ASSESSMENT FOR EVIDENCE OF APOPTOSIS OF MYENTERIC GANGLION CELLS AT THE TRANSITION ZONE IN HIRSCHSPRUNG'S DISEASE AND THE DEVELOPING LARGE INTESTINE

By

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Abstract

Introduction: Hirschsprung's Disease (HD) is the congenital absence of ganglion cells (GCs) within the distal intestine. Our objectives are to determine if apoptosis of myenteric GCs occurs during human development and to determine if myenteric GC apoptosis or injury contributes to HD.

Materials and Methods: Apoptosis of myenteric GCs was assessed in archived fetal intestinal tissue (n = 4; 15-41 weeks gestational age) and in HD at the transition zone (TZ) (n = 6) using anti-cleaved caspase-3. Immunohistochemistry for GFAP, CD68, HLA-DR and APP was used to assess the presence of enteric reactive changes.

Results: No activated caspase-3 expression was present in the myenteric GCs of the developing human intestine or the TZ of HD. No significant increase in GFAP, CD68, HLA-DR or APP expression was present.

Conclusions: Apoptosis does not appear to occur during the development of the human myenteric plexus or, in conjunction with GC injury, in HD.

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List of Abbreviations

ABC .....Avidin-biotin-horseradish peroxidase complex

AS .....Aganglionic segment

APP.....Amyloid precursor protein

BE .....Bull's Eye retrieval buffer

CNS.....Central nervous system

DAB .....Diaminobenzidine

EDN3 .....Endothelin-3

Ednrb .....Endothelin receptor B

ENCDC .....Enteric neural crest-derived cell

ENS.....Enteric nervous system

GA.....Gestational age

GC.....Ganglion cell

GDNF .....Glial cell line-derived neurotrophic factor

GFAP.....Glial fibrillary acidic protein

GFRα-1.....GDNF family receptor α-1

GIT .....Gastrointestinal tract

GPI .....Glycosyl-phosphatidyl-inositol

GS .....Ganglionic segment

H-E .....Haematoxylin and eosin

HD.....Hirschsprung's Disease

HLA.....Human leukocyte antigen

MHC.....Major histocompatibility complex

N.B......Neutral buffered

NCC.....Neural crest cell

PBS.....Phosphate buffered saline

PGP9.5.....Protein gene product 9.5

PNS.....Peripheral nervous system

RET.....Rearranged during transfection

RT.....Room temperature

RTK.....RET receptor tyrosine kinase

TA.....Therapeutic abortion

TUNEL.....Terminal-transferase dUTP nick-end labelling

TZ.....Transition zone

Chapter I: Introduction

Many cells are involved in the normal function of the large intestine and the disturbance of the development of these cells can result in a clinical disease. This thesis will examine the development of myenteric plexus ganglion cells (GCs) of the large intestine which are involved in its normal function. Additionally, the role of myenteric GCs in Hirschsprung's Disease (HD) will be discussed and the possibility of apoptosis of myenteric GCs as a contribution to the disease will be explained.

I.1: Function and Development of the Large intestine

Anatomy and Function

The large intestine consists of several segments and is responsible for water absorption from chyme, which then becomes fecal material. The fecal material accumulates and is stored in the large intestine until defecation takes place. The segments of the large intestine, from proximal to distal, are the cecum (with attached appendix), ascending (right) colon, transverse colon, descending (left) colon, sigmoid colon, rectum and anal canal [50].

Development of Gastrointestinal Tract Wall

The gastrointestinal tract (GIT), which includes the esophagus, stomach, small and large intestine, develops from three main cell types; endoderm, mesoderm and ectoderm [5]. The endoderm gives rise to the epithelial lining of the GIT and the parenchyma of various organs including the liver and pancreas [5]. The mesoderm gives rise to supporting tissues [5] including the circular and longitudinal smooth muscle layers,

submucosa and muscularis mucosae [19]. Lastly, the ectoderm, including the neural crest, gives rise to the neurons and glia that comprise the enteric nervous system (ENS) [5, 19]. Numerous genes, as well as growth factors, are responsible for the development of these three cell types into their respective organs and tissue, and disruption of them can result in organ-specific defects [49].

During human development, the GIT grows in length disproportionately to the rest of the embryo [5], which is the phase of human development from gestational age (GA) 0 to 8 weeks. This is succeeded by the fetal phase lasting until birth. The foregut and hindgut are recognizable in the embryo at GA week 3.5 and the yolk sac is broadly attached to the midgut [28]. At GA week 4 the intestine of the embryo is present as a simple tube and by GA week 5 it lengthens and develops into a loop [28]. Additionally at GA week 5, the duodenum and cecum can be recognized in the embryo followed by the rotation of the small intestine around the superior mesenteric artery at GA week 6 [28]. At GA week 7 the intestine herniates into the umbilical cord, which forms a continuous space with the abdominal cavity, due to its elongation and coiling beyond the capacity of the abdominal cavity [28]. Specifically, the jejunum, ileum, ascending colon and transverse colon herniate whereas the duodenum and large intestine distal to the splenic flexure remain within the abdominal cavity [28]. During the period of herniation, the small intestine continues to elongate and coil and by GA week 10 the intestine re-enters the abdominal cavity [28]. This is followed by the fixation of the cecum in the right lower quadrant of the abdominal cavity and by GA week 38 maturity of the GIT has been established [28]. It is important to note that the rectum is derived from the cloaca and does

not attach to the large intestine until GA week 8 which can lead to developmental abnormalities [28].

The smooth muscle of the GIT develops in a craniocaudal direction [79]. At GA week 8, the circular muscle layer is a thin band in the proximal and distal intestine, with the distal intestine being less condensed [79]. The large intestine has a small amount of circular muscle at GA week 8 as compared to the small intestine and the longitudinal muscle layer is not apparent at this stage of development [79]. By GA week 11, both the circular and longitudinal muscle layers of the GIT are present [17, 19] as broad bands and a mature phenotype is present by GA week 14 [79]. Additionally, the muscularis mucosa is also present as a small band adjacent to the villi by week 14 [79].

I.2: Enteric Nervous System (ENS)

The ENS is the collection of neurons and supporting cells that are located within the wall of the GIT and is the largest division of the autonomic nervous system [20, 23, 25, 51]. Although some neurons of the ENS receive signals from ganglia of the sympathetic and parasympathetic nervous systems as well as the hindbrain [51, 56], the ENS has the capability of functioning autonomously without neural input from the peripheral nervous system (PNS) or central nervous system (CNS) [20, 22, 25]. The ENS has several functions including regulation of the motility reflex of the GIT, mucosal control of water and electrolyte transport [20] and regulation of blood supply to the intestines [22, 77].

ENS Development and Neural Crest Cells

ENS development relies on the migration of neural crest cells (NCCs), their proliferation, differentiation and ganglion formation [5]. Both enteric glia and enteric

neurons are derived from the neural crest [47, 48]. This process begins with the neural crest precursor cells migrating into the GIT at its oral and anal segments then throughout the remainder of the GIT in a craniocaudal direction [49, 51, 54]. The precursor cells are arranged within the developing GIT with respect to its organizational layers (i.e. circular muscle, longitudinal muscle, submucosa and epithelium), then differentiate into neurons and glial cells and finally develop the connections that are essential for the function of the ENS [51]. This process is regulated by several signalling pathways; the two major ones are glial cell line-derived neurotrophic factor (GDNF)/RET (rearranged during transfection) and endothelin-3 (EDN3)/endothelin receptor B (Ednrb) [30].

As previously described, the NCCs migrate into the GIT in a craniocaudal direction [49, 54] and by GA week 7, the NCCs in the large intestine are differentiated into neurons and glia [18, 19, 79]. However, at this stage of development, the ganglion plexuses, both myenteric and submucosal, have not formed and the cells are in a scattered arrangement [18, 19, 79]. The myenteric plexus is formed by GA week 9 in the hindgut from the scattered neurons and glia that are present at GA week 7 [19]. This corresponds to the presence of peristaltic waves and motility starting to occur in the large intestine [28]. The submucosal plexus first forms adjacent to the circular muscle layer by GA week 14 in the hindgut via cell projections of the myenteric plexus [54, 79]. By GA week 20 the submucosal plexus migrates to the inner submucosa away from the circular muscle layer [19]. By GA week 20, GCs and enteric glia display a distribution within the large intestine similar to that seen in the infant gut (i.e. GCs and enteric glia are located throughout the GIT) [19].

Motility

Gastrointestinal motility serves the purpose of moving food through the GIT and mechanically breaks down ingested food into small particles [65]. Motility of the GIT results from the contraction of the smooth muscle contained within its walls [65]. There are two types of contraction of the smooth muscle, peristaltic and segmental [65]. Intestinal peristaltic contractions are responsible for moving a bolus of food through the GIT by contraction of the intestinal segment just proximal to the bolus into a receiving intestinal segment which then contracts to keep propelling the bolus forward [65]. Intestinal segmental contractions of the smooth muscle are responsible for mixing the contents and keeping it in contact with the GIT epithelium [65]. This contracture occurs as a result of the myenteric motor neurons in the ENS [20] which receive signals from the submucosal plexus [65]. In order for the GIT to obtain normal neuromuscular function, the development of the smooth muscle layers, neurons, glial cells and several other cell types must be coordinated precisely [79].

Enteric Glia

Enteric glial cells are similar to astrocytes in the CNS with respect to structure, biochemistry and function [33, 78]. They provide support for enteric neurons [25] and loss of enteric glia results in enteric neuronal cell death because glial cells have an important role in neuronal repair [33]. Additionally, glial cells have an important function in establishing the perimeter of the myenteric plexus as well as divide myenteric ganglia into compartments [24]. This is accomplished by enteric glia forming an incomplete sheath around neurons of the myenteric plexus which partially separates them from extraganglionic connective tissue [21].

Ganglion Cells

Ganglia are clusters of different types of neurons. Adjacent ganglia do not necessarily have identical neuronal types; however, they are usually similar [51]. In different locations within the GIT, the ganglia differ with respect to size, neuronal type, projection patterns and connectivity [22, 51]. ENS ganglia are located in the myenteric and submucosal plexuses [22]. The myenteric (Auerbach's) plexus is between the circular and longitudinal muscle layers of the GIT wall and the submucosal (Meissner's) plexus is within the submucosa internal to the circular muscle layer [51]. Ganglia of the myenteric plexus are located throughout the entire GIT; however, the submucosal ganglia are only located within the small and large intestines [51]. The ganglia are connected to each other by bundles of nerve fibres which have been termed internodal strands [51]. Intestinal segments with an absent or damaged myenteric plexus do not have peristaltic contractions occurring and therefore functional obstruction results [11, 71].

I.3: Hirschsprung's Disease

Abnormalities of the ENS may have a role in prenatal health; however, they become more apparent postnatally and can have severe consequences on the health of newborns [51]. One such disease is Hirschsprung's Disease (HD) and it is considered a neurocristopathy since it is a disease involving cells derived from the neural crest [2, 7].

Epidemiology

HD is a congenital childhood motility disorder affecting 1 in 5000 live births [42, 49] and was first comprehensively described by Harald Hirschsprung [2]. HD is more predominant in males with a male:female ratio of 4:1 [42, 51]. The disease is characterized

by the complete absence of submucosal and myenteric GCs in the distal portion of the large intestine (hindgut), and extends proximally for various lengths [2, 12, 13, 51]. In addition to the lack of GCs in the affected segment, there is also an increase in the number and size of nerve fibres arising from extrinsic neurons in the aganglionic segment (AS) [35, 51]. Situated between the AS and ganglionic segment (GS) is the transition zone (TZ) [2, 81], a region of the large intestine with a decrease in the number of GCs in the myenteric and submucosal plexuses [51, 81]. Additionally, the myenteric GCs of the TZ are irregularly and widely spaced and have an uneven circumferential distribution [81].

Clinical Features

There are several common symptoms of HD including delayed meconium passage, intestinal obstruction, constipation, failure to thrive, abdominal distention and enterocolitis [13, 49]. Intestinal obstruction is due to the persistent contraction of the affected bowel segment which is caused by the lack of the peristaltic waves [11, 51]. This results in the affected segment being devoid of contents and dilation of the proximal segment due to fecal accumulation [42, 51, 71]. The TZ has a diameter greater than the affected contracted AS but smaller than the dilated proximal GS [81].

Within 24 hours of birth, 90% of HD patients will not have passed meconium as compared to healthy neonates, who pass meconium in 95% of cases [13]. Meconium is material that accumulates in the distal ileum and large intestine of the fetus. It consists of intestinal secretions, cellular products and amniotic fluid swallowed during gestation [28]. This cannot be passed in the neonate because of the lack of peristaltic contractions of the intestine [71].

An indication of enterocolitis associated with HD is the presence of diarrhea in neonates without the existence of abdominal distention and vomiting [13]. This is the major contributor to the morbidity and mortality associated with HD [13]. Toxic megacolon is when the intestine becomes gangrenous and has the potential to rupture [42]. This condition is life-threatening and can occur in HD patients if early diagnosis and adequate therapy are not carried out [13]. Toxic megacolon causes sudden abdominal distention, bile-stained vomiting, fever, dehydration and shock [13] due to the loss of neuromuscular activity of the intestine [42]. Additionally, all children with HD also suffer from internal sphincter achalasia which is the inability to relax the internal anal sphincter due to the lack of the recto-anal inhibitory reflex [12].

Diagnosis

HD is most commonly diagnosed in the neonatal period [2, 13] and is confirmed by performing a suction rectal biopsy or full thickness rectal biopsy in addition to comparison with radiographic and anorectal manometry findings [13, 14, 43]. Suction rectal biopsy specimens, which include the epithelium, lamina propria and muscularis mucosae, and full thickness rectal biopsy specimens, which include mucosa and both the circular and longitudinal muscle layers, are examined for the presence of excessively large nerve fibres [13, 43]. Additionally, the full thickness biopsies are evaluated for the presence of GCs in the submucosal and myenteric plexuses to confirm the diagnosis [37, 43]. Once the diagnosis of HD is confirmed, treatment includes the surgical resection of the AS of the large intestine and reanastomosis of the large intestine to the anus [51, 66]. Full thickness biopsies are performed during the resection procedure to determine where the large intestine should be resected; specifically, it is used to establish where the GS begins so

that the TZ and AS are completely excised [37, 51]. The main goal of the resection is to remove the least amount of large intestine but to also ensure that the portion remaining in the patient is physiologically functional [51].

Treatment

Several surgical techniques have been developed to treat HD [61]. There are three main techniques, each with the intention of being less invasive [44, 66]. The first to be conceived was the Swenson procedure and included the removal of the AS of the large intestine and the anastomosis of the remaining GS of the large intestine to the anus [66, 71, 72]. In this procedure, the proximal line of resection of the large intestine is made approximately twelve centimetres proximal to the narrowing segment and the distal line of resection is made as low as possible on the rectosigmoid [71, 72]. The presence or absence of GCs at the proximal line of resection is then determined by intraoperative consultation once the large intestine segment is removed from the patient [72]. If GCs are not present, then additional proximal segments are removed from the patient until a normal distribution is observed in the intraoperative consultation [72]. Importantly, there must be sufficient large bowel remaining in the patient to allow for anastomosis to the anus without tension since associated complications may arise [72]. In this procedure the resection of the rectum is done to the level of the levator muscles and with meticulousness of the surgeon during the procedure the sphincter mechanism of the anus is left intact [71, 72]. The anastomosis of the large intestine segment to the anus is achieved by withdrawal of the remaining rectosigmoid through the anus followed by the proximal large intestine segment [71, 72]. The anastomosis is performed and then is retracted into the pelvis [71, 72].

Secondly, the Duhamel procedure was established to decrease the complications that were associated with the Swenson surgery for HD [15, 66] and includes a retro-rectal transanal approach [13, 15]. In this procedure, the pelvic dissection is minimalized and the rectum is not resected [61]. The rectum is separated from the large intestine at the level of the anterior peritoneal reflection and the AS and TZ are excised [15]. Blunt dissection of the tissue posterior to the rectum allows the pelvic plexus of nerves to not be damaged and the proximal GS of the large intestine is drawn down through the plane of dissection [15]. The GS of the large intestine that remains in the patient is then anastomosed to the lateral or posterior aspect of the rectum [61]. This procedure ensures the preservation of anal sphincter control and motility of the large intestine with the possibility of performing a Swenson procedure if the initial procedure fails [15].

Thirdly, the Soave procedure was developed to incorporate the endorectal pull-through technique into the surgical treatment of HD [66]. This procedure involves the removal of the AS by pulling it through the muscular cuff and then anastomosing the large intestine GS to the rectum [61]. The Soave procedure has been modified to be performed by a transanal approach in which a rectal mucosectomy is carried out proximal to the dentate line and continued to the intraperitoneal rectum [45]. The large intestine is prolapsed through the muscular cuff, intraoperative diagnosis confirms the presence of GCs, and anastomosis is performed with longitudinal incision of the posterior muscular cuff [1, 45].

Both the Duhamel and Soave procedures can be performed as a one-stage or two-stage procedure [44]. The two-stage procedure involves an initial colostomy before the anastomosis of the large intestine to the anus [44]. Complications arise from both

procedures and the one selected is based on multiple factors including preference of the surgeon, presence of perforation, toxic megacolon or enterocolitis, and whether the intraoperative diagnosis of the biopsy is reliable [44]. Furthermore, all three techniques have been adapted to use laparoscopic technology to decrease the invasiveness of each approach [45, 67].

Complications

There are both early and late postoperative complications that can occur after children with HD have been treated surgically. Early complications include bleeding, infection, risks associated with anesthesia, and injury to other organs, which are all complications that can occur with any abdominal surgery [12]. Other complications that can arise are procedure specific. Children who have a staged procedure with a stoma are at risk for stricture, retraction, prolapse and skin breakdown [12, 53]. Complications associated with anastomoses are also seen and include twisting, anastomotic leak, strictures and retraction [12].

Long-term follow up in surgically treated HD patients is important because late complications may occur [83] and consist of obstructive symptoms, incontinence and enterocolitis [12]. Obstructive symptoms include mechanical obstruction, persistent or acquired aganglionosis, associated colonic motility disorder, internal sphincter achalasia and functional megacolon [12]. Due to these complications the patients social and family life may be significantly affected [83]. Most children will overcome the physical and emotional complications and their quality of life will be reasonably normal [83].

Theories of Origin

The exact pathogenesis of HD has not been understood to date. However, the most widely accepted hypothesis is that HD is a disease of the migration of NCCs [2, 51, 55, 80]. It is thought that the NCCs stop migrating in their craniocaudal direction between the fifth and twelfth week of human development [2, 7, 54, 55, 73]. The mechanism by which the migration stops is not well understood since the wall of the aganglionic intestine is capable of supporting NCCs [73]. An alternative hypothesis is that the NCCs migrate to the large intestine; however, they do not survive once they have reached their target site due to apoptotic or non-apoptotic cell death [8, 36, 41, 68, 75, 76].

Genetics

HD has a genetic element as is demonstrated by the fact that there is an increased familial incidence of 4% for siblings as opposed to approximately 0.02% for the general population [4, 51]. As the length of the AS increases, the risk to siblings increases and the sex ratio decreases [4]. HD is an incompletely penetrant autosomal dominant disorder [49] and multiple genes have been demonstrated to be involved in HD [2]. Mutations in RET and Ednrb genes are the most common and therefore the most studied [2, 49]. Additionally, HD patients have demonstrated a mutation of Sox-10, a transcription factor associated with the development of NCCs [49].

RET proto-oncogene mutations are present in approximately 50% of familial cases and 15-20% of sporadic cases of HD [2]. The RET proto-oncogene encodes for a transmembrane tyrosine kinase receptor (RTK) that is present in cells of neural crest origin [3, 55] and is critical for ENS development [3, 63, 73]. It influences cell proliferation, migration, differentiation and programmed cell death [55, 73]. Mutations of RET associated

with HD are commonly loss-of-function mutations and occur throughout the gene [2]. GDNF is a ligand of RTK and is critical for neuronal survival [73] and it binds RET using the co-receptor GDNF family receptor α-1 (GFRα-1) [2]. The binding of GDNF to RET causes it to move to the cell membrane where it undergoes autophosphorylation, which in turn activates downstream effector pathways [2]. These effector pathways are crucial in neuronal development, migration and have anti-apoptotic effects [2, 8, 30]. There are several other known ligands of RET; however, they have not been shown to have a role in HD [2].

Associated Disorders

HD has been associated with other disorders including birth defects, chromosomal abnormalities and other syndromes [55]. One disorder associated with 2.5 – 5% of HD patients is multiple endocrine neoplasia type 2A, which also has a genetic component associated with a RET mutation [2, 55]. Birth defects associated with HD include anomalies of the CNS, cardiovascular system malformations, genitourinary abnormalities and GIT malformations [59, 60]. The most common chromosomal anomaly associated with HD is Down syndrome (Trisomy 21), occurring in 2 – 15% of patients [55]. HD has been shown to be associated with several syndromes including congenital central hypoventilation syndrome, Waardenburg syndrome type 4, Smith-Lemli-Opitz syndrome, and X-linked hydrocephalus [55, 60].

I.4: Apoptosis

Cell death is the result of cell injury due to various causes including ischemia, infection, toxins and immune reactions [42]. Additionally, cell death has a key role in embryogenesis, organ development and homeostasis [42, 70]. Apoptosis is one form of cell

death that occurs due to internal and/or external signals controlling the programmed death of the cell by which the nuclear DNA, nuclear proteins and cytoplasmic proteins degrade due to activation of cellular enzymes [42, 70]. The main purpose of apoptosis is to eliminate cells during embryogenesis, physiological processes and pathological processes [42, 70]. Several morphological features characterize cells undergoing apoptosis. These include cell shrinkage, chromatin condensation, cytoplasmic blebs, formation of apoptotic bodies, and phagocytosis of apoptotic cells or cell bodies [38, 39, 42]. Additionally, there are biochemical characteristics of cells undergoing apoptosis including protein cleavage, DNA breakdown, and phagocytic recognition [38, 42].

Role of Caspase in Apoptosis

Each apoptotic pathway eventually leads to the activation of caspases (cysteinyl aspartate-specific proteases) [52, 69] which are cysteine proteolytic enzymes responsible for cleaving proteins within the cell and are regulators of the initiation of cell death [70]. A minimum of 14 caspases are present in mammals and are initially produced as zymogens, which are inactive enzyme precursors [69, 70]. The caspase zymogens are activated through cleavage at aspartic residues of the caspase by other caspases [69] or by other proteases [52]. There are a variety of internal and external stimuli that cause the activation of caspase to occur including DNA abnormalities, cytotoxic stress, heat shock, radiation, genotoxic damage and many others [38, 52]. The internal and external stimuli activate different apoptotic signalling pathways [38].

There are significant differences in the structures of the various caspases which gives rise to their specific function [52]. Specifically, the structure of caspase-3 causes it to function as an effector caspase that is vital in neural apoptosis [52, 69] and allows it to cleave

itself, other caspase enzymes and caspase substrates [27]. Caspase-3 exists as a zymogen in normal healthy cells but once the apoptotic cascade is initiated, the enzyme is cleaved and is activated [27]. Other effector caspases include caspase-2 and caspase-7 which also will lead to cell apoptosis [52].

RET Signalling Pathway for Apoptosis

There are several pathways that signal apoptosis either intrinsically or extrinsically [70]. One of these pathways is through the RET proto-oncogene. RET is a dependence receptor, meaning that it can induce two signals within a cell. Induction of apoptosis will occur when no ligand is bound to the receptor or, if a ligand is bound, then cell survival is promoted [2, 8]. Thus, when the ligand GDNF is not bound to RET apoptosis is induced [8]. This occurs by cleavage of RET by caspase-3 at sites that are fundamental in apoptosis induction by RET [8]. It is also important to note that certain mutations in the RET gene cause the GDNF apoptosis inhibition function to be impaired [2]. In other words, mutations of RET cause it to be unaffected by the presence or absence of GDNF and thus the mutated RET induces apoptosis [8].

I.5: Immunohistochemistry

Immunohistochemistry is used to identify cell products by the binding of antibodies to the specific cellular components to be studied [42, 85]. A visual marker is linked to the antibody which is visualized using light, fluorescence, or electron microscopy thus identifying where the cellular component is located within a tissue [85].

Anti-cleaved Caspase-3

The activation of caspases is the most precise indicator of apoptotic cells [27]. Caspase-3 is present in the cytoplasm of normal healthy cells and does not react to anti-cleaved caspase-3 antibody because it has not been activated [27]. Once the apoptotic cascade has been activated and caspase-3 has been cleaved, a unique epitope is then exposed and can bind to the anti-cleaved caspase-3 antibody [27]. Since the epitope is unique for the anti-cleaved caspase-3 antibody, positive expression demonstrates that the cell is undergoing apoptosis [27].

PGP9.5

Protein gene product 9.5 (PGP9.5) is a ubiquitin C-terminal hydrolase cytoplasmic protein [46, 82] expressed in neurons, neuroendocrine cells and a variety of other cell types [10, 74]. Its function most likely relies on the tissue type or differentiation of the tissue in which it is expressed [82]. PGP9.5 is used as a marker for enteric neural crest-derived cells (ENCDCs) and migrating NCCs in the developing GIT [64].

GFAP

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed in approximately 50-60% of enteric glial cells [34, 78] and is used as a marker to identify astrocytes and enteric glia [6]. Inflammation in the GIT causes GFAP expression to increase both in the number of enteric glia cells expressing the protein and in intracellular protein expression [58, 78]. This process is similar to astrogliosis that occurs in the CNS [78]. A drawback of using GFAP as a marker for enteric glial cells is that it is also expressed by Schwann cells [24, 84].

CD68

Macrophages are the final differentiation stage of circulating monocytes [31]. They are dispersed throughout the body and are involved in immune functions[31]. The cell surfaces of both macrophages and monocytes have a transmembrane glycoprotein present designated as CD68 [31]. Antibodies to CD68 are used as an immunohistochemical marker to identify monocytes and macrophages [31].

HLA-DR

Class II major histocompatibility complex (MHC) molecules are coded for by human leukocyte antigen (HLA)-D which contains the sub region HLA-DR [42]. MHC class II molecules, and therefore HLA-DR, are present on antigen-presenting cells including macrophages, dendritic cells, B cells and microglia [29, 42, 62]. HLA-DR is used as a marker of microglial activation that can be up-regulated in the CNS following neuronal damage due to inflammation, trauma, ischemia, and neurodegenerative processes [29, 40, 62].

APP

Amyloid precursor protein (APP) is a transmembrane protein that is primarily located at neuronal synapses [57]. Although the function of APP is not completely understood, it is well known that an increase of APP in damaged axons can be detected using immunohistochemistry [32, 57].

Chapter II: Literature Review

II.1: Ganglion Cell Death in Development

During development of the GIT, NCCs divide numerous times in order to populate the GIT with enough neurons to allow it to have normal neuromuscular function [75]. It has been previously established that within the CNS and PNS more neurons are produced than are needed in the mature individual and the excess neurons undergo apoptosis [86].

Gianino *et al*. (2003) conducted a study to look at the factors that influence enteric neuron number using various mouse models [26]. A portion of their study looked specifically at the role of apoptosis in regulating the number of neurons of the ENS by using immunohistochemistry for activated caspase-3 [26]. Gianino *et al*. (2003) looked at the presence of activated caspase-3 in the ENS of wild-type mice at E12, E14, E16, E18, P0, P7 and P14 [26]. They found no presence of activated caspase-3, and therefore no apoptotic cells; however, they wanted to confirm these results to verify that they were not due to inadequate sampling [26]. In order to accomplish this, Gianino *et al*. (2003) used *Bax*$^{-/-}$ and *Bid*$^{-/-}$ mice [26]. Both Bax and Bid are essential for apoptosis in neurons of the CNS and PNS [26, 86] and by using mouse models deficient for each protein, Gianino *et al*. (2003) were able to compare the number of neurons in the wild-type, Bax-deficient and Bid-deficient mice [26]. Gianino *et al*. (2003) found the same number of enteric neurons in wild-type mice as in both of the experimental strains [26]. Therefore, Gianino *et al*. (2003) concluded that apoptosis of neurons is not a major contributing factor in determining the number of neurons comprising the ENS [26].

Consistent with the results of Gianino *et al*. (2003), are the results from a study conducted by Kruger *et al*. (2003) in which they studied the effect of Ednrb on the migration of NCCs [26, 41]. In this study, Kruger *et al*. (2003) looked at NCC death in the ileum and cecum in Ednrb deficient mice and control mice using activated caspase-3 immunohistochemistry [41]. The control mice showed no activated caspase-3 expression in the ileum at E13 or in the cecum at E13.5 – E14 leading Kruger *et al*. (2003) to conclude that the ENS does not undergo apoptosis during development [41].

II.2: Ganglion Cell Death in Hirschsprung's Disease

It has been accepted that HD is a disease of the migration of NCCs that are destined to differentiate into GCs of the submucosal and myenteric plexuses [2, 51, 55, 80]. An alternative hypothesis of the pathogenesis of HD is that cell death of the ENCDCs contributes to the pathogenesis of HD, either through apoptotic or non-apoptotic pathways [8, 36, 75, 76]. These pathways are related to the possible genetic mutations associated with HD including mutations of Sox10 [36, 68], RET [8], Ednrb [41] and GFRα-1 [75, 76].

The Sox10 gene encodes for a transcription factor that is expressed in NCCs and genetic mutations of the gene have been found to be associated with HD [36, 49]. Previous studies have shown that neuronal precursors are not present in the gut of $Sox10^{Dom}/Sox10^{Dom}$ embryos [36, 68] which led Kapur (1999) to look at whether NCC death occurs at an early stage of NCC migration [36]. Kapur (1999) investigated whether apoptosis plays a role in E9 and E10 $Sox10^{Dom}/Sox10^{Dom}$ and $Sox10^{Dom/+}$ embryos using the terminal-transferase dUTP nick-end labelling (TUNEL) procedure which localizes

apoptotic cells [36]. Kapur (1999) found excessive labelling in E9 and E10 $Sox10^{Dom}/Sox10^{Dom}$ embryos at sites where NCCs are normally located [36]. Additionally, Kapur (1999) looked at TUNEL labelling of $Sox10^{Dom/+}$ embryos and found a variable pattern [36]. This labelling surpassed that of wild-type embryos; however, in some cases, it approached the same extent of labelling as that observed in $Sox10^{Dom}/Sox10^{Dom}$ [36]. These results led Kapur (1999) to conclude that there is excessive apoptotic NCC death in Sox10 mutants in the early stages of NCC migration [36]. The results Kapur (1999) attained were in agreement with a previous study by Southard-Smith *et al*. (1998) [36, 68]. Southard-Smith *et al*. (1998) examined the Sox10 gene as a possible contributor to HD and a portion of their study investigated whether apoptosis of NCCs was occurring [68]. They found significant apoptosis occurring in migrating NCCs of $Sox10^{Dom}/Sox10^{Dom}$ and $Sox10^{Dom/+}$ E11.5 embryos using TUNEL [68].

As previously described, the RET proto-oncogene encodes for a transmembrane RTK that is present in cells of neural crest origin [3, 55] and is critical for ENS development [3, 63, 73]. Bordeaux *et al*. (2000) hypothesized that HD is the result of apoptosis of ENCDCs that express RET [8]. This hypothesis was based on the observation that five mutations of the RET gene in HD have been identified to induce apoptosis which do not rely on the presence of a RET ligand [8]. In order to test their hypothesis, Bordeaux *et al*. (2000) had to evaluate several properties of RET including whether RET can induce apoptosis, if the apoptosis is mediated via caspase activation and whether the mutations of RET present in HD cause the induction of apoptosis [8]. Bordeaux *et al*. (2000) demonstrated that RET-induced apoptosis occurs when GDNF is not present in a culture of human embryonic kidney cells that were transfected with human RET and a

neuroblastoma cell line expressing RET endogenously using markers for caspase activation and TUNEL-reactivity [8]. Therefore, Bordeaux *et al*. (2000) concluded that a pro-apoptotic signal was induced by the removal of GDNF in cells that express RET [8]. Bordeaux *et al*. (2000) next demonstrated that RET-induced apoptosis occurs through the activation of caspases and specifically caspase-3 [8]. This was accomplished by co-expression of RET and a caspase inhibitor, baculovirus protein p35, in human embryonic kidney cell culture which together caused a decrease in RET-induced apoptosis [8]. Caspase-3 was determined to cleave RET by performing *in vitro* and *in vivo* studies which indicated that this cleavage is vital to inducing a cell signal for apoptosis [8]. Bordeaux *et al*. (2000) demonstrated that the RET mutations present in HD transformed RET into an inducer of apoptosis by introducing the five HD-associated mutations into the RET coding sequence of cultures of human embryonic kidney cells [8]. All five mutations demonstrated pro-apoptotic activity and the presence or absence of GDNF did not have an effect [8]. These findings allowed Bordeaux *et al*. (2000) to postulate that ENCDCs are not present in HD due to RET-induced apoptosis [8].

Kruger *et al*. (2003) also looked at apoptosis of migrating NCCs using activated caspase-3 to identify apoptotic cells that were Ednrb deficient [41]. Ednrb is a G-protein coupled receptor that when bound to EDN3 maintains ENCDCs in a proliferative state and is required for their migration [30]. However, EDN3 inhibits ENCDC differentiation and when in culture with GDNF, inhibits migration [30]. $Ednrb^{sl/sl}$ and $Ednrb^{+/+}$ mice were used by Kruger *et al*. (2003) to examine both the ileum and cecum at E13 and E13.5 – E14, respectively [41]. Kruger *et al*. (2003) found no evidence of apoptosis in either the

ileum or cecum of Ednrb$^{sl/sl}$ and Ednrb$^{+/+}$ mice leading them to conclude that cell death was not the cause of the arrest of ENCDC migration [41].

Enteric neuronal non-apoptotic cell death has also been studied as having a possible role in the pathogenesis of HD [75, 76]. Uesaka *et al*. (2007) investigated the effect of ablation of GFRα-1 on mice during late gestation and, specifically, if the ablation results in enteric neuronal non-apoptotic cell death [75]. GDNF signalling results from a receptor complex that is comprised of glycosyl-phosphatidyl-inositol (GPI)-anchored cell surface protein, GFRα-1 and RET [2, 73]. Previous studies have shown that GDNF signalling is vital in early ENS development [16]. However, the importance of GDNF signalling in late ENS development has not been demonstrated [75]. Uesaka *et al*. (2007) inactivated GFRα-1 in E15.5 embryos and examined their GIT at E18.5 to see whether enteric neuronal loss results [75]. The GCs showed no difference between control and experimental mice in the small intestine; however, the colon showed HD-like structure in that GCs were almost completely absent and thick nerve fibres were present [75]. This led Uesaka *et al*. (2007) to conclude that GDNF signalling is critical to the survival of enteric GCs and glia during late development [75]. Uesaka *et al*. (2007) next wanted to determine how these cells were dying (i.e. through apoptotic or non-apoptotic pathways) using TUNEL or antibodies for activated caspase-3 and caspase-7 [75]. Both methods did not detect many cells nor were any TUNEL-positive cells also caspase-positive leading to the conclusion that a unique regulation of cell death was occurring [75]. To study the regulation of cell death, Uesaka *et al*. (2007) looked at the biological properties responsible for the death of the colon enteric neurons that were GDNF deprived [75]. They found that neither Bax nor caspases were required for enteric neuronal death and

therefore apoptosis was not the pathway for the death of GDNF-deprived enteric neurons [75].

In addition to examining the role of GDNF signalling [75], Uesaka *et al*. (2008) investigated RET dysfunction and its possible role in inducing death of ENCDCs and thus causing the HD phenotype [76]. Multiple steps were taken to examine the role of RET including its role in colonic neuronal survival, the effect of reducing RET dosage and if this reduction affects the survival of ENCDCs [76]. Uesaka *et al*. (2008) demonstrated that RET was an essential cellular component in order for ENCDCs of the large intestine to survive [76]. This was established by conditional inactivation of the RET gene in a group of mice which would still develop kidneys and enteric ganglia unlike RET-deficient mice that die due to their absence [76]. They found in these knock out mice almost the complete absence of enteric ganglia in the large intestine but normal enteric ganglia in the small intestine, which is characteristic of HD [76]. Uesaka *et al*. (2008) then investigated the effect of reduction of RET on the migration of ENCDCs [76]. They demonstrated that the ENCDCs of the fetuses with RET reduced by half ($RET^{fl/CFP}$) migrated into the GIT in the same manner as the wild-type fetuses [76]. In contrast, fetuses with RET reduced to one third ($RET^{9/CFP}$) of the wild-type showed a delay in migration particularly at the beginning of the hindgut [76]. However the small intestines of $RET^{9/CFP}$ fetuses were colonized identical to the wild-type and the distal large intestines in half of these cases were not colonized [76]. Following this, Uesaka *et al*. (2008) looked at whether neuronal cell death was occurring in the $RET^{9/CFP}$ fetuses [76]. To study this, organ cultures from the gut of E15.5 $RET^{9/CFP}$ fetuses were examined for ENCDCs and after a 6 hour time-lapse observation of the organ cultures, a significant decrease in the number of ENCDCs was

found [76]. In addition to this, Uesaka *et al*. (2008) used electron microscopy to examine the morphology of ENCDCs of $RET^{9/CFP}$ and found them to be smaller, have a sparse distribution and have degenerative changes without the presence of apoptotic figures [76]. They concluded from these results in addition to negative cleaved caspase-3 and TUNEL results, that a substantial number of the ENCDCs experience non-apoptotic cell death [76]. Following this conclusion, Uesaka *et al*. (2008) wanted to ensure that the neuronal death they observed was not a consequence of a deficit in migration that occurred in the $RET^{9/CFP}$ fetuses [76]. Using E14.5 or E15.5 $RET^{9/flox}$ fetuses, which expressed RET at 80% of wild-type but show normal ENCDC migration, Uesaka *et al*. (2008) treated them to decrease the RET expression to one-third of wild-type [76]. This caused the fetuses to have a reduction in the number of ENCDCs once they reached E18.5 [76]. This led Uesaka *et al*. (2008) to conclude that reducing the amount of RET results in non-apoptotic ENCDC death which may be involved in the pathogenesis of HD [76].

Chapter III: Objectives and Hypothesis

There are two main objectives of this study. First, to determine if apoptosis of myenteric GCs occurs during development between GA 15 and 41 weeks and second, to determine if apoptosis or injury of myenteric GCs contributes to HD pathogenesis.

III.1: Development Objectives and Hypothesis

To accomplish the first objective, we used a sensitive immunohistochemical marker for GCs (PGP9.5) to assess their overall distribution in fetal autopsy intestinal tissue between GA 15 and 41 weeks. Secondly, we assessed for apoptosis of myenteric GCs by using anti-cleaved caspase-3 as an apoptosis marker. This allowed us to test the hypothesis that there is post-migrational apoptosis in the development of ganglionic innervation of the human large intestine.

III.2: Hirschsprung's Disease Objectives and Hypothesis

To accomplish the second objective, first we looked for apoptosis of myenteric GCs at the TZ and proximal GS using anti-cleaved caspase-3 as an apoptosis marker. We also assessed possible markers of GC injury, including GFAP, CD68 and HLA-DR. GFAP is up-regulated in damaged brain and the same might occur in the gut. CD68 and HLA-DR are markers of immune activation. APP was used as a possible marker of injury to the GC axons. We compared expression of these markers within the TZ versus the proximal GS and AS where applicable. This allowed us to test the hypothesis that post-migrational apoptosis and cell injury plays a role in HD pathogenesis.

Chapter IV: Materials and Methods

IV.1: Selection of Cases and Tissue Preparation – Development

Fetal intestinal tissue was obtained from three autopsy cases (n = 3; GA 20 – 21, 34 and 41 weeks) and one therapeutic abortion (TA) case (n = 1; GA 15 weeks) from the Winnipeg Health Sciences Centre Pathology Department from September 2007 to February 2008. The examinations of the autopsy and TA cases were performed using standard autopsy and surgical pathology procedures and tissue was submitted for histology accordingly. Sections of intestine and appendix were submitted in each case for overnight dehydration and infiltration with paraffin wax. Tissue sections were embedded in paraffin wax, cut into 4μm sections with a microtome and transferred onto slides. Tissue sections were stained with haematoxylin and eosin (H-E) and reviewed by a pathologist to determine if the tissue was adequate for use in this study.

IV.2: Selection of Cases and Tissue Preparation – Hirschsprung's Disease

Six cases of Hirschsprung's Disease were selected from surgical pathology cases diagnosed at the Winnipeg Health Sciences Centre Pathology Department from June 2007 to January 2008. The cases included five males, ages 3, 4, 5, 8 and 11.5 months, and one female, age 4 years (n = 6). Endo-rectal pull-through specimens from each patient were cut longitudinally and fixed in 10% N.B. formalin overnight. Following fixation, a longitudinal strip of each was cut and sectioned with the proximal end of each section being painted with India ink to mark orientation. Tissue sections were processed overnight for dehydration and infiltration with paraffin wax. The tissue sections were

embedded in paraffin wax, cut into 4μm sections with a microtome and transferred onto slides. Tissue sections were stained with H-E and the slides were reviewed by a pathologist to select the sections containing the TZ and AS slides and, if present, a normal GS slide. Three of the six cases had both a normal GS, TZ and AS slide, and three cases had only TZ and AS slides.

IV.3: Immunohistochemistry

Immunohistochemistry staining was used as follows: anti-cleaved caspase-3 to identify caspase expression in myenteric GCs, PGP9.5 to identify GCs, GFAP to identify enteric glia reaction, CD68 to identify macrophages, HLA-DR to identify microglial and/or monocyte activation, and APP to identify axonal damage in myenteric GCs. **Table 1** lists the dilution of each primary antibody used.

Anti-cleaved Caspase-3

Immunohistochemical studies for activated caspase-3 were performed using the avidin-biotin-horseradish peroxidase complex (ABC). Unstained slides were cut for both GS and TZ for each case when present. Sections were heated and subjected to deparaffinization and hydration using xylene and gradients of ethanol. Tissue sections underwent antigen unmasking using a 10mM sodium citrate solution at pH 6.0 followed by incubation in 1% H_2O_2 for 10 minutes. Tissue sections were washed in dH_2O and flooded with goat serum blocking buffer (20x PBS, 100% goat serum, 10% Triton X-100, 10% Na azide, bovine serum albumin, dH_2O) for two hours at room temperature (RT) to block non-specific binding. Anti-cleaved caspase-3 solution (prepared in goat serum blocking buffer; 1:100 dilution) was applied to tissue sections and incubated overnight in

a cold room. Tissue sections were incubated in the secondary antibody solution, biotinylated goat anti-rabbit, (1:200 dilution) at RT for 2 hours, and incubated in ABC solution for 30 minutes at RT. Following each incubation period, tissue sections were washed with 1x phosphate buffered saline (PBS) with triton. Diaminobenzidine (DAB) substrate was added for 5 minutes for development of peroxidase then tissue sections were submerged in dH_2O for 5 minutes to stop the reaction. Sections were dehydrated using gradients of ethanol and xylene, and the slides were mounted with Permount. Negative controls were prepared from the TZ segments in an identical procedure with the omission of the primary antibody, anti-cleaved caspase-3.

PGP9.5, CD68, GFAP, HLA-DR and APP

The following was performed by the immunohistochemistry staff at the Winnipeg Health Sciences Centre Pathology Department. Tissue sections were deparaffinized and hydrated using xylene and gradients of ethanol. Endogenous peroxidase was reduced by incubation in 3% H_2O_2 in PBS/Tween buffer for 10 minutes. Tissue sections underwent antigen unmasking using a Medicorp Decloaking Chamber and appropriate pre-treatment solution (**Table 1**). Tissue sections were loaded into the DAKO Autostainer Plus Universal Staining System and flooded with primary antibody (see **Table 1** for dilution of each) for 30 minutes at RT. Detection complex, DAKO Envision Plus, containing the secondary antibody was added to the tissue sections for 30 minutes at RT then washed with PBS buffer and distilled water. DAB substrate was applied to the tissue sections for 5 minutes at RT to allow reaction with peroxidase then washed in distilled water to stop the reaction. Slides were placed in 0.5% $CuSO_4$ for 2 minutes to increase contrast of

staining and washed with distilled water. Slides were counterstained with haematoxylin, dehydrated and mounted with Permount.

Table 1: Characteristics of primary antibodies. BE, Bull's Eye retrieval buffer.

ANTIBODY	COMPANY	CLONE	PRETREATMENT	DILUTION
Anti-cleaved caspase-3	Cell Signaling Technology	Polyclonal	Sodium citrate (10mM)	1:100
PGP9.5	DAKO	Polyclonal	BE (1:20)	1:5000
CD68	DAKO	KP1	BE (1:20)	1:4000
GFAP	DAKO	Polyclonal	NONE	1:10000
HLA-DR	DAKO	CR3/43	BE (1:20)	1:2000
APP	Millipore	22C11	BE (1:20)	1:12000

IV.4: Methods of Analysis

PGP9.5

PGP9.5 immunohistochemistry of developing GCs was evaluated for staining intensity, quantity, distribution, overall shape and contour of the ganglion clusters. Analysis was descriptive only and no quantitative methods of analysis were performed.

Anti-cleaved caspase-3

Apoptosis of GCs of the myenteric plexus during fetal development after GA 15 weeks was evaluated by staining of large intestine segments with anti-cleaved caspase-3. Additionally, apoptosis of GCs of the myenteric plexus in HD was evaluated in the TZ and GS, where present, by staining with anti-cleaved caspase-3. The AS in HD was not evaluated for caspase-3 expression. Activated caspase-3 expression was scored in a

binary fashion with positive expression given a score of 1 and no expression a score of 0. The criterion for positive expression was strong nuclear and cytoplasmic expression, comparable to that in apoptotic lymphocytes within the mucosal associated lymphoid tissue. The apoptotic lymphocytes served as an internal positive control and were present in each batch of immunostaining.

GFAP

GFAP expression was evaluated by a single observer in the AS, TZ and GS, where present. Density of GFAP positive cells within the myenteric plexus was scored on a scale of 0 to 1. No expression was scored as 0 and 1 was any observable expression.

CD68

CD68 expression was evaluated by a single observer in the AS, TZ and GS, where present. The density of cells with CD68 expression was scored on a scale of 0 to 5. No expression was scored as 0 and 5 corresponded to a density of CD68 positive cells equivalent to that seen in a lymph node serving as a positive control.

HLA-DR

HLA-DR expression was evaluated by a single observer in the AS, TZ and GS, where present and was scored in a similar fashion to that of CD68. Density of HLA-DR positive cells was scored on a 0 to 5 scale. No expression was scored as 0 and 5 corresponded to a density of HLA-DR positive cells equivalent to that seen within a lymph node serving as a positive control.

APP

APP expression was evaluated by a single observer in the TZ and GS where present. Intensity and amount of axonal staining was graded on a scale of 0 to 4 with 0 being no expression and 4 corresponding to maximum intensity.

Scoring Scales

Both activated caspase-3 and GFAP expression were scored in a binary fashion. Activated caspase-3 expression was scored in a binary fashion because the interest was in establishing whether there is any true expression and whether there was any apoptosis present. GFAP expression was also scored in a binary fashion because a preliminary review of the cases showed only one case with rare focal positive cells and all other cases were negative. Thus, a broader scale for GFAP would have been impractical.

For CD68 and HLA-DR scoring was based on subjective impression of both density of positive cells and intensity of staining within a given region. In order to integrate all information, a five tiered scale was optimal wherein 1 represents minimal staining of few cells and 5 is dark staining of many cells.

For APP, preliminary assessment showed minimal to no variability in density of positive axons from case to case. Since the only changing variable was intensity, a simplified four tiered system was used.

Eosinophils

Presence of eosinophils was evaluated by a single observer using H-E slides at the TZ. Qualitative descriptive analysis was performed to evaluate the presence or absence of eosinophils within the myenteric plexus. Eosinophil presence was graded in a binary

fashion where positivity for eosinophils represented the subjective impression of increased eosinophilic infiltrates within the myenteric plexus.

IV.5: Statistical Analysis

Caspase Expression and GFAP Statistical Analysis

Caspase expression was recorded in a binary fashion and therefore Fisher's Exact Test was used to assess for significant difference between the frequencies of positive expression at the TZ versus the proximal GS. For GFAP it was noted that no case received a score greater than 1. Therefore, for the sake of statistical analysis, it was possible to collapse the values into categorical data and compare frequency of cases with any GFAP expression versus those with no GFAP expression at the TZ, GS and AS using the Fisher Exact Test.

CD68 Statistical Analysis

Scores for CD68 positivity were compared for the GS, TZ and AS using Rank Sum statistics with both the Wilcoxon Rank-Sum Test and the Kruskal-Wallis Test. A one-tailed P test was used to compare the aganglionic segment to the ganglionic segment and the transition zone to the ganglionic segment. Due to a small sample size, paired Rank-Sum Analysis could not be employed and general Rank-Sum statistics were used.

HLA-DR Statistical Analysis

Scores for HLA-DR positivity were compared for the GS, TZ and AS using Rank Sum Analysis for non-parametric statistics with the Wilcoxon Rank-Sum Test and the Kruskal-Wallis Test. A one-tailed P test was used to compare AS to the GS and the TZ

to the GS. Paired Rank-Sum Analysis could not be employed due to a small sample size and general Rank-Sum statistics were used.

APP Statistical Analysis

Scores for APP expression were compared for the GS and TZ using Rank-Sum Statistics for non-parametric variables with the Wilcoxon Rank-Sum Test and the Kruskal-Wallis Test. Paired Rank-Sum Analysis could not be employed due to a small sample size and general Rank-Sum statistics were used.

Chapter V: Results

V.1: Patient Characteristics

Development

The population for the development portion of this study consisted of 4 cases, three of which were from autopsy cases and one from a TA case. Each case represented a different developmental stage including GA 15, 20 – 21, 34 and 41 weeks. **Table 2** lists the characteristics of each patient.

Hirschsprung's Disease

The population for the HD portion of this study consisted of 6 patients. All 6 patients included a TZ and AS; however, only 3 patients had a proximal GS. Five of the patients were male and one was female with ages ranging from 3 months to 4 years. **Table 3** lists the characteristics of each patient.

Table 2: Developmental study patient characteristics. TA, therapeutic abortion.

CASE	GESTATIONAL AGE	CASE TYPE
A	15	TA
B	20-21	Autopsy
C	34	Autopsy
D	41	Autopsy

Table 3: Hirschsprung's Disease study patient characteristics. AS, aganglionic segment. TZ, transition zone. GS, ganglionic segment.

CASE	AGE	SEX	SEGMENTS
A	3 Months	Male	AS, TZ, GS
B	10.5 Months	Male	AS, TZ
C	4 Years	Female	AS, TZ, GS
D	8 Months	Male	AS, TZ, GS
E	4 Months	Male	AS, TZ
F	5 Months	Male	AS, TZ

V.2: PGP9.5 Expression in the Developing Large Intestine

At GA 15 weeks abundant GC clusters are observed in the myenteric plexus (Figure 1*A*). These clusters are large, frequent and in areas show coalescence such that the clusters have a linear band-like appearance.

At GA 20 – 21 weeks the myenteric GC clusters are more widely spaced but are still relatively dense (Figure 1*B*) and in focal areas show some coalescence. Notably, the space between the clusters and band-like areas is widened.

At GA 34 weeks there is an increase in space between the myenteric GC clusters and minimal coalescence (Figure 1*C*). Discrete round clusters are seen at relatively regularly spaced intervals and the space between the clusters occupies approximately the same area as the clusters.

At GA 41 weeks there is a similar appearance to 34 weeks with an apparent increase in space between clusters (Figure 1*D*).

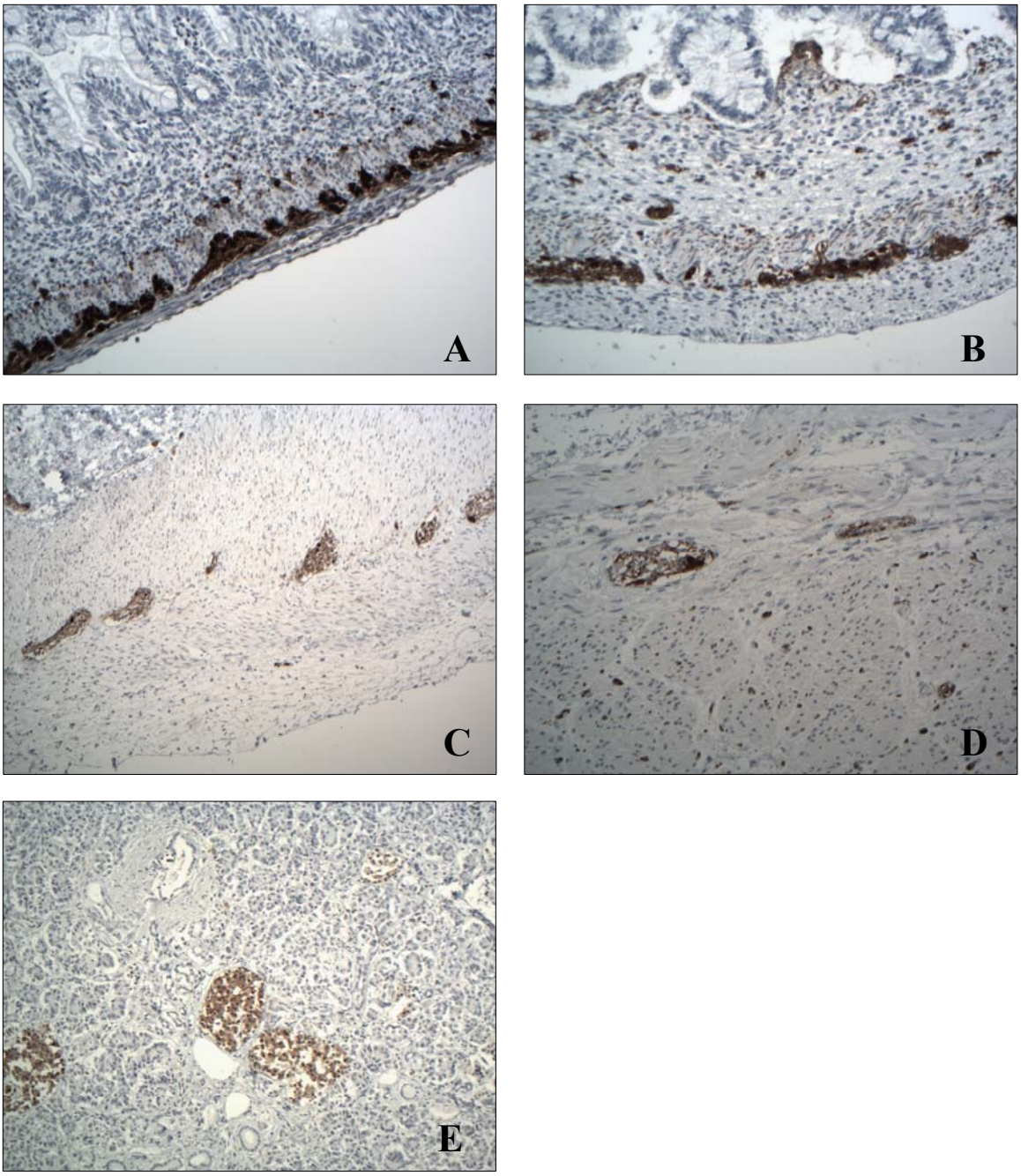

Figure 1: PGP9.5 expression in the myenteric plexus of the developing human large intestine. *A:* GA 15 weeks showing linear band-like appearance of myenteric GC clusters. *B:* GA 20-21 weeks showing myenteric GC clusters beginning to space apart. *C:* GA 34 weeks showing discrete myenteric GC clusters spaced apart. *D:* GA 41 weeks showing increased space between myenteric GC clusters. *E*: Positive control (pancreatic islets). Magnification 200x for *A*-*D*. Magnification 100x for *E*.

V.3: Activated Caspase-3 Expression in the Developing Large Intestine

The large intestine and appendix of all four cases examined showed no expression of caspase-3 in the GCs of the myenteric plexus (**Table 4**). All four cases demonstrated background non-specific caspase-3 staining in the GCs (Figure 2*A-D*) but the intensities were less than positive control lymphocytes undergoing apoptosis within the reactive germinal centres (Figure 2*E*) and were graded as 0.

Table 4: Activated caspase-3 expression in the myenteric plexus of the developing human large intestine.

CASE	CASPASE EXPRESSION
A	0
B	0
C	0
D	0

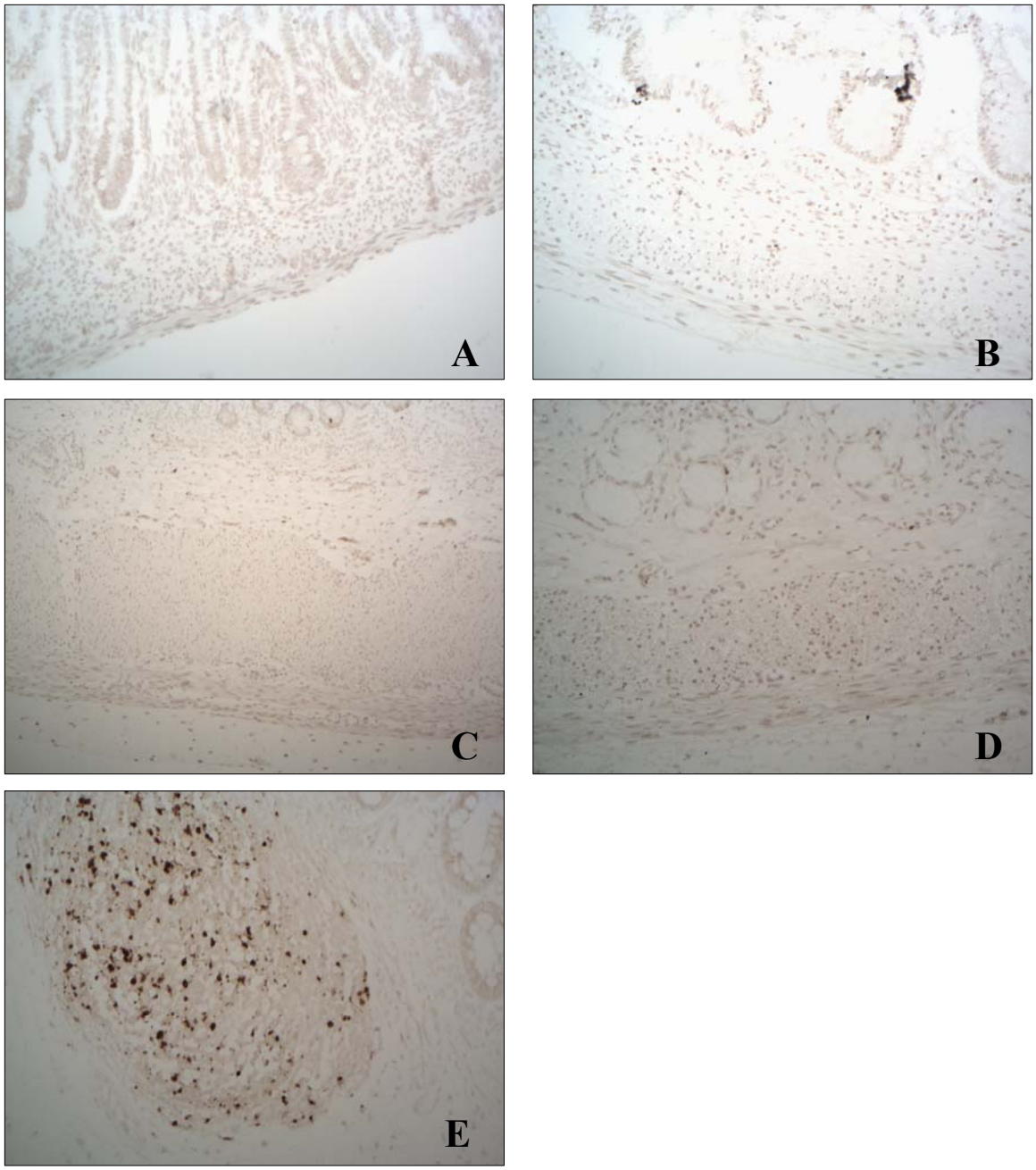

Figure 2: Activated caspase-3 expression in the myenteric plexus of the developing human large intestine. *A:* GA 15 weeks. *B:* GA 20-21 weeks. *C:* GA 34 weeks. *D:* GA 41 weeks. *E:* Reactive germinal centre with apoptotic lymphocytes acting as internal positive control. Magnification 200x.

V.4: Activated Caspase-3 Expression in Hirschsprung's Disease

The TZ and proximal GS of each case, where present, showed no expression of caspase-3 in the GCs of the myenteric plexus (**Table 5**; Figure 3*A-D*). For Case B, the TZ segment slide showed over-staining including the colonic mucosa thus displaying positive background expression (Figure 3*E*). Case B could not be repeated due to time restraints. All six cases demonstrated non-specific background activated caspase-3 expression in the myenteric GCs but the intensities were less than that of lymphocytes undergoing apoptosis within the reactive germinal centres (Figure 3*F*) and were graded as 0. Overall, there was no significant difference in activated caspase-3 expression in the TZ versus the proximal GS using the Fisher Exact Test ($p = 1.0$).

Table 5: Activated caspase-3 expression in Hirschsprung's Disease patients at the transition zone and proximal ganglionic segment.

CASE	TRANSITION ZONE	GANGLIONIC SEGMENT
A	0	0
B	Not interpretable	N/A
C	0	0
D	0	0
E	0	N/A
F	0	N/A

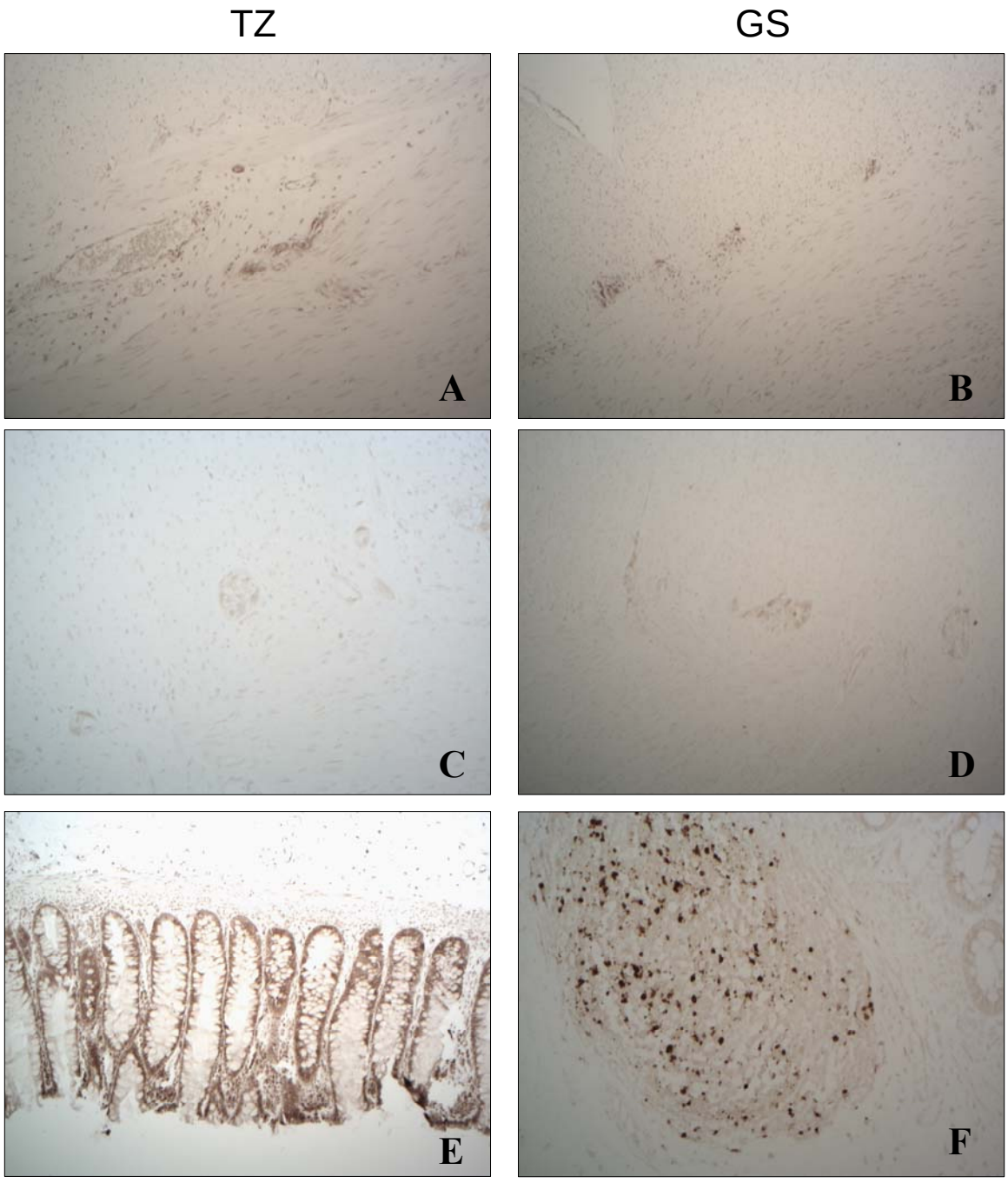

Figure 3: Activated caspase-3 expression in Hirschsprung's Disease patients at the transition zone and proximal ganglionic segment. *A:* Case C at TZ. *B:* Case C at GS. *C:* Case D at TZ. *D:* Case D at GS. *E:* Case B at TZ showing over-staining of colonic mucosa. *F:* Reactive germinal centre with apoptotic lymphocytes acting as internal positive control. Magnification 100x for *A-E*. Magnification 200x for *F*. TZ, transition zone. GS, ganglionic segment.

V.5: GFAP Expression in Hirschsprung's Disease

All of the six cases examined showed no expression of GFAP within the enteric glial cells in the AS and TZ (**Table 6**; Figure 4*A*-*E*). One of three cases with GS present was scored as 1 for GFAP expression on the five point grading scale (Figure 4*F*) with no expression in the other two cases. This case corresponded to a delayed endorectal pull-through in the 4 year old female. Using the Fisher Exact Test, there was no significant difference in GFAP expression for TZ versus GS ($p = 0.333$) or GS versus AS ($p = 0.333$).

Table 6: GFAP expression in Hirschsprung's Disease at the aganglionic segment, transition zone and ganglionic segment.

CASE	AGANGLIONIC SEGMENT	TRANSITION ZONE	GANGLIONIC SEGMENT
A	0	0	0
B	0	0	N/A
C	0	0	1
D	0	0	0
E	0	0	N/A
F	0	0	N/A

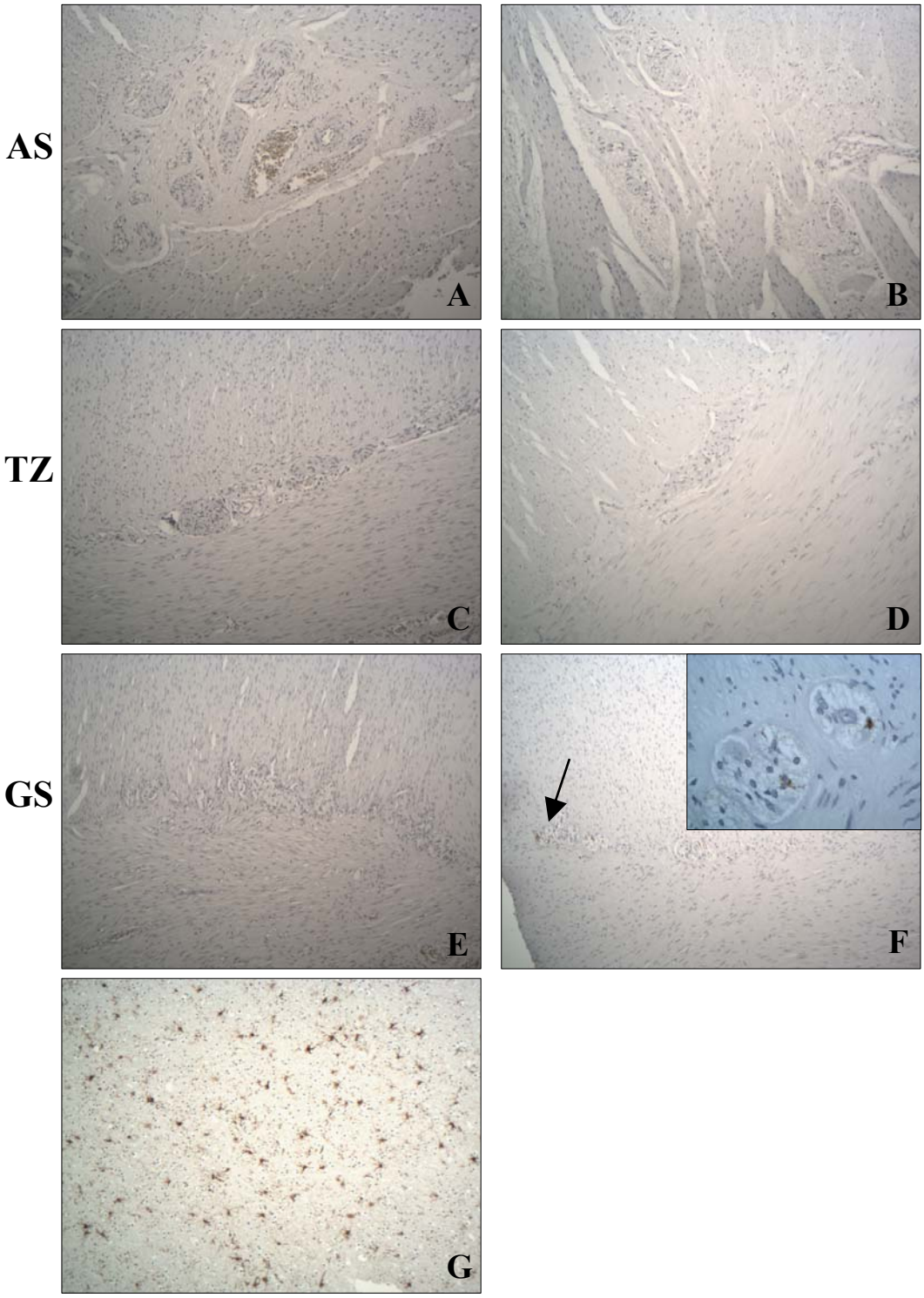

Figure 4: GFAP expression in Hirschsprung's Disease at the aganglionic segment, transition zone and ganglionic segment. *A:* Case A at AS. *B:* Case C at AS. *C:* Case A at TZ. *D:* Case C at TZ. *E:* Case A at GS. *F:* Case C at GS, arrow indicating where insert image (magnification 600x) taken from. *G*: Positive control. Magnification 100x for *A*-*G*. AS, aganglionic segment. TZ, transition zone. GS, ganglionic segment.

V.6: CD68 Expression in Hirschsprung's Disease

All six cases had CD68 expression within the AS and TZ corresponding to 1 or 2 on the five point grading scale (**Table 7**; Figure 5*A-D*). Of the three cases where GS was present, two cases showed CD68 expression corresponding to a value of 1 (Figure 5E) and the third case had no CD68 expression (Figure 5*F*). There were no cases with CD68 expression in the AS, TZ or GS scored as 3, 4 or 5.

Statistical analysis was carried out to assess significance of difference in CD68 expression between GS and AS, and between GS and TZ. Since three of six cases did not have a sufficient GS, paired analysis could not be performed and a non-paired Rank-Sum Analysis was performed. In comparing CD68 expression at the GS versus AS by Rank-Sum Analysis, the sum of scores for the AS was 35 and 10 for GS, with mean scores of 5.83 at the AS and 3.33 at the GS. The Wilcoxon Two-Sample Test one-sided P-value for difference between the groups was 0.1012 and the Kruskal-Wallis Test P-value was 0.1228. In comparing CD68 expression at the GS versus TZ by Rank-Sum Analysis, the sum of scores for the GS was 11 and 34 for the TZ, with mean scores of 3.66667 for the GS and 5.66667 for the TZ. The Wilcoxon Two-Sample Test one-sided P-value for difference between the two groups was 0.1255 and the Kruskal-Wallis Test P-value was 0.1573. Thus, no significant difference was found between the AS and GS and the TZ and GS.

Table 7: CD68 expression in Hirschsprung's Disease at the aganglionic segment, transition zone and ganglionic segment.

CASE	AGANGLIONIC SEGMENT	TRANSITION ZONE	GANGLIONIC SEGMENT
A	2	2	1
B	1	1	N/A
C	2	1	1
D	1	1	0
E	1	1	N/A
F	1	1	N/A

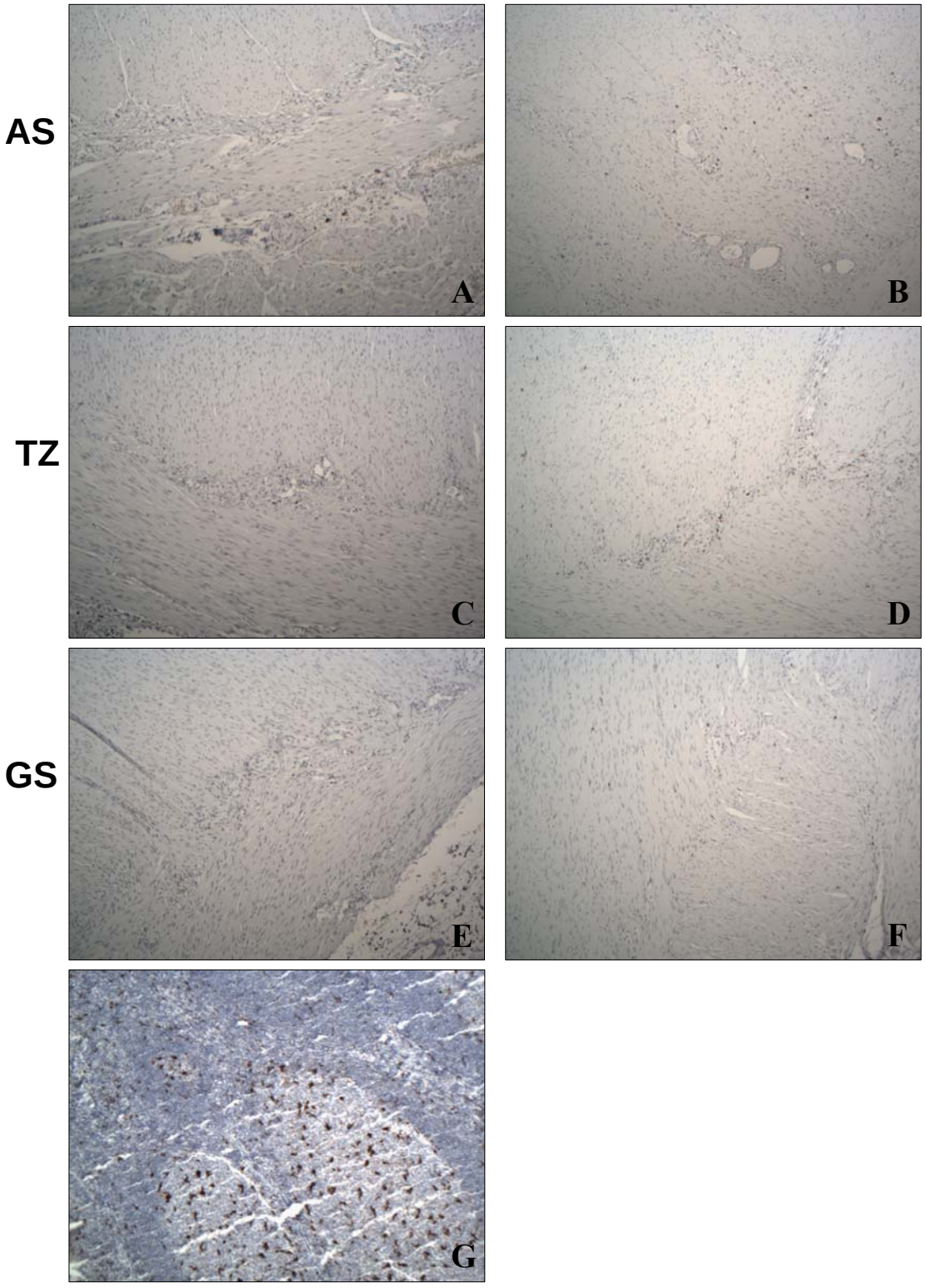

Figure 5: CD68 expression in Hirschsprung's Disease at the aganglionic segment, transition zone and ganglionic segment. *A:* Case A at AS. *B:* Case D at AS. *C:* Case A at TZ. *D:* Case D at TZ. *E:* Case A at GS. *F:* Case D at GS. *G*: Positive control (lymph node). Magnification 100x.

V.7: HLA-DR Expression in Hirschsprung's Disease

Five of the six cases evaluated had HLA-DR expression in the AS corresponding to a value of 1 or 2 on the five point grading scale (Figure 6*A*) with the sixth case having no expression (Figure 6*B*). The TZ in all six cases had HLA-DR expression corresponding to a value of 1 or 2 as did the three cases with GS (**Table 8**; Figure 6*C-E*).

Statistical analysis was carried out to assess for significant difference of density of positive cells in GS versus AS and GS versus TZ. Rank-Sum unpaired analysis was used. The sum of scores of the AS was 30.50 and 14.50 for GS with mean scores of 5.083333 for the AS and 4.83333 for the GS. The Wilcoxon Two-Sample one-sided P-value was 1.00 and the Kruskal-Wallis P-value was 0.8875. In comparing the GS and TZ for HLA-DR expression the sum of scores for the GS was 12.0 and 33.0 for the TZ with mean scores of 4.0 for the GS and 5.50 for the TZ. The Wilcoxon Two-Sample Test one-sided P-value was 0.2387 and the Kruskal-Wallis Test P-value was 0.3711. Thus, no significant difference was found between the AS and GS and the TZ and GS.

Table 8: HLA-DR expression in Hirschsprung's Disease at the aganglionic segment, transition zone and ganglionic segment.

CASE	AGANGLIONIC SEGMENT	TRANSITION ZONE	GANGLIONIC SEGMENT
A	1	2	1
B	2	2	N/A
C	2	2	1
D	2	2	2
E	1	1	N/A
F	0	1	N/A

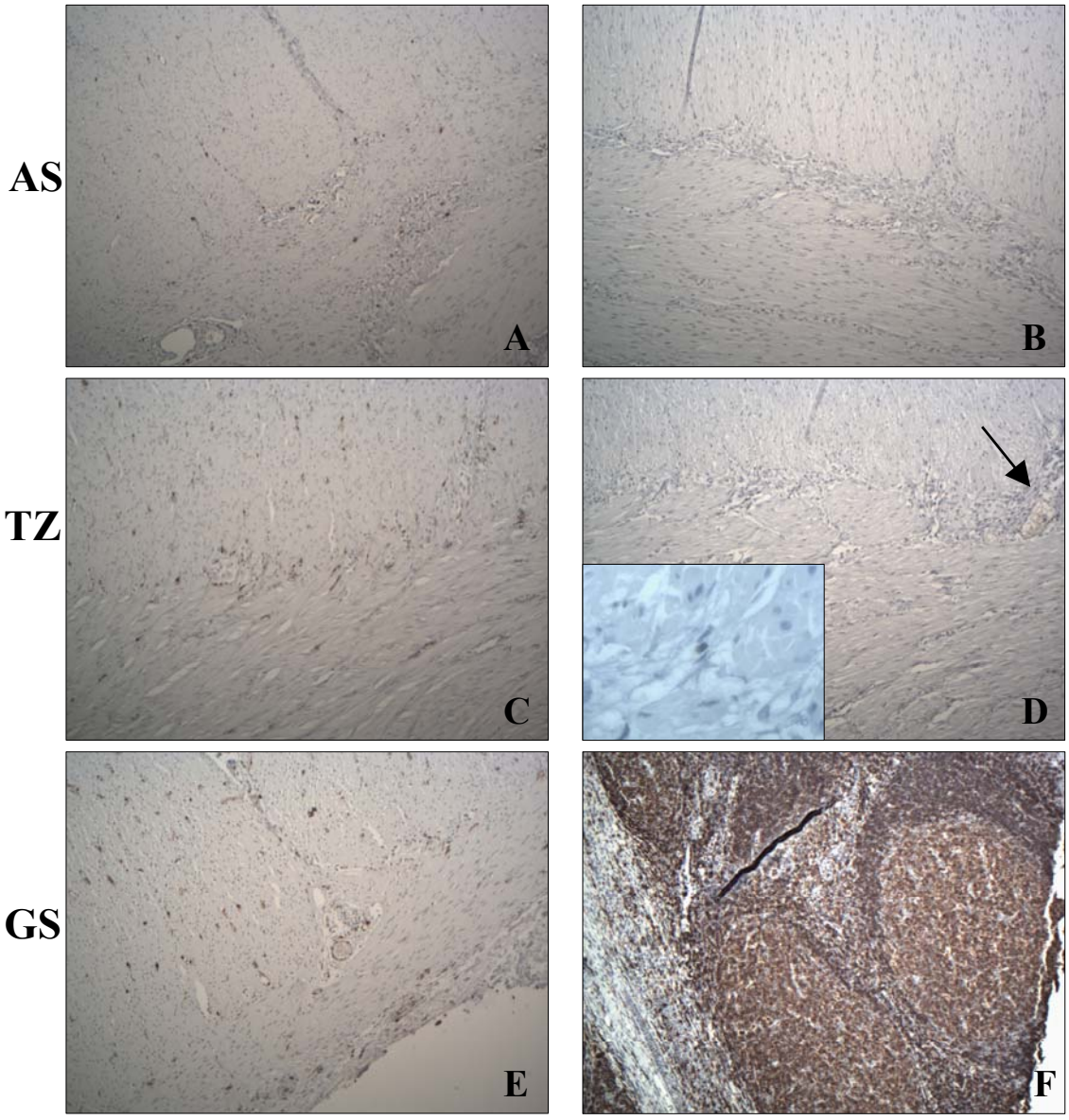

Figure 6: HLA-DR expression in Hirschsprung's Disease at the aganglionic segment, transition zone and ganglionic segment. *A:* Case D at AS. *B:* Case F at AS. *C:* Case D at TZ. *D:* Case F at TZ, arrow indicating where insert image (magnification 600x) taken from. *E:* Case D at GS. *F*: Positive control (lymph node). Magnification 100x. AS, aganglionic segment. TZ, transition zone. GS, ganglionic segment.

V.8: APP Expression in Hirschsprung's Disease

All six cases showed APP expression within the axons of the GCs of the myenteric plexus at the TZ and GS, where present (Figure 7). The intensity of expression corresponded to a value of 1 or 2 on the four point grading scale (**Table 9**). There are no cells to assess in the AS.

Statistical analysis was carried out to assess significant difference between TZ and GS. By Rank-Sum Analysis the sum of scores for the GS was 15 and 30 for the TZ and the mean scores for the GS and TZ were both 5. The Wilcoxon Two-Sample Test one-sided P-value was 0.5 and the Kruskal-Wallis Test P-value was 1.0. Thus, no significant difference in the intensity of expression of APP between the TZ and GS was found.

Table 9: APP expression in Hirschsprung's Disease at the transition zone and ganglionic segment.

CASE	TRANSITION ZONE	GANGLIONIC SEGMENT
A	2	1
B	2	N/A
C	2	2
D	2	2
E	1	N/A
F	1	N/A

V.9: Eosinophils in Hirschsprung's Disease

In one of six cases (Case D), a qualitative impression of increased eosinophils at the myenteric plexus at the TZ was present (Figure 8).

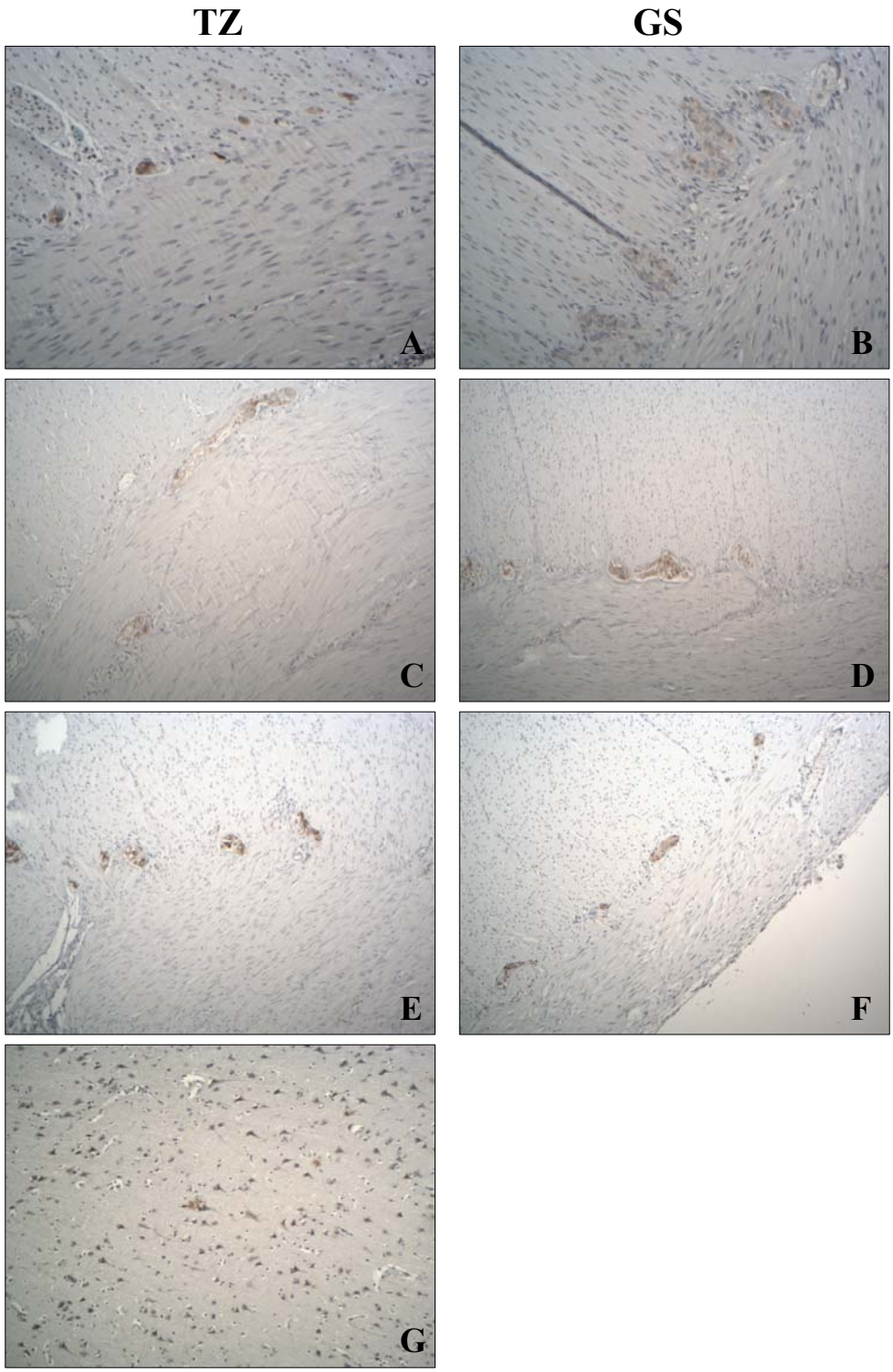

Figure 7: APP expression in Hirschsprung's Disease at the transition zone and ganglionic segment. *A:* Case A at TZ. *B:* Case A at GS. *C:* Case C at TZ. *D:* Case C at GS. *E:* Case D at TZ. *F:* Case D at GS. *G*: Positive control (CNS plaque). Magnification 100x. TZ, transition zone. GS, ganglionic segment.

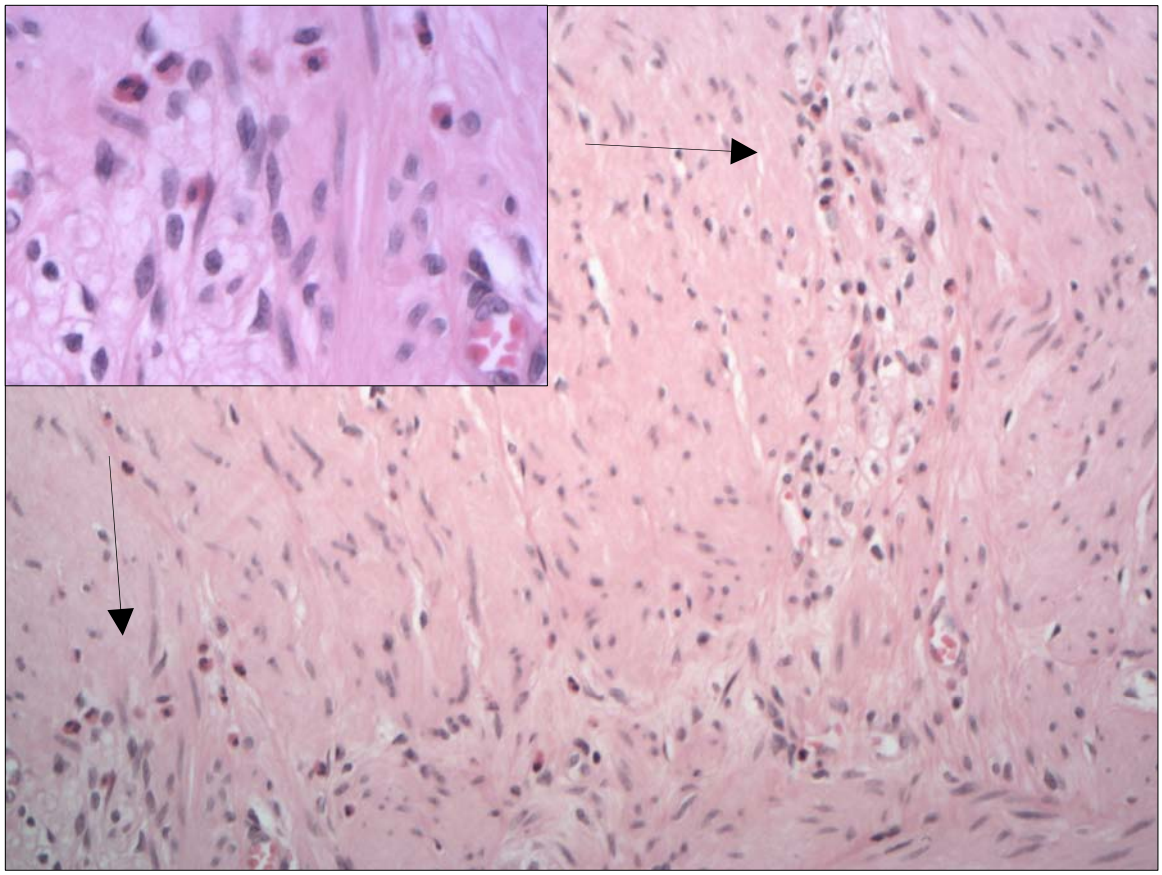

Figure 8: Eosinophil infiltration in Hirschsprung's Disease at the transition zone in an 8 month old male. Arrows indicate infiltration of eosinophils. Magnification 200x. Insert magnification 400x. Haematoxylin and eosin stain.

Chapter VI: Discussion

VI.1: Ganglion Cells in the Developing Large Intestine

Examination of the morphological spectrum of myenteric GCs at various GAs was performed using routine H-E slides in addition to immunohistochemistry for PGP9.5. These stains showed a transition from a linear distribution to closely spaced clusters to widely spaced clusters of myenteric GCs in the developing human large intestine. There are two possibilities for this observation. The change in distribution could be secondary to the disproportionate growth of the large intestine relative to the embryo [5] without a concomitant increase in GC number or due to a decrease in myenteric GC number resulting from GC death. The distribution of myenteric GCs within the human developing GIT changes up until 41 weeks GA; however, the exact mechanism is not certain.

Caspase Expression

To assess whether a decrease in GC number could be responsible for the change in distribution of myenteric GCs, we used immunohistochemistry for activated caspase-3 to determine if caspase dependent apoptosis of myenteric GCs was occurring. Staining of the large intestine and appendix at 15, 20 – 21, 34 and 41 weeks GA showed no evidence of caspase expression and therefore no caspase dependent apoptosis of the myenteric GCs. This result is in agreement with studies by Gianino *et al*. (2003) and Kruger *et al*. (2003) which found no caspase expression in the developing large intestines of wild-type mouse models [26, 41]. It is possible that GC death occurred before 15 weeks GA; however,

due to the lack of cases before 15 weeks GA and difficulty in procuring intestinal tissue at those ages, this was not investigated in our study.

Limitations

Despite the agreement of our results with previous studies, there are some drawbacks of our study that must be considered. In order to sample a myenteric GC when it is undergoing apoptosis, additional tissue sections would be required. The current study only looked at one section when assessing caspase dependent apoptosis which may have restricted our ability to detect them. In addition to the sectioning limitation, the population only consisted of four cases at four different GAs. If additional cases at each GA and more GAs were examined, there would be more evidence to substantiate our conclusions or refute them. Additionally, there are non-apoptotic pathways of cell death which could also be responsible for the possible decrease in myenteric GC number which would not have been identified in this study.

Summary

Overall, within the limitations of our study, we have shown an absence of evidence of caspase dependent apoptosis in the developing human myenteric plexus. This is in agreement with previous studies which also showed that caspase dependent apoptosis does not play a role in normal myenteric GC post-migrational ENS development [26, 41]. Non-apoptotic pathways of cell death now need to be examined to determine if they play a role in the development of the human myenteric plexus.

VI.2: Ganglion Cell Death and Injury in Hirschsprung's Disease

Motivation for this study came from anecdotal reports of patients experiencing constipation after successful endorectal pull-through procedures where GCs were documented to be present within the anastomosed segment. In these anecdotal cases subsequent endorectal biopsies performed several years after the initial surgery showed absence of GCs. Factors that could be responsible for this are insufficient sampling, fibrosis and complications of the regional surgical anatomy. This led us to examine whether apoptosis of the myenteric GCs has a role at the TZ and proximal GS in HD. Furthermore, in Dr. David Grynspan's practice, he has previously observed increased eosinophils at the TZ in HD leading us to consider the possibility that there may be a mechanism of GC injury at the TZ and proximal GS in patients with HD. On evaluation, one case in our population was confirmed to have increased eosinophils at the TZ; however, the exact mechanism leading to this increase is unknown.

Caspase Expression

Our assessment of activated caspase-3 expression of myenteric GCs at the TZ and proximal GS of patients with HD revealed no evidence of caspase dependent apoptosis in postnatal children. This result suggests that at birth the pathology is completely established such that at the time of birth and following subsequent corrective surgery there is no role for ongoing caspase dependent apoptosis in advancing the TZ.

Studies on mouse models of HD by Bordeaux *et al*. (2000), Kapur (1999) and Southard-Smith *et al*. (1998) showed evidence of caspase dependent apoptosis of ENCDCs having a role in the pathogenesis of HD [8, 36, 68], which contradicts our study showing no role of caspase dependent apoptosis. Our study is in agreement with Kruger

et al. (2003) who showed no presence of caspase dependent apoptosis having a role in the pathogenesis of HD [41]. However, the current literature involves only mouse models with various genes that are involved in the pathogenesis of HD and, to the best of our knowledge, no similar studies involving human HD patients. Further studies involving human HD affected intestinal tissue assessing for a role of mutations, such as RET, in promoting post-migrational apoptosis would be needed.

In our experiments, there was no evidence of caspase dependent apoptosis, therefore a possibility that should be investigated further is whether there is non-apoptotic cell death occurring. These include necrosis and autophagy which have been previously suggested by Uesaka *et al*. (2007) and Uesaka *et al*. (2008) [75, 76]. They found non-apoptotic ENCDC death to have a possible role in the pathogenesis of HD in mouse models [75, 76].

A limitation of the methodology of these experiments, is similar to that of the developmental experiments, is that it is difficult to capture a GC undergoing apoptosis in a tissue section. This is made even more difficult given that myenteric GCs at the TZ are irregularly and widely spaced with an uneven circumferential distribution [81]. Therefore, the limited number of tissue sections examined for GC apoptosis may not have been adequate to assess caspase dependent apoptosis of myenteric GCs. In order to overcome this limitation, in further studies, additional tissue sections should be examined for caspase expression of myenteric GCs.

CD68 and HLA-DR Expression

A trend towards increased expression of CD68 within the TZ and AS versus the proximal GS in patients with HD was shown; however, this trend did not reach statistical

significance. The trend observed may truly not be significant, or alternatively, if a larger population was utilized, significance may have been found. If this trend is upheld in further studies, then we will have demonstrated an increase in macrophages in the TZ. However, the significance of such a finding would be uncertain. It could be due to GC injury or, alternatively, be secondary to non-specific inflammatory processes that occur in the AS and the TZ in patients with HD due to constipation and the accumulation of stool.

No evidence of increased expression of HLA-DR was found in the AS or TZ versus the GS in this study suggesting that there is no HLA-DR related microglial activation in the AS or TZ of patients with HD. The absence of up-regulation of HLA-DR does not exclude the possibility of GC death that does not involve microglial activation or the possibility of glial activation without up-regulation of HLA-DR.

GFAP Expression

No evidence of GFAP expression was found in the AS or TZ and only one case with limited expression was found in the proximal GS supporting the lack of activation of enteric glial cells in the AS or TZ. Additionally, this provides indirect evidence against myenteric GC injury. The one case that showed expression in the proximal GS was in a 4-year-old patient with delayed resection. This expression may be secondary to the distension that occurs in the GS and TZ proximal to the obstruction at the AS. There is evidence from studies on inflammatory diseases that sustained colonic dilatation or sustained mechanical insult to the intestine can result in increased GFAP expression [58, 78]. This finding provides credence to the argument of performing early endorectal pull-through surgery since there is a possibility that a delay in surgical treatment could cause glial scarring in otherwise healthy proximal intestinal tissue. A limitation of using GFAP

is that pathways of cell death that do not involve glial activation or pathways that do involve glial activation but without up-regulation of GFAP would not be detected.

APP Expression

There was no evidence of increased APP expression within the TZ versus the proximal GS in patients with HD. APP may be useful for identifying some non-apoptotic pathways of axonal damage; however, the test is probably not comprehensive and would not detect all mechanisms of axonal damage. Thus this experiment specifically shows the absence of the type of axonal damage associated with APP at the TZ in patients with HD but does not exclude other possible mechanisms of GC damage.

Limitations

In order to increase the significance of the results found, several aspects of the methodology of the experiments would need to be improved. Additional tissue sections per case would add to the significance of the results for each case. Using intestinal tissue from age matched controls as opposed to using the proximal GS as a control would be beneficial because we cannot exclude the possibility of subtle abnormalities in the seemingly normal GS of HD patients. Sample size should be increased to assess a larger population of patients with HD to add to the strength of this study. This would also increase the range of ages of patients at time of the operation and increase the number of female patients in the study. Additionally, this study was based on one observer's examination of the various immunohistochemical stains. If the assessment was performed by multiple blind observers the significance of the results would be strengthened.

Summary

Overall, our results show absence of evidence of caspase dependent apoptosis at the TZ of HD patients and essentially absence of evidence of markers of enteric gliosis at the TZ. These results provide evidence that ongoing neuronal injury is not a factor in HD patients after their birth. Further studies looking at a larger population of HD patients and other pathways of neuronal cell death would be beneficial in further characterizing the pathogenesis of HD.

Chapter VII: Conclusion

VII.1: Ganglion Cells in the Developing Large Intestine

This study found a morphological spectrum of change in myenteric ganglion cell distribution from 15 to 41 weeks gestational age with transition from a linear distribution of ganglion cells to closely spaced clusters to widely spaced clusters. Additionally, there was no evidence of caspase dependent apoptosis occurring in myenteric ganglion cells at 15, 20 – 21, 34 and 41 weeks gestational age.

VII.2: Ganglion Cell Death and Injury in Hirschsprung's Disease

This study found no evidence of caspase dependent apoptosis at the transition zone or proximal ganglionic segment in cases of Hirschsprung's Disease. Furthermore, markers of myenteric glial cell activation did not show significant increased expression at the transition zone or aganglionic segment thus demonstrating an absence of evidence of neuronal injury in Hirschsprung's Disease. The presence of eosinophils within the transition zone does indicate a possible ongoing inflammatory process at the transition zone but is not an indicator of ongoing ganglion cell injury.

VII.3: Future Directions

A future study that would be beneficial to perform is to increase the sample size for both the development and Hirschsprung's Disease experiments. Additionally, in order to properly assess for caspase expression more sections, including circumferential sampling of the respective zones, as well as multiple levels per section would be needed.

More rigorous quantitative techniques for assessing levels of expression would be helpful, such as automated analysis or use of a manual grid technique on light microscopy. More advanced techniques, such as dual antibody staining, could potentially be used to verify co-localization of signals; for example caspase expression in neural cells stained with PGP9.5. Expanded antibody panels to assess for other mechanisms of cell death, including autophagy and necrosis, should be considered. Furthermore, examination of whether there are measurable or detectable growth factors or cytokines at the transition zone may help determine what is responsible for the eosinophil or macrophage recruitment.

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