganglionosis in the ‘TashT’ mouse line\textsuperscript{159}. Interestingly, the penetrance of aganglionosis was higher in males than in females, strongly resembling the gender-bias that is observed in HSCR. The inserted transgene was mapped to a gene desert on chromosome 10 that physically interacts with the nearby gene Fam162b. Many X-linked genes were found to be downregulated in ENCCs from ‘TashT’ mouse embryos, and known HSCR genes were differentially expressed.

It has been suggested that the sexual dimorphism in HSCR may result from expression of SRY, which is encoded on the male Y-chromosome. The transcription factor SOX10 positively regulates RET expression by binding to its promoter region. In contrast, SRY represses RET transcription and can competitively replace SOX10 on the RET promoter\textsuperscript{160}. Since SRY is expressed exclusively in males, the gene could contribute to HSCR in males by reducing RET expression.

The Notch, WNT, TGF-β and FGF signaling pathways are involved in the specification of numerous lineages during embryonic development, including the development of the ENS. Notch is required for maintaining the progenitor state of ENCC by regulating SOX10 expression and conditional inactivation of Notch signaling in neural crest cells results in premature neurogenesis\textsuperscript{161}. Wnt signaling is involved in multiple aspects of ENS development. Non-canonical signaling by Wnt11 regulates the specification of the neural crest, proliferation of ENCC depends on Wnt1 and Wnt3a, and Wnt5 is required for axon guidance\textsuperscript{162–164}. BMP proteins are part of the TGF-β superfamily and have been implicated in the development of the ENS. BMP2 and 4 are required for the migration of ENCCs along the gut and loss of BMP signaling causes hypoganglionosis\textsuperscript{165,166}. Moreover, ENCCs fail to organize into ganglia in the absence of BMPs. FGF signaling is required for specification of the neural crest and FGF2 regulates proliferation of neural crest cells\textsuperscript{167,168}.

**COMMON, RARE AND DE NOVO VARIANTS IN HSCR**

As summarized in Table 1, a large number of genes have been associated to HSCR. Most of these genes have been identified through linkage analysis in HSCR families,
the analysis of syndromic HSCR cases and by genetic studies in genetically isolated populations. These cases show a Mendelian mode of inheritance, either recessive or dominant with reduced penetrance. However, the majority of HSCR cases are sporadic and non-syndromic, and show a non-Mendelian mode of inheritance. Consequently, only ~25% of the heritability in HSCR can currently be explained. Part of the missing heritability is likely due to genetic variation in yet unidentified HSCR genes. The introduction of GWAS has allowed the identification of predisposing variants in sporadic HSCR cases on a genome-wide scale and has implicated the neuregulin and semaphorin class 3 pathways in the etiology of HSCR. In addition to the common variants associated with HSCR, targeted sequencing has revealed that rare variants contribute to sporadic HSCR as well.

Next-generation sequencing (NGS) has revolutionized the genome-wide analysis of rare variants. However, it remains challenging to assess the contribution of rare variants to sporadic, non-Mendelian disease. Although rare variants with a relatively large effect size may be identified through exome sequencing, their allele frequency is usually too low for statistical significant associations. Moreover, the large number of genotyped base pairs in exome sequencing requires a large multiple-testing correction, further reducing statistical power. Thus far, exome sequencing studies in HSCR have focused on known and candidate disease genes or have been performed on familial HSCR cases. When it comes to discovering pathogenic rare variants in sporadic disease, exome sequencing has been most successful for de novo mutations. De novo mutations are newly occurring genetic variants that are caused by errors in DNA replication or environmental factors. When de novo mutations occur in the germline they can be passed on to the next generation. Consequently, germline de novo mutations can be found in the somatic cells of a child, but not in somatic cells of its parents. De novo mutations are a major cause of syndromic HSCR, particularly in the HSCR-associated syndromes Waardenburg-Shah (de novo mutations in SOX10), congenital central hypoventilation syndrome (PHOX2B) and Mowat-Wilson syndrome (ZFHX1B). In sporadic HSCR, mutations in RET and EDNRB have been reported to occur de novo, mainly in patients with long-segment HSCR. Given its high heritability, it is conceivable that long-segment HSCR can be caused by de novo mutations in other, not previously associated genes as well. Genome-wide screens for de novo mutations in neurodevelopmental disorders found that de novo mutations contribute to the genetic etiology of these disorders and have led to the identification of new disease
genes\textsuperscript{174}. A genome-wide screen for \textit{de novo} mutations in sporadic HSCR cases may therefore identify new genes for HSCR and help our understanding of HSCR genetics, which could have important implications for genetic counseling.

\textbf{DE NOVO MUTATIONS}

\textbf{Patterns and rates of \textit{de novo} mutations in the human genome}

The rate at which \textit{de novo} mutations occur in the human genome is $1.20 \times 10^{-8}$ per nucleotide per generation for single nucleotide variants (SNVs)\textsuperscript{175,176}. This comes down to an average of 63.2 \textit{de novo} SNVs in the human genome\textsuperscript{175}. Measurements of \textit{de novo} SNV rates in the exome (the coding regions of the genome) are slightly higher, with observed rates of $1.31-2.17 \times 10^{-8}$ (176). This difference may be partly explained by the high GC content of the exome and by transcription-induced mutagenesis\textsuperscript{177}. The \textit{de novo} mutation rates of small insertions and deletions and large copy number variations (CNVs) are orders of magnitude lower\textsuperscript{178,179}. However, due to their large size, the total number of base pairs affected by \textit{de novo} CNVs exceeds the number of \textit{de novo} SNVs. \textit{De novo} mutations are not randomly distributed over paternally and maternally inherited chromosomes. In fact, 75\% of \textit{de novo} SNVs are located on a chromosome that is inherited from the father\textsuperscript{175,176,180}. Moreover, the number of germline \textit{de novo} mutations increases with the father’s age. For every added year to the father’s age at time of conception, the child will have 2 additional \textit{de novo} mutations\textsuperscript{175,180}. The age of the mother at time of conception does not affect the number of \textit{de novo} mutations in the offspring. The high number of cell divisions during male spermatogenesis can explain the gender and age bias, which continues to increase with age.

The Genome of the Netherlands (GoNL) consortium has analyzed the patterns of \textit{de novo} mutations in 250 Dutch parent-offspring trios in great detail\textsuperscript{181}. The GoNL study found that the genomic position of \textit{de novo} mutations is not random and depends on the father’s age. \textit{De novo} mutations in offspring from young fathers reside in late-replicating genomic regions. With increasing paternal age, \textit{de novo} mutations are more likely to be found in early-replicating loci\textsuperscript{181}. In addition, \textit{de novo} mutations in offspring from older fathers are more often located in the proximity of genes, suggesting that older fathers not only transmit a higher number of \textit{de novo} mutations, but their \textit{de novo} mutations are also more likely to have a functional impact\textsuperscript{181}. In line with other reports, the GoNL study found
clusters of 2-3 de novo mutations within 20 kb\textsuperscript{182,183}. Interestingly, the clustered de novo mutations contained few C>T substitutions in CpG islands and many C>G substitutions compared to non-clustered de novo mutations\textsuperscript{181}. This finding suggests that the clustered de novo mutations arise by a different mechanism than non-clustered de novo mutations.

\textbf{De novo mutations in human disease}

The introduction of new genetic variation by de novo mutations is a requirement for evolution. However, only a fraction of all de novo mutations will be advantageous for an individual. In fact, de novo mutations are an important cause of human diseases\textsuperscript{174}. Somatic de novo mutations are involved in cancer and many genetic diseases are caused by germline de novo mutations. Rare developmental disorders are generally caused by de novo mutations in a single gene. These include CHARGE syndrome (mutations in CHD7), Schinzel-Giedion syndrome (SETBP1), Kabuki syndrome (MLL2), Bohring-Opitz syndrome (ASXL1) and KBG syndrome (ANKRD11)\textsuperscript{184–188}. Besides rare developmental disorders, de novo mutations can also contribute to more common genetic diseases. De novo CNVs have been identified in a wide range of neuropsychiatric disorders and the frequency of CNVs in these patients is far higher than in the general population\textsuperscript{189}. Targeted sequencing of known disease genes for autism spectrum disorders (ASD), schizophrenia and intellectual disability (ID) showed that de novo SNVs in multiple, different genes play a role in these diseases\textsuperscript{190,191}.

The introduction of NGS has allowed the genome-wide study of de novo SNVs. In reality, researchers often limit their study to the exome, because exome sequencing is less expensive than whole genome sequencing and non-coding variants remain difficult to annotate and interpret. Four large exome sequencing studies on ASD found de novo SNVs in many interconnected genes that act together in neuronal development\textsuperscript{192–195}. The fraction of de novo mutations that was deleterious was higher in ASD patients than in their unaffected siblings\textsuperscript{192,195}. An excess of nonsynonymous de novo SNVs was also reported in schizophrenia\textsuperscript{196}. Another exome sequencing study on schizophrenia reported an increased de novo mutation rate in patients\textsuperscript{197}. However, it must be noted here that the de novo mutation rate in schizophrenic patients was not compared to unaffected controls, but to publically available whole-genome sequencing data. Since mutation rates from exome sequencing data are higher than those from whole-genome sequencing data\textsuperscript{176}, it is unclear whether the increased de novo mutation rate is
attributable to the phenotype or the sequencing technology. A whole genome sequencing study on patients with ID found de novo SNVs and CNVs in known ID genes in 20 out of 50 patients\textsuperscript{198}. Combined with other technologies, these authors reported de novo events in ID genes in 60% of their patient cohort. The largest de novo mutation screen by exome sequencing was performed on 1,213 patients with congenital heart disease (CHD)\textsuperscript{199}. The de novo SNV rate in these patients was not different from controls, but patients carried significantly more damaging de novo mutations. The enrichment for damaging mutations was even more pronounced in patients that were diagnosed with neurodevelopmental disability or other congenital anomalies in addition to CHD. In line with the phenotypes, heart- and brain-expressed genes carried an excess of damaging de novo mutations.

In general, patients with ASD, schizophrenia or CHD with neurodevelopmental involvement are enriched for deleterious de novo mutations, but do not show an increase in the total number of de novo mutations\textsuperscript{192,195,196,199}. Nevertheless, offspring from older fathers are at increased risk of developing psychiatric disorders, including autism, attention-deficit/hyperactivity disorder (ADHD) and bipolar disorder\textsuperscript{200}. This finding suggests that psychiatric patients carry increased numbers of de novo mutations, a hypothesis that thus far has not been confirmed by exome sequencing studies.

Owing to its time- and cost-efficiency, NGS has made its way into clinical genetic diagnostics. A report has been published on 250 patients with suspected genetic disorders with Mendelian inheritance, who had been subjected to exome sequencing. A molecular diagnosis could be made for 25% of the cases. This study found that 83% of autosomal dominant mutations and 40% of X-linked mutations were de novo, emphasizing the contribution of de novo mutations to genetic disorders\textsuperscript{201}.

AIMS AND OUTLINE OF THIS THESIS

The aim of the work described in this thesis is to assess the contribution of rare variants to the development of HSCR. The advent of NGS has allowed the genome-wide identification of rare variants, including de novo mutations. The role of de novo mutations in long-segment HSCR is described in chapter 2. Since genes carrying de novo mutations were not linked to ENS development based on
bioinformatics prediction, we tested the functional contribution of these genes to
ENS development in a zebrafish model.

The interpretation and selection of candidate genes in HSCR relies on our
understanding of the biological processes underlying ENS development. To better
understand these processes, we analyzed the gene expression profiles of enteric
neural crest cells and the long-term effect of RET signaling in these cells. As
described in chapter 3, predicted regulatory genes in ENS development included
known HSCR genes and provided novel candidate genes for HSCR.

Although NGS offers new possibilities in the genetic dissection of HSCR
and other genetic disorders and traits, the application of the technology remains
challenging. The identification of de novo mutations in exome sequencing data is
best described as looking for a needle in a haystack, since the number of technical
artefacts in NGS data is orders of magnitude larger than the true number of de novo
mutations. Chapter 4 describes how sequencing quality parameters can be applied
to discriminate de novo mutations from false positives and allow their efficient
detection.

In addition to de novo mutations, the exome contains many rare, inherited
variants. Association studies for rare variants generally suffer from a lack of
statistical power. In chapter 5 we describe how a combination of strategies can be
applied to approach this problem and to extract useful data from such studies.

The incidence of HSCR is over a hundred times higher in Down syndrome
patients than in the general population, suggesting that the trisomy of one or more
genes on chromosome 21 predisposes to HSCR. We overexpressed genes from
chromosome 21 in a transgenic zebrafish model to identify chromosome 21 genes
linking HSCR to Down syndrome, as described in chapter 6.

Finally, chapter 7 summarizes and discusses the work presented in this
thesis. Moreover, future perspectives for the genetic dissection of HSCR are given.
REFERENCES


