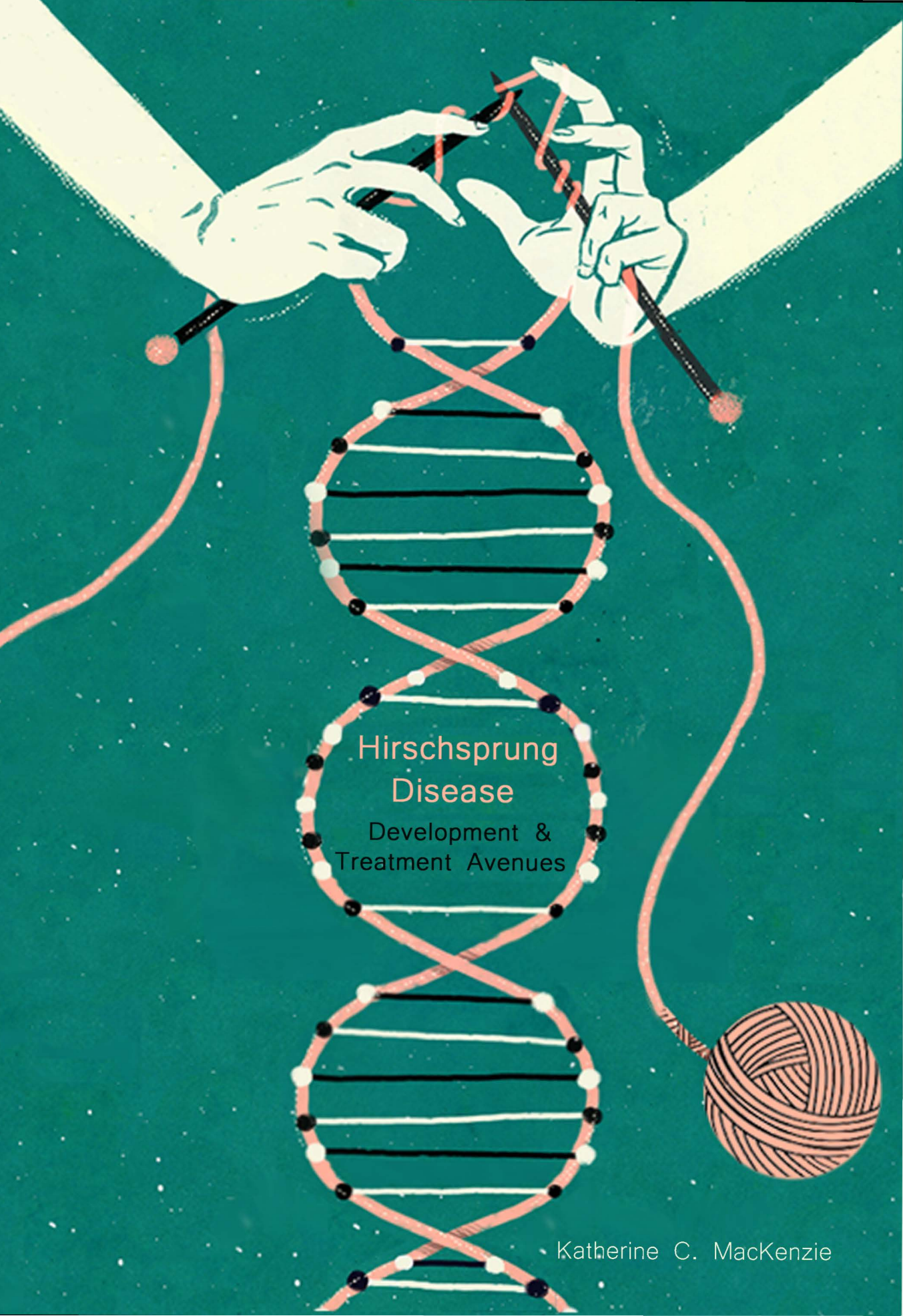
Hirschsprung
Disease
Development &
Treatment Avenues
Katherine C. MacKenzie

Hirschsprung Disease:

Development & Treatment Avenues

Katherine Christa MacKenzie

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ontwikkeling & behandelingsmogelijkheden

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by

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List of Abbreviations

ARTN	Artemin
BP	Base Pair
CN	Copy Number
CNP	Copy Number Polymorphism
CNS	Central Nervous System
CNV	Copy Number Variation
DOF	Degree of Freedom
E	Embryonic Day
EAS	External Anal Sphincter
ECM	Extracellular Matrix
EDN3	Endothelin 3
EDNRB	Endothelin Receptor Type B
ENC	Enteric Neural Crest
ENCC	Enteric Neural Crest Cell
ENS	Enteric Nervous System
ESC	Embryonic Stem Cell
FCS	Foetal Calf Serum
FI	Faecal Incontinence
GABBR1	Gamma-aminobutyric acid B receptor 1
GDNF	Glial cell line-derived Neurotrophic Factor
GFRα1	Glial cell line-derived Neurotrophic Factor Receptor alpha 1
GI	Gastrointestinal
GNL1	Guanine nucleotide binding protein like 1
GOSHS	Goldberg-Shprintzen Syndrome
HEK	Human Embryonic Kidney
HSCR	Hirschsprung Disease
IAS	Internal Anal Sphincter
iPSC	Induced Pluripotent Stem Cell
KIF1BP	Kinesin Binding Protein/KIF1 Binding Protein
LOF	Loss of Function
MCAP	Megalencephaly-capillary malformation
MSC	Mesenchymal Stem Cell
MWS	Mowat-Wilson Syndrome
NCC	Neural Crest Cell
NGS	Next Generation Sequencing
NRG1	Neuregulin 1
NRTN	Neurturin
PI3K	Phosphatidylinositol-3-kinase
PGRS	Polygenic Risk Score

PSC	Pluripotent Stem Cell
PSPN	Persephin
RET	Rearranged During Transfection
RoH	Runs of Homozygosity
SCG10	Superior Cervical Ganglia 10
SEMA3A	Semaphorin 3A
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
TCA	Total Colonic Aganglionosis
TEPT	Transanal Endorectal Pull-Through
USP32	Ubiquitin Specific Peptidase 32
WES	Whole exome sequencing
WS4	Waardenburg-Shah Syndrome
WT	Wild Type


1

Chapter 1

General Introduction

1

Hirschsprung disease (OMIM #142623) is a congenital malformation of the nervous system of the gastrointestinal tract, the Enteric Nervous System. The work in this thesis focuses on modes of development of this disease, *in vitro* disease modelling and possible therapeutic options.

The Enteric Nervous System

The gastrointestinal (GI) tract is the core of the digestive system and has many functions including the mixing and breaking down of ingested material, water and nutrient extraction and absorption, secretion of enzymes and propulsion of ingested material through the body for waste expulsion in defecation[1]. These functions require the input of various cell types communicating together in order to effectively digest food and avoid nutritional deficit or inflammatory responses from poor gut function. At birth, the GI tract, including the oesophagus, stomach, small intestine and colon, is approximately 3 meters in length, and increases to approximately 7 meters in adults[3]. The development of such an organ is complex and requires extensive elongation of the primitive gut tube, as well as migration of a wide variety of cell types along the gut, to ultimately result in co-ordinated gut activity[4]. Proper functioning of the GI system is regulated by the enteric nervous system (ENS).

The ENS is one of the three main divisions of the autonomic nervous system, along with the sympathetic and parasympathetic divisions. The ENS is also colloquially known as the second brain or the brain in the gut, due to its ability to function independently from the central nervous system (CNS). The basic functions of the ENS do not require input from the sympathetic and parasympathetic divisions, although it is influenced by both[5]. The ENS is made up of neuronal cells and supportive glial cells

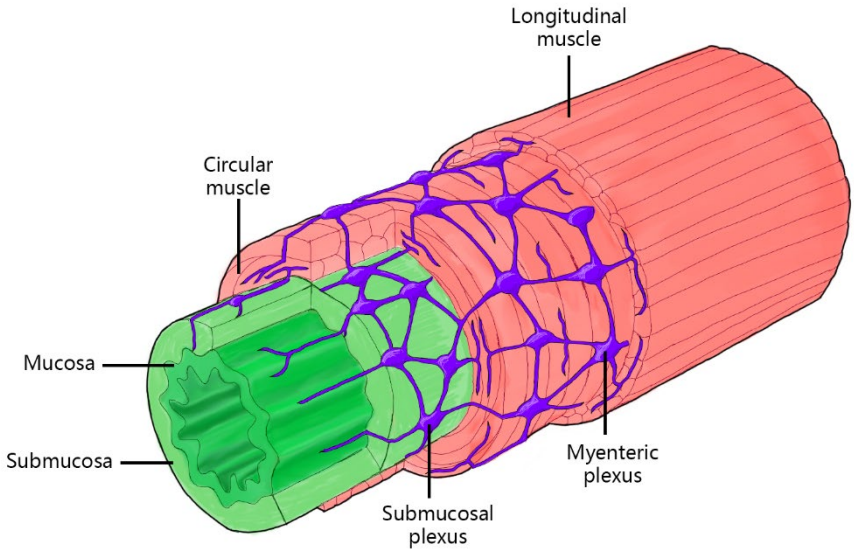

Figure 1. Schematic Cross-section of the adult GI tract showing the submucosal and myenteric plexuses either side of the circular muscle layer. Axons extend radially from the plexuses for communication between plexuses and into the gut mucosa.

located within ganglia, which form two distinct mesh-like plexuses in the wall of the GI tract (Figure 1).

The myenteric plexus spans the entire length of the GI tract and lies between the longitudinal and circular muscle layers. It controls muscle contraction and relaxation which generates the peristaltic movement of the gut. The submucosal plexus, located between the circular muscle and mucosa, is only prominently seen in the small and large intestines. It regulates fluid absorption and secretion, modulates blood flow, and responds to stimuli from the mucosa to support gut function and homeostasis[6].

Development of the ENS

The ENS is derived from the neural crest which arises at the border between the neural plate and the non-neural ectoderm. The majority of cells that are fated to become the ENS originate at the level of the vagal neural crest. These precursor cells, known as enteric neural crest cells (ENCCs), migrate to the cranial end of the gut tube at week 4 of human development (embryonic day [E]9.5 in mice)[7,8], and rapidly proliferate, migrate and differentiate to colonise the entire length of the gut by week 7 (E13.5 in mice) (Figure 2)[7,8]. A smaller population of neural cells also arises from the sacral region of the neural tube, but contributes to the ENS mainly in the distal hindgut[9,10]. In addition to the uniform directional (oral to anal) migration of vagal ENCCs along the gut, Nishiyama and colleagues showed that, in mice, ENCC migration halts

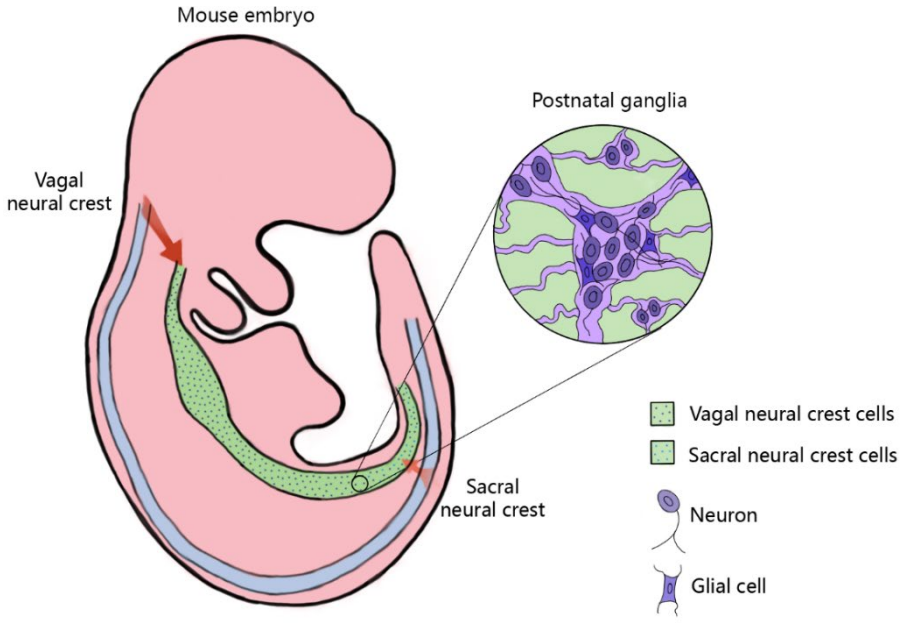

Figure 2. Schematic of vagal neural crest cell (NCC) migration into the embryonic gut tube and the contribution of sacral NCCs in the hindgut. The final ganglia contain glial and neuronal populations.

at the midgut to hindgut boundary to allow for trans-mesenteric migration to occur before the wave-front of ENCCs advances into the hindgut region[11]. It is unclear whether this trans-mesenteric migration occurs in other species, including humans, however given that Hirschsprung disease (HSCR) is mostly limited to the distal colon, this migratory path of ENCCs is interesting for further study.

As the gut is growing and elongating during embryogenesis and foetal development the ENCCs are highly migratory, with cells migrating in all directions rather than just towards the caudal end of the gut. The leading wavefront of migrating cells sets the tracks for the other cells to follow, determining the position of the ganglionic network[12,13]. Migratory pathways are dependent on the expression of surface receptors in order for the cells to recognise environmental signals, if present. For example, GDNF, expressed in the gut mesenchyme, is recognised by GFRα1, expressed on the migrating NCCs and acts as a chemoattractant for the migrating wavefront[7]. Studies in avian models have established that migration in the pre-umbilical stage, through the foregut and midgut, occurs before smooth muscle formation. As the cells migrate along this mesenchymal gut tube, the circular muscle layer begins to develop, creating a different migration environment for cells in the hindgut[10,13]. Additionally, cell adhesion molecules and appropriate extracellular matrix (ECM) components, such as collagen 18 and agrin proteins, secreted at the migrating wavefront, play a crucial role in these migratory pathways[12,14]. Intercellular and extracellular signals from surrounding cells and the ECM also influence cell fate decisions and components of the ECM have been studied *in vitro* to assess their effect on ENCCs and other gut cells. It has been shown that on tissue engineering matrices, nitrergic neuronal populations are

enriched with presence of collagen IV. Presence of laminin and/or heparan sulphate gives balanced relaxant and contractile motor neuron populations[15].

As cells stop migrating and find their final positions within the gut wall, they form connections and synapses with each other and the neural cell bodies group into ganglia[16,17]. The ENCCs differentiate into multiple neuronal subtypes as well as enteric glia. At least 20 enteric neuronal subtypes have been identified, varying in function, electrophysiology, neuro-transmitters and morphology[18].

Multiple trophic factors, morphogens, and transcriptional regulators control and influence enteric neural subtype specification within the ENS[19]. However, the mechanisms determining specific differentiation patterns are poorly understood and a fate map of ENCC differentiation does not yet exist. Factors influencing ENCC proliferation, migration or differentiation may alter the ratio of subtypes within the GI tract, depending on when and where a neuron is 'born' during ENS development, as evidenced by its exit from the cell cycle. The timing of cell cycle exit has been linked with lineage commitment and this could mean that a slowing of migration or proliferation of ENCCs would not similarly slow-down their differentiation and cell cycle exit[20]. It is possible that this could lead to insufficient naïve ENCCs reaching the distal colon. Neural activity also influences ENCC differentiation and migration, as treatment with neurotoxins that inhibit vesicular signalling has been shown to alter the ratio of neuronal subtypes and slow cell migration[21]. Thus, alterations in developmental signals can occur through many modes and pathways, which create opportunities for physiological and/or anatomical malformations of the ENS.

Hirschsprung Disease

Hirschsprung disease (HSCR) is a congenital neuropathy of the GI tract characterised by an absence of enteric ganglia in a variable length of the distal gut (Figure 3). This defect is usually confined to the colon but, although rare, total intestinal aganglionosis can occur. The length of the

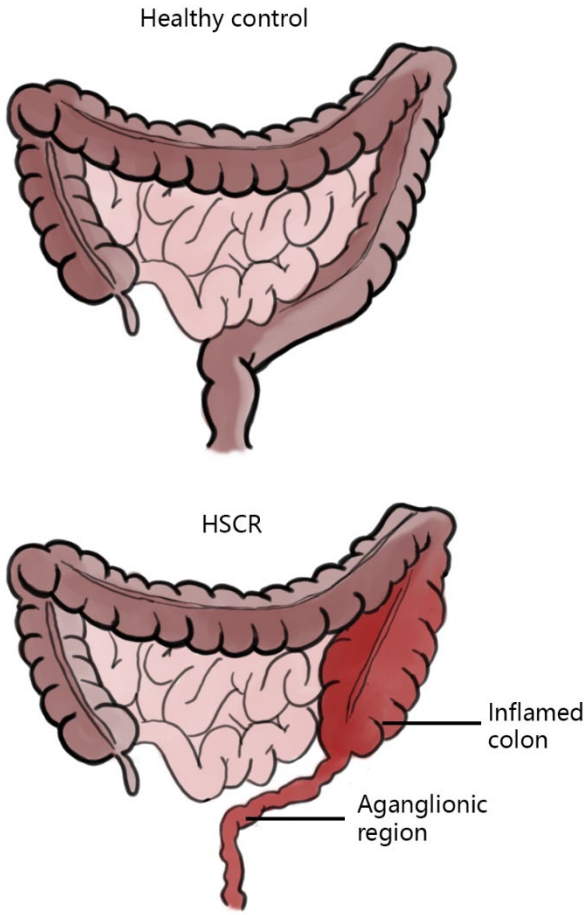

Figure 3. Schematic of the intestines of a healthy individual compared to a patient with HSCR. The tonic constriction of the distal colon in the patient leads to functional obstruction. The inability of faecal matter to pass causes inflammation and expansion of the proximal regions of the colon, observed by abdominal distension in the patient.

aganglionic segment varies from only the rectal regions, or rectal and sigmoid regions, (short segment), extended towards the splenic flexure or transverse colon (long segment), or further to the cecum (total colonic aganglionosis; TCA). The affected region of gut lacks intrinsic neural input with the result that the smooth muscles of the gut wall contract, causing life-threatening obstruction and preventing passage of stool. Complications from HSCR include infections, enterocolitis, abdominal swelling and potential rupture of the colon[6,22-24].

HSCR results from a failure of ENCCs to colonise the full length of the GI tract, which could be caused by functional deficit within the ENCCs themselves or in the local environment that the ENCCs encounter during their migration along and within the gut. The appropriate proliferation, migration, differentiation and survival of these cells is therefore essential for colonisation. The basic pathophysiological feature in HSCR is functional obstruction caused by the tonic contraction of smooth muscle of the aganglionic segment, and absence of the peristaltic motion of the gut. Despite extensive research, the pathophysiology of this is not fully understood. There is no clear explanation for the occurrence of tonic contraction of this smooth muscle other than the absence of signals from the ENS.

Genetics of HSCR

Genetics of Isolated HSCR

HSCR is a congenital disorder and can be present as an isolated feature or part of a syndrome. Isolated HSCR has been shown to have familial recurrence, but it most commonly occurs sporadically[2,25]. The incidence of HSCR is estimated at 1 in 5000 live births, although this varies between populations[6]. Developmentally, HSCR has a genetic

component and there is a sex-linked bias in classical and short segment cases with a male:female ratio of approximately 4:1[6]. To date, at least 17 genes have been found to play a role in isolated HSCR development, in patients and animal models (Table 1)[2,26,27]. Of these, the Rearranged during Transfection gene (*RET*) has proved to be the most important. This can be concluded from several genetic linkage analyses which have shown that, even in the absence of pathogenic coding variants in *RET*, over 90% of familial HSCR is linked to the gene[28,29]. Mutations affecting

Table 1. HSCR Associated genes

Gene	Location	Phenotype	Incidence	Pathway
RET	10q11.2	HSCR	50% familial; 20% sporadic	RET
GDNF	5p13	HSCR	Very rare	RET
GFRα1	10q26	HSCR	Very rare	RET
NTN	19p13	HSCR	Very rare	RET
PSPN	19p13	HSCR	Very rare	RET
EDNRB	13q22	HSCR; WS4	3-7%	Endothelin
EDN3	20q13	HSCR; WS4	<5%	Endothelin
SOX10	22q13	HSCR; WS4	>5%	Transcription factor
L1CAM	Xq28	HSCR x-linked hydrocephalus	Rare	-
NRG1	8p12	HSCR	6%	ERBB2; RET
NRG3	10q23.1	HSCR	-	ERBB2; RET
DENND3	8q24.3	HSCR (zebrafish)	-	-
NCLN	19p13.3	HSCR (zebrafish)	-	-
NUP98	11p15.4	HSCR (zebrafish)	-	-
TBATA	10q22.1	HSCR (zebrafish)	-	-
IHH	2q35	Hypoganglionosis (zebrafish)	-	Hedgehog; RET
GLI3	7p14.1	-	-	Hedgehog; RET

HSCR: Hirschsprung disease; **WS4**: Waardenburg-Shah syndrome.

its coding and non-coding regions have been described[30,31]. When screening for mutations, pathogenic coding variants in *RET* are identified in ~50% of familial and 15-35% of sporadic HSCR cases[25,32].

A number of loci in or around non-coding regions of *RET* have been identified, in several studies, to be linked with susceptibility to or protection from HSCR[33-41]. An enhancer variant in *RET* intron 1, a C>T SNP, was found to have a higher HSCR contribution risk than coding sequence variants. The frequency of the T allele in the European population is approximately 20% and is as high as 50% in the Chinese population, which could contribute to the higher incidence of HSCR in Asian populations[25,39]. Common variants in *RET* are well established as a susceptibility factor for HSCR[30,42]. All these studies point towards a central role for *RET* in the development of isolated HSCR and the ENS.

RET signalling balance

RET encodes for a receptor tyrosine kinase, RET, which is involved in several intracellular signalling cascades, regulating cell differentiation, migration, proliferation and survival. RET activation depends on binding with either glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) or persephin (PSPN) and with one of the four GDNF family receptor alphas (GFRα1-4) respectively to form a complex[43]. Disturbance of RET or any of these binding partners will in turn affect the action of downstream pathways, which can also disturb the development of other components of the peripheral and central nervous systems[44]. It has been hypothesized that, because *RET* proves to be the key player in isolated HSCR development, *RET* signalling is the fulcrum of ENS formation, with other pathways and the action of ENS-related proteins being dependent upon correct *RET* expression[2]. This

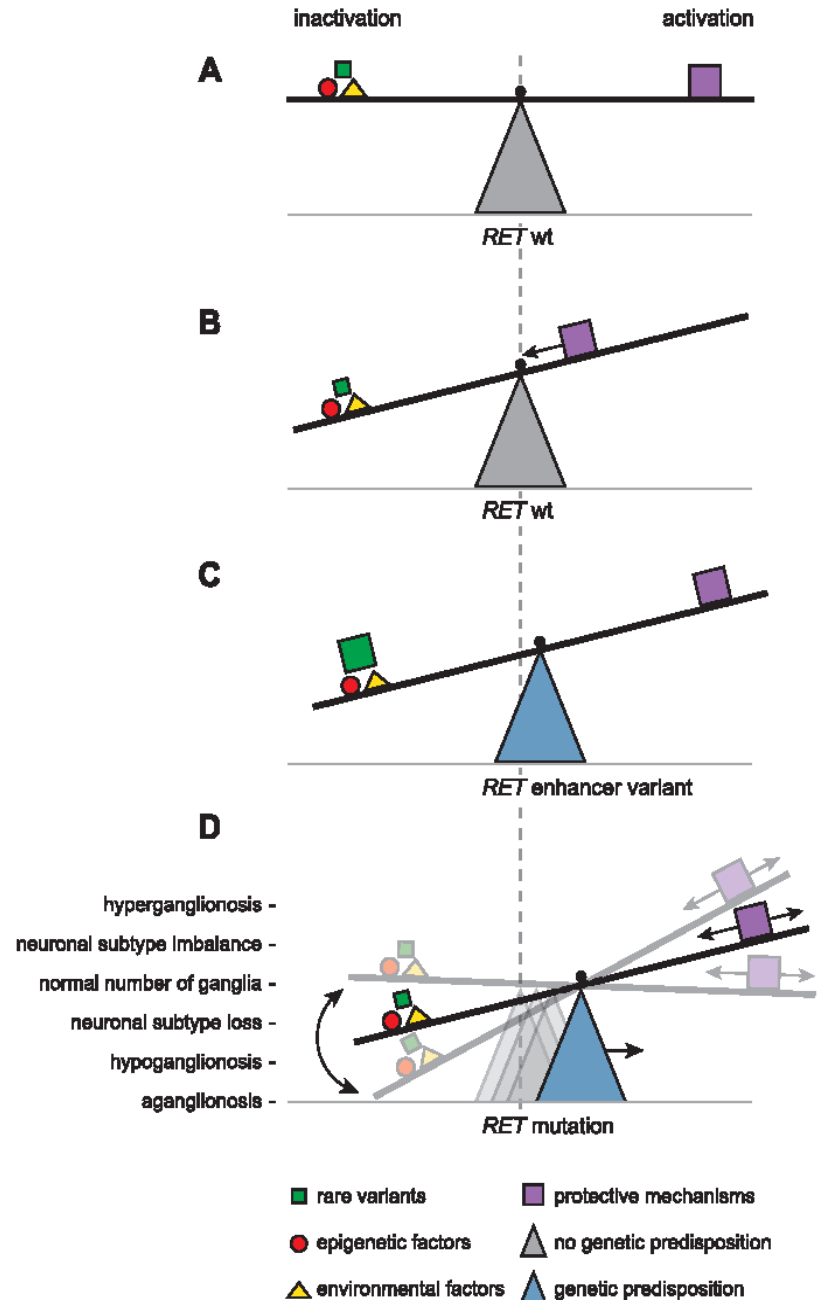

Figure 4. The *RET* signalling balance theory proposes *RET* as the fulcrum of ENS development. Other protective or predisposing factors can contribute in varying amounts towards the tipping of the normal balance towards hyperganglionosis or aganglionosis[2].

model proposes *RET* as the primary influencing factor in the multifactorial development of the ENS, the balance of which can be shifted by genetic or non-genetic factors to lead to a spectrum of ENS phenotypes, ranging from total aganglionosis to hyperganglionosis (Figure 4). It is likely to be a combination of (inherited) genetic and non-genetic factors that cause HSCR.

Genetics of Syndromic HSCR

HSCR occurs as an isolated phenotype with no associated anomalies in the majority of patients[45]. However, due to the genetic nature and the interaction of pathways in this disease it is inevitable that if ENS development is impaired there may be associated impairments in other cell types. Approximately 12% of HSCR patients have an associated chromosomal abnormality and 18% have additional congenital anomalies[2,25]. These associated anomalies most commonly affect other ectoderm or neural crest derivatives, but there are crossovers to other systems that may have links with cell migration or proliferation signals[46].

There are a number of defined syndromes that have HSCR as a feature and are generally explained by Mendelian inheritance (Table 2). In some of these it is a variable feature and its presence or absence may be influenced by modifying factors that either predispose a patient to HSCR or protect against its development[25,46]. Goldberg-Shprintzen syndrome (GOSHS) is caused by truncating variants in the KIF1 Binding Protein gene (*KIF1BP*)[47]. It is characterised by dysmorphic facial features, microcephaly, developmental delay, intellectual disability and short stature and has HSCR as a variable feature among other associated phenotypes. The presence or absence of HSCR in GOSHS patients is highly variable, even in members of the same family, with the same

Table 2. Characterised syndromes with HSCR as a mandatory or frequent feature.

Syndrome	Gene(s)	HSCR	Other features
WS4 MWS	*SOX10; EDNRB; EDN3* *ZEB2*	100%	Pigmentary anomalies; sensorineural deafness
GOSHS	*KIF1BP*	>70%	Craniofacial dysmorphia; microcephaly; polymicrogyria; developmental delay
DS	Tri21	~7%	Characteristic facial dysmorphism; intellectual disability; developmental delay
CCHS	*PHOX2B*	~20%	Autonomic respiratory failure
BBS	Several		Pigmentary anomalies; renal anomalies; intellectual disability; polydactyly
MKKS	*MKKS/BBS6*	~10%	Cardiac anomalies; polydactyly; hydrometrocolpos
SLOS	*DHCR7*	-	Developmental delay; intellectual disability; microcephaly; craniofacial dysmorphism; syndactyly
CHHS	*RMRP*	~10%	Metaphysial dysplasia; dwarfism; fine, sparse, blonde hair; anaemia; immunodeficiency
MEN2	*RET*	~2%	MTC; parathyroid tumours; pheochromocytoma

HSCR: Hirschsprung disease; **WS4**: Waardenburg-Shah syndrome; **CCHS**: Congenital Central Hypoventilation Syndrome; **MWS**: Mowat-Wilson Syndrome; **GOSHS**: Goldberg-Shprintzen Syndrome; **BBS**: Bardet-Biedl Syndrome; **MKKS**: McKusick-Kauffman Syndrome; **SLOS**: Smith-Lemli-Opitz Syndrome; **CHHS**: Cartilage-Hair Hypoplasia Syndrome; **MTC**: Medullary Thyroid Cancer.

causative variant[46,48,49]. *KIF1BP* is associated with microtubule dynamics, cargo trafficking and axonal outgrowth, but its precise functions in development are not well known[49-52].

In **chapter 3** we introduce new truncating variants in *KIF1BP* and add two patients with missense variants, one with classical GOSHS features and the other with an alternative phenotype. Functional studies of the missense variants indicate that a threshold of *KIF1BP* expression is necessary to avoid GOSHS development.

Copy Number Variation in HSCR

As well as defined monogenic syndromes, there are many patients with complex phenotypes and multiple associated developmental defects that are yet unexplained. Chromosomal abnormalities may explain part of these clinically complex patients. Changes in the number of copies of DNA present in the genome are termed Copy Number Variations (CNVs). These can contribute to phenotypes, diseases or syndromes that are influenced by gene dosage[53]. CNV is also known to contribute to HSCR disease aetiology. Chromosomal band deletions[54,55] and duplications[54,56-58] have been described in HSCR patients, most of these being syndromic patients. Deletions of chromosomes 10 and 13 were instrumental in the identification of *RET* and *EDNRB* as major HSCR genes[25,59]. Patients with Down Syndrome, trisomy 21, have a 100 times higher incidence of HSCR than the general population[25], implying that genes or regions on chromosome 21 are sensitive to dose increase and may increase susceptibility to ENS disorders. Large CNVs are more frequent in individuals with developmental anomalies compared to healthy controls[60]. In addition, more common CN polymorphisms (CNPs) are thought to be modifiers of the HSCR phenotype[61,62]. Therefore, we believe that rare CNVs could contribute significantly to syndromic patients with HSCR as a feature, where no pathogenic variant can be identified. In **chapter 4** this phenomenon is explored in HSCR patients with and without other congenital anomalies in order to find new genes or regions that may be causative for the HSCR phenotype.

Other factors in HSCR development

Known HSCR genes have only been implicated in ~30% of cases[25]. There has been no implicated high penetrant causative variant found in the remaining ~70% of cases and in sporadic, non-familial, HSCR genetic

counselling is challenging. There are a number of possible reasons for the missing heritability. There may be an epigenetic component affecting protein expression of the known, implicated pathways, or there may be other genes involved in pathways up or downstream of, or otherwise linked to, these pathways that have not yet been elucidated. Changes in expression of HSCR related genes, either due to variation in methylation regulating genes, or methylation changes to promotor or enhancer regions themselves, have been shown to influence ENS development[63-65]. Environmental factors including maternal diet and use of prescribed drugs can also play a role and may influence epigenetic patterns. This is most likely limited to being a small influencing factor rather than being significant enough alone to cause any damaging phenotype[66-69].

A further theory that warrants investigation is the existence of somatic cell variations affecting a subset of cells important in the ENS colonisation of the GI tract. During the accelerated cell division and growth of embryonic development there are many chances for mistakes to be made in DNA replication. If there is a failure of DNA repair mechanisms to identify and correct these mistakes this would lead to subsets of cells containing variants that are not present in the remaining embryonic cells. Dependent on the temporal and spatial identity of a cell in which this variation occurs this may lead to a whole system, organ or tissue containing a mosaic variant. However, as discussed in **chapter 2**, such defects would prove difficult to identify in patient material.

Diagnosis & Treatment

HSCR is suspected when a newborn infant fails to pass meconium within the first 48 hours of life, which is generally the case for >90% of HSCR

cases[22]. Patients are usually diagnosed before 3 months of age, although some within the first year or upon weaning from breastmilk. Rarely, an older patient will be diagnosed, but they generally have a history of chronic obstipation and the aganglionic segment is short[22]. When HSCR is suspected, the diagnosis is usually confirmed by taking a rectal suction biopsy. The length of the aganglionosis is established using histological examination for presence/absence of enteric ganglia.

The current standard of care for HSCR is surgical removal of the affected aganglionic region and anastomosis of the ganglionic bowel region to the anus. A colostomy may be fashioned prior to surgery. This allows the obstruction to be bypassed, and allows the gut to grow and inflammation to diminish. The most common surgical mode for resection is transanal endorectal pull-through (TEPT), which minimises the invasive nature of the surgery as the abdominal cavity does not need to be opened. TEPT can take place entirely transanally. However, it is commonly performed in combination with laparoscopy or laparotomy, to visualise and mobilise the colon abdominally[70]. Common surgical procedures are the Swenson, Soave, Duhamel and Rehbein procedures which are adaptations of similar pull-through approaches with differences in anastomosis[6,70,71].

Although surgical intervention is generally effective in preventing obstruction, it may not prevent further complications for the patient, including enterocolitis, faecal incontinence or chronic constipation, and an increased risk of infections[23,72,73]. TEPT may avoid some of the risks of open abdominal surgery, however the rates of faecal incontinence as well as the psychological and psychosexual side-effects of this surgery may negatively impact on the quality of life of HSCR patients[24,72,74,75]. It

has also been shown that TEPT can cause long-term damage to the anal sphincter, due to the prolonged and significant period of being stretched. This can be minimised if a combined laparotomy technique is implemented[6]. If complications arise following surgery, it may be necessary to have a redo surgery. A redo surgery is primarily for patients with persistent constipation and may involve removal of a further section of colonic tissue to ensure that the transition zone between the ganglionic and aganglionic regions is totally removed, as this is likely to have disturbed function and may have been inaccurately identified at the time of surgery[76]. Correction may also be necessary if there is a twisting of the bowel in the anastomosis which leads to discomfort or abdominal pain[77]. Patients who undergo corrective transanal surgery have a higher risk of damage to the anal sphincter, so this is only undertaken if the benefits outweigh this risk.

Additional, non-surgical, treatment options for HSCR include continued use of a colostomy, modified diet, laxatives and/or anti-diarrhoeal drugs, electrical nerve stimulation, hospital visits for rectal/colonic irrigation and injection of bulking agents to thicken the anal sphincter. These options, as well as the above-mentioned surgeries, leave a lot to be desired in terms of patient care. Quality of life can be negatively affected and, while paediatric patients are resilient to some psychological impacts, their frequent hospital visits and the social implications of faecal incontinence could complicate educational and social commitments in later life[24,72,75]. Due to normal life expectancy in HSCR patients the need for prolonged treatment can generate large healthcare costs[78-81]. New treatment avenues that are being explored in the field include nerve cell-replacement or transplantation therapies, discussed here and in **chapter 5**. Other, less well explored options can

be multidisciplinary and effective for a greater range of patients, as discussed in **chapter 6**.

Cell-based therapies for HSCR

One of the major drives in ENS translational research is to develop a cell replacement therapy for enteric neuropathies such as HSCR. HSCR is the primary focus for cell replacement therapy as cells could be transplanted into the aganglionic region in an attempt replenish and rescue the absent ENS. There are a number of cell types that are generally considered for any cell transplantation therapy: cells that are obtained from the same tissue as the cells that are to be replaced; ameliorative cells, such as mesenchymal stem cells (MSCs), that facilitate natural recovery within the body and decrease inflammation; or stem cells, either of embryonic, postnatal or reprogrammed origin, that are differentiated towards the desired cell lineage or type.

Sources of cells for ENS therapy

A number of options for a viable cell source for transplantation therapies have been explored[82-86]. The discovery of ENS stem cells that persist within the postnatal gut, and the assessment of their proliferative potential in mice[13,87], led to multiple attempts to isolate them from human colon and characterise them *in vitro*[88]. Although they provide an ideal cell source for functionally investigating the ENS, their proliferative capacity may be inadequate for generating required numbers of cells, especially when obtained from postnatal gut[89]. Current culture methods of ENCCs in neurospheres maintain a progenitor state in some cells, and these cells are able to integrate and form a functional network upon transplantation[85,90-92]. However, the area covered remains low in mouse

models and considering the scale-up to human gut, it is likely that many more cells would be required in a human therapy.

Pluripotent stem cells (PSCs) were initially thought to be ideal stem cell source for treating a variety of diseases. First studied from embryonic origin, embryonic stem cells (ESCs) offered a potentially unlimited source of cells to study differentiation pathways[93,94]. However, ethical considerations of using human embryos for this purpose, as well as the need for immunosuppression in transplantation, created significant barriers for their use[95]. The generation of induced PSCs (iPSCs) from human fibroblast cells by Takahashi and Yamanaka in 2006 led to a huge advance in developmental biology research. These cells have comparable differentiation potential to ESCs and can be created with somatic cells from the intended patient, circumventing source and immune rejection issues[96,97].

However, persisting issues include the genomic stability of the iPSCs, which have been shown to accumulate chromosomal aberrations after a number of passages in culture, and the ability to differentiate the cells into the correct lineage whilst avoiding the introduction of tumorigenic cells to a patient[98-100]. An additional consideration when thinking of a transplantation therapy for a genetic disease, is the genetic background of the patient and whether the cells' function will be negatively affected by the pathogenic variant that initially caused the disease.

A number of protocols have been developed for the differentiation of PSCs towards an ENS lineage[101-104]. Most of them initially achieve a vagal neural crest expression pattern and the study from Fattahi, et al., shows promise in transplantation and rescue of a mouse model of HSCR[103]. However, if iPSC-derived neural crest cells (NCCs) are to be used for

disease modelling and transplantation, a standardised method for creation and characterisation should be developed to ensure replicability. Particularly in the case of using HSCR patient derived iPSC-NCCs for genetic characterisation, standardised and controlled methods would ensure that any differences measured are due to genetic background rather than protocol variation. The parameters for measuring cell function, to assess the need for genetic correction, as well as the necessary stage of differentiation for ideal integration efficiency following transplantation, remain to be determined[105].

Aims & Outline of this Thesis

The aims of the work described in this thesis were to investigate the missing heritability that is seen in HSCR in both isolated and syndromic cases, understand more about the development and differentiation of ENCCs, assess suitability of various cell sources for transplantation therapy, and explore possible treatment avenues for current and future HSCR patients.

The possibilities of somatic variants contributing to the development of, and accounting for the missing heritability in, HSCR are discussed in chapters 2.1 and 2.2. In **chapter 2.1** we outline the need for appropriate distinction between inherited parental mosaicism and true somatic mosaicism, proposing an appropriate experimental design to truly differentiate between the two in HSCR patient tissue. In **chapter 2.2** this experimental design is utilised to look for ENCC specific variation in patient tissue. We further discuss the mechanisms of somatic variation in ENCCs and why, due to the developmental patterning of the ENS, they are unlikely to be detected, even if they have contributed to the phenotype.

In syndromic cases, HSCR can be a variable feature, the presence of common *RET* variants as well as the type of causative variant may influence HSCR development. The presence of HSCR in cases of GOSHS is discussed in **chapter 3**. Pathogenic variants in *KIF1BP* lead to GOSHS, in which HSCR is a variable feature. Given that HSCR is not a mandatory feature, it is likely that predisposing factors can be found to have involvement in its development. A number of patients have been reported in the literature, and truncating variants in *KIF1BP* have been found to be causative in all sequenced cases. In this chapter, we report nine new patients with *KIF1BP* variants, and functionally investigate, for the first time, three new missense variants identified in two patients with differing phenotypes. We also look at the presence of *RET* common SNPs, as a determining factor for the presence or absence of HSCR.

Another possible genetic factor for HSCR in syndromic cases is the presence of CNVs that affect dosage sensitive HSCR loci. In **chapter 4** we compare the size and number of CNVs between syndromic and non-syndromic HSCR cases, to find new candidate genes/loci.

To further look into the underlying pathogenesis of HSCR development we created iPSC lines from four patients with different pathogenic variations. These cells offer possibilities for disease modelling, functional investigation of variants as well opening the door to future iPSC-enteric neuron transplantation options. In **chapter 5** we present the characterisation of these patient-derived iPSCs and explore variations in their function compared to iPSCs generated from healthy controls. We also discuss their genomic stability and viability with a view to their use in cell therapy.

In order to bring an expedient option for the treatment of HSCR patients who continue to suffer from gastrointestinal problems following the current surgical standard of care, we may have to think beyond biological interventions. The technological world has arguably been able to advance at a faster rate than the development of purely biological treatment options. With the advances in microelectronics and prosthetic technologies incorporating sensory input, the opportunities for developing transplantable devices may provide a more elegant solution than the more primitive prostheses currently available. These ideas are discussed in **chapter 6,** together with a patent proposal for an artificial prosthetic sphincter with an anatomically relevant mechanism and design which could provide therapeutic options for HSCR patients, but also to others suffering from faecal incontinence, or loss of anal sphincter control.

Chapter 7 summarises the work in this thesis and discusses future prospects in the field of ENS development, as well as possibilities for treating patients with HSCR and related disorders.

All supplementary material can be found here.

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2

Chapter 2.1

Do *RET* somatic mutations play a role in Hirschsprung disease?

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Refers to:
Jiang Q, Liu F, Miao C, Li Q, Zhang Z, Xiao P, Su L, Yu K, Chen X, Zhang F, Chakravarti A, Li L. "RET somatic mutations are underrecognized in Hirschsprung disease" Genet Med. 2017. doi:10.1038/gim.2017.178.

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Dear Editor,

We have read the manuscript from Jiang et al.,[1] recently published in your journal, with great interest. In this, a contribution of *RET* somatic mutations for Hirschsprung disease (HSCR) is hypothesized.

HSCR is a complex inherited disorder characterized by the absence of enteric ganglia in the distal part of the colon. Several genes and loci have been described to underlie disease pathogenesis. However, variants in these genes explain no more than 20% of all cases[2]. This missing heritability seen in HSCR is a common feature of many complex disorders and explaining it remains challenging. Considering that HSCR develops during embryogenesis as a result of either impaired migration, proliferation or differentiation of enteric neural crest cells (ENCCs), it is tempting to consider that somatic mutations occurring during the development of the enteric nervous system, and specifically affecting ENCCs, can also contribute to HSCR genetics. Jiang and colleagues think that this is possible, and we share the same opinion, as we believe that somatic variants could be underrecognized in HSCR, and thus, possibly account for some of the missing heritability. However, the results described by the authors do not, in our opinion, fully support the conclusions of the manuscript. This is mainly because routine genetic testing on DNA derived from blood or saliva would not find these ENCC specific mutations, nor would easily detect low mosaic variants. With this letter, we intend to further discuss our concerns and highlight the difference between causative

somatic mutations and germline mosaicism resulting in seemingly *de novo* mutations in the next of kin.

In their study, the authors screened 152 HSCR patients by targeted exome sequencing and direct gene screening. In eight patients they identified putative deleterious *de novo* variants in *RET*. Since *RET* is the major HSCR causing gene,[3] they assumed that these variants were responsible for the disease phenotype. However, only six patients carried a truly *de novo RET* mutation, as in the other two the variants identified were also found in one of the parents. The authors continued the genetic analysis of the six remaining patients, and described based on their findings, that the *RET* mutations identified were somatic. This result led to the conclusion that *RET* somatic mosaicism is present in 75% of the HSCR cases and is, therefore, underrecognized. However, in four of the six patients studied, germline mosaicism was identified in the parents. As these variants are transmitted to the affected next of kin as heterozygous variants, these cannot represent somatic mosaicism. In addition, the parents are not affected, leading us to speculate that their ENCCs are either not affected by the mutation or that the mutational load in their ENCCs does not cross a threshold for abnormal ENS development. Therefore, although these four families are likely to represent germline mosaicism resulting in seemingly *de novo* mutations in the next of kin, they do not support the conclusion that *RET* somatic mutations are underrecognized. These heterozygous mutations would be detected (and are detected in these patients) in routine genetic screening and as such, do not resolve missing heritability due to "recognizing" somatic mosaicism.

For the remaining two patients, the RET variants identified were present in blood, saliva, and colon, in family 1 (39/39/44%) and family 2 (44/35/39%). The authors validated the sequencing data with TA cloning, but the results are variable even within the same tissue, and the differences seen in the amplicon-based sequencing test are well within the normal range for detection of a heterozygous variant. Moreover, the samples tested—blood, saliva, and colon—derive from tissues originating from different germ layers. Blood, mostly lymphocytes, is derived from mesoderm; saliva, lymphocytes, and epithelial lining of the mouth are derived from mesoderm and ectoderm; and the colon has contributions from all three germ layers. The high allele frequencies identified in all three samples for both patients (>30%) does not allow for discrimination between very early developmental stage somatic mutations or de novo variants present as a germ-line mosaicism in one of the parents. Although the authors acknowledge this fact in the discussion, it is more fitting to conclude that the variants identified in these two remaining patients are likely to be de novo heterozygous variants.

In conclusion, we agree with the hypothesis of Jiang et al. and think that somatic variants might well play a role in HSCR development. However, based on the results presented we think that it is not possible to make such a conclusion, as no true somatic RET mutations were identified in any of the presented patients. We believe that to draw the conclusions stated, the allele frequency of the mutations in ENCCs, surface ectoderm, and, for instance, blood or fibroblasts would need to be compared because these represent different germ layers and include the cells involved in HSCR. An experimental setup that would isolate cells specifically from each germ layer, as well as ENCCs,

or that would separate the colon into various cell types, would be necessary to determine whether true somatic mutations impact HSCR development. Only if differences were found under such conditions could the authors show that RET somatic mutations are indeed underrecognized in HSCR.

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Chapter 7

General Discussion

Hirschsprung disease (HSCR) is a rare congenital malformation of the enteric nervous system (ENS) characterised by an aganglionosis in a variable length of the distal gastrointestinal (GI) tract. Aganglionosis results in constriction of the smooth muscle in the affected region, leading to a functional obstruction[1]. During development, the cells that form the ENS bud from the neural crest and rapidly proliferate and migrate along the developing gut tube. These neural crest cells (NCCs) eventually form the enteric ganglia and differentiate into neuronal subtypes and glial cells located in the submucosal and myenteric plexuses of the ENS. A disturbance to proliferative, migratory, differentiative and/or survival functions in these cells could contribute to the pathogenesis of HSCR[2]. The current treatment for HSCR consists of surgical resection of the affected gut region, however most patients continue to have prolonged GI tract complications following surgery[3,4]. Understanding the development and pathogenesis of HSCR is vital to provide improved treatment options for these patients.

Factors involved in the development of HSCR

The disruption of ENS development is thought to be broadly under genetic control. A number of key NCC regulating genes have been implicated in HSCR pathogenesis. However, pathogenic variants in these genes only explain ~50% of HSCR cases[2]. It is therefore likely that other strong pathogenic variants in yet unknown genes, and/or combinations of pathogenic variants and weaker modifying variants, contribute to the aetiology and pathogenesis of HSCR[5]. Of the genes known to be involved in HSCR pathology, *RET* is understood to be the major influencer, acting as a fulcrum in the balance between aganglionosis and hyperganglionosis[5]. If there is a strong *RET* or other causal variant then the presence or absence of risk alleles[6-8] at common

SNP locations is unlikely to influence disease development substantially. The *RET* balance is therefore more representative in cases where a combination of variants and other risk factors have the cumulative effect of a HSCR phenotype.

If a patient presents with a multi-feature syndromic form of HSCR then the risk alleles may slightly influence presence or absence of HSCR, but more likely large copy number variations and chromosomal displacement affect its presence or absence. It could also be that combinations of missense variants and modifying SNPs have a cumulative influence on pathology in both non-syndromic and syndromic forms of HSCR.

Genetics of isolated HSCR

In non-syndromic isolated HSCR cases *RET* is the major genetic risk factor. However, the inheritance in sporadic non-syndromic HSCR cases is considered complex[2]. In this group, many genes other than *RET* have been identified. The identified genes are primarily involved in either the *RET* signalling pathway or the endothelin signalling pathway, and it is likely that other up- or down-stream influencers of these are yet to be identified. Due to the frequency of sporadic isolated cases and the unexplained genetic origin in many familial cases, it can be safely assumed that there are further disease genes yet to be identified, and/or factors other than the genomic DNA sequence of a patient that may play a role in disease pathogenesis[9,10]. Susceptibility to HSCR could be further influenced by stochastic effects on gene expression, additional variants in other genes that influence regulatory elements, and environmental effects on gene expression[6]. Additionally, the effects of

variants may be subject to epigenetic factors and changes in methylation patterns, which could also affect the complex heritability.

Genetics of unexplained syndromic HSCR

Approximately 12% of HSCR cases are associated with chromosomal anomalies, and approximately 18% of cases present in combination with other defects or features[11]. In contrast to non-syndromic HSCR, in syndromic HSCR we assumed that large copy number variations (CNVs) could explain part of the missing heritability. In **chapter 3** we discuss CNV and how large CNVs may influence HSCR pathology if they overlap dose-sensitive genes that affect ENS development. HSCR can present as one symptom in patients with multiple associated anomalies, these can be recognised syndromes or newly presenting multi-feature presentations[11,12]. In multi-feature patients with known strong pathogenic variants CNVs have little influence on HSCR development. We demonstrate that CNVs in multi-feature patients, without a known pathogenic variant, tend to be longer and affect regions that contain genes expressed in the developing ENS. This data needs to be replicated in independent cohorts to confirm if candidate genes within large CNVs are seen in multiple patients. Investigations in zebrafish models, to knock down these genes or express multiple copies, may help to confirm new candidate genes present within CN loss/gain regions.

Missense variations in known syndromic HSCR

Missense variants can go unnoticed and be present in the healthy population, but still disturb protein folding, binding or other functions. They can have the same pathogenicity as a loss of function variant, specifically in recessive diseases in which carriership does not result in a disease phenotype. In **chapter 4** the identification of missense variants

in Goldberg-Shprintzen syndrome (GOSHS), as well as the presence or absence of HSCR as a variable clinical feature in GOSHS is discussed. Truncating variants in the KIF1 binding protein gene (*KIF1BP*) are known to cause GOSHS[13]. The presentation of two patients with missense variants in *KIF1BP* was interesting given the lack of knowledge of protein folding and interactions. There is no crystal structure available of KIF1BP and the interactive sites are not well understood. Given that loss-of-function variants have been reported in all 7 exons, it is likely that there are many regions, even in the terminal regions of exon 7 that are vital to RNA or protein stability. The two patients presented with different phenotypes and the finding of missense *KIF1BP* variants in patient NL1 was surprising, given their clinical presentation. The homozygosity of the missense variant in patient CYP3, or the region affected, may have been a tipping factor in the development of classical GOSHS with HSCR. The expression of KIF1BP in CYP3 is lower than in NL1, despite the compound heterozygous variants in exons 1 and 7. A CNV analysis in these patients, particularly patient NL1, may be informative to determine if there are other candidate regions that have influenced neural development. As no other pathogenic variant was identified in diagnostic screening of patient NL1, and given their syndromic features, it may be that CNV of dose dependent genes contributes to their neurological phenotypes. This is the case in HSCR patients with associated syndromic features and would be interesting to investigate in other multi-feature patients.

Missing heritability in HSCR

As previously mentioned, in many HSCR cases a genetic cause for the disease cannot yet be identified. This can be partly explained by the complexity of inheritance patterns, presence of low-penetrant non-

coding variants and the influence of risk alleles[14]. In **chapter 2** we discussed somatic mosaicism as an influencer of HSCR, and the difficulty of identifying this phenomenon in relation to a HSCR phenotype. The developmental patterning of the ENS is such that a variant originating early has a high chance of being out-competed by other "healthy" cells. Unless this somatic variant gives a competitive advantage, such as higher proliferation or migration rate. This could lead to these cells reaching the distal colon and being unable to differentiate to the correct lineages and/or survive. We conclude that it is therefore extremely difficult to prove that true somatic variants contribute to HSCR aetiology, but it cannot totally be ruled out as a mode of missing heritability or a cause of some sporadic cases.

As somatic mutations appear unlikely to play a major role in HSCR, the question of what might explain these unsolved cases remains. A possible problem could be the genes that are selected as being candidates for HSCR. Current filtering criteria selects genes that have a clear role in neuronal development, are expressed in the developing central nervous system (CNS) or ENS, or expressed in NCCs[15]. It can be assumed that there are HSCR causing variants that are not expressed in ENCCs themselves, but in other developing GI tract tissues. Variants in genes expressed in smooth muscle or connective tissue could change the local gut environment through which ENCCs migrate[16]. These changes could leave the distal colonic segment unable to support the migration, incorporation or survival of the ENCCs, an example of this is variation in *EDN3*[17,18].

Moreover, the timing of cell-cycle exit has been shown to be of importance to ENS subtype specification[19], the current filtering criteria

could also exclude cell cycle genes that may influence ENS differentiation. In order to fully understand the enteric neural subtypes derived from ENCCs it would be beneficial to utilise single-cell RNA sequencing technologies to build a control database from healthy gut at various developmental stages as well as postnatally. This could be built of the ENS as well as other GI tract cell types. Once a baseline of expression at various points along the GI tract is established then comparisons can be made with different disease states. This could also help with the identification of causal genes and further understand links in the developmental pathways that are disrupted in HSCR development.

Non-genetic influencing factors

Other than inherited and somatic alterations, external factors can also influence ENS development by changing the epigenetic landscape[9,20]. Using a similar strategy to that mentioned above, it would be beneficial to create a control database for the methylation state of various regions and developmental stages of the GI tract. Initial efforts could focus on isolation of ENCCs from control gut in order to establish methylation patterns of the ENS. Eventually other cell types and full gut sections could be included to be able to compare HSCR patient tissue and find large methylation changes. The creation of expression maps of the ENS, both with RNA and methylation patterns, would be pivotal to linking known pathways together and finding new players in the network of ENS development.

The ability of clinical geneticists to give reliable genetic counselling to the families of patients relies on an understanding of the heritability of the condition. This is dependent on a knowledge of the genetic

background of HSCR pathogenesis as well as the non-genetic influencing factors. However, it may not be possible to explore every avenue, and there will still be sporadic cases that are difficult to explain.

Modelling HSCR

Proliferation, migration, differentiation and survival of ENCCs is a major focus of research and deviations of these processes are shown to cause aganglionosis in animal models of HSCR[21]. There are cases where the causative variant is known to be disruptive in these mechanisms and is shown to have expression in ENCCs specifically. However, for many patients the remaining ganglionic gut functions to a manageable degree and no other physiological problems are reported other than malfunction of the anal sphincter region. Animal models for developmental disease can only truly be useful if the genetic variant is known, and there is a practical limit to the number of genes that can be investigated in one model. The use of patient-specific cells to create a model circumvents this issue as the genetic background does not need to be fully known to functionally test for defects.

In **chapter 5** the creation of induced pluripotent stem cells (iPSCs) from HSCR patients was presented. These iPSC lines each harbour variants for known causative HSCR genes and were investigated for differences in function that may influence their ability to rescue the ENS using cell transplantation strategies. These iPSCs could also be utilised for disease modelling at the cellular level.

The development of a reliable *in vitro* model for HSCR might help to dissect small cellular changes in ENCCs and/or the local gut environment. A 3D organ-on-chip system for GI tract development and function would be ideal to investigate the interplay of the different cell-

types in the gut that are necessary for normal ENS development. Initial establishment of such a system with iPSC lines from healthy controls will allow the optimisation of cell-type ratios and flow of nutrients. Such a model, and generation of iPSCs from patients, will allow for the investigation of individual patient gut and help to determine the functions that may be disrupted during development. Moreover, it will also be help to determine whether ENCCs, smooth muscle, mucosal interaction or extracellular matrix (ECM) components were altered. Although gut organoids also offer a method of creating a 3D gut-like environment for cell transplantations, the spheroid structure is random which will influence intercellular signalling[22]. Gut organoids form a self-organising lumen system which creates multiple signalling gradients that are difficult to compare between organoids. An organ-on-chip system would offer a more organised and replicable model and is already being used with mucosal models to create an epithelial layer that can support a microbiota[23].

Cell transplantation therapy

As discussed in **chapter 5**, the possibilities for cell replacement therapy in HSCR have been explored for a number of years. The ability of transplanted cells to find the appropriate positioning, into either submucosal or myenteric plexus, and to form functional connections, both with other transplanted cells and with target cells within the gut, is vital to transplantation success. The appropriate cell type for this purpose, and an optimal transplantation strategy still need to be determined when scaling up from mouse to human gut[24]. No human trials have yet been attempted, but initial transplants into HSCR mouse models have been encouraging. Both injection of postnatal enteric neural crest cells (ENCCs)[25,26] and of more proliferative pluripotent stem

cells (PSCs) differentiated towards neural crest[27] have shown an integration and spread of transplanted cells.

Initial transplantation studies established the potential of postnatally derived ENCCs to integrate and survive in explanted colonic segments[28,29]. *In vivo* transplantation of ENCCs of embryonic and postnatal origin has been shown to lead to the engraftment of donor-derived cells within recipient colon[25,30]. Additionally, it was shown that ENCC-derived neurons adopt the appropriate localisation within the gut and can give rise to various enteric neurons, including the main subtypes for excitation (ChAT, VAChT, Calretinin and Calbin-din) and relaxation (nNOS and VIP)[25,30]. The transplanted cellular networks were also shown to closely localise with the endogenous ENS, suggesting functional integration of the transplanted neurons

Methods of transplantation

Current protocols for transplantation of cells into *in vivo* gut involves injection of cells in suspension with saline or matrigel[26,27]. Injection of ENCCs has proven safe in longer term follow-up and no migration of cells to ectopic sites was observed. Using PSCs, it is uncertain how many of the injected cells remain at the injection site and which other locations cells may reach. It is a recognised pattern following injection of PSCs to other organs that, although beneficial effects may be seen in the target tissue, cells are found in other organs[31]. It is also likely that many injected cells will die before making cell-cell contact[32]. Therefore, the already high numbers of cells required with this method, given the size of the target organ, may be even higher than anticipated when accounting for cell viability. Cellular scaffolds, injectable gels or other devices may make the environment more amenable to cell invasion,

lead to easier introduction of the cells, increase cell survival and avoid the migration of cells beyond the desired location[33,34].

Over-invasion and mass migration and proliferation is a known issue in PSC transplantation[31]. The study by Fattahi et al., considered as a landmark paper, shows transplantation of PSC-derived NCCs to wild-type and Ednrb$^{-/-}$ mouse models[27]. The study shows a promising start to integration of PSC-derived NCCs and migration of transplanted cells along the GI tract. However, it has yet to be determined whether these cells also migrate to other regions in the body, especially connecting abdominal organs and this was not investigated with the reported transplantations. The extensive migration that they present is contrary to ENCC transplants reported in literature, which show a more modest migration to form small ENS-like plexuses in mouse gut[25,26,28,30,35,36]. The interesting factor in these studies is how many injection sites would be necessary in the human gut in order to effectively form a functional ENS. The highly proliferative nature of the PSC-derived cells could circumvent this, although it is vital to ensure that all transplanted cells are adequately differentiated to at least a multi-potent single germ lineage progenitor state as opposed to retaining ability to form other lineages. The slowing of the cell cycle to a point of normal turnover for tissue maintenance is required after the desired integration of cells, otherwise the tumour-risk from the transplanted cells is higher[37].

Numbers matter

It has been shown that in development critical threshold numbers of NCCs are required for full colonisation of the GI tract[38]. It is also known that cells benefit in culture from contact with other cells producing "friendly cytokines"[39]. A similar phenomenon may translate in

transplantation where both adequate cell number and density are vital to transplantation success. A disadvantage of the significant cell expansion needed to create large numbers of cells is the propensity of cells to acquire genetic and epigenetic changes upon long-term culture and expansion[40-42]. Such changes may reduce the efficacy of generating specific cell derivatives, or could potentially compromise safety, for example by promoting tumour growth. ENCCs offer less tumorigenic risk than pluripotent alternatives[37], and a more primed ability to form these enteric neural subtypes directly[25]. However, they may not be proliferative enough for expansion to required numbers[43]. Current culture methods do not generate enough cells for both characterisation and transplantation. Pluripotent cell types expand more quickly in culture than postnatal stem cells, which would decrease expansion time for generating sufficient numbers.

Safety of Cell Transplantation

Human PSCs are becoming more popular as a therapeutic tool and are currently being investigated in clinical trials for a number of conditions, including macular degeneration, spinal cord injury, diabetes, heart disease and Parkinson's disease[44]. However, before these cells can safely be used in routine therapies, a better understanding of their behaviour, and understanding the possible genetic changes that may have occurred during their processing, is required.

Safety discussions to date have focused mainly on possibility of teratoma formation from transplanted cells, migration of cells beyond the tissue of interest and the occurrence of genetic variation arising during cell culture. To mitigate the possibility of teratoma development from transplanted cells the appropriate differentiation stage would

need to be reached. Cell therapy should consist of administering progenitors or differentiated derivatives rather than undifferentiated stem cells, and the accidental transplantation of undifferentiated cells should be avoided[37]. With the appropriate checks and characterisation of cells the chance of teratomas would be minimal. Another safety issue is the spread of the transplanted cells outside the tissue of interest, as discussed briefly above. When cells integrate into non-target tissues, possibly in combination with (epi)genetic changes, this could have profound consequences[31]. Monitoring the spread of cells is therefore crucial and the investigation into methods to ensure that transplanted cells remain at the transplantation site is necessary.

The primary focus of attention should be on the potential (epi)genetic changes that may have arisen during creation and culture of PSCs. It is these somatic (epi)genetic changes that may have a substantial impact on the behaviour of the PSCs and may even lead to malignant transformation of the mutated cells[45]. Monitoring the PSCs for such genetic changes is therefore crucial. Discussions are still ongoing on how to screen cells and how to interpret the results in order to evaluate their significance for the safety of therapeutic applications[46]. Due to the risks associated with significant cell expansion *in vitro*, the time in culture and number of passages of cells should be kept as low as possible. Cells should be checked genetically, preferably by exome sequencing, before use. As long as the effects of individual genetic variants on the PSCs or differentiated cell types are uncertain, and while cells are likely to spread from the target tissue, the introduction of a conditional suicide gene could provide a fail-safe strategy for eliminating cells after transplantation if a problem were to arise[46].

Other challenges to overcome

Eventually, with a greater understanding of the development of enteric neural subtypes at all levels of GI tract development, reliable induction of ENCC-specific differentiation will be possible with iPSC lines. A non-integrating viral transduction system to iPSCs will further reduce the inter-line variability and allow for the patient specific defects to be more readily compared. The therapeutic potential of iPSCs is great, they are arguably the most valuable tool for personalised regenerative medicine, but for their full potential to be realised it will be necessary to recognise and correct for their disadvantages.

As the gut receiving transplantation has developed without ganglia the ECM and cellular environment of the tissue may be less receptive to ENS cell transplantation. As mentioned above, the creation of a suitable transplant agent or cell scaffold system may create a more receptive environment for the transplanted cells[33,47]. It is yet to be established if cytotoxicity from inflammation or fibrotic regions of scar formation at the anastomotic region in HSCR could make tissues less permissive to cell transplant invasion. In spinal cord injury the fibrotic tissue is known to be an issue for transplantation and scar ablation is common prior administration of cells[48].

Multi-disciplinary Treatment for HSC

Current standards of care for HSCR patients leave many with poor anal sphincter control[4,49], and this has been highlighted as a target region for initial cell therapy[50]. Given the safety and other challenges with the use of cell therapy that are yet to be overcome, such an approach is unlikely to reach the clinic for a number of years, and could have variable success rates within patients. In **chapter 6**, we introduce a novel treatment

possibility with the description of a device that could mimic the physiological function of the anal sphincter. In HSCR patients the constriction of the sphincter can lead to chronic constipation, and the damage to the musculature from surgery can lead to continued incontinence and anal leakage. There is need for a solution to allow for sphincter control for both opening and closing. Current solutions for this problem focus on closure, with no solution that will address both contraction and relaxation of the muscles[51].

The anal sphincter complex is comprised of the internal anal sphincter (IAS) and the external anal sphincter (EAS). It is the combined function of these sphincters that allows normal physiological defecation[52]. Function of the IAS is entirely involuntary and controlled by parasympathetic nerve fibres and the myenteric plexus whereas the EAS is mainly innervated by the pudendal nerve and is under voluntary control[53]. During defecation the increased pressure and stretch from bowel contents on the internal sphincter signals for it to relax, which is picked up by sensory nerves more distally and by the EAS. The pressure is sensed and these combined signals let us know that we need to defecate. The voluntary control of the EAS allows us to choose when to defecate. The lack of this control is the primary cause of adult faecal incontinence, and a number of pathologies can contribute to this[54]. The proposed solution of an electronic prosthetic anal sphincter is a multi-disciplinary approach, combining surgical techniques with microelectronics and physiological function in order to provide patients with a modern and convenient solution to anatomical malfunction.

Conclusions

HSCR development is complex and multifaceted. Although the overarching *RET* pathway can account for much of HSCR pathophysiology, there remains much to be learned from genetic studies. Collection of patient material and inclusion of parents and other family members in sequencing and functional cell-based research approaches will be instrumental in discovering new candidate genes and pathways that contribute to HSCR aetiology. The advancing of technologies to reliably sequence small amounts of DNA, and identification of methylation and other epigenetic marks will help to answer many questions that remain concerning the missing heritability observed in familial and sporadic HSCR cases. This, together with the understanding of HSCR as a variable symptom in multi-feature and syndromic cases, will enable more informative genetic counselling to patients and their families.

Treatment options for HSCR have been stagnant for the past decades, and ongoing research is necessary before novel cell therapy approaches can be applied in a clinical setting. The differentiation of PSCs to appropriate lineages is progressing quickly and, given a concerted effort in safety and efficacy trials, clinical application is approaching. A further possible option that may be suitable to more patients is the use of an electronic prosthetic that could mimic anatomical function. In the future this could be used in combination with cell transplantation therapy and allow for the training of surrounding tissue and conditioning of transplanted cells to function within the anal sphincter complex.

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Appendix

Summary

Hirschsprung disease (HSCR) is a disease of the intestines. It is characterised by an absence of the enteric nervous system (ENS) in a distal portion of the colon causing a contraction of the muscles and a functional obstruction. This results from defects in the differentiation, proliferation, migration and/or survival of ENS progenitors during development. HSCR can be an isolated trait or be part of a multi-feature syndrome. There are multiple developmental pathways which contribute to these defects and the genetic background of HSCR is complex, ~70% of cases cannot be explained by known genes.

The possibilities of somatic variations contributing to the development of, and accounting for the missing heritability in, HSCR are discussed in chapters 2.1 and 2.2. In **chapter 2.1** we outline the need for appropriate distinction between inherited parental mosaicism and true somatic mosaicism, proposing an experimental design to differentiate between the two in HSCR patient tissue. In **chapter 2.2** this experimental design is utilised to look for ENCC specific variation in patient tissue. Although somatic variants were present in all included patients, somatic variants in HSCR related genes were not. Due to the nature of ENS development it is likely that somatic variants could not be identified in the distal colon. If damaging somatic variants were to occur in ENCCs, these cells would likely be out-competed to ENS niches before reaching these distal regions.

In syndromic cases, HSCR can be a variable feature, the presence of common *RET* variants as well as the type of causative variant may influence HSCR development. The presence of HSCR in cases of Goldberg-Shprintzen syndrome (GOSHS) is discussed in **chapter 3**.

Pathogenic variations in KIF1 binding protein (*KIF1BP*) lead to GOSHS. A number of patients have been reported in the literature and truncating variations in *KIF1BP* have been found to be causative in all sequenced cases. We report nine new patients with *KIF1BP* variations, and functionally investigate three new missense variants which were suspected to be pathogenic in two patients with differing phenotypes. The three missense variants were found to result in a decrease in KIF1BP expression. The variant resulting in the lowest expression was present in the patient with the classical GOSHS and HSCR. Common predisposing HSCR SNPs were not found to have correlation with the presence or absence of HSCR in GOSHS patients.

Another possible genetic factor for HSCR in syndromic cases is the presence of copy number variations (CNVs) that affect dosage sensitive HSCR loci. In **chapter 4** we compare the size and number of CNVs between syndromic and non-syndromic cases of HSCR to find new candidate genes/loci. Syndromic HSCR patients with an unknown genetic aetiology have more and larger CNVs than isolated HSCR cases with a known pathogenic variant. These large CNVs overlap with dose-sensitive genes which may help to identify candidate genes for HSCR.

To further look into the underlying pathogenesis of HSCR development we created iPSC lines from four patients with different pathogenic variations. These cells offer possibilities for disease modelling, functional investigation of variants as well opening the door to future iPSC-enteric neuron transplantation options. In **chapter 5** we present the characterisation of these patient-derived iPSCs and explore variations in their function compared to iPSCs generated from healthy controls. Three out of four of the patient-derived lines differentiated

effectively towards a vagal neural crest lineage and expressed appropriate markers. The less successful line also showed a markedly higher proliferation rate suggesting immaturity in differentiation. High seeding density was an important factor in successful differentiation, implicating cell-cell contact as vital for neural crest formation. Together our data suggest that for many HSCR patients, gene correction may not be necessary before transplantation trials.

In order to bring an expedient option for the treatment of HSCR patients who continue to suffer from gastrointestinal problems following the current surgical standard of care, we may have to think beyond biological interventions. The technological world has arguably been able to advance at a faster rate than the development of purely biological treatment options. With the advances in microelectronics and prosthetic technologies incorporating sensory input, the opportunities for developing transplantable devices may be provide a more elegant solution than the more primitive prostheses currently available. These ideas are discussed in **chapter 6,** together with a patent proposal for an artificial prosthetic sphincter with an anatomically relevant mechanism and design which could provide therapeutic options for HSCR patients and others suffering from faecal incontinence or loss of anal sphincter control.

In conclusion, the work of this thesis investigates the development of, and treatment options for, HSCR. We explore the missing heritability that is seen in HSCR, modes of development and differentiation of the progenitors of the ENS, suitability of various cell sources for transplantation therapy, and explore other possible treatment avenues for current and future HSCR patients.

Samenvatting

De ziekte van Hirschsprung (HSCR) is een aangeboren neurologische aandoening van het maagdarmkanaal. De ziekte wordt gekenmerkt door de afwezigheid van ganglia in het uiteinde van de darm. Ganglia zijn groepjes zenuwcellen en gliacellen die behoren tot het enterische zenuwstelsel (engels: ENS). Daar waar de ganglia ontbreken trekken de spieren samen en veroorzaken zo een verstopping. Het ontbreken van ganglia kan het gevolg zijn van fouten in de differentiatie, proliferatie, migratie en/of overleving van ENS voorlopercellen tijdens hun ontwikkeling. De aanleg / ontwikkeling van het enterische zenuwstelsel is complex en dat kan meestal ook gezegd worden van het ontstaan van HSCR. De belangrijkste oorzaak voor het ontstaan van HSCR zijn fouten (mutaties) in het erfelijk materiaal. Er is één gen dat het meest gemuteerd voorkomt en dat is het *RET*-gen. We vinden mutaties van het *RET*-gen die genoeg veranderingen kunnen veroorzaken om de ziekte doen ontstaan, maar, veel vaker vinden we ook variaties die de kans op de ziekte verhogen, maar niet veroorzaken. Echter, in ~70% van de gevallen kan de volledige oorzaak nog niet worden verklaard door mutaties.

HSCR kan als een op zichzelf staande ziekte voorkomen, maar kan ook in combinatie met andere afwijkingen worden gediagnosticeerd; dan noemen we het een syndroom.

Zoals gezegd is de oorzaak van de ziekte veelal erfelijk, en mutaties in een groot aantal genen zijn al gevonden. Deze mutaties erft de patiënt vaak over van één van de ouders. Soms ontstaan de mutaties in de geslachtscellen (dit noemen we ook wel kiembaan mozaïcisme) en heeft alleen het kind de genetische afwijking. Maar zelfs als we alle genen

screenen vinden we niet altijd een duidelijke verklaring. Een hypothese die we hebben onderzocht was of er mutaties voorkomen alleen in het ENS. Die mutaties moeten dan tijdens de ontwikkeling van het kind ontstaan. We noemen dergelijke mutaties somatische mutaties of variaties. Deze hypothese, dat somatische varianten bijdragen aan de ontwikkeling van HSCR, wordt besproken in de hoofdstukken 2.1 en 2.2. In hoofdstuk 2.1 schetsen we de noodzaak voor een duidelijk onderscheid tussen kiembaan mozaïcisme en werkelijk somatisch mozaïcisme, en stellen we een toepasbaar experimenteel plan voor om in weefsel van HSCR patiënten dit onderscheid ook daadwerkelijk te kunnen maken. In hoofdstuk 2.2 wordt van dit experimentele plan gebruik gemaakt om te onderzoeken of er in het ENS van patiënten inderdaad sprake is van specifieke somatische variaties in de zenuwcellen. Alhoewel somatische varianten aanwezig waren in alle onderzochte patiënten, vonden we er geen in de genen waarvan bekend was dat ze HSCR kunnen veroorzaken. Ook beschrijven we dat, gezien de manier waarop het ENS wordt aangelegd, de kans op het vinden van somatische varianten niet heel waarschijnlijk is; we denken namelijk dat als somatische varianten zouden voorkomen in het ENS, deze cellen vermoedelijk weg zouden worden geconcurreerd door gezonde ENS specifieke cellen voordat ze deze plek bereiken.

Zoals gezegd, kan HSCR onderdeel zijn van een syndroom. Eén van de bekende syndromen is Goldberg-Shprintzen syndroom (GOSHS). We bespreken dit syndroom in hoofdstuk 3. Pathogene varianten in het KIF1 bindingseiwit (KIF1BP) veroorzaken GOSHS, met HSCR als een variabel kenmerk. Alhoewel HSCR geen criterium is voor de diagnose GOSHS, zijn er mogelijk wel factoren die er voor zorgen dat deze patiënten HSCR

ontwikkelen. Een aantal patiënten zijn beschreven in de literatuur en in al die patiënten waarbij DNA geanalyseerd werd, werden varianten gevonden die een verkort KIF1BP eiwit tot gevolg hebben. We beschrijven negen nieuwe patiënten met varianten in KIF1BP. Ook hebben we functioneel onderzoek gedaan naar drie nieuwe missense varianten (missense: vervanging van een aminozuur door een ander aminozuur), t.w. varianten waarvan we denken dat ze ziektes kunnen veroorzaken. We hebben deze varianten gevonden in twee patiënten met verschillende fenotypes. De drie missense varianten bleken een verlaagde KIF1BP expressie tot gevolg te hebben. De variant met de laagste expressie werd gevonden in de patiënt met klassiek GOSHS en HSCR. We hebben geen veelvoorkomende veranderingen gevonden die gecorreleerd zijn aan de aan- of afwezigheid van HSCR in GOSHS patiënten.

Een andere mogelijk genetische factor voor HSCR in syndromale gevallen, is de aanwezigheid van grote variaties in het DNA, d.w.z. grote stukken van een chromosoom die extra of juist minder aanwezig zijn. In hoofdstuk 4 vergelijken we de grootte en het aantal chromosoomafwijkingen (CNVs) tussen syndromale en niet-syndromale HSCR patiënten om nieuwe kandidaat genen/loci te vinden. Syndromale HSCR patiënten met een onbekende genetische oorzaak hebben meer en grotere CNVs dan patiënten met alleen HSCR en een bekende ziekteverwekkende mutatie. Deze grote CNVs overlappen met dosis-gevoelige genen. Deze bevindingen kunnen helpen bij het identificeren van nieuwe kandidaat genen voor HSCR.

Om de onderliggende pathogenese van HSCR verder te onderzoeken, hebben we cellijnen gemaakt van geïnduceerde pluripotente stamcellen

(engels: iPSCs) van vier patiënten met mutaties in verschillende genen. Deze cellen bieden de mogelijkheid om het ziektebeeld te modelleren en de varianten functioneel te onderzoeken. Dit type onderzoek opent de deur naar toekomstige therapie. We denken dat daar waar de neuronen ontbreken deze cellen mogelijk gebruikt kunnen worden voor transplantatie in de darm. In hoofdstuk 5 laten we de karakterisering, van deze van de patiënt afgeleide cellijnen zien. Ook gaan we na of de variaties die we hebben gevonden effect hebben op de cellijnen (we vergelijken de patiënten cellijnen met controle cellijnen). Drie van de vier van de patiënt afgeleide lijnen lieten geen echte verschillen zien. Eén cellijn was anders. De cellijn vermeerderde zich aanmerkelijk sneller dan de rest, hetgeen duidt op een onrijpe differentiatie. Een belangrijke factor voor het goed groeien is een hoge celdichtheid bij het opgroeien, wat er op duidt dat cel-cel contact van vitaal belang is bij de vorming van deze cellen. Omdat de meeste cellen geen groot verschil lieten zien lijkt het erop dat deze cellijnen geschikt zouden moeten zijn voor therapie.

Om met een doeltreffend alternatief te komen voor de behandeling van HSCR patiënten die last blijven houden van gastro-intestinale problemen na de huidige standaard operatieve behandeling, moeten we verder denken dan de biologische interventies. In de technische wereld is de vooruitgang sneller gegaan dan de ontwikkeling op het gebied van puur biologische behandelmogelijkheden. Met name de vooruitgang in de micro-elektronica en de prothetische technologieën die gevoelssensoren weten in te bouwen, bieden mogelijkheden om transplanteerbare hulpmiddelen te ontwikkelen. Deze ideeën worden besproken in hoofdstuk 6, samen met een patent protocol voor een kunstmatige sluitspierprothese met een anatomisch toepasbaar

mechanisme en ontwerp. Deze zou kunnen voorzien in therapeutische behandelmogelijkheden voor HSCR en andere patiënten die leiden onder fecale incontinentie of het gebrek aan controle over de sluitspier.

Samengevat, het werk gepresenteerd in dit proefschrift, onderzoekt de ontwikkeling van, en de behandelmogelijkheden voor, HSCR. We hebben gezocht naar erfelijke factoren in HSCR, de wijze van ontwikkeling en differentiatie van de ENS voorlopercellen, de geschiktheid van cellen met verschillende origine voor transplantatie therapieën én andere behandelmogelijkheden voor huidige en toekomstige HSCR patiënten.

Curriculum vitae

Personal Information

Name:	Katherine Christa MacKenzie
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Place of Birth:	Alexandria, Scotland

Professional Experience

2014-2019	PhD student with Prof. Dr. Robert Hofstra, in the Department of Clinical Genetics, Erasmus MC, Rotterdam

Education

2013-2014	Master of Science *Stem Cell Technology* University of Nottingham, England
2008-2013	Bachelor of Science with Honours *Anatomical Sciences* University of Dundee, Scotland

Internships

2013	Nurture Fertility Clinic, Nottingham, England
2011-2012	Defence Science and Technology Laboratory, Kent, England
2011	Scottish Centre for Regenerative Medicine, Edinburgh, Scotland

PhD Portfolio

Courses		
Genetics course	2014	**3**
Laboratory Animal Science (Artikel 9)	2014	**3**
Research Integrity	2015	**0.3**
OIC Functional Imaging	2015	**1.5**
Safely Working in the Laboratory	2016	**0.3**
Special Topics course: Epigenetics	2016	**3**
Special Topics course: CRISPR	2017	**3**
Biomedical English Writing	2017	**2**

Seminars & Workshops		
Sophia Research Days	2014-18	**1**
Clinical Genetics Meetings	2014-18	**2**
Clinical Genetics Seminars	2014-18	**1**
Mouse Models course	2015	**2**
MGC workshop: Maastricht	2015	**1**
MGC workshop: Dortmund	2016	**1**
Journal Club	2016-18	**1**
MGC workshop: Texel	2018	**2**

Conferences & Symposia		
MGC Symposia	2014-18	**2**
International Symposium on Development of the ENS: Rotterdam	2015	**1**
Mouse Models Symposium: Leiden	2015	**0.5**
Stem Cell Symposium: Utrecht	2016	**0.5**
European Society of Human Genetics: Copenhagen	2017	**1**
International Symposium on Development of the ENS: Boston	2018	**2**

Teaching		
Supervisor Bachelor student	2016	**1**
Teaching Assistant Journal Club	2016-17	**2**
Teaching Assistant Philosophy & ethics	2016-18	**3**
Total		**40**

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