IDENTIFICATION AND CHARACTERISATION OF MUCIN ABNORMALITIES IN THE GANGLIONIC BOWEL IN HIRSCHSPRUNG'S DISEASE

By Ruth Marian Speare

Submitted for MD (Res)

Abstract

Hirschsprung's disease (HD) is a congenital abnormality of unknown origin, characterised by a lack of ganglion cells in the distal colon which results in functional colonic obstruction. The major cause of morbidity and mortality in these children results from an inflammatory condition called enterocolitis.

Mucins are large glycoproteins produced by intestinal cells which are a vital part of the colonic defensive barrier to infection. Previous work has found that this barrier is deficient in children with HD in the aganglionic colon and in the immediately adjacent ganglionic colon and that this is related to the risk of developing enterocolitis.

This study aimed to further investigate the mucin defensive barrier in a greater region of the ganglionic colon in HD, to establish the extent of any mucin deficiencies and whether these were confined to a limited region close to the aganglionic colon. Mucosal biopsies were collected at intervals along the colon at the time of corrective surgery or colostomy closure in the controls. Organ culture with radioactive mucin precursors was performed and the mucin produced was purified and analysed, results quantified by DNA content in the sample. Lectin binding studies were also carried out.

Patients were found to produce lower levels of new mucins most distally, but much higher levels five centimetres proximal when compared to controls. The rest of the colon studied also showed changes in mucin production, with a lack of production of gel-forming mucins and sulphated secreted mucins in Hirschsprung's disease higher up the colon. Lectin binding studies, which indicate the presence of existing mucins as well as those produced during organ culture, demonstrated greater levels of binding in patients compared to controls for wheat germ agglutinin and *Maackia amurensis* agglutinin.

CONTENTS

List of Figures

List of Tables

Acknowledgements

I would like to thank Adil Aslam for the idea behind this research and for helping me to establish, fund and carry out this project. Thank you to Professor Ian Sanderson for all of his help and support at various stages along the way.

Many thanks to Ian McFarlane whose laboratory experience and advice was invaluable at all stages of this project from helping me learn how to make up reagents to retrieving and analyzing the data and who coped with my lack of laboratory experience and mathematical short comings with patience and humour. Thank you to Dr Tony Corfield for his advice and for coming all the way over from Bristol to help set up some of the methodology in our department and the research department in Bristol for providing the gel filtration columns.

Thank you to the other consultants within the Paediatric Surgical Departments of Addenbrooke's Hospital and Norwich and Norfolk Hospital for allowing their patients to be approached and obtaining biopsies at surgery and to the parents of the children who agreed to take part in this research.

I would also like to thank Roger Lee in the medical equipment department at Addenbrooke's for making the metal grids for the organ culture and to the laboratory staff who allowed me space to work without complaint. I would also like to thank Jacqui Frith in the administration department for her help.

Thank you to my family for their proof reading and grammar and spelling suggestions! Finally a big thank you to my husband Damian for his love and support who has lived through this research and subsequent write up with me and put up with a lot in the process, as well as providing an ear and fresh take on various aspects of this work and using his computer talents to help put the final touches together!

Abbreviations

ANS	-	autonomic nervous system
Asn	-	asparagine
BMP	-	bone morphogenetic proteins
BSA	-	bovine serum albumin
Ca^{2+}	-	calcium ion
Cad	-	blood group antigen
cAMP	-	cyclic adenosine monophosphate
CDX	-	caudal type homeobox gene
CK	-	cysteine knot
CIF	-	cellular insoluble fraction
COREC	-	Central Office for Research Ethics Committees
CSF	-	cellular soluble fraction
D	-	aspartic acid
D-	-	disulfide-rich domain
Da	-	daltons
DAB	-	diaminobenzamidine
DNA	-	deoxyribonucleic acid
Dom	-	dominant megacolon
DPM	-	disintegrations per minute
DTT	-	dithiothreitol
EC	-	enterocolitis
ECE	-	endothelin-converting enzyme 1
EDNR	-	endothelin receptor

EDTA	-	ethylenediaminetetraacetic acid
EGF	-	epidermal-growth-factor
ENS	-	enteric nervous system
ER	-	endoplasmic reticulum
ET	-	endothelin
FIM-B.1	-	frog integumentary mucin
Fuc	-	fucose
G	-	glycine
Gal	-	galactose
GalNAc	-	*N*-galactosamine
GDNF	-	glial cell line-derived neurotrophic factor
GFRα1	-	co-receptor for GDNF
GFRα2	-	receptor for NTN
GlcNAc	-	*N*-acetylglucosamine
GRP	-	gastrin-releasing peptide
H	-	histidine
HCL	-	hydrochloric acid
HD	-	Hirschsprung's disease
HMG	-	high-mobility group transcriptional regulators
ICC	-	interstitial cell of Cajal
Ig	-	immunoglobulin
Ihh	-	Indian hedgehog
IL	-	interleukin
Kit	-	tyrosine kinase receptor
Le/Ley	-	Lewis antigen
ls	-	lethal spotting
MAA	-	*Maackia amurensis* agglutinin

MASH	-	mammalian achaete-scute homologue 1
mRNA	-	messenger ribonucleic acid
MUC	-	mucin
Na^+	-	sodium ion
NCAM	-	neural cell adhesion molecule
NE	-	neuroendocrine
Neu5Ac	-	*N*-acetylneuraminic acid (sialic acid)
NK	-	natural killer cell
NO	-	nitric oxide
NT	-	neurotrophin
NTN	-	neurturin
OAT	-	*O*-acetyl transferase
P	-	proline
PAPS	-	3'-phosphoadenosine 5'-phosphosulphate
PBS	-	phosphate buffered saline
PMSF	-	phenylmethylsulphonyl fluoride
PMX2B	-	paired mesoderm homeobox 2b transcription factor
PSM	-	porcine sub-maxillary mucin
PVDF	-	polyvinylidine Fluoride
RET	-	rearranged during transfection (RET) tyrosine kinase receptor
RET	-	c-RET proto-oncogene
Ser	-	serine
Shh	-	sonic hedgehog
S^l	-	piebald lethal
sl	-	spotting lethal
SRY	-	sex-determining region Y
SSF	-	secreted soluble fraction

ST	-	sulphotransferase
TF	-	trefoil factor
Thr	-	threonine
Tris	-	Tris(hydroxymethyl)aminomethane
TNF	-	tumour necrosis factor
TZ	-	transition zone
UEA	-	*Ulex europaeus* agglutinin
VIP	-	vasoactive intestinal peptide
VWF	-	von Willebrand's factor
WGA	-	wheat germ agglutinin
xg	-	centrifugal force

Chapter 1: Mucins

1.0 Mucins – Basic Structure

Mucus is a highly hydrated slippery gel containing many different constituents, including immunoglobulins, sloughed cells and bacteria.[1,2,3] It is secreted by specialized epithelial cells lining the lumen of the gastrointestinal, respiratory and genitourinary tracts. In the colon this gel layer serves as a lubricant in the passage of luminal contents, protects against mechanical trauma and acts as the first line of defence against potential colonic pathogens, foreign compounds and toxins by providing both a physical and a diffusion barrier.[4,5,6,7,8] The thickness of mucus covering the mucosa gradually increases along the colon reaching a maximum of 285 μm in the rectum.[9]

Mucus glycoproteins, otherwise known as mucins, are the main component of mucus.[10] They are very large, high molecular weight glycoproteins between 500,000 and 30,000,000 daltons in size.[11] Mucins can occur in secreted or membrane bound forms, the former being subdivided into gel-forming or non-gel forming.[11,12] They are all identified by the abbreviation MUC.

The basic structure of mucins consists of highly glycosylated repetitive sequences of threonine and/or serine residues.[11] These oligosaccharide chains are linked via *O*-glycosidic bonds, a linkage through an oxygen atom, a feature which characterises mucins and differentiates them from other glycoproteins.[13,12]

Individuals make several structurally different mucins and a given mucin may be found in more than one organ.[14] At least ten different mucin peptides are expressed in the gastrointestinal tract in a tissue-specific fashion, implying functional differences

between different mucins.[15] The main mucins expressed in the colorectum in humans from birth onwards are MUC2 and MUC4.[16] MUC1 and MUC3 are also found in the colon but at lower levels.[17]

To date at least twenty human mucin genes have been identified. [10,18, 19] The majority of these can be divided on the basis of their structural characteristics into three distinct subfamilies; membrane-bound, non-gel forming and gel-forming.

Membrane-bound Mucins

The membrane-bound mucin family is composed of eleven mucins. (Figure 1.1) These mucins have a transmembrane domain, a short cytoplasmic tail, and an extended extracellular domain that forms the glycocalyx of epithelial cells.[11] They are normally anchored to epithelial cells but can also be found in soluble form (proteolytic cleavage of the anchored form), secreted form (alternative splicing variants) and in a form which lacks the characteristic tandem repeat region (alternative splicing variants).[20,21,22]

Because of shedding and direct secretion, cell-surface mucins can also be seen as components of secreted mucins.[23] MUC3 is expressed in low levels in the colon but is more abundant in the small intestine.[14] It is only expressed on mucosal surfaces and the upper parts of the colonic crypts.[24] MUC4 is widely expressed in the colon and small intestine.[14]

Membrane mucins appear to have several functions. They form part of the cell-surface glycocalyx, protect the mucosa from pathogens, they can function as anti-cell adhesion molecules by virtue of their large negative charge and conversely can act as ligands (e.g. for P and E-selectins).[11,25,26] MUC1 may also aid in the maintenance of epithelial

integrity, through its interaction with beta-catenin, which in turn interacts with other molecules important in cell-cell junction formation.[27,28]

Secreted Non-gel Forming Mucin

MUC7, MUC8 and MUC9 belong to this subgroup, although MUC8 has not been fully characterized.[19, 29] These mucins have both a simpler structural organisation and lower molecular weight than the other human mucins.[30,31]

Secreted Gel-forming Mucins

This sub-family of mucins are secreted by goblet cells or other secretory cells and have the ability to form a gel which gives mucus its viscoelastic properties. Five mucins make up this group. (Figure 1.1) These mucins are encoded by a cluster of four highly related genes on chromosome 11, region p15.5 and a similar gene on chromosome 12.[32,19]

MUC2 is the main gel-forming mucin in the colon[33, 34] and immunohistochemistry has suggested that it is goblet cell specific.[14] It is virtually absent from the soluble mucin fraction,[35] but is almost the sole component of the insoluble mucin in the human colon.[36]

In general all mucins share a common basic structure and this is crucial in conferring on them their functional characteristics.[12]

Mucin	Distribution	Locus	Reference
Membrane-bound			
MUC 1	Colon, stomach, breast, gall bladder, cervix, pancreas, respiratory tract, duodenum, kidney, eye, B & T cells, dendritic cells, middle ear	1q21-24	37, 10 , 38
MUC 3A/B	Colon, small intestine, duodenum, gall bladder, middle ear	7q22.1	19, 39, 40
MUC 4	Colon, stomach, respiratory tract, cervix, eye, middle ear	3q29	19, 41
MUC 11	Colon, respiratory tract, reproductive tract, urinary tract, liver, thymus	7q22.1	42
MUC 12	Colon, small intestine, stomach, pancreas, lung, kidney, prostate, uterus	7q22.1	42
MUC 13	Colon, small intestine, stomach, appendix, trachea, kidney, middle ear		23
MUC 15	Colon, small intestine, spleen, thymus, prostate, testis, ovary, peripheral blood leukocyte, bone marrow, lymph node, tonsil, breast, foetal liver, lungs, middle ear	11p14.3	29
MUC 16	Peritoneal mesothelium, reproductive tract, respiratory tract, eye, middle ear	19p13.2	43
MUC 17	Colon, small intestine, duodenum, stomach, middle ear	7q22.1	44
MUC 18	Melanocytic naevi		45, 46
MUC 20	Colon, kidney, placenta, lung, prostate, liver, middle ear		46
Gel-forming			
MUC 2	Colon, small intestine, respiratory tract, eye, middle ear	11p15.5	19, 47, 48
MUC 5AC	Stomach, respiratory tract, cervix, eye, middle ear	11p15.5	48, 49
MUC 5B	Respiratory tract, salivary glands, cervix, gall bladder, seminal fluid, middle ear	11p15.5	48, 50
MUC 6	Colon, Stomach, duodenum, gall bladder, pancreas, seminal fluid, cervix, middle ear	11p15.5	48, 49, 51 ,
MUC 19	Sublingual gland, submandibular gland, respiratory tract, eye, middle ear	12	52
Non-gel forming (soluble)			
MUC 7	Salivary glands, respiratory tract, middle ear		53, 54
MUC 8	Trachea/bronchus (not fully characterized)		19, 55
MUC 9	Fallopian tube	1p13	29, 56

Table 1.1: Human mucins identified to date

1.1 Mucins – Polypeptide Structure

Mucins are polymers containing many subunits. Each subunit consists of a central polypeptide core surrounded by radially attached oligosaccharide chains, giving a structure which has been likened to a bottle brush.[13] (Figure 1.2) The polypeptide region is coded for by MUC genes [57] and consists of central domains and flanking domains.[10]

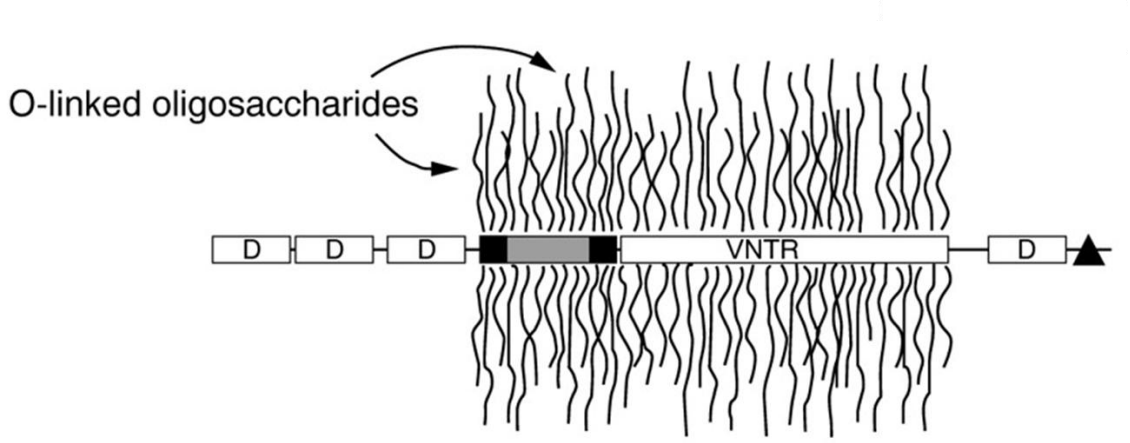


Figure 1.1: A schematic representation of a MUC2 subunit showing the 'bottle brush' appearance.[58]

The heavily glycosylated nature of mucins has made determination of their polypeptide structure very difficult due to the fact that the conditions necessary to deglycosylate them also cause a significant degree of proteolysis.[4] Most of the information on mucin polypeptide sequencing has been obtained from recombinant DNA methods.[12]

The first central domain is characterised by tandem repeat peptide sequences. These sequences are rich in threonine and/or serine amino acids, creating potential sites for oligosaccharide attachment [31,38,40,59,60] and contain at least one proline residue per repeat and normally many more.[11] Proline appears to be important in governing the specificity of the initial galactosamine transferase responsible for mucin oligosaccharide synthesis.[61]

Different mucins exhibit variability in both the length and sequence of these domains. Tandem repeat sequences may be as short as eight residues as in MUC5AC, or as long as 169 residues in MUC 6.[34, 62] Repeat sequences may also differ in their content of threonine and serine; from 75% in MUC5AC to only 25% in MUC 1.[11] A lower percentage of these amino acids results in fewer potential sites for glycosylation and affects the mucin's physical properties by making it less rigid.[11] MUC2 contains two different tandem repeat regions, the central one containing 23 amino acid tandem repeats and a second upstream 347 residue domain of irregular repeats.[60, 63, 64] The two domains are rich in threonine, serine and proline.[60, 64]

In addition to variations between different mucins in the nature and length of their tandem repeat domains, individual mucins can also exhibit polymorphism in the number of their tandem repeats.[65, 66] In MUC1 mucins, tandem repeat domains as short as 20 nucleotides and as long as 125 nucleotides have been reported.[38] Mucin genotype may confer different physical properties on the resulting mucin by varying the underlying structure of its protein product.[11] Tandem repeats can also vary in their repeatability. Some mucins have identical sequences, for example MUC7,[67] whereas others demonstrate a more degenerate repeat sequence.[12] In those mucins whose genomic organisation is known, the tandem repeat domain is encoded by a unique exon varying in size from 2.2kb (MUC7) to 21kb (MUC4).[68, 69]

Secreted Gel-forming Mucins

The other type of central domain is found in gel-forming mucins. These domains are rich in cysteine, poor in threonine and serine and may be interspersed by further tandem repeat domains.[63, 70] In MUC2, two of these sequences have been identified flanking its second upstream repeat region,[64] whereas in MUC5AC and MUC5B, these domains are

more numerous and are separated by further repeat domains.[51, 70] These 'cysteine-rich' domains in MUC2 contain potential *N*-glycosylation sites.[63] *N*-linked glycosylation occurs between an oligosaccharide group and the amide nitrogen of an asparagine residue. It can only occur if this residue lies within the tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except proline.[61] These linkages confer stability on secreted mucins and may assist in ensuring correct tertiary protein formation.[61]

Central domains are flanked on either side by other types of polypeptide regions.[63] Domains found particularly in gel-forming mucins are known as disulfide-rich D-domains. These were first described in von Willebrand's factor (VWF) and have since been found in several other proteins.[71, 72] They can occur at either end of the polypeptide chain and are found in a similar number, position and size to those in prepro-VWF.[11, 63] MUC2, MUC5AC and MUC5B [73] contain four of these regions at the amino terminal end (N), designated D1, D2, D'and D3 and also one, D4, at the carboxyl (C) terminal.[12, 74,75] The D domains have far fewer carbohydrate links than the tandem repeat domains and contain up to 30 ½ cysteines.[12] (Figure 1.3)

Downstream of D4 at the C-terminal in MUC2, MUC5AC and MUC5B are three other domains which show homology to further domains found in VWF namely, B-domain, C-domain and a cystine-knot (CK)-domain.[10, 56] (Figure 1.3) The C-domain consists of 100-130 residues and has a similar make-up to those in the nearby tandem repeat domains, for example MUC2 contains 29% threonine, 18% serine and 20% proline.[63] This region is therefore sometimes known as 'mucin-like'. The terminal 90-120 residues are rich in cysteine and show sequence identity to those in the CK-domain in

VWF in common with transforming growth factor β2, nerve growth factor and platelet derived growth factor.[12, 73]

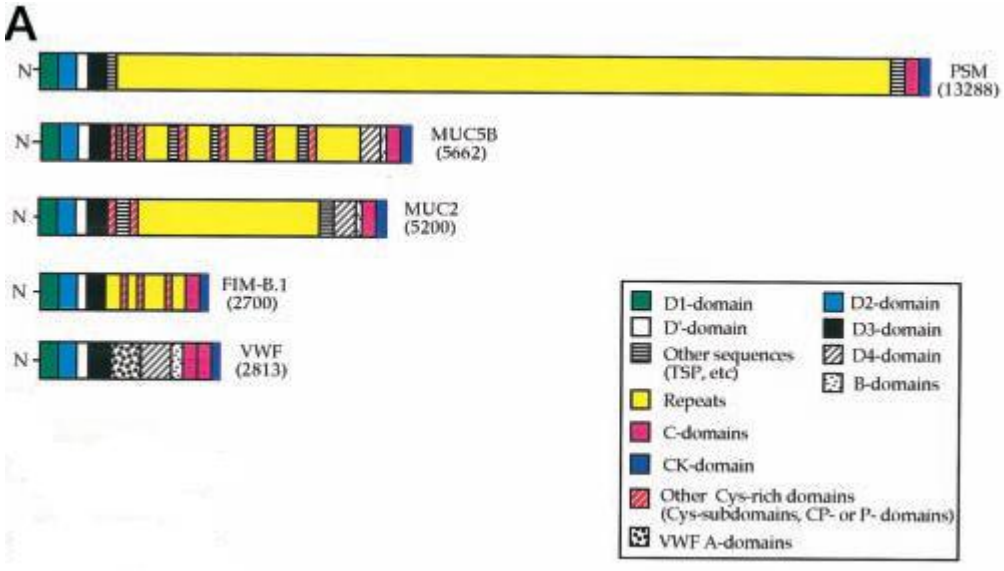


Figure 1.2: A schematic representation of the polypeptide domains of gel-forming mucins and their similarities to von Willebrand's factor (VWF), porcine sub maxillary mucin (PSM) and frog integumentary mucin (FIM-B.1) 12

The ability to form mucin-type gels results from oligomerization of mucin core proteins mediated by D-domains.[76, 77] Disulphide bonds form between cysteine residues; dimers at the C-terminal end and trimers at the N-terminal end.[78,79] *N*-glycosylation in the region of the CK-domain influences the folding rate of mucin monomers to allow the structural maturation and stability of MUC2 dimers.[80] Cysteine domains may also be involved in other intermolecular bonds through non-covalent linkages.[81] The gel-forming properties of this group of mucins is due to the complex, branching network of covalently linked mucin molecules and can be destroyed by reduction of disulphide bonds.[77]

Within the cysteine-rich D4-domain of the MUC2 protein is an autocatalytic protein-cleavage site at the motif GDPH (G = glycine, D = aspartic acid, P = proline, H = histidine).[77, 81] Cleavage here produces a subunit called a 'link protein' and produces a new, reactive C-terminal which has the potential to link to *O*-glycans of other mucin molecules.[81] This may explain the previously uncharacterized non-disulphide covalent bonds found between MUC2 monomers.[81] This cleavage site is also found in those mucins containing D4-domains, MUC5AC and MUC4.[81] It can be speculated that MUC4, a membrane bound mucin, could link to the gel-forming mucin complex via this linkage method aiding the complexity and viscosity of the mucus gel layer.[77]

Membrane-bound Mucins

Membrane-bound mucins also contain tandem repeat domains but have neither D-domains nor cysteine domains.[82] However, they do exhibit two other regions, a transmembrane domain and a relatively short cytoplasmic tail which associates with cytoskeletal elements and cytosolic adaptor proteins and may participate in signal transduction.[74]

There are also domains in the extracellular juxtamembrane region. One of these is the SEA domain (sperm protein, enterokinase, agrin), a long extracellular *O*-glycosylated region.[83] During intra-cellular post-translational processing, a cleavage occurs at the SEA domain yielding two subunits.[84] Although these subunits remain associated during biosynthesis, the α-subunit can be shed from the cell surface.[85] The other domain found is one with close homology to the epidermal-growth-factor (EGF) family.[86] Its function is not established but they are postulated to allow the interaction between cell-surface mucins and members of the EGF receptor family in regulation or signalling of functions

such as inflammation or differentiation.[86, 87] Other domains which have been described include P-domains and domains similar to those found in the trefoil factor family.[88]

The separation of mucins into membrane-bound and secreted is not absolute. In addition to membrane-bound mucins being able to link with gel-forming mucins, as discussed above, cell-surface mucin genes appear to undergo alternative splicing and encode isoforms, lacking transmembrane and cytoplasmic domains.[21, 89] Due to secretion of these isoforms and the ability of subunit shedding, membrane-bound mucins can also be considered to be components of secreted mucins.[19, 23]

1.2 Mucins - Oligosaccharide Structure

Oligosaccharides not only account for 80% of the dry weight [90] of a mucin molecule, they play a crucial role in its function, gel-like properties and stability. The highly glycosylated protein domains form long extended structures, which have a much greater solution volume, giving aqueous mucin solutions a higher viscosity than unglycosylated proteins.[12] It is the oligosaccharide chains which aid this structural formation by limiting the rotation around peptide bonds and also by mutual negatively charged repulsion forces between groups.[12] The dense array of oligosaccharides also helps to protect the underlying polypeptide core from proteolysis by bacterial enzymes. Although there are variations in oligosaccharide chain length and antigenic structure between different mucins, they all share a similar basic structural pattern.[91]

Mucin oligosaccharide chains contain, on average, 8-10 monosaccharide residues of five different types; fucose (Fuc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-galactosamine (GalNAc) and sialic acid.[44] All mucin monosaccharides form six-membered pyranose ring structures.[44]

Oligosaccharides of both secreted and cell surface glycoproteins, are classified by the nature of their linkage to the protein core.[92] They can be *N*-linked, via an amide nitrogen of an asparagine residue, (*N*-acetylglucosamine-asparagine) or *O*-linked, a linkage through an oxygen atom.[13, 93, 94] In mucins the majority of the oligosaccharides are *O*-linked, the *O*-glycosidic linkages occurring between N-acetylgalactosamine and the hydroxyl amino acids of threonine and serine of the protein core.[91]

O-glycans form a very heterogeneous group with an extremely large potential for structural diversity.[94, 95] Monosaccharides can undergo substitution at one or more of positions 2, 3, 4 and 6 of their pyranose ring with either an α or ß linkage.[44] When this is considered alongside the possible differences in chain length, chain branching and combinations of individual monosaccharides, the resulting potential for variety is enormous.[44, 94, 95]

Detailed studies on the composition of colonic mucin oligosaccharides have been achieved through cleavage of the *O*-glycosidic linkage by alkaline degradation of mucin.[96, 97] Many different distinct oligosaccharides have been identified but, despite the enormous heterogeneity this reveals, most represent variations on a limited number of basic structural types.[96, 97]

O-linked oligosaccharides can be described in terms of their core, backbone and terminal regions.[95] Core structure refers to the primary linkage to the polypeptide chain and backbone to the subsequent monosaccharide sequence. Eight types of core structure have been classified depending on the branching and binding of the initial *N*-acetylgalactosamine residue at position 3 and/or 6 (Figure 1.4).[95, 98]

Core 1	Gal β(1-3)-GalNAc α(1-O)-Ser/Thr
Core 2	Gal β(1-3)-[GlcNAc β(1-6)]-GalNAc α(1-O)-Ser/Thr
Core 3	GlcNAc β(1-3)-GalNAc α(1-O)-Ser/Thr
Core 4	GlcNAc β(1-3)-[GlcNAc β(1-6)]-GalNAc α(1-O)-Ser/Thr
Core 5	GalNAc α(1-3)-GalNAc α(1-O)-Ser/Thr
Core 6	GlcNAc β(1-6)-GalNAc α(1-O)-Ser/Thr
Core 7	GalNAc α(1-6)- GalNAc α(1-O)-Ser/Thr
Core 8	Gal α(1-3)- GalNAc α(1-O)-Ser/Thr

Figure 1.3: Difference *O*-linked oligosaccharide core structures found in mucins.[95, 98]

In human colonic mucins the majority are based on a core 3 structure, although 1, 2 and 4 core-based oligosaccharides have been described, as well as small amounts of core 5 and core 6 mucins.[99]

Colonic mucins have backbones composed of repeating galactose and N-acetylglucosamine units (Gal-GlcNAc) either linear or with branching side chains at the galactose residues.[95, 100] Backbones can be defined as either type 1 chain (Galβ1-3-GlcNAc) or type 2 chain (Galβ1-4-GlcNAc) and consist of one or more of these disaccharide repeats.[101, 102]

Mucin oligosaccharide chains are terminated by blood group substances. These are specific carbohydrate complexes identified on the surface of red blood cells and are classified as either type 1 or type 2.[102] The underlying backbone structural type dictates which blood group substance type is attached and the variability within types is genetically determined, centring on the Lewis and the Secretor genes.[102] Type 2 is predominant in secretory mucins and includes the substances A, B, H, Le^b, Le^a and

Sle^a.[102] Other intestinal mucins are predominately type 1 and include Lewis x and y (Le^x, Le^y) and SLe^x and show little expression in the normal gastrointestinal tract.[102] Sialylation precedes the final and terminating step of fucosylation in the biosynthesis of the blood group substances Sle^a and Sle^x.[102] Other blood group determinants include Sd^a/Cad antigens which are other major structural features in human colonic mucin glycans.[24, 99]

Blood group probes and lectins have been used to identify terminal residues.[103, 104, 105] Lectins are proteins isolated from animal, plant and microorganisms which bind to specific non-reducing terminal monosaccharides determinants. They include wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin (UEA) and *Maackia amurensis* agglutinin (MAA) which bind to sialic acids and N-acetylglucosamine residues, fucosyl residues and α(2-3) linked sialic acids respectively.[103, 106, 107] A terminal sugar can be part of a blood group determinant or a *O*-sulphate ester or sialic acid residue which has been added to a terminal blood group substance.[95, 100]

Mucins can be classified into neutral and acidic subtypes. The latter are further differentiated into sulphated (sulfo or sulpho mucins) or non-sulphated (sialomucins) groups. Neutral mucins appear to be the predominant subtype expressed in gastric mucosa, whereas acidic mucins are expressed throughout the intestinal epithelium and dominate in the large intestine. [108]

Colonic mucin is particularly rich in sialic acids (sialomucins) which differ from other sugars within mucin oligosaccharides by having an acidic carboxyl group and a three carbon side arm which can exist in a number of variant forms.[102] Sialic acid is largely limited to the secretory mucins of the small and large intestine where it can be added to short core structures, the backbone or terminal blood group substances. [102] The most

common form of sialic acid in humans and the common precursor for all sialic acids, is *N*-acetylneuraminic acid (Neu5Ac), a nine carbon pyranose amino sugar which has a carboxyl group at its anomeric carbon. This monosaccharide is found in many variant forms including *O*-acetylated and *N*-glycolyl. [109]

Neu5Ac is most commonly is found as a single terminal residue joined to an underlying galactose or GalNAc by an α-2,3- or α-2,6- glycosidic linkage.[99, 100] Exceptions to this usual pattern are α-2,8- linked sialic acids which can be attached to an underlying α-2,3 sialic acid residue.[99] Almost 50% of mucin sialic acids are *O*-acetylated, approximately 30% substituted by di-or tri-*O*-acetyl esters and 15% as mono-*O*-acetyl esters.[100] *O*-acetyl groups can be substituted within the carbon side arm at C4, C7, C8 or at C9. [110] Differing levels of *O*-acetylation between individuals is likely to be due to genetic variation in the gene coding for *O*-acetyl transferase (OAT). Approximately 9% of Caucasians, for example, are homozygous for the inactive version of this gene and consequently only produce non-*O*-acetylated sialic acids.[102]

Mucus glycoproteins are also rich in carbohydrate *O*-sulphate esters (sulfomucins). [111] Sulphation is most frequently found on Gal and GlcNAc residues in *N*-acetyl-lactosamine sequences, specifically at the 3-position of Gal and the 6-position of GlcNAc of *O*-glycan cores and their elongated structures, either as a terminal residue or in a more internal position.[44, 112] The majority of oligosaccharides in the colonic mucosa consist of between 4 – 12 monosaccharides and are both sialylated and sulphated, sulphation being particularly characteristic of colonic mucins.[100] Sulphation not only adds negative charge to mucins, it also protects the mucin from bacterial degradation.[111, 113] Sulphate can also be added to the mannose residues of N-glycan chains [114] and to sialyl Lewis^x determinants attached to core 2 *O*-glycans.[115]

Sialic acids and sulphate esters ionise at all but the lowest pH values resulting in negatively charged residues. In colonic mucins the high density of sialic acids and sulphate esters and the resulting repulsion between these negatively charged groups causes the expansion of their tertiary structure and a rise in their viscous properties.[44]

1.3 Mucins - Multimeric Structure and Assembly

It has proved difficult to elucidate the multimeric structure of mucins. Their large molecular size and high carbohydrate content mean that conventional protein chemistry investigative tools do not work.[12] As previously mentioned, mucins show significant homology to VWF in their terminal regions, the D-domains, where a high cysteine content creates potential for di-sulphide linkages.[116,117] The combination of information obtained from work on porcine sub maxillary mucin (PSM) using mucin encoding plasmids with assumptions based on what is already known about VWF tertiary structure has led to a proposed model for mucin polymerization.[116, 117, 118 , 119]

The assembly of mucin multimers involves several stages, some in the endoplasmic reticulum (ER) and some in the Golgi apparatus. The current model for mucin oligomerization is that mucin monomers undergo dimerization in the endoplasmic reticulum by the formation of disulfide linkages between their CK-domains within minutes of their biosynthesis.[48, 79, 117] This occurs preceding or concomitant with *N*-glycosylation which also takes place in the ER and is thought to be important in ensuring the correct folding of mucin dimers.[19, 79]

The incorporation of *O*-linked oligosaccharides begins after dimerization and *N*-glycosylation. [69] In cells expressing secreted mucins, e.g. intestinal goblet cells, *O*-glycosylation is initiated in the *cis*-Golgi, although in other mucin-producing cell lines

this starts in the ER. [79, 120] This process continues in the *medial*- and *trans*-Golgi compartments, where the glycosyltransferases necessary for elongation and termination of the oligosaccharides are located.[12] Glycosyltransferases transfer sugar residues from an activated donor substrate, usually a nucleotide sugar, to a growing carbohydrate group.[121] Terminal glycosylation sequences are likely to reflect the glycosyltransferases which are expressed in the cell.[121]

The control of *O*-glycan biosynthesis is not well understood but is thought to involve many different factors.[15] Heterogeneity of *O*-glycans is likely to be dictated by various factors: regulation of glycosyltransferase genes, availability of acceptor and sugar nucleotides, enzyme competition for acceptor intermediates and compartmentalization.[15, 94, 95]

The first stage in mucin *O*-glycosylation is the α link addition of *N*-acetylgalactosamine to the hydroxyl group of a serine or threonine residue in the polypeptide core.[95, 122] The enzyme involved in this process is UDP-GalNAc:polypeptide GalNAc transferase and the presence of proline in a triprolyl sequence nearby appears to be important .[61]

The next stage is elongation, where further *O*-linked oligosaccharides are added individually to the polypeptide chain, the initial elongation step determining the type of core the oligosaccharide will have.[95] In *O*-glycosylation all the sugars are added by individual transfers (glycosyltransferases) from their nucleotide derivatives on the luminal surfaces of the ER and/or Golgi complex, in contrast with *N*-linked oligosaccharides which require preformed oligosaccharide intermediates.[94, 95] The expression and compartmentalization of glycosyltransferases may be the predominant factor in determining final oligosaccharide structure, although the position and structure

of previously added oligosaccharides is also known to influence subsequent glycosylation.[123, 124]

Sugars are sequentially added until a terminal sugar residue (to which no other sugars can be added) ends the process. This is commonly sialic acid (Neu5Ac), sulphate or fucose, but can be *N*-acetylgalactosamine, α-galactose or a blood determinant.[95]

In common with all monosaccharides, the formation of Neu5Ac begins with glucose [125] which is phosphorylated to glucose-6-phosphate. (Figure 1.5) This is then converted to fructose-6-phosphate by the action of an epimerase and an amino group is transferred from L-glutamine to create glucosamine-6-phosphate; the precursor for N-acetyl glucosamine, N-acetyl galactosamine and N-acetyl neuraminic acid. Radiolabeled [H^3]-glucosamine, used in organ culture, can be incorporated during these stages.[126]

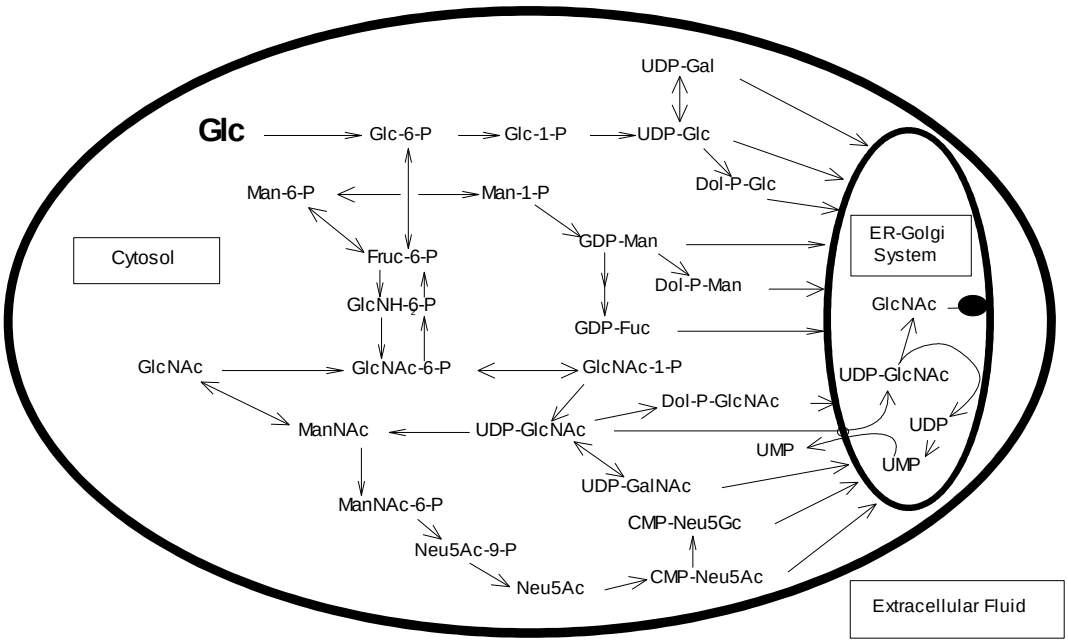


Figure 1.4: Metabolic pathways for the generation of nucleotide sugars including sialic acid. (Modified from Varki, 1994) 127

The addition of sialic acid to an *O*-glycan chain occurs in the *trans*-Golgi. The sialyltransferases which mediate this action require the sialic acid to be in its sugar nucleotide form, CMP-Neu5Ac, which is first formed in the nucleus before being transported into the Golgi apparatus.[128]

A particular feature of colonic mucins is the presence of sulphate esters with the most highly sulphated mucin population being found in the rectum.[112] In secreted mucins core structures 1 - 4 and 6 can serve as potential substrates for sulphotransferases (STs).[129, 130] Two families of sulphotransferases have been identified which transfer sulphate from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to either the 3-position of Gal (Gal3ST) or the 6-position of GlcNAc (GlcNAc6ST) residues within *N*-acetyl-lactosamine sequences.[112, 131] This is the most frequently found form of sulphation in mucins.[112] In addition other sulphated epitopes may be found on mucins, catalysed by different sulphotransferases, for example GalNAc 4-sulphate and Gal 6-sulphate linkages and can be added to core sugars or to N-glycan chains.[112]

Sulphotransferases are located in the *trans*-Golgi.[112] Sulphation can block certain pathways and prevent oligosaccharide branching and elongation e.g. once core 1 is 3-sulphated, core 2 cannot be formed.[112, 132]

High molecular weight mucin multimers are created from dimers after N-glycosylation and initial *O*-glycosylation.[133] Interchain disulphide bonds form between cysteine residues in the amino terminal D-domains of dimers.[116, 117] All of the amino terminal D-domains in continuity appear necessary for multimerization although the disulphide bridges are actually established between two D3 domains.[118] The other D-domains are thought to prevent inappropriate multimerization in the ER and the *cis*- and *trans*-

Golgi.[12] Instead of a linear polymer structure, MUC2 appears to form into trimers via its N-termini suggesting that it will form branched networks.[78]

Once in a polymerized state, mucin gels can exist in either a condensed phase or an expanded hydrated phase.[134] Condensing granules bud off the *trans*-Golgi apparatus and form into mature secretory granules within the apical cytoplasm.[135] While in a secretory granule mucins exist in a condensed state. As the secretory granule docks on to the plasma membrane, a secretory pore is formed and Ca^{2+} from inside the pore is exchanged with extracellular Na^{+}, driving water into the granule and converting the mucin to its hydrated phase. The resulting massive swelling of the granular content drives the release of the mucin into the extracellular space and its formation into a gel.[134] The dense glycosylation causes an extended conformation allowing the molecules to occupy large volumes.[12, 78]

MUC2 occurs as an insoluble complex that resists extraction with guanidinium chloride unless disulfide bonds are cleaved.[35, 36,] The formation of insoluble MUC2 gel is likely to involve "cross-links" in addition to those that join mucin monomers into larger structures.[136] Within the polypeptide C-terminus of MUC2 is an autocatalytic protein-cleavage site between the D and P residues. Cleavage at this point yields a subunit which is believed to have a role in cross-linking mucin core proteins to the oligosaccharide side chains of MUC2, establishing higher order structures in the mucus gel.[81] Further intra and intermolecular disulphide bonds are also likely to be involved.[81] Proposed other intermolecular interactions include hydrogen bonding, hydrophobic interactions and physical entanglements.[137]

Different forms of MUC2 have been identified; N-glycosylated protein core in monomeric or dimeric forms in the cell layer, partially glycosylated oligomerized

intermediates and the fully glycosylated oligomerized form in both the secreted medium fraction and the cell layer.[138]

Exocytosis involves two distinct processes, baseline secretion and compound exocytosis. Baseline secretion or simple exocytosis which occurs under normal conditions involves a continuous low rate release of newly synthesized mucin granules, present in the apical regions of the goblet cells.[139] Compound exocytosis is an accelerated secretory event regulated by endocrine, paracrine, neurocrine and immune pathways.[135] It results in the acute release of centrally stored mucin granules and occurs in response to a stimulus which may be a neuroendocrine agent (e.g. vasoactive peptide), a cytokine (e.g. interleukin 1) or other compounds (e.g. adenosine triphosphate.[135, 140, 141] Only a certain fraction of the total mucin produced in the colonocyte and the goblet cells in a twenty four hour period is secreted out of the cell.

1.4 Mucins - Role and Function

The colonic epithelial barrier consists of a highly specialized columnar cells arranged in a single layer lining the crypts and flat mucosal surface. These cells have several functions as well as providing a simple physical barrier to infection and damage. They secrete water, ions and macromolecules, the constituents of mucus, which washes away and dilutes potentially harmful agents.[135]

Mucus is produced mainly by goblet cells, specialised secretory epithelial cells, but also by enterocytes.[24] Mucus consists of water (up to 95% by weight), mucins (no more than 5% by weight), inorganic salts, carbohydrates and lipids.[142] In the colon the mucus barrier consists of an inner insoluble layer, next to the goblet cells, almost completely devoid of bacteria and an outer viscous soluble layer containing a mixture of bacteria,

mucins and cell debris.[9, 143] In addition, a layer of surfactant lipids secreted by epithelial cells coats the surface of the mucus gel preventing penetration by water soluble toxins.[144]

One of the main roles of mucus is to provide a protective barrier between the gut lumen and the epithelium by physically covering cell surface glycoconjugates thereby preventing the adhesion of enteric pathogens. [145] Within this layer a stable microenvironment is formed which, while being readily permeable to ions and low molecular weight solutes, is a physical barrier to larger molecules, such as proteases and toxins, which could cause epithelial damage. Pathogens must bind to the mucus layer and then penetrate through it to gain access to the epithelial cells.[146]

The mucus matrix can also serve as a highly selective barrier possibly by locally concentrated oligosaccharide chains functioning in a similar way to ion-exchange or gel filtration and facilitating or restricting diffusion.[77] In the stomach bicarbonate ions secreted by the epithelium are held within the mucus creating a pH gradient from pH 2 at the stomach lumen to pH 6-7 at the cell surface. At a pH above pH 4, hydrochloric acid (HCL), can form channels within mucus, allowing it to pass through. However, below pH 4 it cannot penetrate the mucus layer. This allows secreted HCL to pass into the stomach lumen while simultaneously preventing luminal HCL access to the epithelium. This function may be in part influenced by sialic acid structure.[77, 147]

Mucin gels bind other biologically active molecules. One important group of such molecules is the trefoil factors (TF), a family of peptides containing the trefoil motif.[148] They are expressed by most mucin-producing epithelia, including the gastrointestinal tract, and bind to the D domains of mucins.[149] As well as contributing to mucus

viscosity they also promote cell motility and differentiation, have an anti-apoptosis effect and promote wound healing and mucosal repair.[150, 151]

Cytokines are also found in mucus gels bound to oligosaccharides. Disruption to the mucin barrier, by physical or chemical means, could cause their release, leaving them free to instigate an inflammatory reaction.[77] Secretory immunoglobulin A (sIgA) is retained at high concentrations within mucus where it can trap the progress of pathogens. The exact nature of this binding is not clear but mucin integrity is important for its efficacy.[19] Mucins also bind antimicrobial molecules (e.g. histatin-1) and their oligosaccharides may even have direct antimicrobial activity.[152, 153]

Many pathogens need to bind or penetrate epithelial cells in order to cause pathology. This is reflected by the widest diversity of cell-surface mucins being found in the colon, the most microbe rich environment.[19] Membrane bound mucins are likely to be able to provide signals to epithelial cells in response to alterations in the mucin layer, through changes in conformation or ligand status of their extracellular domains. This may cause changes in epithelial cell proliferation, differentiation or cell-adhesion.[77] It is also probable that these cell-surface mucins act as releasable decoy ligands for microbes trying to attach to the glycocalyx.[19] Individuals with MUC1 alleles which have smaller extracellular domains have a greater risk of developing gastritis after *Helicobacter pylori* infection.[154]

Secreted mucins express many oligosaccharide structures which are also found on the epithelial cell surface which could allow mucins to act as decoys for bacterial adhesins.[19] *Streptococcus pyogenes* and *enteroviruses* bind to sialic acids which are found on the epithelial surface and on mucins.[19, 155] Some mucins also act as viral agglutinating agents.[19]

Mucus offers several ecologic advantages to intestinal bacteria. Colonization of mucus may allow the bacteria to avoid rapid expulsion and confer a growth advantage.[19] Nutrients such as vitamins and minerals may be available within the mucus matrix as well as the enzymatic digestion of mucins providing a potential source of carbohydrates and peptides.[145] Bacterial colonization may also confer advantages to the host by retaining pathogens in the mucus layer, away from the epithelium and by providing a favourable environment prevent pathogenic bacteria gaining a niche.[19]

Mucus secretion is typically enhanced in response to intestinal microbes.[156] Cholera toxin of *Vibrio cholerae*, triggers massive mucin release via a cAMP-dependent mechanism[157] but conversely, *Clostridium difficile* toxin A exerts an inhibition of compound mucin exocytosis, although without altering baseline (constitutive) mucin secretion when added to HT29-Cl.16E cells.[158] Bioactive factors released by mucosal immune cells also regulate the composition of mucins.[19] For example TNFα increases α-2,3-sialyltransferase expression in HT29 cells suggesting that it triggers an increased synthesis of sialomucins.[159] Other factors which seem to enhance mucin secretion include pro-inflammatory cytokines and prostaglandins.[160, 161]

At the level of the mucus barrier there is a constant dynamic equilibrium between mucin synthesis and mucin degradation. The normal enteric bacterial flora secrete a range of enzymes which act to continually break down the mucin gel as more mucus is produced.[100] A balance between these conflicting processes is necessary to maintain a functional defensive barrier at the mucosal surface.[90] While mucus shedding and degradation rates exceed colonization rates, pathogenic bacteria are eliminated.[146] The structure of colonic mucins is specifically adapted to cope with the degrading activity of the micro flora.

The breakdown of mucins occurs in a stepwise manner that begins with proteolysis of the non-glycosylated "naked" regions of the mucin glycoproteins by host and microbial proteases.[162] This initial step markedly reduces mucin gelation and viscosity and results in the accumulation of highly glycosylated subunits (>500 kDa) that are resistant to further proteolytic attack.[163] Before the remainder of the protein core can be hydrolysed, the oligosaccharide chains must first be broken down.[90] Bacterial enzymes breakdown the mucin oligosaccharide chains sequentially, starting from the peripheral non-reducing end of the chain.[90] The rate of further degradation is therefore dependent on the efficient removal of these terminal residues.[100]

In colonic mucins up to 50% of these terminal residue positions are occupied by *O*-acetylated sialic acids.[24, 164] Bacterial sialidases act to remove sialic acids but they are slowed down or blocked by the presence of two or more *O*-acetyl esters on the C7-C9 tail of these monosaccharides.[24, 165] These specific terminal residues acting in this way control the overall rate of mucin degradation. Removal of these *O*-acetyl esters, by sialic acid *O*-acetyl esterase, allows the abundant sialidase activity, normally present in the colon, to desialylate the oligosaccharide chains thus freeing up sites for further degradation by other glycosidases.[166] Some enteric bacteria secret a specific sialic *O*-acetyl esterase to overcome this block.[167]

The other type of acidic mucins, sulfomucins, may also protect against bacterial activity. Sulphated mucins appear less degradable by bacterial glycosidases and host proteases.[168] Sulphate esters may mask antigenic or lectin binding sites, may also regulate the biosynthesis, half-life and biological roles of glycoproteins and may control lymphocyte homing and inflammation, so sulphation is also an important regulatory feature in colonic mucins.[112, 169] The presence of mucin oligosaccharide-specific

sulphatases in the colon has been established [170], in particular bacterial enzymes with specificity for galactose-6-O-sulphate and GlcNAc-6-O-sulphate.[100]

Sulphated glycans add negative charge to mucins which affects their physical and chemical properties including hydration and metal ion binding.[113] They are also involved in functions such as cell signalling, cell adhesion and inflammation.[171]

The highly heterogeneous nature of *O*-glycans may be an important aspect of mucosal defence. *O*-glycans may mediate specific binding of antibodies and microbes and be important in host-pathogen interactions as well as inflammation and cancer metastasis.[24] Oligosaccharide chains are known to mimic cell surface receptors and microbes bind to them, rather than reaching the mucosal surface.[91] Their diverse nature means that they are able to trap a broad diversity of microorganisms and the terminal residues are the primary targets for this interaction.[24, 94, 101] In pathogenic bacteria their ability to bind to a target residue appears to be related to its enzymatic capability. For example, *Salmonella typhimurium* binds preferentially to a glycoprotein containing sialic acid and possesses a sialidase.[172, 173]

Binding to specific O-glycans by commensal bacteria could also aid mucosal defence by facilitating their mucus colonization and therefore providing them with a growth advantage over pathogenic bacteria.[174] Multiple potential attachment sites for commensals may be a further way in which mucin structure acts as a major influence in dictating the composition of the enteric bacterial flora.[15]

Different glycosylation patterns are known to exist along the length of the intestine. A gradient has been demonstrated from ileum to rectum with a pattern of decreasing levels of fucose and ABH blood group expression and increasing levels of sialic acids and

acidity.[24] Sd^a/Cad antigens are absent in the ileum and increasingly expressed along the length of the colon.[24, 99] This gradient of variability may help to explain the region-specific bacterial colonisation in the colon, the different glycans available enabling a given microbe to establish a niche that it can occupy.[24] In a similar way, resident colonic flora may differ among individuals because of variations in the specific carbohydrate composition of their intestinal mucins.[175, 176]

Increased concentrations of sulphated mucins have also been found in the more distal areas of the large bowel.[104, 177, 178] This increase in the proportion of acidic mucins along the intestine appears to mirror the increase in microbial population.[179]

It has been shown that the structure of mucins, in particular their post translational modifications, are crucial in limiting their own degradation. The rate of mucin breakdown must equal the rate of mucin production in order to maintain mucosal defence. Alterations in mucin structure and composition may therefore be integral in the pathogenesis of gastrointestinal disease.

1.5 Mucins – Alterations in Disease

Mucins are an important area of study for many diseases. Quantitative and qualitative alterations have been documented. Changes in mucins structure and production have been implicated in the pathogenesis of conditions as varied as cystic fibrosis and gastric cancer.[37, 180]

A reduction in the ability to produce mucus will lead to a thinner protective layer. In ulcerative colitis which affects the colorectum, there is a reduction in the thickness of the mucus layer compared to the normal healthy colon. This appears to be a

consequence of a depletion of the number of goblet cells in the affected mucosa.[57, 181] The inner layer of mucus also appears to be reduced over the surface of colonic carcinomas and polyps, allowing closer contact between epithelium and luminal contents, including lectins which are known to affect mucosal proliferation.[182, 183] Mucin derived from inflamed rabbit colons shows a reduced inhibition of adherence of *Escherichia Coli*, using an in vitro binding assay, compared to mucin derived from normal colons.[156]

Differing levels of mucin gene expression is one of the ways that disease may manifest. In normal stomach mucosa MUC1, MUC5AC and MUC6 are ordinarily expressed. In the transition from early to later stages of intestinal metaplasia, there appears to be a gradual change in mucin type, from the additional of MUC2 to the normal mucin make-up, to the replacement of stomach mucins with MUC2 and MUC3 alone.[37] In ulcerative colitis it seems that alterations in MUC2 expression are closely related to inflammation as during active disease. Reduced levels of biosynthesis of MUC2 precursors and total MUC2 are found, while normal levels are detected during remission.[184] MUC2 is the most prominent mucin in the colon but in colonic adenocarcinoma, MUC5AC is in abundance.[185] Some microbes can directly alter mucin gene expression, e.g. *H. pylori* suppressing MUC1 and MUC5AC gene expression in a human gastric cell line.[186]

Mucin production is altered in a similar way in the inflammatory bowel condition Crohn's disease. This has not only been found in the diseased bowel, where MUC1 is reduced, but also in healthy ileal mucosa, which expresses lower levels of MUC3, MUC4 and MUC5B than normal controls.[187] As Crohn's disease often involves or starts in the terminal ileum and MUC3 and MUC4 are the main mucins expressed in this

region of the intestine, this may represent a primary, or at least very early, mucosal defect in these patients.[187]

In gastrointestinal disease, changes are found at the level of mucin oligosaccharide chains. Mucins produced by colorectal cancer cells have a reduced number and length of carbohydrate chains, suggesting a general decrease in glycosylation or premature terminal of the glycan chains.[188] Alterations in the length of oligosaccharides can result in the formation of new antigenicities, for example Tn, T and sialyl Tn.[57, 102] All three of these epitopes are expressed in over 90% of colorectal cancers but are absent from normal mucosa.[189] There appears to be a progression in their appearance with T antigen being found in well and moderately differentiated tumours and Tn in more poorly differentiated carcinomas.[57] However, other authors have found no strong evidence for the presence of T antigen in either normal colon or adenocarcinoma.[190]

Differences in amounts of sulphated and sialylated mucins and alterations in the levels of *O*-acetylesterase and sulphatase activity are associated with gastrointestinal pathology.[191, 192] The terminal oligosaccharide, sialyl-Lewis^x, has also been associated with malignancy and metastases.[77, 193] This antigenic site is normally masked by *O*-acetylation.[191] Loss of sialic acid *O*-acetylation occurs throughout the whole sequence of transformation from adenoma to carcinoma and is present at the earliest stages of premalignancy.[126] It is therefore likely that the appearance of the sialyl-Lewis^x epitope, as well as other epitopes, is secondary to these changes.[102] S-fimbriated *Escherichia coli* which cause sepsis and meningitis in newborn infants have been shown to bind to certain sialic acid containing structures on human erythrocytes.[194]

Loss of colonic mucin sulphation has been found in ulcerative colitis.[192] Lack of sulphation may affect the viscoelastic properties of the mucins and increase the rate of

bacterial degradation.[100] Increased levels of faecal sulphatase activity, in particular those which cleave 6-sulphated sugar linkages, has been documented and may contribute to the reduced sulphation found in this disease.[164] Reduced turnover of sulphate in ulcerative colitis has also been demonstrated [111] and this, in combination with a reduction in fucosylation, is found in European colitics with severe disease.[181]

Alterations in enzyme activity are key features underlying mucin changes in colonic cancers. Reduced levels of mucin sulphation is a common finding in adenocarcinoma.[111] Cultured cancer cells have shown a loss of sulphotransferase activity suggesting that this may be the primary defect.[100] Further detailed work on colonic cancer cultured cell lines has shown typical changes in the activity of a number of key glycosyltransferases.[192]

The normal pathway in colonic mucin production is through core 3 and core 4 pathways. In malignancy, alterations of these key glycosyltransferases block certain pathways resulting in a switch from core structure 3, to core 1 and core 2.[196] Alterations in core structure can have a knock-on effect on the rest of the oligosaccharide structure and a reduction in branching and a modification of sialylation have also been noted.[102, 195] Increased expression of specific glycosyltransferases can also lead to the aberrant expression of blood group determinants in gastrointestinal malignancies. One example is fucosyltransferase-4 which is associated with increased expression of the Ley blood substance.[196]

Mucin alterations appear to play a major part in pathogenesis of many diseases, whether as a primary defect or as a consequence of a pathological process.

1.6 Mucins – Alterations in Children

There is little information known about the role of mucins in disease of childhood and the variations which occur with age.

Immediately after birth, the gut is practically sterile but during the first 12 to 24 hours of extra uterine life becomes colonised with maternal and environmental flora.[143] *Escherichia coli* and *Enterococcus* sp appear first followed by obligate anaerobes, *Bifidobacterium* and some *Bacteroides* sp in breast fed babies. In babies fed formula milk, the opposite occurs: *Bacteroides* sp. predominate and are accompanied by some *Bifidobacterium* sp.[143] The infantile flora gradually evolve toward a normal adult flora during the next two years of life but during this time the flora are not stable or diverse and so are easily subject to disturbances and imbalances which is reflected in the highest incidence of enteropathies and food allergies occurring between 0 and 2 years of life.[143]

The first bacteria to colonize the neonatal gastrointestinal system depend on the innate repertoire of bacterial adhesion sites, which varies according to genome. If these bacteria have glycolytic potential, new binding sites will appear so new bacteria can colonize, resulting in every person having their own unique intestinal flora.[143] Resident bacteria can also influence the carbohydrate repertoire of mucins by sending messages to host cells which interfere with the expression or activity of cell glycosyltransferases.[197]

Mucin genes and mRNA have been found to be expressed in the early stages of the development of the human gastrointestinal system.[76] The MUC5AC gene is not normally expressed in the adult intestinal tract and the MUC4 gene is only found in low levels in the adult small bowel.[76] However, both have been detected in foetal gut,

MUC5AC at 12 to 13 weeks gestation and again at 27 weeks and MUC4 at high levels in foetal small intestine at 12 and 13 weeks gestation.[76] Some authors have suggested that mucin genes may be involved in the development of enterocytes.[76] The adult pattern of major MUC2 and MUC4 gene expression and low to background MUC1 and MUC3 predominates from birth onwards.[198]

In situ hybridisation studies have shown the pattern of mucin mRNA expression in the foetal colon.[198, 199] MUC2 mRNA was seen by 12 weeks gestation in the colon, MUC3 and MUC4 at around 13 weeks, the former at high levels, the latter low levels.[199] These decreased towards term and by 18 weeks MUC1 was detected in the colon.[198, 199] MUC5AC mRNA was also transiently seen in colonic goblet cells at week 17.[199]

Mucin sulphation within the colon starts as early as the 14th gestational week in the lower crypts and at 20 weeks in the upper crypts, but *O*-acetylated sialomucins do not appear until after the 23 week.[200, 201] In the foetus and at birth, colonic goblet cells are mainly sulphated.[163] Glycoprotein synthesis appears to be completed by 23 weeks gestation. No affinity for the lectin *UEA* has been detected in foetal or neonatal colon.[201]

In the mouse colon from day 14 of life, sialylated mucin containing goblet cells were only found in colonic crypts and sulphated mucin containing goblet cells were found on colonic cuffs.[163] Acidic mucins predominate at birth and the ratio of neutral to acidic mucins increases between birth and weaning and decreases after weaning.[163] However, under germfree conditions, the ratio of neutral to acidic mucins in the mouse colon is higher, and sulphated mucins appear to increase at the expense of sialylated mucins which suggests that exposure to bacteria may influence mucin structure.[163] The human

adult pattern of sialylated mucins in goblet cell crypts and sulphated mucins in the cuffs becomes more distinct in the distal infantile colon after weaning.[163]

Studies on weaning pigs have found an increase in mucin secretion with age as well as an increase in the sulphate content of the mucins in the older animals.[202] Further lectin binding studies on the structure of membrane bound mucins in these animals have shown differences. The expression of membrane glycoconjugate moiety Neu5Ac α-2,6 recognized by *Sambucus nigra* agglutinin-1, was high in newborn pigs, declined in suckling animals and was very low in weaned animals. Conversely, the expression of the membrane bound moiety Neu5Ac α-2,3 recognized by *Maackia amurensis* agglutinin-2 was low after birth but increased in the older animals.[203]

Robbe-Masselot *et al* looked at lower gastrointestinal foetal oligosaccharides and found that the majority were based on core 2 structures and in contrast to adult mucins, Sd^a/Cad determinants were not expressed on foetal *O*-glycans and there was not an acidic gradient along the intestinal tract.[204] During foetal life there is expression of type 2 blood group substances A, B, H and Le^b by secretory mucin throughout the gastrointestinal tract. [102] After birth these blood group sequences are restricted to the foregut and midgut. [102]

Organ culture experiments measuring mucin production by colonic mucosal biopsies have shown that older children incorporate a greater proportion of sulphate compared to glucosamine, especially in the secreted mucin fraction.[205] Children under 3 months produced the greatest amount of mucin per unit DNA and those children between 3 months to 3 years the lowest. Further studies measuring both radiolabeled and unlabeled mucins have shown similar results with a high turnover in the youngest

children, a drop in turnover in the middle age group followed by a rise in the oldest children.[205]

Looking at these age groups further, there was a statistically significant increase in the ratio of mucin sulphation incorporation between the youngest and the oldest age groups and a linear increase in the sulphation with increasing age. Other experiments have shown that this was due to an increase in sulphate and not a reduction in glucosamine incorporation. [205] *O*-acetylation of sialic acids appears to occur at similar levels to adults.[169]

Mucins are an important part of mucosal defence and their alterations in quantity and quality have been implicated in many different disease processes. Colonic mucin defence evolves during infancy and childhood so it is logical to assume that the pathogenesis and disease progression of conditions affecting the colon in children may be influenced by changes in mucin integrity. One such condition is Hirschsprung's disease.

Chapter 2: Hirschsprung's Disease and the Enteric Nervous System

Hirschsprung's disease (HD), or congenital intestinal aganglionosis, is a congenital condition of unknown origin characterised by an absence of ganglion cells in the distal colon. It usually presents in childhood and can cause considerable morbidity and even mortality in those children affected.

2.0 Hirschsprung's Disease – Historical Background

In 1691, Frederick Ruysch a Dutch anatomist, described a child with intestinal obstruction who was probably suffering with HD. However, it was not until almost two hundred years later in 1886 that this condition was formally characterised by Harold Hirschsprung. He initially presented the case of two children to the Paediatric Congress in Berlin, the title of his presentation being "Constipation in newborns due to dilatation and hypertrophy of the colon". He described the post mortem findings of colonic distension and muscular hypertrophy occurring proximal to a smaller normal size rectum.[206] The two patients described died at eight and eleven months respectively, with unrelenting constipation, malnutrition and enterocolitis.[207, 208] Hirschsprung went on to describe the clinical consequences of this disease in ten additional children in case reports in 1904 calling the condition congenital dilatation of the colon.

The pathogenesis of this disease was not immediately apparent and three basic theories were proposed. The first suggested that there was a malfunction in the hypertrophied colon; the second that there was a mechanical obstruction in the colon or rectum. The last theory was the spastic theory, where the distal colon contracted spastically leading

to a functional obstruction. Tittel first proposed that the distal colon was the actual site of the abnormality and not the more proximal dilated colon and he identified an absence of ganglion cells in a Hirschsprung's child as far back as 1901. Unfortunately confusion remained as to the cause of the disorder until the late 1940s due to the failure to separate Hirschsprung's children from those with primary constipation.[207, 208]

Ehrenpreis in 1946 was the first to appreciate that the colon became dilated as a secondary process to the distal functional obstruction and two years later two separate groups definitively documented the absence of ganglionic cells in the myenteric plexus and normal innervation in the proximal dilated colon was also described. [209, 210]

In the late 1940s the clinical observation was made of improved health in children with HD after a colostomy in the ganglionic bowel followed by deterioration when the colostomy was later closed.[211] The combination of this observational evidence with the histological findings established the absence of ganglion cells as the main abnormality in the pathogenesis of HD.[212] This led to the development of rectal biopsy as a diagnostic test and a treatment strategy aimed at removing the abnormal segment of colon.[213, 214]

The first description of a reconstructive operation was by Swenson, the principle being resection of the abnormal aganglionic rectum and re-establishment of bowel continuity by a pull-through operation which brought the normal ganglionic bowel down to the anus, where an anastomosis was performed.[214, 215] In subsequent years a variety of surgical procedures have been developed, the commonest being variations in pull-through technique, most notably by Duhamel and Soave.[216, 217]

The Duhamel procedure was designed to prevent dissection anterior to the rectum and as a method appropriate for use in infants. It involves bringing the ganglionic bowel

down to the anus behind the aganglionic rectal stump, an anastomosis being formed with the back wall of the anal canal above the dentate line.[216] Martin's modification, commonly used today, adds a proximal suture anastomosis between the rectal stump and the pulled-through bowel. The resulting septum between the two lumens is either crushed, as in the original description, or nowadays divided with a stapling device. [218] This creates an expanded rectum, the anterior wall aganglionic and the posterior wall ganglionic.

In the Soave procedure the mucosa is stripped off the aganglionic bowel leaving the muscular sleeve of aganglionic rectum in situ. The normal ganglionic colon is then pulled-through the rectum wall and either left hanging out of the anus to form adhesions with the anal canal as originally described [217] or sutured to the anus.[219]

2.1 Hirschsprung's Disease – Incidence and Aetiology

Hirschsprung's disease occurs in approximately 1:5000 live births.[208, 220] The male to female ratio varies depending on the length of colon affected, being 4:1 for the most common type, rectosigmoid Hirschsprung's disease, and 2:1 to 1:1 for longer segment disease. [221,222, 223] In the majority, approximately 75-80%, the area of aganglionosis is limited to the rectosigmoid region [3, 224, 225, 226] but it can occur throughout the colon, extending proximally in continuity and in a few cases involves the small bowel.[3, 223, 224, 227] Total colonic aganglionosis occurs in about 15% of cases.[222, 228]

Eighty percent of HD cases are sporadic but the remaining twenty percent are inherited as an autosomal dominant trait with incomplete penetrance and variable expression.[229, 230, 231]

Children with HD can also be affected by other congenital abnormalities, these being present in 18-29% of patients.[221, 232] In premature infants the incidence of other congenital abnormalities is higher, up to 47%.[233] In particular Hirschsprung's disease is known to have an association with congenital heart disease and Down's syndrome, [234] as well as trisomy 18, Ondine's curse, Currarino's triad and Smith-Lemli-Opitz syndrome. [235,236,237] Intestinal atresias, in particular colonic atresias, have been found in Hirschsprung's patients.[238]

2.2 Hirschsprung's Disease – Clinical Presentation

Hirschsprung's disease results in a functional obstruction of the colon which can manifest clinically in several ways. The commonest presentation is a failure to pass meconium within 48 hours of birth. Around 90% of neonates with Hirschsprung's do not achieve this compared to 98% without the disease who do.[225, 239, 240] Failure of meconium passage can progress to acute intestinal obstruction with abdominal distension and bilious vomiting. Consequently obstructive enterocolitis can occur or even bowel perforation if the condition is not diagnosed and treated. [232]

Not all children with HD present as neonates in this manner. In 5% of patients constipation is the sole symptom [232] with these children tending to be diagnosed later, in infancy or early childhood, with chronic constipation. [232] Often these children only open their bowels with the aid of suppositories or enemas and classically produce an explosive passage of faeces after rectal examination. Late presenters may also have a history of vomiting, abdominal distension and failure to thrive. [232]

2.3 Hirschsprung's Disease – Pathophysiology

The underlying abnormality in Hirschsprung's disease is an absence of enteric ganglion cells in the distal intestine. (Figures 2.1 & 2.2) This results in the failure of relaxation of the affected bowel causing it to remain in a contracted state. The proximal ganglionic bowel consequently dilates and hypertrophies, becoming thicker and longer than normal. The taeniae disappear and the longitudinal muscle appears to completely surround the intestine. [241] The distal aganglionic colon appears macroscopically normal.

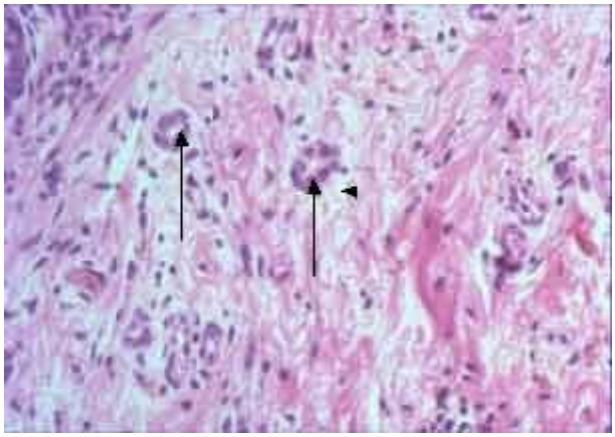


Figure 2.1: Histology section from a rectal biopsy from normal bowel demonstrating ganglion cells (arrows). (Farr G. The Autonomic Nervous System. BecomeHealthyNow.com.)

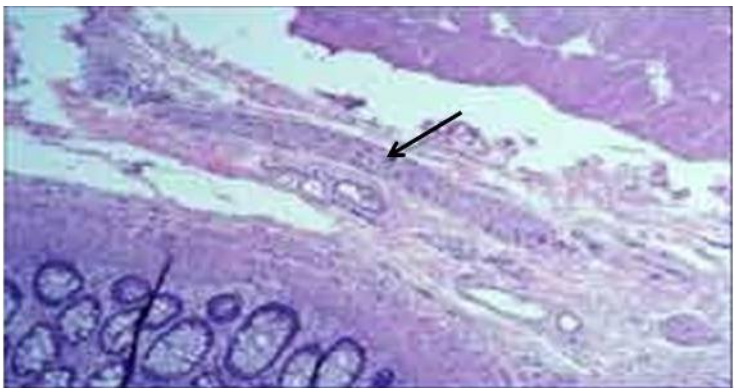


Figure 2.2: Histology section from a rectal biopsy from a Hirschsprung's patient showing no ganglion cells and thickened nerve trunks (arrow). (Farr G. The Autonomic Nervous System. BecomeHealthyNow.com)

The mechanisms which lead to the development of HD are unclear and probably multi-factorial. A disruption in one of the many pathways necessary in the development of the enteric nervous system could result in an absence of ganglion cells. Cells may fail to migrate to the intestine correctly or once there may not be able to proliferate, differentiate or survive. A familiarly with the normal function and development of the enteric nervous system is necessary in order to appreciate the different factors involved and how these may be altered in HD.

2.4 The Enteric Nervous System – Structure

The enteric nervous system (ENS) is defined as the system of neurons and their supporting cells within the walls of the gastrointestinal tract.[242] It is part of the autonomic nervous system which also includes the parasympathetic and sympathetic nervous systems. The ENS is the most complex of the autonomic systems in terms of the number of different functional types of neurones found, their interconnectivity and in their expression of neurotransmitters.[243, 244] ENS neurones can differ in the direction their axons project (orally, anally, radially or circumferentially) in the target of their axon (e.g. other neurones, smooth muscle) and in the neurotransmitter that they use.[245]

The ENS not only mediates gut motility, it also plays an important role in the secretory activity and water and electrolyte transport by the mucosa and in the regulation of intestinal blood supply.[246] Many ENS neurones receive input from the para and sympathetic nervous systems but there are also complex systems within the ENS which allow it to function independently.[242]

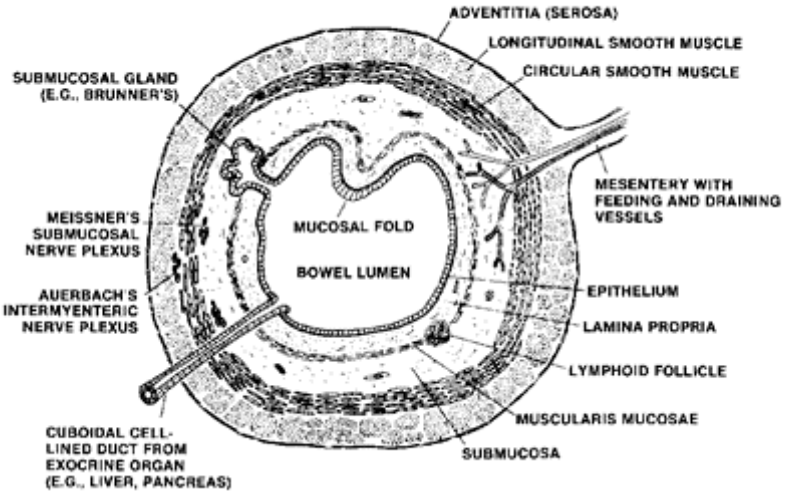


Figure 2.3: Schematic representation of the layers of the intestine showing the nerve plexi. [247]

The basic organisation of the ENS is numerous ganglia connected by bundles of nerve fibres, called internodal strands, lying in two main plexuses.[248] The intermuscular myenteric (Auerbach's) plexus, lying between the circular and longitudinal muscles and the submucosal (Meissner's) plexus internal to the circular muscle layer.[242, 248] (Figures 2.3 & 2.4) These are continuous around the circumference and along the small and large intestine.[248] There is also a much smaller mucosal plexus.[248] Each enteric ganglion is a cluster of many different neurones and all ganglia contain similar types of neurones, although there is some regional variation in this as well as in ganglia size and connectivity.[242]

Functionally the neurones of the ENS can be divided into three subgroups; sensory, which receive information from the gut lumen, inter-neurones which process this and pass it to the third subgroup, motor neurones, the effector cells which innervate the smooth muscle and secretory glands.[249]

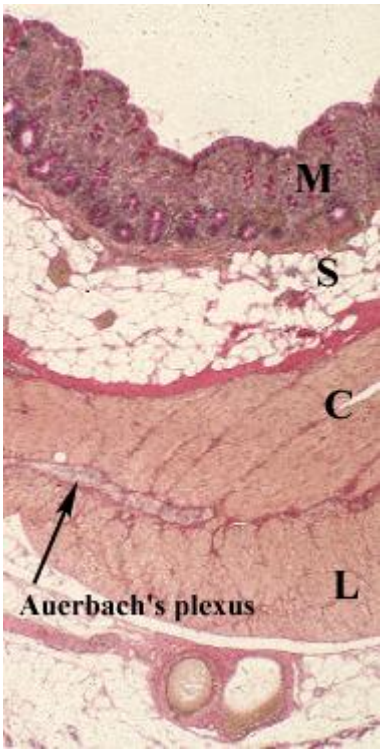


Figure 2.4: Histological section showing the layers of the intestine wall and their relation to Auerbach's plexus. M = mucosa, S = submucosa, C = circular muscle, L = longitudinal muscle. (The Digestive System. www.med.mun.ca/anatomyts/digest.digtut.htm)

Within the ENS are networks of cells associated with the external muscles of the gut and with the nerve fibres innervating the intestine. These are called interstitial cells of Cajal (ICC) and form into subpopulations with differing physiological functions.[250] They are derived from local gut mesenchyme and not from neural crest cells.[251] Some ICCs act as 'pacemaker cells', generating slow electrical waves that cause rhythmical muscle contractions and others are intermediaries in neuromuscular transmission.[250] ICCs expression the receptor tyrosine kinase (Kit) and their development is dependent on the stem cell factor-Kit signalling pathway.[252] Their absence or lack in Hirschsprung's disease is unclear as separate studies have demonstrated contradictory results.[253, 254, 255, 256]

These elements act together in an integrated manner to control the functions of the gut, primarily independently, but also under the influence of the extrinsic nervous system.

2.5 The Enteric Nervous System – Function

The ENS is composed of adrenergic and cholinergic nerves and also non-adrenergic, non-cholinergic nerves.[248] The main inhibitory non-adrenergic, non-cholinergic neurotransmitter is nitric oxide (NO).[257] NO is released in response to nerve stimulation of the myenteric plexus causing smooth muscle relaxation. It is synthesized by the activation of NO synthase.[258]

Many other nerve fibres of intramural origin have been identified with various different neurotransmitters and messengers, mainly neuropeptides, including substance P, vasoactive intestinal peptide (VIP) etc.[259] In normal circumstances there is a fine balance of those forces controlling relaxation and contraction of smooth muscle, with a state of relaxation predominating.[241] An intrinsic gut reflex allows intestinal content to travel to the anus as the mild distension the bolus causes initiates a contraction proximally and a relaxation distally. In the absence of ganglion cells this fails to occur.[241]

Extrinsic control is through both preganglionic cholinergic fibres and postganglionic adrenergic fibres. Parasympathetic cholinergic fibres release acetylcholine and cause muscle contraction. Sympathetic adrenergic fibres mainly inhibit contraction, although may also act via some excitatory pathways and use norepinephrine as their mediator.[259]

2.6 The Enteric Nervous System - Hirschsprung's Disease

In Hirschsprung's disease histological examination of the affected segment shows an absence of intramural ganglion cells in Meissner's and Auerbach's plexuses.[239] The loss of ganglion cells results in the loss of the intrinsic, predominantly inhibitory, enteric nerve function. The major mediator of this system is nitric oxide and a loss of the enzyme required for its production, nitric oxide synthase, has been found in the myenteric plexus of aganglionic bowel.[258] It has been suggested that this is due to a decreased or absence expression of neuronal nitric oxide synthase gene at the mRNA level.[260]

The extrinsic nervous system seems to respond to this by markedly increasing its innervation of adrenergic and cholinergic nerve fibres in the aganglionic segment of bowel.[261, 262] Thickened nerve trunks are also seen in between the smooth muscle layers and extend into the submucosa.[208] These fibres are detected by acetylcholinesterase staining.[260]

Several other neuronal abnormalities in aganglionic colon have been reported, especially in peptide-containing fibres. There is a virtual absence of enkephalin and gastrin-releasing peptide (GRP) – containing nerve fibres in the aganglionic colon [263] and a markedly reduced supply of others such as substance P and vasoactive peptide.[263, 264] A release of substance P can cause vasodilatation, an inflammatory response and modulate mucosal secretion.[265] In contrast a hyper-innervation of neuropeptide Y-containing fibres in aganglionic colon has been found.[266]

Other documented abnormalities include an increased level of neuroendocrine cells [267] and altered gene expression of certain types of muscarinic acetylcholine receptors in the smooth muscle of aganglionic bowel.[268]

It appears that the combination of a loss of the intrinsic inhibitory nerves coupled with an excess of excitatory extrinsic stimulus leads to an imbalance of normal smooth muscle contractility resulting in a failure of the distal bowel to relax and dilate, resulting in a functional obstruction.[241] The proximal bowel dilates with faeces and appears abnormal but has normal innervation and occurs as a consequence to the contracted aganglionic bowel. Proximal to the aganglionic bowel is an area of hypoganglionosis called the transition zone.

2.7 The Enteric Nervous System – Development

In normal embryologic development the enteric nervous system arises from the migration of neural crest cells.[269] These arise between the neural plate and the epidermal ectoderm, along the whole length of the body axis.[245] Ablation studies in chick embryos at various axial levels by Yntema and Hammond revealed that only disruption in one small region prevented enteric nervous system development. This they termed the vagal level which was level with somites 1-7. They concluded that vagal neural crest cells gave rise to the ENS.[269, 270] This was confirmed by studies transplanting neural segments between chick and quail.[271]

The contribution of neural crest cells from the lumbosacral region to the ENS has been contentious but recent work has concluded that this is the source of some hindgut ENS neurons and glial cells.[272] As vagal crest cells are able to give rise to a range of differentiated neurones and glial cells, this suggests that the purpose of cells originating

from the sacral region, is to perform a specific function not fulfilled by vagal derived cells.[245] Other recent work has suggested that cells caudal to the vagal crest also contribute to the foregut. [273]

Neural crest cells migrate to the upper end of the alimentary tract and proceed in a distal direction.[274] The first nerve cells arrive in the oesophagus at the fifth week of gestation, the midgut by the seventh week and have migrated to the distal colon by the twelfth week.[245] After these cells have colonised the gut, they must migrate radially to form firstly the myenteric plexus and then subsequently the submucosal plexus.[275]

Neural crest cells appear to be guided in their migration by glycoproteins, including fibronectin and hyaluronic acid (laminar protein). The development of these glycoproteins precedes neural crest cell migration and these supporting fibres progress down the gastrointestinal tract, through the basement membrane and end in the muscular layer.[276] Neural crest cells then use these structures to migrate to the correct destination.

Once neural crest cells have reached the correct region of the gut they need support to survive there which may be provided in the form of trophic factor signalling. Next they need to proliferate in order to form the vast number of cells necessary for the entire gut from a relatively small population of neural crest cells and then differentiate with accurate orchestration so that the ENS can acquire the appropriate number and types of neuronal and glial cells.[249] These cells then cluster to form ganglia. The normal distribution of ganglion cells is present by 24 weeks gestation and these continue to develop *in utero* and into childhood. [277] Failure of one component during any of these processes could lead to aganglionosis.

RET is a cell surface molecule encoded by the *RET* proto-oncogene. It contains an extracellular ligand-binding domain, a transmembrane segment, a cytoplasmic kinase domain and a carboxy-terminal.[249] All classes of RET have been shown to play a critical role in a variety of developmental processes and some have been implicated in human disease.[278, 279] *RET* transcripts are detected in enteric neural crest cells prior to invasion of the foregut mesenchyme and are maintained at high levels throughout migration and colonisation of the gut wall.[271] Studies looking at the site of *RET* expression in the developing embryo clearly suggest that *RET* encodes for a receptor which interacts with a growth factor involved in the survival, proliferation, migration and differentiation of neuronal cell lineages.[249, 280, 281]

Glial cell line-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor – β super-family. It is GDNF which acts via its receptor, RET and co-receptor GFRα1 on neural crest cells, activating the tyrosine kinase domain of RET.[249, 282] It is not only a mitogen and survival factor for these cells but it also induces their differentiation into neurones.[244, 283] GDNF is widely expressed during embryogenesis by gut mesenchyme cells. Neurturin (NTN), a member of the GDNF ligand family, also activates RET by binding to GFRα2 and plays a role in the support and proliferation of a subset of enteric neurones.[284] (Figure 2.5)

The caudal directional movement of crest cells is thought to be due to a least two factors. Firstly the response of these cells to contact with each other tends to stimulate movement in a direction away from that contact, i.e. towards a region with a lower density of cells.[285] Secondly crest cells are chemotactically attracted to GDNF *in vivo* and if these cells use up GDNF as they migrate this could produce a gradient for chemotaxis to occur.[245, 286]

SOX-10 (Sex-determining Region Y (SRY)-box 10) is a member of the SRY-related family of HMG -containing transcriptional regulators (high-mobility group). It appears important for neural crest cell survival as absence leads to apoptosis of peripheral ganglia derived from these cells.[287,288] PAX-3 (paired box 3) is another transcription factor involved in ENS development. It functions with Sox-10 to regulate RET expression in mice.[289] Initially those neural crest cells destined for the ENS express SOX-10. They are then induced to express RET and under the influence of GDNF they maintain their multi-potential capacity, proliferate and colonise the entire bowel.[249]

Another important system in ENS development is the ET_B/ET-3 signalling system. The endothelins are a family of 21-amino acid peptides (ET-1,-2,-3) which act on two cell-surface receptors, ET-A, ET-B, coded for by EDNR-A and EDNR-B genes respectively.[242, 249, 290] They are intercellular local messengers and are first produced as precursors called pre-proendothelins which are cleaved by endothelin-converting enzyme 1 (ECE -1) to their biologically active forms.[290] The ET_B receptor gene is on chromosome 13q22 and codes for a G protein-coupled receptor with seven transmembrane domains.[242] In adults this system is important in the regulation of blood pressure by controlling tone in the microvasculature but is also involved in regulating morphogenesis during early embryologic development.[242]

Animal studies by Baynash et al found evidence for the involvement of ET-3 in ENS development by demonstrating that in mice, targeted disruption of the gene encoding ET-3 caused a phenotype of white spotting and megacolon and that in the lethal spotting mouse ET-3 is mutated. [277, 291]

The ET_B receptor is expressed on neural crest cells before their emigration and throughout their migration.[292] ET-3 and its activating enzyme ECE-1, are produced by gut mesenchyme cells and act via this receptor as a brake on the differentiating role of GDNF without affecting the proliferative role.[293] ET-3 may therefore maintain cells in the mitotic and migratory pool, thus allowing a large pool of enteric neuronal precursor cells to build up.[294] (Figure 2.5)

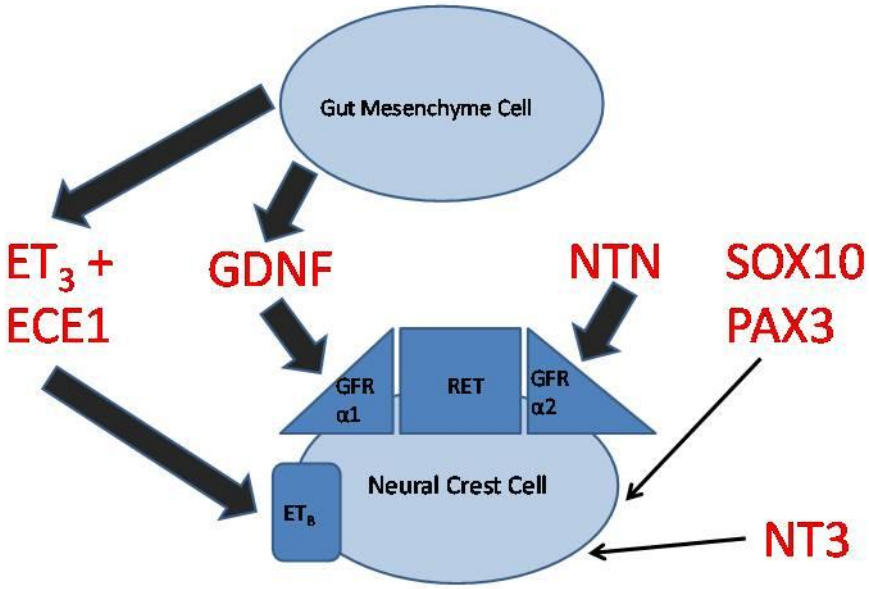


Figure 2.5: Schematic representation of some of the signalling pathways involved in enteric nervous system development. Receptors are shown in dark blue.

As the cell population in the gut increases, cells are allowed to mature and aggregate to form ganglia, perhaps due to reduced amounts of neurotrophic factors or reduced response by the cells.[249] ET-3 may exert this action by inhibiting the activity of the transcription factors SOX-10 and PAX-3 as these act synergistically to promote differentiation in ENS neurones.[245] ET-3 also promotes the development of enteric smooth muscle and this pathway is also intrinsic to melanocyte development.[293]

There are many other signalling pathways thought to be important in ENS development. MASH-1 (mammalian achaete-scute homologue 1) is a member of a family of transcriptional regulators involved in peripheral nervous system cell differentiation. It appears to be required for the development of the serotonergic sub-lineage of the ENS.[249, 295] Other transcription factors intrinsic to ENS formation include PHOX-2a and 2b, HOX-1, and IL-1. [241, 248, 296]

NT3 is a neurotrophin synthesized by intestinal epithelial and mesenchymal cells.[297] It is thought to regulate the survival and differentiation of enteric neurones mainly by interacting with TrkC receptor tyrosine kinases.[297, 298] Studies have suggested that GDNF and NT3 are components of two independent signalling systems which operate sequentially during embryogenesis to promote neurogenesis of the gut, GDNF being needed earlier in the sequence.[249]

Hedgehog proteins are secreted proteins which participate in development processes and are known to influence ENS development in models.[242] Indian hedgehog (Ihh) and Sonic hedgehog (Shh) in particular seem to influence ENS development. Shh is thought to be important for the radial patterning of the gut so that the smooth muscle cells and neurones differentiate in the outer layers.[242]

Recent work has also suggested a role for bone morphogenetic proteins (BMP), as these were strongly expressed in the ENS and their inhibition led to hypoganglionosis and failure of enteric ganglion formation, with crest cells unable to cluster into aggregates.[299]

The development of the ENS from neural crest cells involves many different pathways. It is also a very interactive process with signalling occurring between mesenchymal cells and enteric neurones. For example, ICC are seen in the absence of enteric neurones

but at much lower levels suggesting that neurones produce substances which play a role in ICC development.[300]

Mutations within any of these pathways could affect the stem cells ability to migrate, proliferate, differentiate or survive. Defects could occur within the cells themselves or within their environment; at their site of origin, along their migration pathway or at their destination, within the intestine.[242] Many of the genes coding for key peptides at various stages of these pathways are associated with Hirschsprung's disease.[277]

2.8 Hirschsprung's Disease - Pathogenesis

Neural crest cells contribute to many other structures throughout the body. They differentiate into diverse cell types including neurons and glia of the sensory, sympathetic and parasympathetic ganglia, pigmented cells, neuroendocrine cells, adrenal medulla and facial cartilage.[301] Diseases which arise from the neural crest are grouped together and given the name neurocristopathies. These are divided into two basic forms; simple involving a single pathological process, which are generally unifocal and localized and the more complex neurocristopathy syndromes which are multifocal.[183] HD is classified as a simple neurocristopathy and can occur in isolation or associated with other conditions, for example Shah-Waardenburg syndrome, which involve other systems originating from abnormal development of the neural crest.[183]

In 1967 Okamoto *et al* suggested that the absence of ganglionic cells in HD was due to failure of neural crest cell migration. Evidence for this came from animal studies where transection of a chick embryo at the hindgut level resulted in distal aganglionosis, which could be corrected by subsequent implantation of pigeon ganglion cells distal to the transection level.[241]

Although migration failure may be part of the underlying mechanism, neural crest cells must also proliferate, survive and differentiate in order to give rise to fully fledged enteric ganglia.[230] The intestinal environment must be conductive to these processes.

HD has a strong genetic component. The risk of the disease in a further sibling is 4% compared to 0.02% in the general population and this risks increases with the length of the affected bowel.[242] Several specific gene mutations have been identified in human and animal models of HD but many cases remain unexplained. Known gene mutations account for less than 50% of distal aganglionosis cases [302] suggesting that some cases are either not genetic in origin or their genetic basis is still unknown. To date at least eight HD genes have been identified but it remains a complex inheritance disorder.[291] Often a single mutation may not be enough for the disease to manifest. Clinical expression of HD appears to require the interaction of multiple susceptibility genes.

Two of the important signalling systems involved in ENS development have been implicated in the pathogenesis of HD. These are the GDNF-RET/GFRα1 and ET-3-ET_B. Mutations affecting any of the aspects of the RET/GDNF system could affect neural crest cell migration, differentiation and survival.

RET was first implicated in the pathogenesis of HD by linkage studies showing that an autosomal dominant gene for HD mapped to a region where *RET* had been previously localised. Subsequent mutational analysis showed that around 50% of patients with the inherited form of HD had mutations in *RET* and they were found in 70-80% of those with long segment disease.[226, 277, 291] Mutations identified range from whole gene deletions to missense mutations, base pair deletions and splicing alterations and are the commonest forms of mutations found in HD patients.[279] Further mutations may

indirectly affect the production of RET, for example those involving promoter or other regulatory regions.

Mutations in the GDNF component of the RET signalling system have also been reported in HD in up to 4.6% of patients.[291] In functional assays the absence of GDNF or GRFα1 results in a reduction or absence of RET signalling.[303] There is also some evidence that the expression of GDNF may be reduced in the absence of an identifiable genetic mutation. Martucciello *et al* demonstrated an absent immunoreactivity for GDNF in the aganglionic segment compared to the normoganglionic segment in HD patients.[291] This could represent an important co-factor in the pathogenesis of HD, where even in the absence of a mutation in the *RET* or *GDNF* genes, a lack of GDNF expression may prevent the necessary RET activity and cause neuroblast migratory arrest.[291]

Much of the information on ENS development and the genetic basis of HD has come from animal studies. As melanocytes are also derived from the neural crest, many of these models also exhibit pigmental changes. The lethal spotting (*ls*) and piebald lethal (S^l) mouse strains have aganglionic colons and pigmentation defects, the former allows reproduction, so as a consequent has been more extensively studied. [304] The rat model of spotting lethal (*sl*) also demonstrates total colonic aganglionosis. [305] The dominant megacolon (*Dom*) mouse has an autosomal dominant inheritance, the homozygous genotype being lethal in early embryogenesis, the heterozygote dying early in life and exhibiting distal aganglionosis.[306]

Homozygous mice with defects in *RET*, *GDNF* or *GFRα1* die at birth and have kidney agenesis and intestinal aganglionosis.[307] Studies on knock-out mice, where the gene encoding for *GFRα1* was missing, resulted in the absence of all mid and hindgut

neurones except for a few in the distal colon.[307] Assuming these are neurones derived from the lumbosacral region, it indicates that the vagal neural crest cells are dependent on the GDNF/RET signalling system in a way that the sacral crest cells are not.[307]

Approximately 10% of patients with HD have a mutation in one of the genes involved in these endothelin signalling pathways, [277, 291] about 3-5% of HD cases appear to stem from ET_B mutations.[308] This is a dominant effect with incomplete penetrance. In the piebald S^l mouse and also the *sl* rat, there is a mutation in the *ET-B* receptor gene.[291] The main ligand for the receptor in the gastrointestinal tract is ET-3.[309] Mutations in the *ET-3* gene, on chromosome 20q13.2-13.3, are found in about 5% of HD patients as well as in the *ls* mouse model.[310] The gene encoding for endothelin converting enzyme (ECE-1), necessary for production of active ET-3, has been found to be mutated in certain complex abnormalities which involve aganglionosis.[311]

Mutations within the endothelin system appear to affect specific subpopulations of enteric neural cells and do not cause the catastrophic elimination of the entire ENS as occurs in GDNF/RET mutations. Two groups have proposed the theory that deficiencies in the ET-3/ET-B system may cause premature differentiation of the neural crest cells, resulting in a smaller population to migrate and proliferate in the distal intestine.[293, 312] Animal studies looking at blocking this pathway and its affect on distal colonization have been contradictory.[292, 313]

Transcription factors are proteins which bind to regulatory elements of DNA, thereby promoting or repressing the transcription of the gene. In this way they regulate gene expression.[242] Phox2a and Phox2b are transcription factors which are expressed in enteric neurons, some parasympathetic neurons and all noradrenergic neurons.[314] Both regulate the expression of RET as well as the noradrenaline synthetic enzymes, tyrosine

hydroxylase and dopamine β hydroxylase.[315, 316] In Phox2b knock-out mice, neural crest cells failed to migrate beyond the foregut and certain polymorphisms of this gene in humans have been associated with HD.[315, 317]

Other transcription factors have been linked with HD. In the *Dom* mouse model, a mutation of the gene encoding Sox-10 results in distal aganglionosis.[287] Sox-10 gene targets are unknown but mutations affecting its function have been reported in HD. [318] Pax-3 is also thought to be needed for the initiation of RET expression by enteric neuron precursors.[289] Mash-1 appears to be necessary for neuronal cell differentiation but not their migration and this shows very close homology to the human version Hash-1.[295] Other possible candidates for involvement in ENS development and HD pathology include the Hox transcription factor family and SIP1, a repressor of transcription.[319]

Defects in the neurotrophin (NT) family of neurotrophic factors may also play a role in HD aetiology. Targeted mutation of the genes encoding NT-3 and its receptor, TrkC, resulted in a decreased number of enteric neurones in mice and reduced levels of TrkC expression have been described in HD.[298, 320] Other genes which have been implicated include PMX2B.[321]

There are other abnormalities that have been reported in HD which could influence neuroblast migration. Abnormal distributions of fibronectin and laminin have been demonstrated in the aganglionic intestinal wall and these are thought to provide a framework for neural crest cell movement.[322, 323] In order for neuroblasts to migrate, they must also adhere to the intestine cells. Neural cell adhesion molecule (NCAM) has been found to be reduced in the aganglionic colon compared to both controls and ganglionic tissue from Hirschsprung's patients.[324]

Vascular anomalies have been documented in HD. Abnormal arteries have been found in the HD colon where there was a proliferation of smooth muscle cells in a thickened adventitia.[325] Functional defects of vascular endothelin B receptor have also been found. [326] Vascular defects resulting in ischemia may interrupt ganglion cell migration or affect their survival once migration has occurred.

HD is a multigenic disorder, principally caused by dysfunction in either of the two main signalling pathways involved in ENS development; RET/GDNF and ET3/ETB. A single mutation in ones of the genes involved is likely to add a variable contribution to the final disease phenotype, each one adding a degree of genetic susceptibility.[291]

2.9 Hirschsprung's Disease – Treatment

The underlying principle of management of Hirschsprung's disease is to relieve the functional obstruction caused by the aganglionic segment.[239] The definitive treatment is surgical and there are two main ways of surgical management. The first option is to perform a diverting colostomy in ganglionic bowel, confirmed by intra-operative frozen section, soon after birth, once the diagnosis has been confirmed by rectal biopsy. [327] Following this at the age of 3-6 months the definitive corrective surgery is performed. This is called a 'pull-through' procedure and involves the removal of part of the aganglionic section and suturing of the ganglionic colon to the rectum. During surgery intra-operative sections are sent for immediate histological examination to confirm that only ganglionic colon is being used. This is referred to as a staged pull-through. The colostomy is either closed at this point (two stage) or in a separate operation at a later date (three stage).

The second option is known as a primary pull-through.[328] This dispenses with the intermediate colostomy and instead relies on regular rectal wash outs to keep the colon deflated and clear of a build up of faeces until the child is old enough (usually 3 months) for pull-through surgery. Some surgeons perform a primary pull-through within weeks of birth although this is not one of the management methods practiced at the centres involved.[329, 330]

Several difference pull-through procedures have been described and are used in difference institutions but all employ the same underlying principles of relieving the functional obstruction without impairing rectal continence or damaging pelvic nerves.[214, 215, 239] The most widely used are the retrorectal transanal pull-through (Duhamel) operation where a neorectum is created with an anterior aganglionic half and a ganglionic posterior half and the endorectal pull-through where the proximal normal colon is pulled through the aganglionic segment from within after first stripping it of its mucosa.[216, 217, 219, 239] (Figure 2.6)

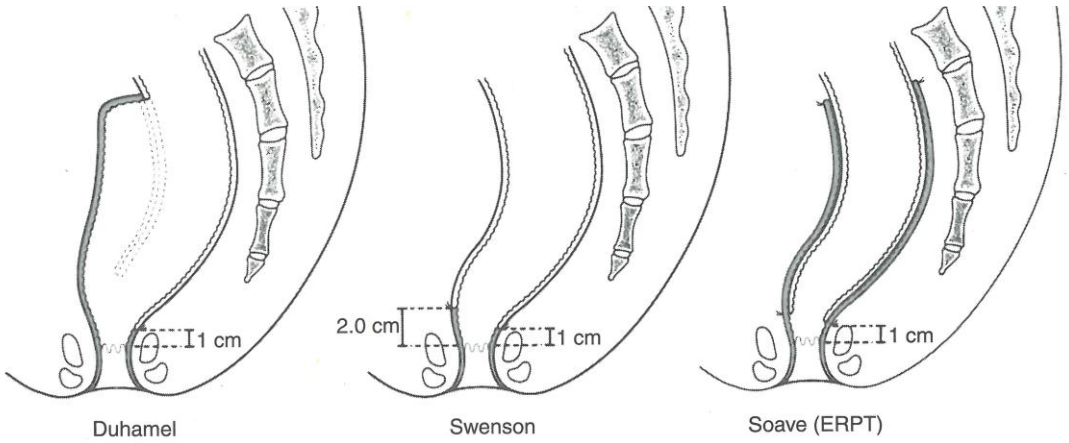


Figure 2.6: Schematic representation of the different types of pull-through procedure for Hirschsprung's disease. The dark lines represent the retained aganglionic bowel. (Modified from Teitelbaum DH. Hirschsprung's disease) [331]

2.10 Hirschsprung's Disease – Outcome

Corrective surgery for Hirschsprung's disease can cause complications, resulting in morbidity and occasional mortality in some children.[241] These can be classified into early, occurring soon after surgery and late. Some of the more common early complications include anastomotic leak or breakdown (2%), rectal pouch problems, faecal fistula, intestinal obstruction which requires further surgery (8–13%) and anal stenosis (10-20%) which can often be managed by rectal dilatations.[241, 332, 333] The overall reported incidence of these early complications ranges from 1.1- 20%.[241, 332,333]

Post operative deaths (within 30 days of procedure) have been reported at between 0 to 1.5% and late deaths due to either complications of the operation or post operative enterocolitis from 1-2.1%.[332]

Surgical correction of HD should ideally allow normal stooling but despite corrective surgery many children have ongoing problems with faecal continence or constipation. Long term outcome is variable and establishing reliable data is problematic due to different criteria being used by different authors.

Typical incontinence rates seem to range from between 3-8%, [241] although higher rates have been reported, [334] but these seem to improve as the patients become older [335] and if neurologically impaired children are excluded, continence rates are similar to age-matched controls. [241, 336]

In one study the outcome was reported as very satisfactory in 67%, in 27% occasional enemas and laxatives were needed and 8% had constipation or soiling.[335] They also found that function improved with time and at 15 years follow-up 88% were considered to have normal bowel function.[335] Yanchar and Soucy found similar improvements with

only 8% having fair to poor continence in those over 15 years compared to 50% in younger children.[334]

Soave reported the outcome as 83% good and 17% as unsatisfactory.[337] The constipation rate after the various procedures has been recorded as 54% after a Duhamel, 43% after a Soave and 4% after a Swenson.[338] Others have found that only 64% of patients had a normal stooling habit but 90% of parents were moderately or very satisfied with their child's outcome.[334] Continence in children over five years of age has been reported at 86%.[339]

2:11 Hirschsprung's Disease - Transitional Zone

In the colon of HD patients there exists a region of hypoganglionosis in between the aganglionic and ganglionic colon.[209] This is referred to as the transition zone (TZ) and usually extends over a few centimetres.[209] This area is known to be abnormal and is characterized by diminished numbers of ganglion cells.[209] Other documented abnormalities include changes in Auerbach's plexus which grows more irregular, tapering towards the aganglionic colon [340] and occasional acetylcholinesterase staining hypertrophic bundles of extrinsic nerves.[227, 340]

The histological features of the TZ are subtle [341] and its margins along the bowel are not symmetrical. White and Langer demonstrated that the circumferential distribution of the TZ is uneven and has a leading edge of ganglion cells which extends into the aganglionic bowel.[342] This leading edge was most often seen on the anti-mesenteric border and ganglion cells tended to cluster within it, in contrast to a complete absence of such cells in the remainder of the circumference.[341] A single biopsy could therefore

give misleading results.[343] In order to ensure the best surgical results the TZ should be included in the resected specimen.[341]

The remaining colon with its complement of ganglionic cells is considered to be 'normal', however this distal section can retain other structural and functional features which may be abnormal or suboptimal.

Sandgren, Larsson and Ekblad compared the number of enteric neurons and the expression of various neurotransmitters along the ganglionic sections of colon in the homozygous (short rectal aganglionosis) and heterozygous (phenotypically normal) lethal spotted mouse model of HD. They reported many differences including a reduction in number of total neuron cell bodies and an increase in VIP expression in both plexi in the homozygous mice.[256]

Abnormalities have also been reported in humans. Neuropeptide nerves are found in normal numbers in the proximal ganglionic colon in HD but in a disordered arrangement.[264] Others have found that proximal ganglionic samples have little immunoreactivity for general neuropeptide markers.[344] It appears that even where ganglionic cells are present other neuronal elements may be deficient resulting in abnormal function of the enteric nervous system.

RET mRNA expression levels were examined in HD patients.[260] Compared to the ganglionic colon, a significantly lower signal was found in the aganglionic segment and also in the hypoganglionic transitional zone, although this signal was higher than that of the aganglionic.[260] Levels of mucin production have also been found to be low in the ganglionic colon nearest the aganglionic colon.[345]

2.12 Hirschsprung's Disease – Enterocolitis

Enterocolitis (EC) is the most serious and potentially life-threatening complication which can affect children with HD. The incidence in the literature varies considerably affecting from 17-50% of HD children, with a mean incidence of 25% and can be the presenting feature of HD.[346, 347, 348, 349, 350, 351] Reported mortality figures also widely differ ranging from 0–39%.[332, 349] Although the mortality for EC has fallen over time, presumably due to improved recognition and critical care, it still remains the major source of morbidity and mortality for HD patients and can lead to lengthy and complicated hospital admissions.[352, 331, 346, 353]

The association between HD and enterocolitis was first recognized in 1956, [354] but the clinical aspects of EC were first accurately characterized by Bill and Chapman who described the classic features of abdominal distension, fever and foul-smelling stools.[346] Symptoms and signs can range from mild to life-threatening and there still remains some discrepancy in the diagnostic criteria used.[348] The natural progression of the disease does, however, show discrete histological phases which have been used to establish a grading system based on the pathological severity. [355] Within the histopathological changes noted, the retention of mucin is particularly characteristic.[355]

The greatest risk of developing EC is before diagnosis of HD and can be the presenting feature of the disease.[332, 351] A delayed diagnosis of HD beyond the first week of life substantially increases the risk of EC.[347] Other factors have also been associated with increased risk of EC. Some authors have found a higher incidence of EC in children with long segment aganglionosis,[347] although other studies have refuted this.[332, 346] Children with HD who also have associated anomalies appear to have a higher risk of developing EC, an incidence of 29% compared to 47% in some studies. [349]

Genetic factors may be important as there is an increased risk of EC occurring in children with a family history of HD [332, 356] although long-segment disease is known to be associated with both a family history and greater risk of EC and these variables have not been studied independently.[351] Once a child has had an episode of EC, their risk of further episodes may be greater.[357]

The aetiology of EC remains unknown. One of the earliest theories postulated was that functional obstruction of the aganglionic bowel led to stasis of faeces causing bacterial overgrowth and infection leading to epithelial destruction, ulceration and subsequent bacterial translocation.[351] However, the fact that EC occurs in 5-35% of patients after corrective surgery, even in the absence of post operative stenosis, [145] and even, if only rarely, in the presence of a defunctioning colostomy, suggests that there must be other factors involved.[339, 346, 347]

Several studies have found evidence for an infectious aetiology, both in abnormal pathogen carriage and impaired immunity. *Clostridium difficile* (C. *difficile)* is one such bacterium which has been implicated. An increased incidence of C. *difficile* in the stools of patients with EC compared to those without and to controls has been reported.[358] Also this bacterium has been detected in the stools of HD patients beyond 12 months of age, demonstrating prolonged carriage, unlike the typical absence of this organism in the gastrointestinal tract of normal children at this age.[359] A further study found that in 39% of children with pre-pull-through EC had enterocyte adherence by bacteria on histological examination, the organisms involved being *Escherichia coli, C. difficile* and *Cryptosporidium*.[353]

Despite this evidence the significance of *C. difficile* in the aetiology of EC remains in doubt. Not only is the bacteria and its toxin found without ill effect in the faeces of

healthy infants and children but other studies have not replicated these results, finding no variation in stool bacterial flora in children with or without enterocolitis.[355, 360, 361] Another proposed infectious candidate is rotavirus, increased levels being found in those children affected with EC by Wilson-Storey *et al.*[360] It is possible that several different infectious agents have the potential to cause EC in the presence of defective mucosal integrity and immunity.

An impairment in host immunity could increase susceptibility to EC. Infants with Down's syndrome (trisomy 21) and HD have a 50% risk of developing EC compared to 29% in all other HD children.[356] Children with trisomy 21 are known to have reduced levels of humoral and cellular immunity, suggesting that immunodeficiency may be involved in the aetiology of EC.[347, 362]

Various impairments in cellular and humoral immunity have been described in HD patients.[351] Alterations in neutrophil response and decreased serum complement levels have been reported.[123] Wilson-Storey and Scobie measured immunoglobulin A (IgA) levels in HD patients. The presence of secretory IgA in saliva was presumed to indicate its presence at the luminal surface of the bowel. Those patients who previously or subsequently developed EC, had increased levels of IgA in the colonic mucosa, lower serum IgA levels and virtually all had an absence of salivary IgA.[363] They hypothesized that a failure of transport of IgA from its point of manufacture across the mucosal cell and into the gut lumen resulted in a susceptibility to EC.[363]

Further studies have also shown a reduction in luminal staining of IgA in EC.[363, 364] Other groups have found low levels of IgA in colonic mucosa.[365] Studies on the piebald mice found higher levels of immunocytes and IgA containing cells in those mice with EC compared to controls.[366]

Natural killer (NK) cells have anti-tumour and antiviral activity as well as potentially being regulators of antibody production.[364] An increase in NK cells infiltrating the ganglionic bowel in EC compared to aganglionic bowel and ganglionic bowel in controls has been reported.[364] This may be related to abnormalities in peptidergic nerves causing regional abnormalities in neuropeptides.[364]

Caudal type homeobox gene-1 and 2 (CDX-1, CDX-2) encode transcription factors in endoderm derived tissues of the intestine.[167] CDX genes control proliferation and differentiation of intestinal mucosal cells. The involvement of mucosal abnormalities in EC has already been suggested by presumed inefficient transfer of IgA across the gut mucosa. The expression of both CDX genes has been found to be reduced in patients with EC which could result in a loss of mucosal integrity and function.[167]

A further feature which is important in mucosal integrity and immunity is the mucus gel layer and its main constituents, mucins. Mucins have several functions including helping to prevent bacterial colonisation of mucosal cells.[5] Enterocolitis complicating HD is still a cause of morbidity and mortality. Its aetiology remains poorly understood but is likely to be multi-factorial.[351] The presence of a robust mucus defence barrier may be important in its prevention.

2.13 Hirschsprung's Disease - Mucin Alterations

Alterations in the sulphate and sialic acid content of mucins can render the mucus layer more susceptible to degradation by pathogens.[164] A reduced secretion of sulphated mucins allows bacteria to adhere more easily to enterocytes, thus facilitating bacterial invasion and infection.[164, 355] Changes in mucins have been reported in HD. Teitelbaum *et al* found a relative increase in neutral mucins and a decrease in acidic sulphated

mucins in those patients with EC compared to those without.[355] Others have reported an increase in sulphated mucins in patients with HD, especially those with EC.[367]

Animal studies on the piebald mice have also demonstrated mucin alterations in HD and EC.[366] A marked depletion of sulphomucin and neutral mucins combined with an increase in sialomucin secretion was found in all piebald mice, those with and without EC, although the changes were greater in those mice without EC.[366]

MUC2 is the main gel-forming mucin in the colon.[352] Levels of MUC2 protein in stool have been shown to be much lower in children with HD compared to controls and undetectable in those who had a current episode of EC. [368] The fact that the levels were lower even in those without EC suggests that there is a true deficit in MUC2 production rather than a secondary response to an infective process. Although mRNA levels for MUC2 have not been found to be significantly different in HD compared to normal controls, posttranslational changes in mucins could result in the abnormal expression of this glycoprotein in HD.[16]

Episodes of enterocolitis are unrelated to the type or timing of surgery [332] and can occur after corrective surgery, 92% of children having their last bout of EC requiring hospital admission within two years of pull-through.[332, 369 , 370] The frequency of these admissions tends to decrease with time. [371] That most children outgrow their episodes of EC could be explained by the fact that in normal children the mucus defensive barrier is at its weakest between 3 months to 3 years, an increased turnover of mucins occurring with increasing age.[205, 352]

Neuroendocrine (NE) cells modulate gut function by endocrine, paracrine or neurocrine pathways. Decreased levels of these have been found in the ganglionic colon of patients with EC compared to those without EC.[165] As mucus secretion is partly under the

control of the ANS, the weakened mucus barrier could result from an underlying ANS abnormality and corresponding reduction in NE cell populations.[165, 364] This would help to link and explain poor mucus secretion and motility disturbances which can persist after surgery.[351] Alternatively NE deficiencies may occur as a secondary response to infection.

Studies by Aslam *et al* investigated the mucin turnover of the aganglionic and adjacent ganglionic colon in HD patients and in controls.[345] The quantity of radioactive labelled mucins produced in 24 hours per µg DNA was measured and the ratio of incorporation of the labelled precursors determined. Not only was the turnover of mucin in both the cellular and secreted fractions of the aganglionic samples reduced compared to controls, this was also the case in the 'normal' distal ganglionic bowel.[345]

What is not clear is whether this abnormal production of mucins is localised to a region close to the transition zone or reflects a more generalised deficiency. Little is known about the mucin production of the 'normal' ganglionic colon in HD. If poor mucin turnover was found to only affect a short segment of ganglionic colon, this additional length could be resected along with the aganglionic colon and transition zone. Presuming that a defective mucin barrier is a major contributor to the development of enterocolitis, this alteration in surgical technique could result in fewer cases of post operative enterocolitis. This hypothesis is strengthened by the finding that in those HD children who underwent corrective surgery, a correlation has been found between a low turnover of mucin precursors in distal ganglionic colonic biopsies and an increased incidence of post-operative enterocolitis.[372]

Further studies have looked in more detail at the colonic mucins in the distal colon in children with HD and have found that the protective modifications of mucins appear to

be present in similar amounts to controls, patients showing matching staining patterns for sulphated mucins within the colonic crypts and similar levels of *O*-acetylation. [16] Similarly the expression of mucin genes has not been found to be altered in HD; in all age groups and in both ganglionic and aganglionic colon MUC1, 2, 3, 4 and 5B were detected in similar quantities.[16]

Lectin binding study results with *Maackia amurensis* demonstrated 50-70% mucin binding with no significant differences seen between aganglionic bowel, ganglionic bowel and normal controls.[16]

The aim of this study was to further investigate the ganglionic and presumably 'normal' colon in children with Hirschsprung's disease.

Chapter 3 - Materials and Methods

3.0 Subject Recruitment and Study Groups

Local ethical approval for the study was obtained through the COREC system. The study was approved for Research Governance purposes by the local Research and Development Directorate of Addenbrooke's Hospital and of Norfolk and Norwich Hospital. All subjects were recruited from paediatric surgical units from one of two hospitals (Addenbrooke's Hospital or Norfolk & Norwich Hospital).

The study group consisted of children with a diagnosis of Hirschsprung's disease made on clinical grounds and confirmed by an absence of ganglion cells on suction rectal biopsy specimens.

The majority of controls were children born with an anorectal malformation who had a diverting colostomy sited soon after birth.

Parents of suitable children were approached before surgery and given a parent information leaflet to read. Consent was obtained using existing ethical guidelines. General practitioners were informed of participation in the study. (Appendix 1 for copies of forms)

3.1 Retrieval of Specimens

The children with Hirschsprung's disease in this study underwent surgical correction, involving removal of the aganglionic segment of colon,[239] by one of two established management options; a three stage approach, with an intermediate colostomy, or by a deferred primary pull-through procedure.[328] In the latter, the colon was kept decompressed by rectal washouts until the time of surgery.

Mucosal biopsies were taken intra-operatively at the time of definitive pull-through surgery in the study group or at the time of colostomy closure in the control group. The most distal biopsy (A) was taken just above the transition zone. In primary pull-through cases this was assumed to be just above where the collapsed distal colon met the dilated proximal colon and in some instances was further identified by sequential intra-operative frozen sections. In the control group and in those in the study group with a colostomy, the most distal specimen was taken from just inside the proximal end of the stoma. Subsequent biopsies were then removed more proximally at 5cm intervals and lettered according to the diagram below. (Figure 3.1)

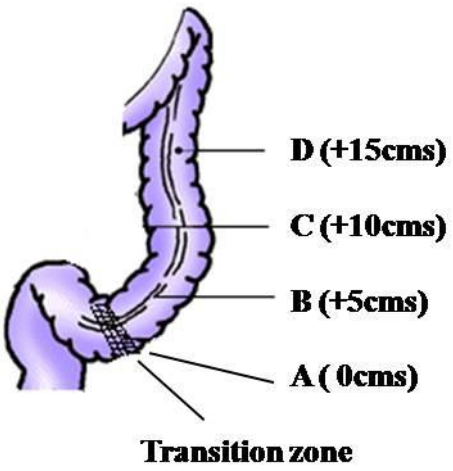


Figure 3.1: Schematic representation of the distal colon and biopsy sites.

The investigator was present in theatre at operation to supervise the collection and labeling of specimens. In those patients recruited by the team at Norfolk and Norwich Hospital, this involved driving sixty miles there, with an appropriate tissue retrieval kit, collecting and labeling the specimens in theatre and then driving back a further sixty miles to the laboratory at Addenbrooke's Hospital. The return journey took at maximum of 1 hour 40 minutes. The laboratory bench space was set up in readiness prior to the journey in order to reduce the time taken to commence organ culture. Biopsies were transferred to the laboratory in PBS at room temperature, and placed into organ culture within a maximum of 2 hours.

3.2 Reagents

Reagents were supplied from Sigma (Sigma, Poole) unless otherwise stated. All tissue culture reagents were obtained from GIBCO (GIBCO, location).

Culture Medium

A working stock of 100mL of culture medium was prepared.

88.9mL Dulbecco's modified Eagles medium

10mL foetal bovine serum (10%)

1mL penicillin (10,000U/mL) invitrogen/gibco

1mL streptomycin (10,000µg/mL) invitrogen/gibco

0.1mL gentamicin (50mg/mL) invitrogen/gibco

The tissue culture medium was sterilized using a 0.22µm filter and stored in 10mL aliquots at -20^0C.

Purification of Guanidine Hydrochloride

Guanidine hydrochloride (reagent grade) was made up to an approximately 7M solution in phosphate buffered saline (PBS) by adding 1kg guanidine, HCl to 1.5L PBS (GIBCO) and stirring at room temperature for 24 hours. The guanidine solution was de-ionised by the addition of 15g activated charcoal and stirred at 4ºC for 24 hours. The suspension was filtered through two pieces of Whatman No 1 filter paper (Labsales, UK) and a further 15g of activated charcoal was added and stirred at room temperature for 24 hours. The suspension was filtered as before and finally through a 0.22μm filter (Milipore). The solution was diluted with sufficient extra PBS to give a final concentration of 6M.

Tris Buffer

A 0.5M Tris/HCL pH 8.0 buffer was prepared; 30.25g Tris base was added to 400mL of distilled water. The pH was adjusted to 8.0 with addition of 50% HCL (v/v) and further distilled water was added to give a final volume of 500mL.

Extraction Buffer

0.5M Tris/HCL buffer 8.0 pH containing 6M guanidine HCL, 10mM dithiothreitol and 5mM EDTA

Inhibitor Cocktail

The cocktail was made up fresh in 10mL batches as required.

15.4mg benzamidine	10mM
6.3mg N-ethylmaleimide	5mM
1mg soy bean trypsin inhibitor	0.1mg/mL
100µL 1M DTT	10mM
100µL 5M EDTA	3mM
250µL 6.8mg/mL PMSF in propan-2-ol	1mM
Final volume	10mL

The cocktail was kept on ice and used immediately.

Gel filtration buffer – 10mM Tris/HCl pH 8.0

Stock solution 5M

50 x dilution = 1 mL in 50mL

Need 500mls = 10mL 5M stock and 490mL water

Gel filtration buffer – 0.5M guanidine HCL/PBS

Stock solution 6M

12 x dilution = 1mL in 12mL

= 100mL in 1200mL

= 100mL 6M stock and 1100mL PBS

Preparation of Dialysis Tubing

Dialysis tubing (visking size 1 inf dia $^{8}/_{32}$: MWCO – 12-14,000 Daltons) was prepared by boiling the tubing for an hour in distilled water containing sodium bicarbonate and EDTA. Subsequently the tubing was stored in 0.1% sodium azide (w/v) until use.

3.3 Organ Culture

Mucosal samples obtained at surgery were prepared for culture using established organ culture methods. [372, 373, 345] After initial dissection in theatre any remaining extra-mucosal layers were dissected off using a sharp scalpel blade so that macroscopically a pure mucosal sample was obtained and this was further divided into $2mm^2$ pieces.

These mucosal pieces were placed in a model type tissue culture dish, (Becton Dickinson) luminal surface up, on a raised steel grid covered with lens tissue overlying the culture dish well. (Figure 3.2)

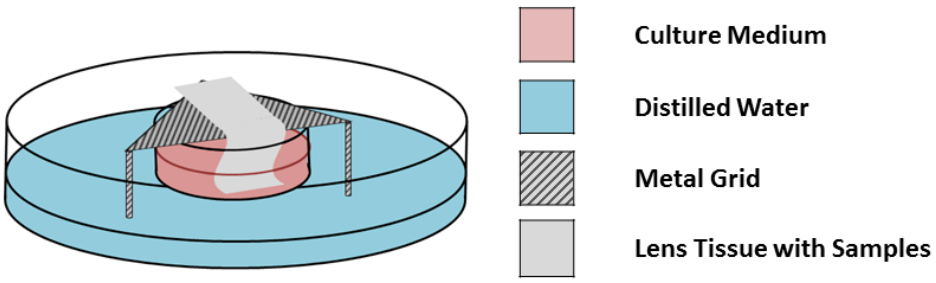


Figure 3.2: Schematic representation of the organ culture equipment.

Two mL of culture medium was placed in the central well of the dish, in contact with the lens tissue and one mL of distilled water was added to the outer rim of the culture dish to prevent drying of the sample. Once the organ culture was assembled 10µCi of each of the radioactive precursors [3H]-glucosamine and [35S]-sulphate (Amersham)

were added to the medium. The mucosal cultures were incubated at 37ºC in a humidified atmosphere at 5% CO_2 for 24 hours

3.4 Cellular Fractionation

Following incubation the culture medium was collected and the mucosal tissue was washed with 1mL ice cold PBS. The medium and PBS wash were combined and dialysed against 5L distilled water over 24 hours. The dialysis step was then repeated for a further 24 hours.

The dialysate was centrifuged at 12,000xg at 4ºC for 10 minutes and the supernatant separated from any formed pellet; this formed the **Secreted Soluble Fraction (SSF)** and was stored at -80ºC until chromatography. Any remaining pellet was retained and was processed with the cellular soluble fraction.

The mucosal tissue was homogenised in 1mL of PBS containing protease inhibitor cocktail along with any pellet remaining from the secreted soluble fraction. Homogenisation was carried out in a manual glass Potter-Elvejhem homogenizer. Approximately 20 strokes were required after which a smooth homogenate was obtained with only non-reducible connective tissue remaining visible. A 20μL aliquot of the resulting homogenate was removed for DNA analysis. The homogenate was centrifuged at 12,000xg at 4ºC for 10 minutes and the resulting supernatant retrieved formed the **Cellular Soluble Fraction (CSF)**, which was stored at -80ºC until chromatography.

The pellet was re-suspended in 1mL extraction buffer and incubated for 15 hours at room temperature. Proteins were denatured by guanidine. Remaining insoluble mucin

was solublised; firstly the disulphide bridges were disrupted by DTT and then their reformation prevented by reduction through the addition of solid iodoacetamide to give a final concentration of 25mM and incubated for 5 hours at room temperature in the dark. The sample was then centrifuged at 12,000xg at room temperature for 15 minutes. The supernatant was retrieved and any remaining pellet underwent a maximum of 2 further extractions in 1mL of extraction buffer for 2 hours. The solutions were centrifuged at 12,000xg at room temperature for 15 minutes. The supernatants obtained at each step were pooled and collectively formed the **Cellular Insoluble Fraction (CIF)**, which was stored at -80ºC until chromatography.

3.5 Gel Filtration

The fractions obtained after the various extraction procedures carried out on the mucosal samples were analysed by gel filtration. Glass columns 1cm x 30cm (Dr Tony Corfield, Bristol Mucin Research Group) were packed with Sepharcryl S-500 HR(Amersham) and equilibrated and eluted with the appropriate buffer. For the secreted soluble and cellular soluble samples the buffer used was10mM Tris/HCL pH 8.0 and for the cellular insoluble samples 0.5M guandine/HCL/PBS.

The stored fractions were thawed, centrifuged for 5 minutes at 12,000xg and loaded carefully onto the top of each column. The total volume of the cellular soluble and cellular insoluble fractions and a maximum of 2mL of the secreted soluble fraction were used. The volume of the remainder of the secreted soluble was measured and re-frozen at -80ºC.

A high molecular weight dye, blue dextran, which has a similar molecular weight to mucin molecules (2000 kD compared to 500 – 30,000 kDa) and is therefore too large to enter the gel, was run through the column to establish the void volume or exclusion volume (the volume of buffer that elutes from the column first which is too large to enter the gel). The fraction numbers in which the blue dextran appeared would correspond to the fraction numbers containing the mucin. (Figure 3.8)

Fractions of 1mL were collected manually to a total of 40mL. The mucin samples were obtained at around fractions 13-16. All fractions were stored at 4ºC for later analysis. Each column was washed through twice with the appropriate buffer after a gel filtration run and clamped and covered between experiments. Columns were repacked after 5 runs.

3.6 Determination of Radiolabel Incorporation

A 100µL aliquot of each 1mL fraction obtained from gel filtration was placed in a scintillation vial (pico prias vials) with 1mL of scintillation fluid (Optiphase 'Hisafe' 3) and the vials thoroughly mixed. Counts were measured for up to 4 minutes per sample in a scintillation counter which was programmed to count disintegrations for ^{35}S and ^{3}H labels. Counts were automatically converted to disintegrations per minute (DPM) by the scintillation counter and the results retrieved from a print out of the data. Quenching curves were obtained to correct for any factors which cause energy loss in the scintillation solution by using 1µL of ^{35}S with serial dilutions of acetone or by the manufacturer supplied quench curve for ^{3}H.

Example calculation Patient L SSF sample A:

Raw dpm H^3 at fraction 13	=	70.13
Decay corrected dpm	=	raw dpm ÷ (exp (- decay constant x (date of count – incubation date)))
	=	a
Dilution factor corrected dpm	=	a x 10
Volume SSF	=	2.525mLs
Volume corrected dpm	=	(a x 10) x (volume SSF ÷ 2)
	=	(a x 10) x (2.525 / 2)
	=	a x 10 x 1.26
DNA content sample A	=	37.59ng/mL
Final calculation for corrected dpm	=	**a x (10 x 1.26) ÷ 37.59**

Radioactivity Calculations

Mucosal samples were cultured in the presence of 10µCi of [3H]-glucosamine and 10µCi of [35S]-sulphate. [35S]-sulphate has a short half-life and therefore the volume of stock solution required needed to be recalculated for each experiment.

Example Calculation:

[35S]-sulphate stock	=	250µCi on 03/05/2004
Half-life	=	87.4 days
Experiment date	=	06/05/2004 (i.e. 3 days from starting date)

$$R_t = R_o e^{-kt}$$

R_t	=	radioactivity at time of experiment
R_o	=	radioactivity at initial date
e	=	exponential
k	=	constant
t	=	time (days)
ln	=	log natural
t½	=	half-life

$$k = \frac{ln½}{-t½}$$

$$k = \frac{ln½}{-87.4}$$

$$k = 7.93e^{-3}$$

$$R_t = R_o e^{-kt} = R_o e^{-(7.93e^{-3}\ x\ 3)} = 244\mu Ci$$

At start date there were 250µCi in 250µL solution, therefore 1µCi/µL. At time of the experiment there were 244µCi left in the stock.

$$\frac{244}{250} = 0.97\mu Ci/\mu L \therefore$$

10µCi required for the experiment,

$$\therefore \quad \frac{10}{0.97} = 10.3\ \mu L\ of\ [35S] - Sulphate\ Stock$$

3.7 DNA analysis

DNA analysis was carried out using 20µL aliquots of homogenized material from each of the samples. A dye-binding fluorometric assay kit, PICO green (Invitrogen) was used.

A stock solution of 2µg/mL DNA was made by diluting the standard stock dsDNA provided in the kit with TE (Tris/HCL, EDTA). The solution was used to generate a 5 point standard curve from 1ng/mL to 1µg/mL as recommended by the manufacturers. The dilutions were made to a final volume of 100µL as in the table below. Each was placed in duplicate wells in a multi-well plate (Labsales, UK).

Well	DNA solution (µL)	TE (µL)	Concentration	DNA content(ng)
A	100	0	1µg/mL	200
B	25	75	25ng/mL	50
C	10	90	10ng/mL	20
D	2.5	97.5	2.5ng/mL	5
E	1	99	1ng/mL	2
F	0	100	Blank	0

Table 3.1 : DNA Content of Wells

Each 20µl patient sample was run in four wells diluted with TE as follows:

- 2 x 5µl with 95µl of TE (total 100µl)
- 2 x 2µl with 98µl of TE (total 100µl)

A 200 fold dilution of the PICO green reagent was prepared in TE. 100µL of this was added to each well. The multi-well plate was placed in the fluorometer and the

Picogree.sec program was used. The reading from the reagent blank was subtracted from all the results.

The results from the standard DNA samples were plotted on a graph (x axis = ng DNA, y axis = fluorometer reading). (Figure 3.3) A linear trend line was produced and from the line equation the DNA content of the unknown patient samples was calculated. This was converted into ng DNA/µL and a mean for each patient sample obtained.

3.7.1 Example calculation - Patient Q @ sample C:

Linear trend line equation: $y = mx + c$

DNA standard sample gave results of

$\mathbf{m}$ = 2.81 $\mathbf{c}$ = 4.95

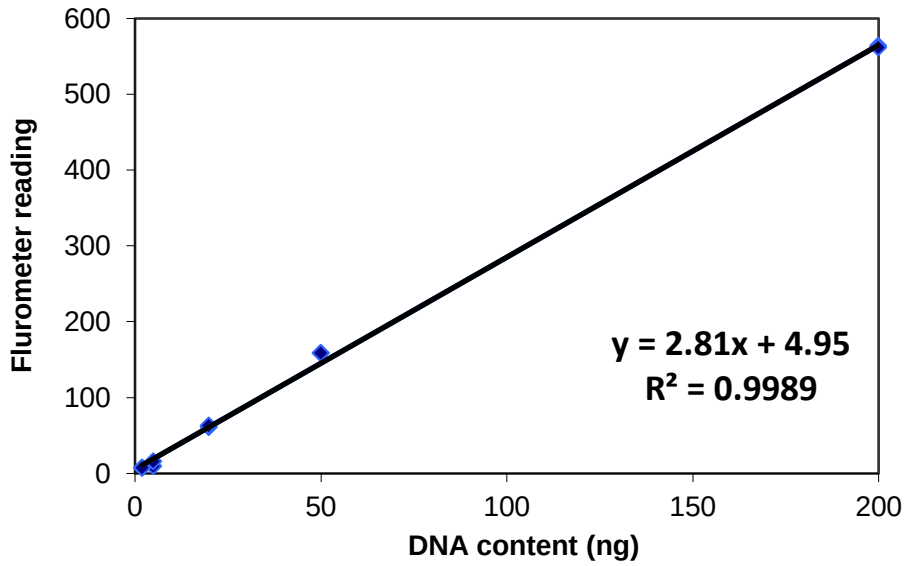


Figure 3.3: Standard DNA curve used with Patient Q

Fluorometer reading for Sample C of Patient Q = 450.26

$$\therefore x = \frac{(y-c)}{m} = \frac{(450.26-4.95)}{2.81}$$

$$= 158.47 \text{ ng DNA in Sample C}$$

3.8 Slot Blot Analysis of Gel Filtration Fractions

The fractions obtained from gel filtration were subjected to slot blot analysis. A standard protocol (Dr Tony Corfield, Bristol Mucin Research Group) was used as a starting point and adapted to a working format. The development of the final methodology required several preliminary experimental stages.

3.8.1 Establishment of Protocol

Assembly and loading of slot blot apparatus

The slot blot apparatus (minifold 2, Whatman) was assembled using nitrocellulose membrane (Amersham) on top of two sheets of blotting paper. All were pre-soaked in PBS. Trial 600µL aliquots of PBS were loaded into the wells and vacuum was applied but the PBS remained in the wells even after one hour.

The method was modified by using a more capable vacuum pump which improved the transference of samples through the nitrocellulose but only in those wells nearest the vacuum inlet. Reduced resistance offered by the emptied wells compared to the remaining full wells, resulted in the vacuum acting preferentially through these. To try

to counter this once the wells were empty they were covered. Parafilm, plasticine and autoclave tape were all trialed, the latter being the most effective in promoting well clearance, through several wells still did not empty. The final modification was to run the slot blot using a single piece of blotting paper. This resulted in complete and rapid clearance of all the wells without the need to cover any of the wells.

Trial Run of Slot Blot with Standard Mucins

The slot blot was loaded as above with serial dilutions of serum and saliva. Nitrocellulose was separated from blotting paper using forceps and placed face up in a tray. The membrane was blocked in PBS with 0.05% (v/v) polyoxyethylene sorbitan mondaurate (Tween-20 (Sigma, Poole)) and 1% bovine serum albumin (BSA) (Sigma, Poole) for 4 hours at room temperature on a rocking platform covered with cling film.

Wheat germ agglutinin-horse radish peroxidase conjugate (EY laboratories Inc) was added to the blocking solution to give a final dilution of 1:15,000, covered with cling film and incubated for a further 4 hours at room temperature on a rocking platform. The nitrocellulose membrane was washed three times with 20mLs of PBS and 0.05% Tween-20 for 8 minutes and then a final wash with 20mLs of PBS for 9 minutes.

The slot blot membranes were developed with a perioxidase reaction using diaminobenzamidine (DAB) reagent (Sigma) but no colour change signal was detected.

A failure at one or more stages was suspected:

1. Mucin sample – failure to bind to the nitrocellulose or dilutions too weak to allow detection
2. Lectin – failure to bind to mucin or inadequate amount of lectin present for detection
3. Colour detection – DAB detection system not working

Modifications to Slot Blot Method

The efficiency of the DAB detection system was tested by applying neat lectin dots directly on to the nitrocellulose. After blocking and washing as before, the membranes were developed with DAB. The dots of lectin were found to stain although they appeared quite grainy.

Higher concentrations of the standard serum and saliva mucins were used and these were loaded on to the slot blot in 4 sets of identical dilution series; neat, 1:2, 1:4, 1:6, 1:8. The nitrocellulose was then divided into 4 pieces, blocked as before and then different concentrations of the lectin were added to each; 1:1000, 1:2000, 1: 5000, 1:10,000. These were incubated for four hours, washed and developed. All samples stained although the appearance was grainy and non-uniform within each slot. There was no improvement with the more concentrated WGA solutions so a concentration of 1:10,000 was used for further experiments.

The method above was tested for the ability to detect the lower mucin concentrations expected in patient samples. Using the same WGA dilution, 20µL samples of serial dilutions of serum and saliva (neat to 1:3125) were added in triplicate to the blot.

Patient samples from gel filtration fractions representing the mucin peak and the eluted volume and a blank of PBS were also loaded. The samples all stained with DAB, including the blanks, with no gradation between the serial dilutions. The appearance of the blot remained very grainy.

To try to improve the differentiation between mucin concentrations a different detection system, enhanced chemiluminescence was used. As above serum and saliva dilutions were loaded in duplicate on to a blot, to yield two replicate membranes and these were blocked as before and WGA added. One was developed with DAB and the other with chemiluminescence. Both gave similar results showing a lack of differentiation between the dilution levels.

PVDF membrane (Amersham), was compared to nitrocellulose. PVDF was wet with methanol and then placed in PBS. Serum and saliva dilutions were loaded in duplicate onto PVDF and nitrocellulose membranes. The membranes were cut into two and were blocked and WGA added. Half of each membrane was detected using the DAB system and half by chemiluminescence. The nitrocellulose membrane showed better staining results.

To try to improve the blot appearance a more purified form of BSA was used as the blocking agent (Sigma A9647). Also concentrations of 0.05% and 0.5% Tween-20 were compared. Neither alteration made a significant difference.

In the original protocol a small amount of bleach was added after the DAB detection system had caused a colour change, to stop the reaction. The bleach was omitted and the blot removed from the DAB agent and washed instead. This resulted in the

resolution of both the grainy appearance of the staining and a clear difference in colour uptake could be noted between the different mucin concentrations.

Application of Method to Subject Samples

Preliminary slot blots were performed to determine whether the peak of radioactivity coincided with the WGA staining peak. Aliquots of 50µL were taken from each of the gel filtration fractions from 1 to 40 and loaded onto a blot. A separate experiment was performed for each of the mucin fractions, SSF, CSF and CIF. Peak staining occurred at the same gel filtration fractions as the peak radioactivity counts. In order to determine the volume of sample required to give optimum staining, different volumes of sample were loaded onto a blot. The optimal volume was found to be 300µL.

3.8.2 Final Method Protocol

A piece of blotting paper and a piece of nitrocellulose were cut to size and soaked in PBS at room temperature. These were loaded into the slot blot apparatus (minifold 2, Whatman), care taken to only handle the nitrocellulose with forceps.

A separate slot blot was performed for each subject. The fractions obtained from gel filtration previously analysed for radioactivity resulted in a bi-peaked distribution. Pools were made from 200µL aliquots of each of the three highest front peak and back peak samples with the highest scintillation counts, resulting in each subject having 24 separate samples, i.e.

A	SSF Front peak	CSF Front peak	CIF Front peak
	SSF Back peak	CSF Back peak	CIF Back peak
B	SSF Front peak	CSF Front peak	CIF Front peak
	SSF Back peak	CSF Back peak	CIF Back peak
C	SSF Front peak	CSF Front peak	CIF Front peak
	SSF Back peak	CSF Back peak	CIF Back peak
D	SSF Front peak	CSF Front peak	CIF Front peak
	SSF Back peak	CSF Back peak	CIF Back peak

Table 3.2: Samples used for slot blot analysis for each subject

A mucin standard curve was loaded onto every blot in the following dilutions; 1:10, 1:50, 1:100, 1:250, 1:500. PBS was used as a blank. Samples were loaded in duplicate in 300µL aliquots.

The membrane was blocked in PBS with 0.05% Tween-20 and 1% BSA for 4 hours at room temperature. The lectin was added to this solution in the appropriate dilution and was incubated for a further 4 hours at room temperature. The dilution of lectin required was determined by repeated experimentation and was 1:10,000 WGA, 1:100 MAA (EY laboratories Inc). UEA (EY laboratories Inc) was used in the following dilutions; 1:10,000, 1:5000, 1:1000 and 1: 100.

The membrane was washed with PBS/Tween-20 (0.05%) for three eight minute washes. Just before use, 1 ml of the DAB reagent was added to 9 mLs of the DAB buffer and added to the membrane. When a colour change had occurred the reagent was removed and the membrane washed in distilled water. (Figure 3.4)

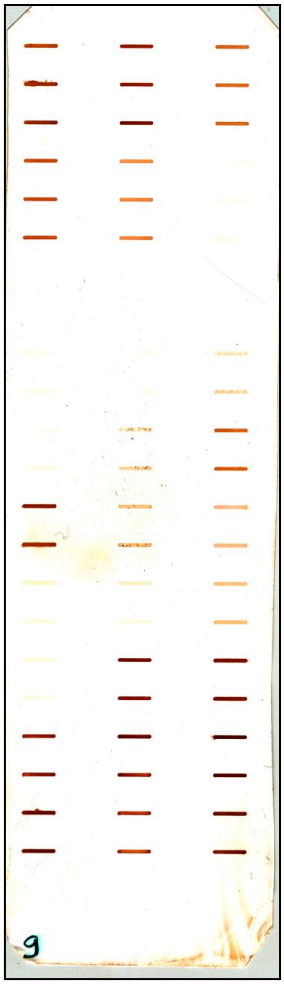


Figure 3.4: Example of a slot blot obtained using WGA lectin.

Slot blots were scanned and the densitometry was calculated using Image J (internet resource, National Institute of Health, USA, Research Services Branch). (Figure 3.5) Corrections for background reactivity were performed. Inter-membrane variation was controlled for by comparison against the standard mucin curves present on each membrane.

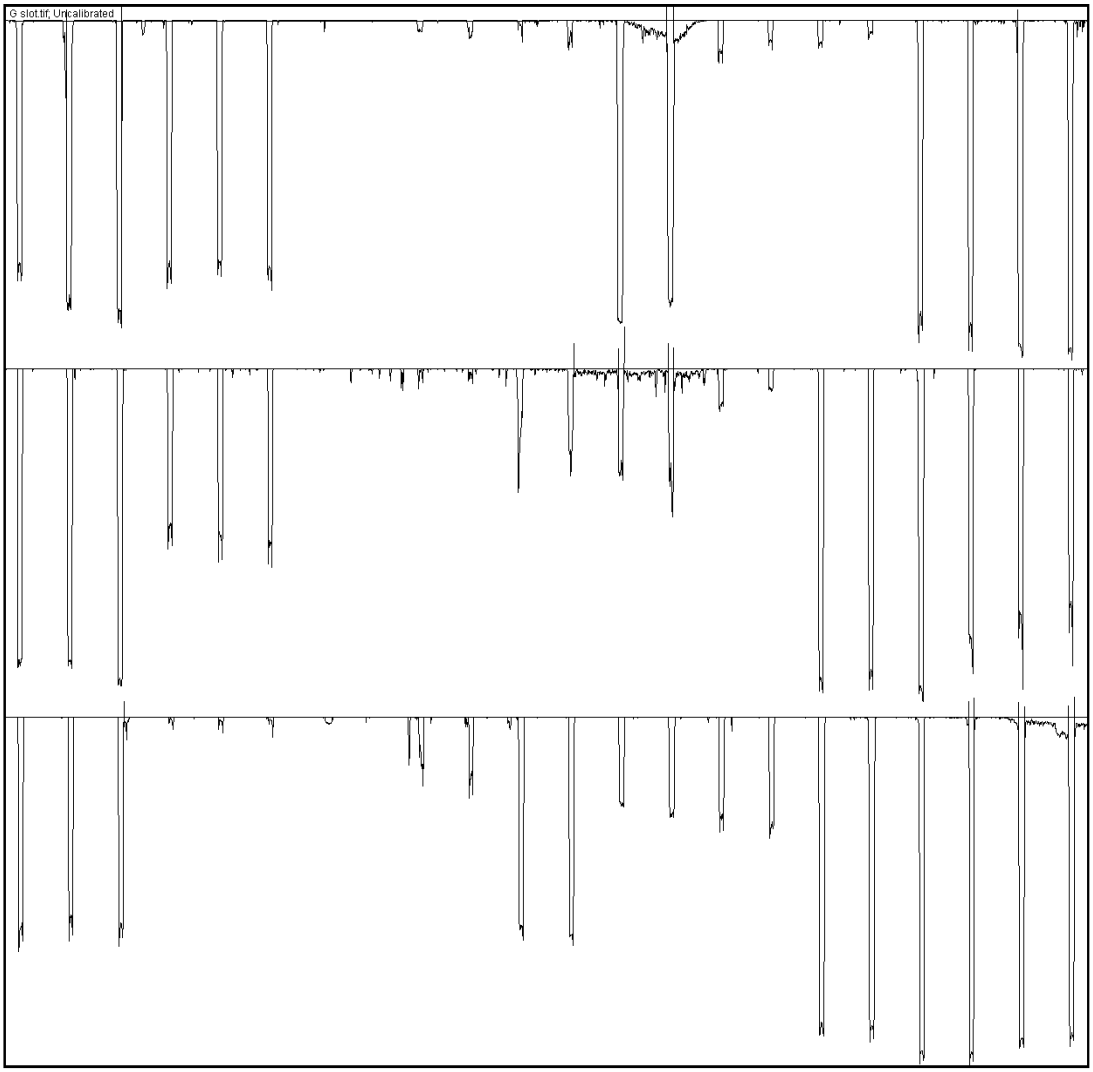


Figure 3.5: Densitometry trace from a WGA slot blot obtained using the Image J program.

The dot intensities were plotted on a graph and a logarithmic curve fitted. (Figure 3.6) The relative mucin concentration of the patient samples was interpolated from the curve equation. As each subject sample was loaded in duplicate a mean result was obtained.

3.8.3 Example WGA slot blot calculation - Patient H @ sample B:

Logarithmic Curve equation: $y = m \ln x + c$

Standard Mucin Curve sample gave results of

$\mathbf{m}$ = 346.08 $\mathbf{c}$ = 320.16

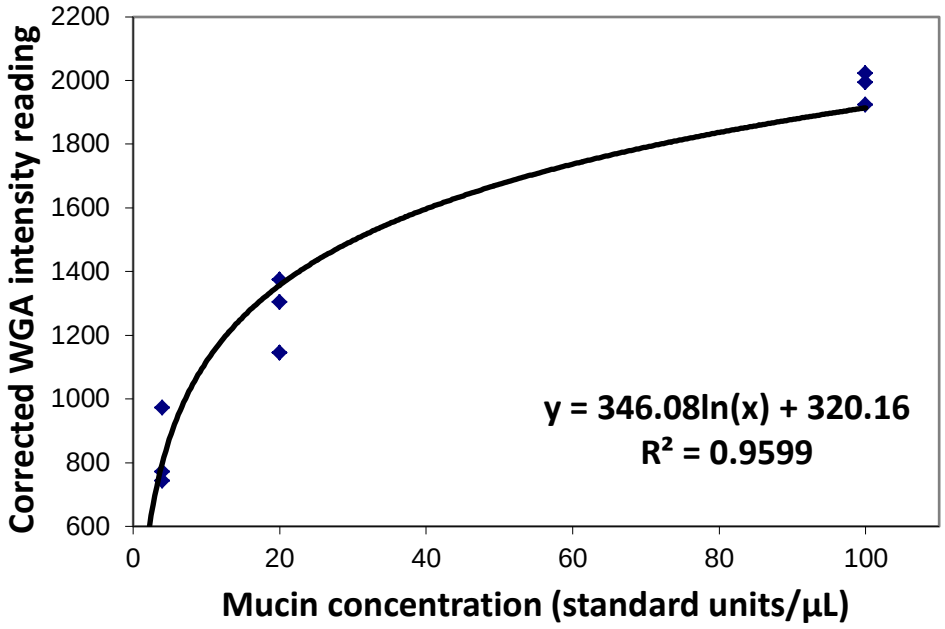


Figure 3.6: Standard mucin curve obtained from a WGA slot blot.

Densitometry reading for Patient H @ Sample B (CSF Fraction) = 1491

$$\therefore x = e^{\frac{(y-c)}{m}} = e^{\frac{(1491-320.16)}{346.08}}$$

= 29.46 WGA Binding

3.8.4 Example MAA slot blot calculation - Patient T @ sample B:

The slot blots performed with the lectin *Maackia amurensis* agglutinin (MAA) also had standard mucin dilutions run along side subject samples. These generated a hyperbolic curve. (Figure 3.7)

Standard MAA Curve sample gave results of

$\mathbf{a}$ = 2119.63 $\mathbf{b}$ = 4147.56

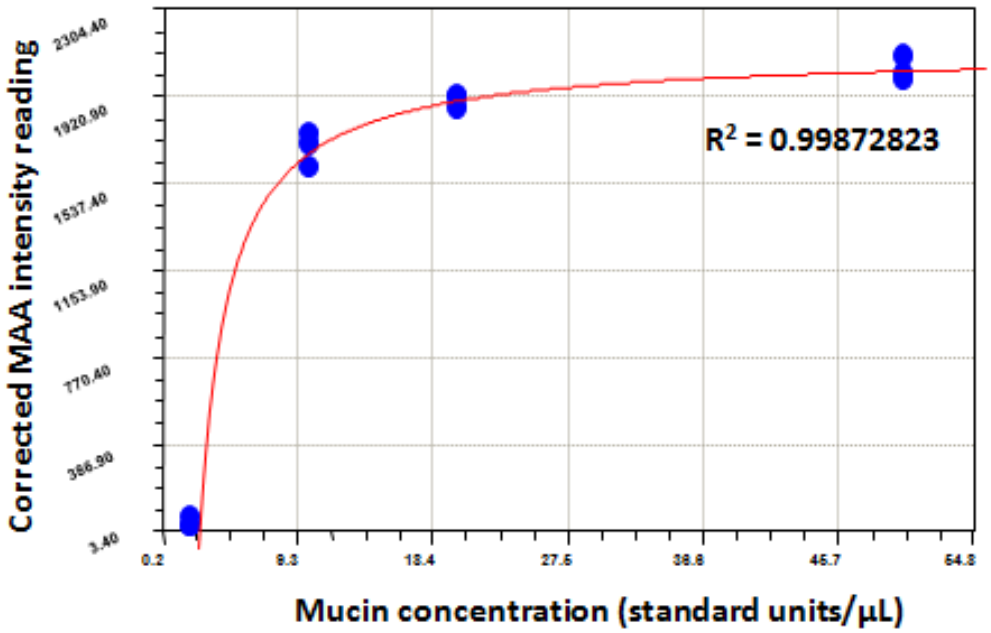


Figure 3.7: Example of a standard mucin curve obtained from a MAA slot blot.

Densitometry reading for Patient T @ Sample B (CSF Fraction) = 1859

$$\therefore x = \frac{b}{(y-a)} = \frac{4147.56}{(1859-2119.63)}$$

= 15.91 MAA Binding

3.9 Analysis of data

Data obtained from the scintillation counter print out was manually entered onto Excel workbook sheets (Microsoft Office). Disintegrations per minute were calculated separately for [3H] and [35S] for each of the 40 gel filtration fractions obtained from the secreted soluble, cellular soluble and cellular insoluble mucins fractions respectively. DPM were corrected for the following; background radioactivity (by subtracting the mean DPM from the fractions collected prior to the void volume from the other fractions), radioactive decay and total DNA content of the mucosal sample. In addition the secreted soluble samples were also corrected for the fraction of the total volume run on the column. The incorporation of label in each fraction was expressed as dpm/ng DNA.

For each biopsy point a single set of incorporation values for each precursor was generated by adding together the figures for the secreted soluble, cellular soluble and cellular insoluble generated at fractions 1 to 40. The incorporation results from the separate extraction fractions and the combined results were plotted graphically against the gel filtration fraction number giving three individual graphs and one combined graph for each patient or control for each biopsy taken and each precursor used. (Figures 3.8 & 3.9)

The resulting graphs of disintegrations per minute for the gel filtration fractions showed a double peaked configuration. Mucins are very high molecular weight molecules so are elucidated early on in the gel filtration process at a similar fraction number to blue dextran. [11] Slot blot analysis of samples taken from each of the gel filtration fractions with wheat germ agglutinin stained in the region of the first peak and also within the second peak. Based on these confirmatory results and on previous similar work with

gel filtration columns, the front peak was designated the mucin peak and the other peak nominally called the back peak.[345, 372]

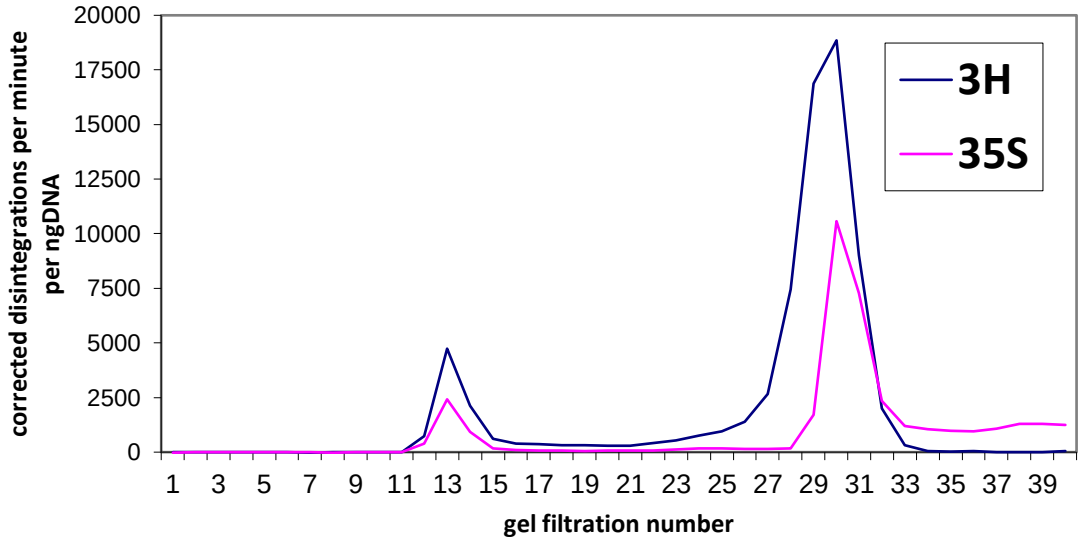


Figure 3.8: Example of a Patient graph of gel filtration fraction number and incorporation

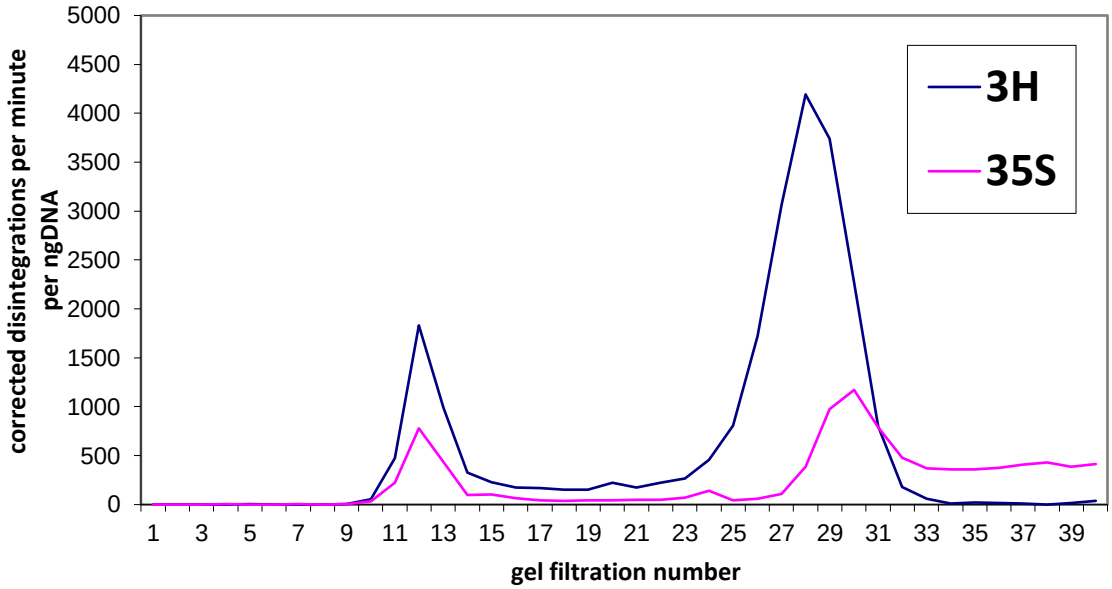


Figure 3.9: Example of a Control graph of gel filtration fraction number and incorporation

A single incorporation value for each precursor was calculated by taking the sum of the three highest values within each peak.

Example: **Patient J @ Biopsy level A**

Total incorporation values (SSF + CSF + CIF) - peak values in red

Fraction	3H	35S
5	2.48	-3.95
6	1.41	4.66
7	-6.96	-0.53
8	-7.46	-0.61
9	-13.83	-6.23
10	-13.16	0.31
11	28.32	21.88
12	**502.92**	**228.02**
13	**2127.13**	**1013.06**
14	**1088.79**	**452.79**
15	383.78	120.96
16	319.54	86.15
17	316.82	84.99
18	323.24	73.81
19	342.54	69.63
20	340.77	64.30
21	372.43	55.37

Table 3.3: Incorporation results from a subject demonstrating the mucin peak values

Single figure results for patient J at point A are therefore:

3H = **502.92 + 2127.13 + 1088.79**

= 3719

35S = **229.02 + 1013.06 + 452.79**

= 1695

The data for this analysis for each patient and control can be summarized thus:

Patient J

	A		B		C		D	
	mucin	**back**	**mucin**	**back**	**mucin**	**back**	**mucin**	**back**
3H	3719	9628	1539	10853	2180	6175	1342	3464
35S	1694	3615	428	4896	654	1289	491	786

Table 3.4: Summary results for incorporation for a subject

Mean values for the patient and control groups were calculated for total incorporation within the peaks and for incorporation within the separate extraction fractions peaks for each precursor and corrected for DNA content. Relative incorporation was derived by dividing [35S]-sulphate incorporation by [3H]-glucosamine incorporation.

Overall incorporation values were calculated by adding the result from each biopsy and dividing by the total number of biopsies taken to yield a single figure per subject. i.e. (A + B + C + D)/4

Lectin binding results were calculated as mentioned above from the generation of a standard curve. Mucin peak densitometry mean values were calculated for each separate biopsy level and for each separate extraction fraction studied for patients and controls. Addition of the binding values for the separate extraction fractions gave total mucin binding values. Overall densitometry results were calculated by adding the combined result from each biopsy and dividing by the total number of biopsies taken as above.

Densitometry results were further quantified by DNA content of the sample and expressed per 100ngDNA.

3.10 Statistical Analysis

Statistics were generated using the In Stat 3 program (GraphPad Software). An unpaired t-test was used where data passed a normality test, Kolmogorov and Smirnov method. Welch correction was applied when the difference between standard deviations was statistically significant. The Mann-Whitney test was used where the data did not follow a normal distribution pattern. A p value of < 0.05 was considered significant.

Chapter 4 –Results

4.0 Study subject data

A total of 10 patients with Hirschsprung's disease and 6 controls were recruited over a period of 15 months. There were no withdrawals from the study.

All of the Hirschsprung's patients had their diagnosis established by rectal biopsy and had histological confirmation of their diagnosis at operation. None had any evidence of enterocolitis at the time of surgery or diarrhoea of unknown origin. Eight had Hirschsprung's disease affecting the rectosigmoid colon, two affecting the descending colon. (Table 4.1) None of the controls had diarrhoea at the time of surgery and none had any evidence of immunodeficiency.

Six patients underwent the Duhamel operation as part of a two or three stage procedure [328] and four had a laparoscopic-assisted Soave procedure as a single stage procedure.[329] (Table 4.1) Five controls had an anorectal malformation, one had sustained a rectal injury. All had a colostomy performed as part of their initial treatment. (Table 4.2)

Mean number of biopsies taken per patient was 3.6 and in the control group 3.3. Within the patient group seven had four biopsies retrieved, two had three biopsies and one had two biopsies. In the control group three had four biopsies taken, two had three biopsies and one had two biopsies.

Gestation and birth weight were comparable between the two groups ($p = 0.35$ and $p > 0.99$). There were five episodes of pre-operative enterocolitis affecting three patients and six episodes of post-operative enterocolitis affecting four patients. Two patients

had pre and post-operative enterocolitis. (Table 4.1) There were no complications arising from the study. Follow-up was ongoing in the majority of patients at the time of write up.

Patient Identifier	Demographics at operation		Length of disease	Type of Surgery	Episodes of enterocolitis		Pre-operative use of washouts	
	Age (days)	Weight (kgs)			Pre - op	Post - op	Elective	Emergency
G	106	7820	Rectosigmoid	Duhamel	0	0	No	No
H	349	8660	Descending colon	Duhamel	0	0	No	No
J	169	6950	Rectosigmoid	Duhamel	0	3	No	No
K	220	8200	Rectosigmoid	Soave	2	0	Yes	No
L	502	14300	Rectosigmoid	Soave	2	1	Yes	Yes
M	97	6920	Rectosigmoid	Soave	1	1	Yes	Yes
Q	97	7010	Rectosigmoid	Soave	0	0	Yes	No
R	78	6290	Rectosigmoid	Duhamel	0	0	No	No
T	177	8790	Descending colon	Duhamel	0	0	No	No
E	61	5160	Rectosigmoid	Duhamel	0	1	No	No

Table 4.1: Demographics and clinical features of patients.

Control Identifier	Demographics at operation		Underlying Diagnosis	Type of Surgery
	Age (days)	Weight (kgs)		
F	409	12300	Anorectal malformation	Closure of colostomy
N	92	8800	Anorectal malformation	Closure of colostomy
P	155	8270	Anorectal malformation	Closure of colostomy
S	212	8500	Rectal perforation	Closure of colostomy
X	345	8700	Anorectal malformation	Closure of colostomy
Y	186	7500	Anorectal malformation	Closure of colostomy

Table 4.2: Demographics and clinical features of controls.

	Patients		Controls		
	Median	**Range**	**Median**	**Range**	**p value**
Age at operation (days)	137.5	61 - 502	199	92 - 409	0.43
Weight at operation (kg)	7415	5160 - 14300	8600	7500 - 12300	0.12

Table 4.3: Age and Weight of Subjects

4.1 Gel Filtration Graphs

The incorporation of [3H]-glucosamine and [35S]-sulphate in each sub-fraction was expressed as dpm/ng DNA. The majority of the graphs, especially those from the cellular soluble mucin fraction, showed a characteristic pattern of two peaks, the first at around 12-14 fractions and a second at around 28-31 fractions. The two peaks for both radioactive precursors coincided and were separated by a region of minimal turnover. In all graphs the back peak was higher than the mucin peak and the [3H]-glucosamine incorporation peaks were greater than those of [35S]-sulphate. Patterns yielded were similar in patients and controls.

Two other patterns of graph were found to occur. In some the first peak was minimal or absent. This was the most common pattern found in the secreted soluble mucin graphs and occurred in both patients and controls. The third pattern occurred predominantly in the cellular insoluble fraction where the two peaks were linked by levels of incorporation well above base line, giving rise to a continuous curve linking the two peaks. Patients and controls again displayed similar patterns.

The number of the fractions where the two peaks was elucidated was slightly different in the three mucin samples. In the secreted soluble the peaks tended to occur earlier at around 10-12 and 24-26 respectively, whereas in the cellular soluble and cellular insoluble they occurred at 12-14 and 29-31. This was true for patients and controls.

The sum of the corrected incorporation for the three mucin sub-fractions was calculated for [3H]-glucosamine and [35S]-sulphate to give a single set of figures and a single graph for each radioactive precursor and for each point along the colon for every patient and control.

These showed the same double peaked configuration. (Figures 4.1 & 4.2) The front, mucin peak occurred at around fraction number 13 and was in most a sharp peak, with the highest value at the centre and the second and third highest values either side. This was the same in patients and controls and for both precursors. The back peak was of a similar configuration for [35S]-sulphate incorporation but more broad based for [3H]-glucosamine incorporation.

The front peak was designated the mucin peak due to confirmatory results using blue dextran and WGA binding as detailed in the methodology.

Comparing the graphs along the colon from A to D for individual subjects, although there was no uniform pattern or progression, most controls showed a higher mucin peak at A compared to B whereas in most patients the reverse occurred. (Figures 4.1 & 4.2)

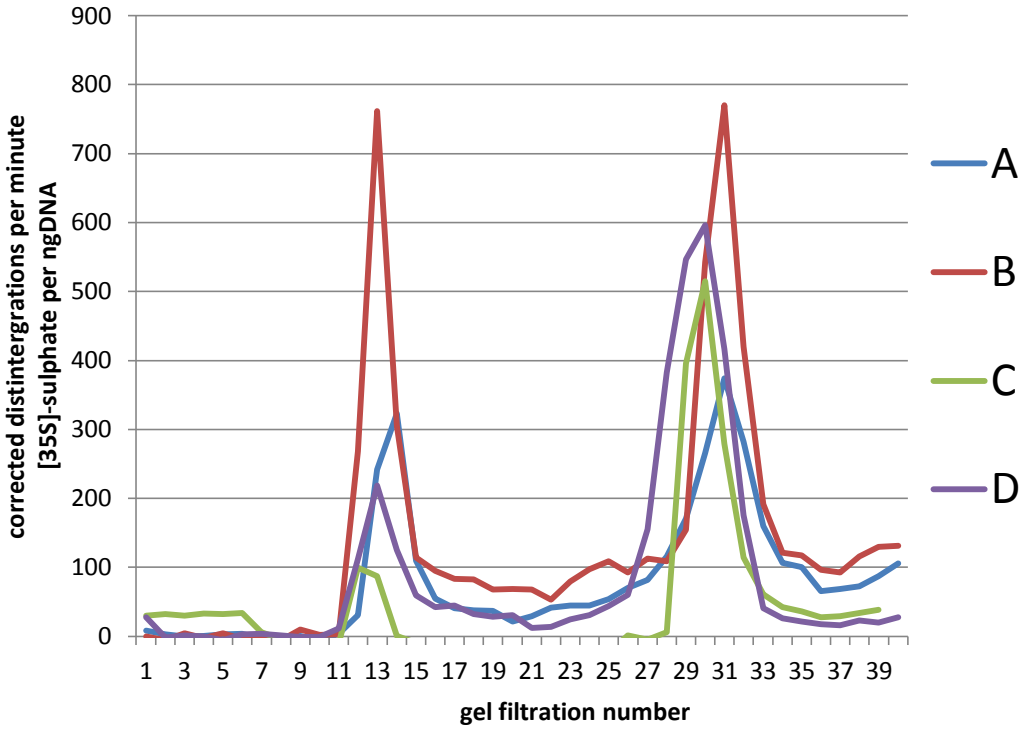


Figure 4.1: Example Patient graph showing mucin and back peaks

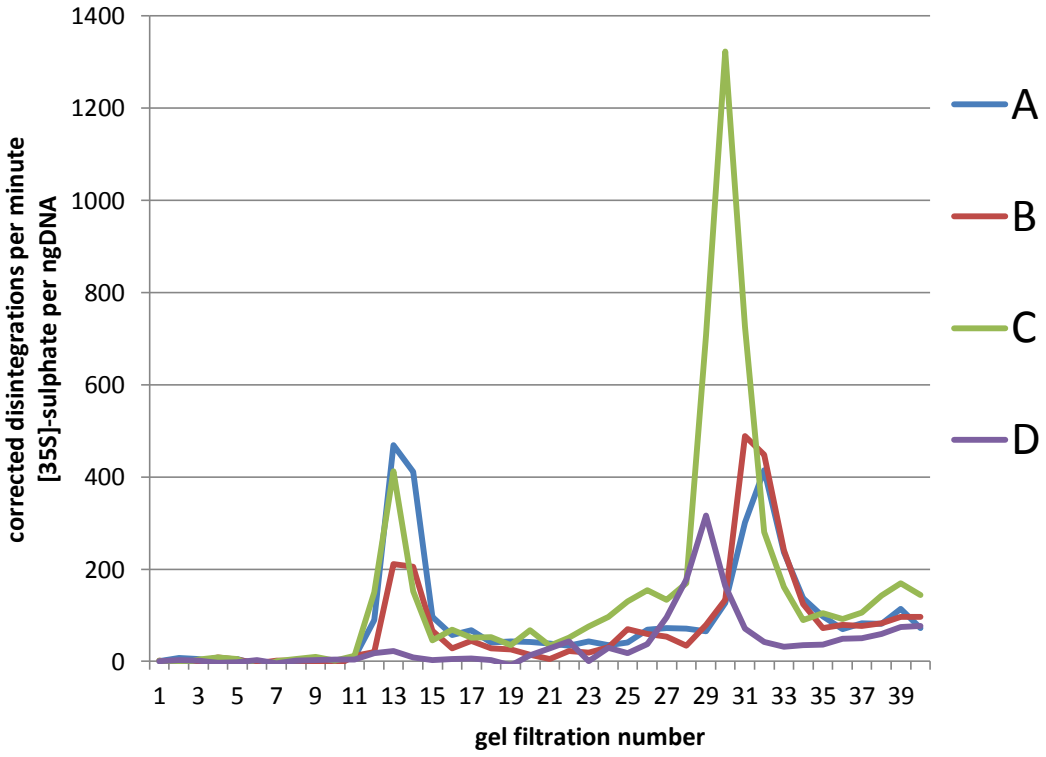


Figure 4.2: Example Control graph showing mucin and back peaks

4.2 Total Mucin Peak Incorporation

The incorporation of [3H]-glucosamine and [35S]-sulphate within the mucin peak was expressed as dpm/ng DNA and samples were incubated for 24 hours. Any statistically significant results are shown in red. Overall incorporation values were calculated by adding the result from each biopsy and dividing by the total number of biopsies taken to yield a single figure per subject. i.e. (A + B + C + D)/4

Mucin Peak [3H]-glucosamine Incorporation		
	Mean	**Standard Deviation**
Patients	4079	±1926
Controls	3530	±1037
		(p value 0.53)

Table 4.4: Mean Total Overall [3H] Incorporation within Mucin Peak

Mucin Peak [35S]-sulphate Incorporation		
	Mean	**Standard Deviation**
Patients	1369	±773
Controls	1206	±562
		(p value 0.66)

Table 4.5: Mean Total Overall [35S] Incorporation within Mucin Peak

4.2.1 Total Mucin Incorporation at the Different Colonic Points

Total [3H] Incorporation					
Subject		**A**	**B**	**C**	**D**
Patients	G	2674.34	8348.31	6997.41	5001.91
	H	3819.74	3130.65	5085.69	3129.34
	J	3718.84	1538.66	2180.05	1342.48
	K	1854.52	3841.20	993.83	1164.83
	L	1267.47	7086.22	9261.22	
	M	8057.17	9711.85	6413.25	6750.56
	Q	653.20	1955.63	7257.24	3074.48
	R	2544.99	2893.74	1919.16	1027.92
	T	5989.24	2633.88	5846.06	5019.56
	E	4348.98	2212.36		
Controls	F	2914.30	3600.63	5332.45	3354.50
	N		5683.77	3658.90	
	P	5612.37	3480.13	7979.81	1934.16
	S	6101.16	1839.79	272.68	
	X	4909.98	1090.40	2755.43	
	Y	5688.21	2108.42	511.16	897.50

Table 4.6: Total Individual [3H] Incorporation within Mucin Peak

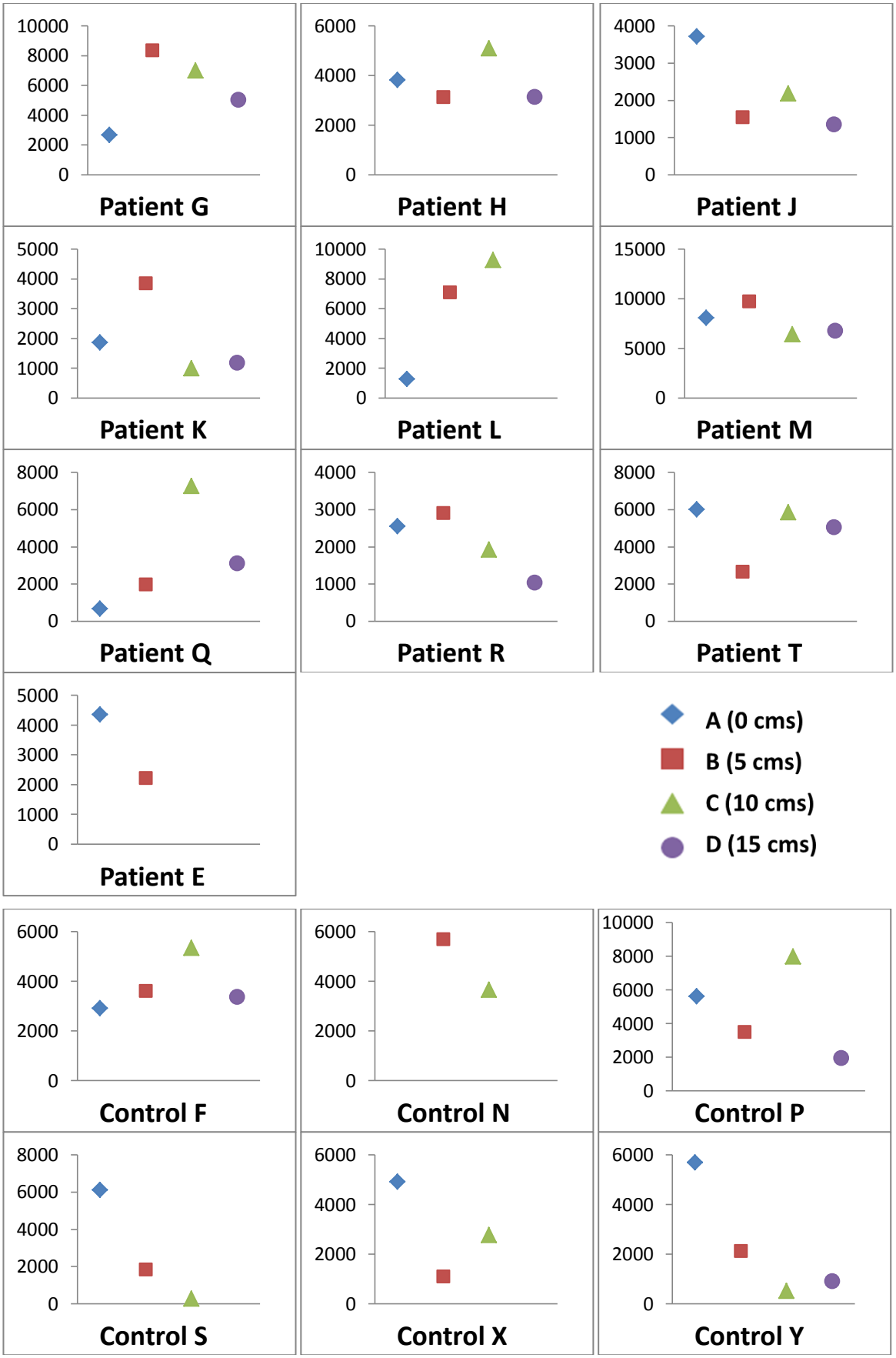


Figure 4.3: Individual subject graphs of total mucin peak [3H]-glucosamine incorporation (corrected disintegrations per minute/ngDNA) at colonic points A to D

Total [35S] Incorporation					
Subject		**A**	**B**	**C**	**D**
Patient	G	737.60	4047.10	652.05	2182.62
	H	1756.22		2648.70	1482.65
	J	1693.86	427.75	654.08	490.71
	K	673.72	1334.99	175.45	456.20
	L	194.42	649.95	2329.76	
	M	2824.27	4289.38	1217.12	1083.05
	Q	34.68	144.18	240.59	568.07
	R	750.02	881.65	497.62	340.47
	T	1955.22	982.86	2425.80	1675.17
	E	2925.89	1691.79		
Control	F	1581.61	1528.93	2313.99	1476.93
	N		1731.56	996.93	
	P	976.18	481.97	714.54	49.96
	S	1783.08	483.61	111.39	
	X	3393.15	462.09	2009.09	
	Y	672.39	1844.03	321.85	545.21

Table 4.7: Total Individual [35S] Incorporation within Mucin Peak

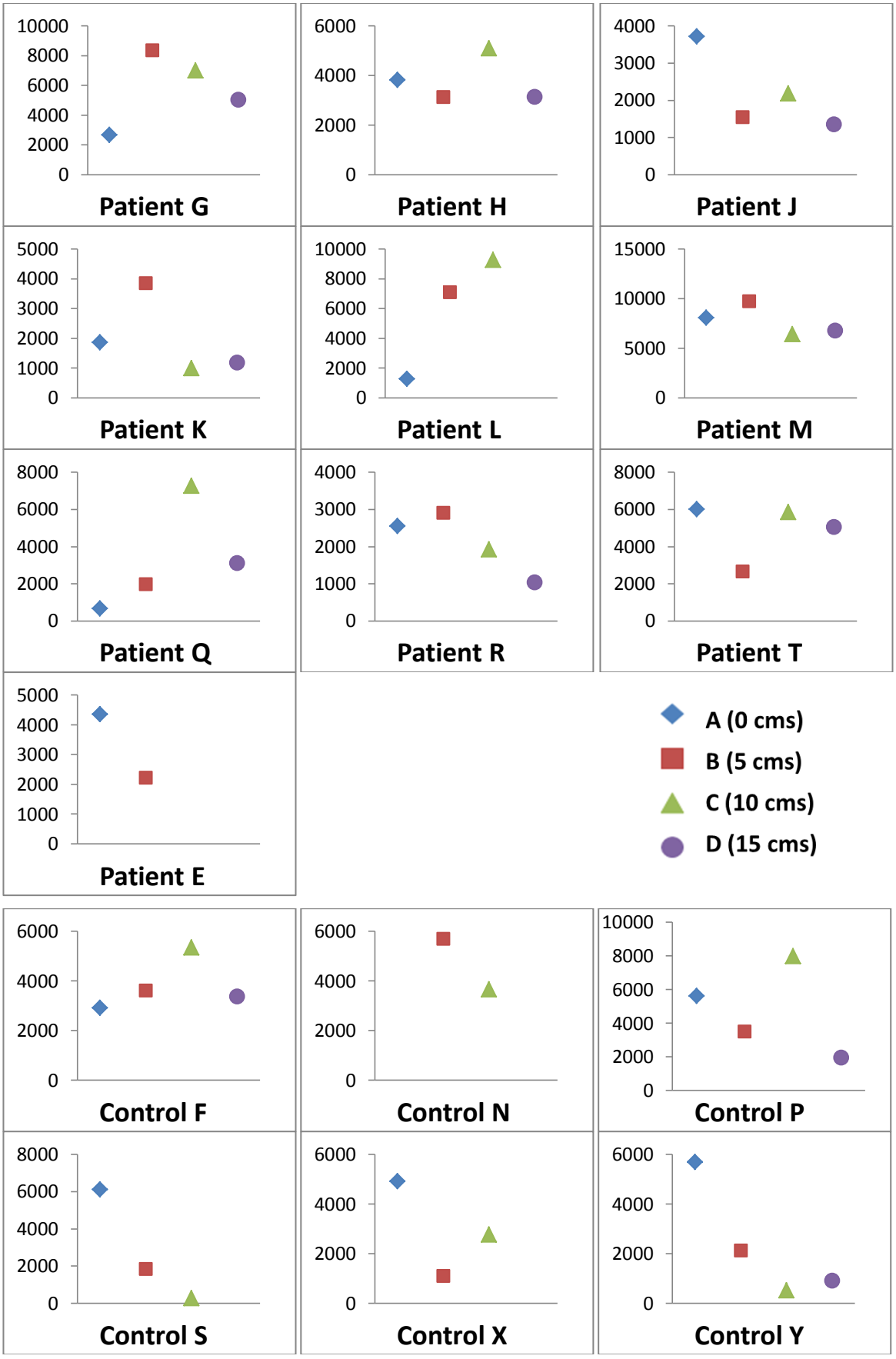


Figure 4.4: Individual subject graphs of total mucin peak [35S]-sulphate incorporation (corrected disintegrations per minute/ngDNA) at colonic points A to D

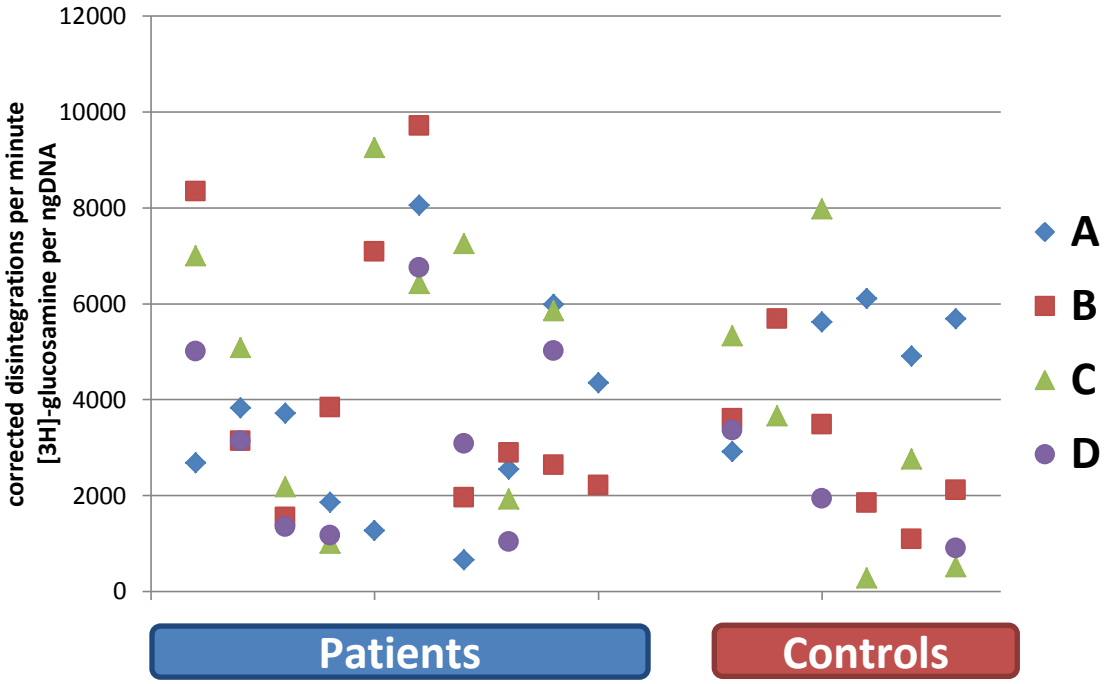


Figure 4.5: Scatter graph of Mucin Peak [3H]-glucosamine incorporation

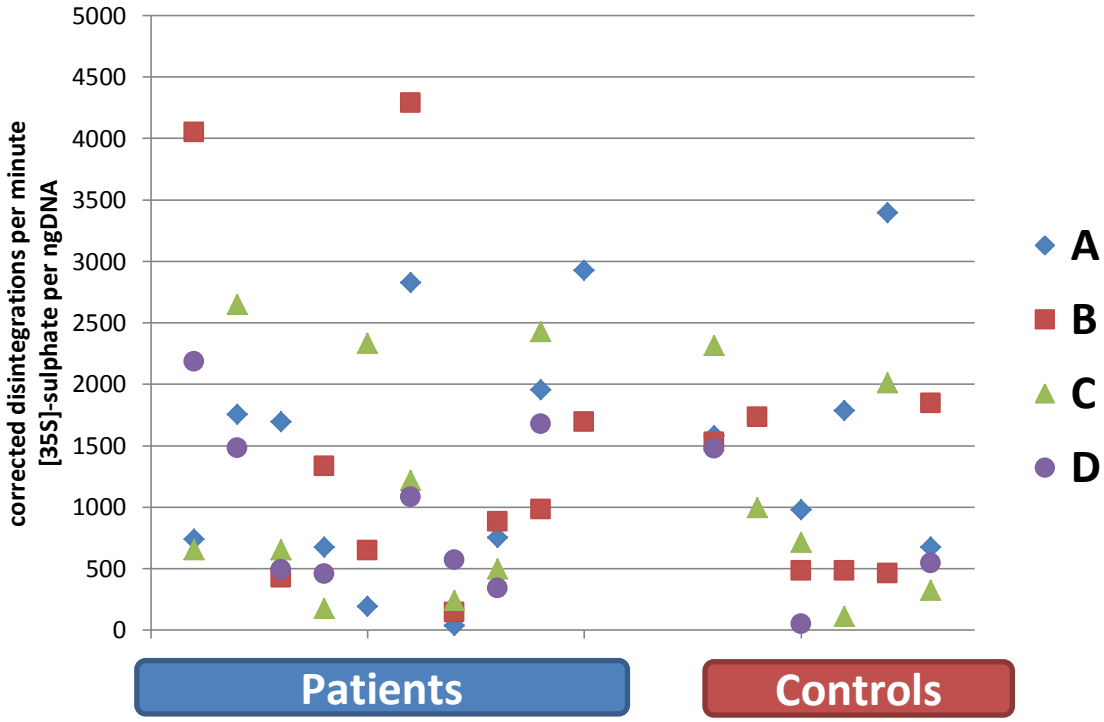


Figure 4.6: Scatter graph of Mucin Peak [35S]-sulphate incorporation

4.2.2 Mean Mucin Peak Incorporation at the Different Colonic Points

Mean Mucin Peak [3H]-glucosamine Incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	3493	±2242	5045	±1266	0.18
B	4335	±2930	2967	±1648	0.32
C	5106	±2814	3418	±2943	0.28
D	3314	±2118	2062	±1234	0.37

Table 4.8: Mean [3H] Incorporation within Mucin Peak at the Colonic Points

Mean Mucin Peak [35S]-sulphate Incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	1354	±1031	1681	±1057	0.58
B	1606	±1525	1089	±679	0.39
C	1205	±996	1078	±899	0.81
D	1035	±683	1011	±725	0.48

Table 4.9: Mean [35S] Incorporation within Mucin Peak at the Colonic Points

4.3 Incorporation within the Sub-Fractions of the Mucin Peak

The following tables show the incorporation results in dpm/ngDNA obtained from the three sub-fractions within the mucin peak of both patients and controls.

4.3.1 Secreted Soluble Fraction - [3H]-glucosamine incorporation

Subject		A	B	C	D
Patients	G	11.00	271.34	100.47	124.44
	H	802.60	50.40	67.18	13.76
	J	9.22	372.28	250.78	41.03
	K	25.10	50.28	55.35	42.07
	L	55.90	176.52	1702.29	
	M	175.52	614.60	163.24	586.77
	Q	2.88	25.57	23.78	5.75
	R	96.88	48.23	74.07	11.91
	T	11.06	7.55	7.41	0.00
	E	52.45	30.70		
Controls	F	116.99	117.60	174.67	97.72
	N		72.01	194.37	
	P	5.12	2.45	32.59	5.57
	S	11.77	191.93	13.14	
	X	14.14	8.91	27.64	
	Y	134.68	28.58	71.96	24.49

Table 4.10: Individual [3H] Incorporation within SSF fraction

Mucin [3H]-glucosamine incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	124	±244	57	±64	0.43
B	165	±200	70	±74	0.20
C	272	±542	86	±79	0.34
D	103	±199	43	±49	0.63

Table 4.11: Mean [3H] Incorporation within SSF fraction

4.3.2 Cellular Soluble Fraction - [3H]-glucosamine incorporation

Subject		A	B	C	D
Patients	G	2555.38	7600.23	5509.65	4135.02
	H	3087.16	2853.23	4601.90	3350.00
	J	3450.07	1260.07	1828.75	1196.78
	K	1522.85	3119.51	809.50	846.47
	L	1155.89	6223.23	7056.73	
	M	7522.55	9047.40	5578.06	6340.32
	Q	632.61	1897.96	7125.19	3011.62
	R	2394.73	2480.64	1732.11	988.53
	T	5691.78	2286.73	5514.69	4738.81
	E	3812.12	1769.94		
Controls	F	2480.91	3294.10	4185.03	2736.29
	N		5183.10	3098.16	
	P	5219.82	3206.38	6755.27	1382.07
	S	5094.39	1480.72	243.08	
	X	4670.50	952.36	2142.72	
	Y	5237.03	1877.98	404.35	797.23

Table 4.12: Individual [3H] Incorporation within CSF fraction

Mucin [3H]-glucosamine incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	3183	±2108	4541	±1174	0.21
B	3854	±2737	2666	±1548	0.35
C	4417	±2372	2805	±2464	0.23
D	3076	±1981	1639	±995	0.27

Table 4.13: Mean [3H] Incorporation within CSF fraction

4.3.3 Cellular Insoluble Fraction - [3H]-glucosamine incorporation

Subject		A	B	C	D
Patients	G	132.59	785.83	1410.67	790.48
	H	214.87	450.50	703.36	345.79
	J	338.06	785.83	31.71	138.05
	K	311.49	777.36	187.81	287.41
	L	76.62	865.02	1220.20	
	M	632.57	897.93	688.42	636.73
	Q	22.25	39.88	138.83	88.28
	R	79.18	358.50	98.09	35.12
	T	288.48	344.79	329.05	359.02
	E	625.51	297.18		
Controls	F	367.29	421.65	1184.01	520.49
	N		428.66	336.83	
	P	326.56	292.47	1334.88	610.94
	S	443.37	229.93	23.77	
	X	319.14	42.40	592.50	
	Y	316.50	159.48	36.63	73.80

Table 4.14: Individual [3H] Incorporation within CIF fraction

Mucin [3H]-glucosamine incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	272	±216	355	±54	0.28
B	560	±297	262	±151	**0.04**
C	534	±506	585	±565	0.86
D	335	±265	402	±288	0.72

Table 4.15: Mean [3H] Incorporation within CIF fraction

4.3.4 Secreted Soluble Fraction - [35S]-sulphate incorporation

Subject		A	B	C	D
Patients	G	2.76	208.97	6.05	34.73
	H	320.78		9.76	0.00
	J	3.20	6.43	12.63	0.05
	K	0.00	0.44	5.42	3.79
	L	8.56	8.97	63.80	
	M	7.67	137.68	15.16	4.04
	Q	2.09	2.67	11.50	0.00
	R	20.79	0.00	0.00	3.75
	T	1.52	0.00	0.00	0.00
	E	12.67	0.00		
Controls	F	36.29	21.99	98.04	52.46
	N		0.00	0	
	P	2.30	0.00	0.00	0.00
	S	0	0.00	0	
	X	60.46	9.36	2.14	
	Y	7.14	11.21	25.26	4.14

Table 4. 16: Individual [35S] Incorporation within SSF fraction

Mucin [35S]-sulphate incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	38	±100	21	±26	0.63
B	41	±77	7	±9	0.23
C	14	±19	21	±36	0.69
D	6	±12	19	±29	0.53

Table 4.17: Mean [35S] Incorporation within SSF fraction

4.3.5 Cellular Soluble Fraction - [35S]-sulphate incorporation

Subject		A	B	C	D
Patients	G	716.41	3735.91	571.02	1977.53
	H	1458.70		2435.93	1563.11
	J	1576.99	421.87	619.64	443.45
	K	584.87	1170.39	155.57	367.37
	L	190.32	619.30	2147.60	
	M	2665.32	4135.37	1066.89	1117.78
	Q	39.25	141.85	554.68	582.93
	R	708.29	836.93	526.37	345.74
	T	1892.13	899.09	2325.14	1626.43
	E	2632.85	1508.68		
Controls	F	1407.45	1433.81	2084.21	1271.36
	N		1624.09	924.91	
	P	937.30	459.67	686.43	39.36
	S	1535.04	509.76	121.10	
	X	3245.15	442.70	1788.78	
	Y	688.14	1739.63	291.53	488.00

Table 4.18: Individual [35S] Incorporation within CSF fraction

Mucin [35S]-sulphate incorporation					
	Patients		Controls		
	Mean	SD	Mean	SD	p value
A	1247	±948	1563	±1001	0.56
B	1497	±1442	1035	±626	0.41
C	1156	±893	983	±797	0.71
D	1003	±654	600	±624	0.38

Table 4.19: Mean [35S] Incorporation within CSF fraction

4.3.6 Cellular Insoluble Fraction - [35S]-sulphate incorporation

Subject		A	B	C	D
Patients	G	38.32	251.74	83.81	193.56
	H	101.69		316.46	156.05
	J	133.55	104.56	37.26	64.95
	K	94.75	214.27	35.33	94.98
	L	0.00	27.52	162.00	
	M	227.74	429.37	155.95	207.72
	Q	0.00	1.81	15.26	15.46
	R	21.14	70.87	0.00	5.64
	T	63.23	88.05	103.14	74.65
	E	339.22	167.70		
Controls	F	140.65	142.08	229.57	186.82
	N		124.37	93.04	
	P	37.07	31.61	44.81	6.62
	S	106.24	0.00	0.00	
	X	150.03	16.02	295.78	
	Y	0.00	52.31	1.13	47.09

Table 4.20: Individual [35S] Incorporation within CIF fraction

Mucin [35S]-sulphate incorporation					
	Patients		Controls		
	Mean	SD	Mean	SD	p value
A	102	±109	87	±66	0.78
B	151	±133	61	±59	0.10
C	101	±100	111	±124	0.87
D	102	±77	80	±95	0.71

Table 4.21: Mean [35S] Incorporation within CIF fraction

4.3.7 Ratios of [35S]/[3H] within Mucin Peak Sub-Fractions

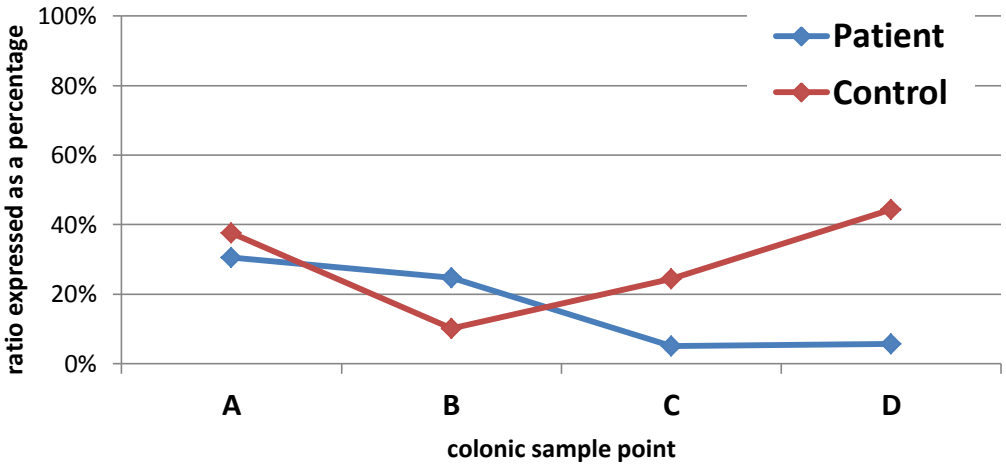


Figure 4.7: Mean Ratio of [35S]/[3H] Incorporation within SSF

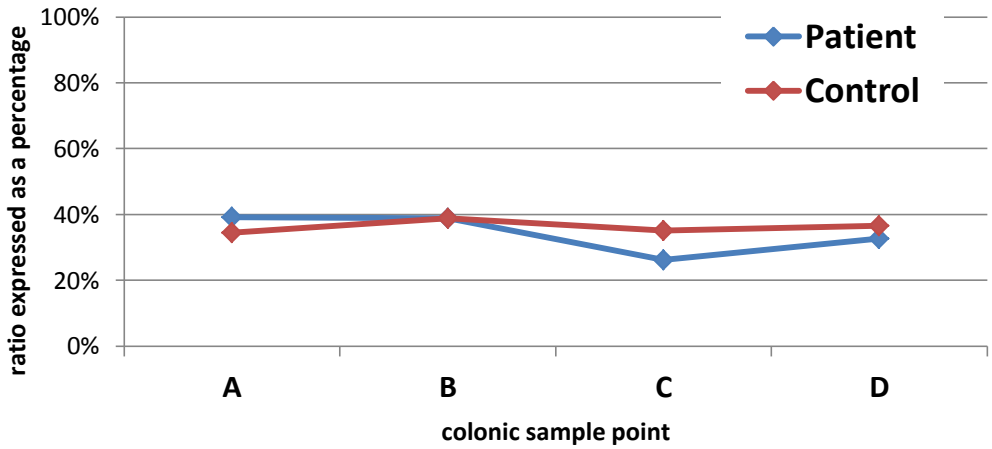


Figure 4.8: Mean Ratio of [35S]/[3H] Incorporation within CSF

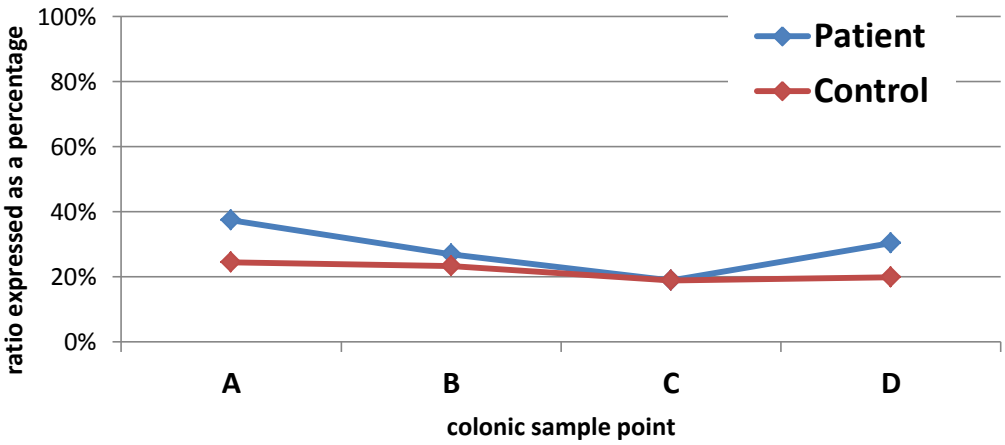


Figure 4.9: Mean Ratio of [35S]/[3H] Incorporation within CIF

4.4 Total Back Peak Incorporation

Mucin [3H]-glucosamine incorporation		
	Mean	**Standard Deviation**
Patients	11841	±6459
Controls	13022	±5759

(p value 0.72)

Table 4.22: Mean Total Overall [3H] Incorporation within Back Peak

Mucin [35S]-sulphate incorporation		
	Mean	**Standard Deviation**
Patients	3100	±2278
Controls	3403	±2406

(p value 0.81)

Table 4.23: Mean Total Overall [35S] Incorporation within Back Peak

4.4.1 Total Back Peak Incorporation at the Different Colonic Points

Total [3H] Incorporation					
Subject		**A**	**B**	**C**	**D**
Patients	G	6434.21	46247.05	7669.76	24944.47
	H	11368.60	9479.32	13168.95	10763.73
	J	9628.48	10852.93	6174.53	3464.17
	K	6262.98	8062.80	4506.99	4305.19
	L	2315.35	11225.50	34096.95	
	M	19632.45	30776.48	17542.04	19478.15
	Q	3416.82	4428.99	8263.71	4487.68
	R	9015.76	7516.07	5449.03	4921.01
	T	7790.02	6994.20	13577.41	16135.10
	E				
Controls	F	6675.01	12154.93	14386.72	12730.60
	N		12940.49	11004.03	
	P	8312.31	21363.22	41145.54	25027.81
	S	14447.36	14068.46	3442.42	
	X	15359.84	4769.28	19232.04	
	Y	9726.67	9896.37	2525.15	5591.24

Table 4.24: Total Individual [3H] Incorporation within Back Peak

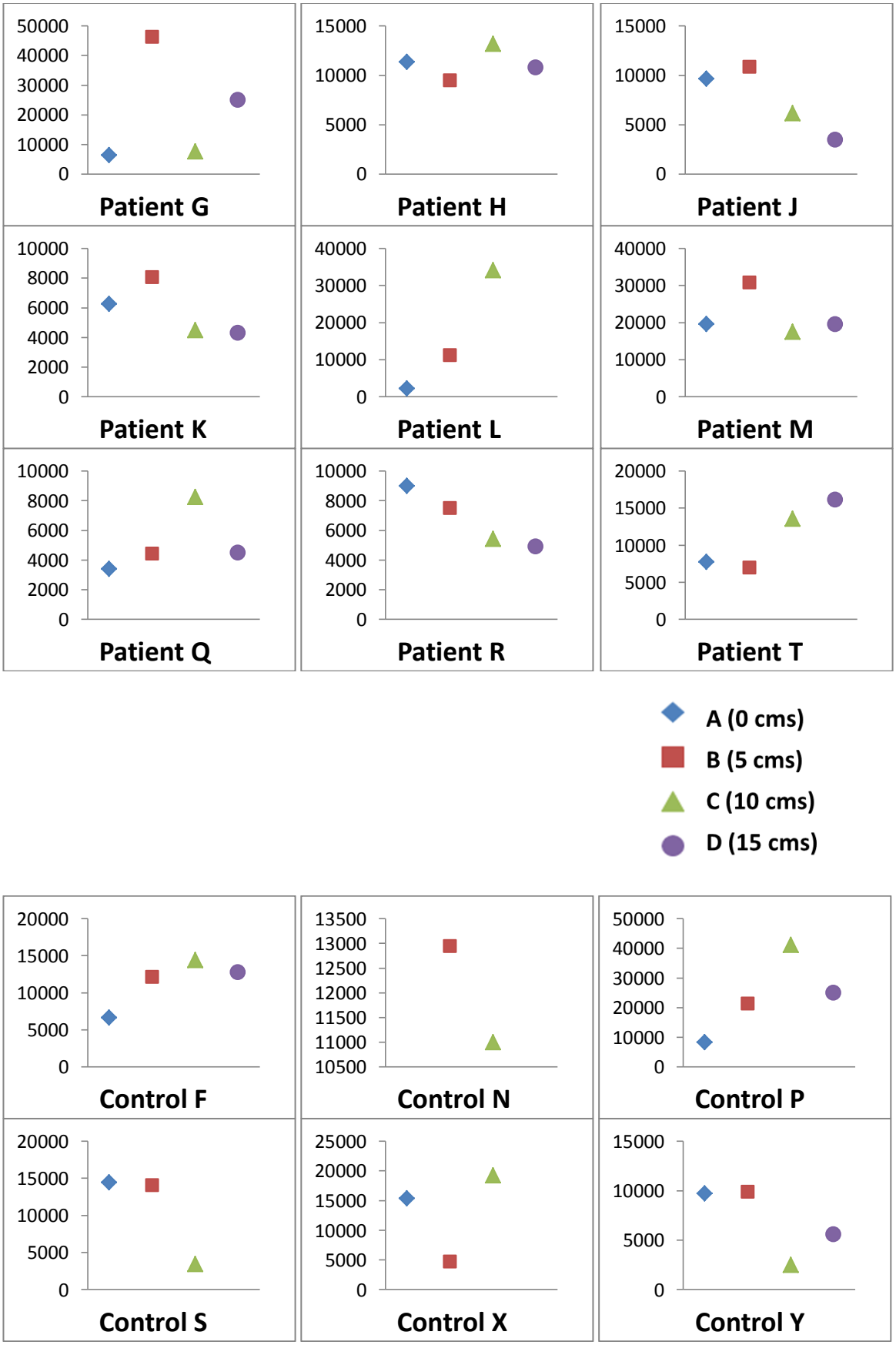


Figure 4.10: Individual subject graphs of total back peak [3H]-glucosamine incorporation (corrected disintegrations per minute/ngDNA) at colonic points A to D

Total [35S] Incorporation					
Subject		**A**	**B**	**C**	**D**
Patient	G	1664.26	20100.69	2194.36	9402.01
	H	2290.55		1801.99	1793.81
	J	3614.96	4895.57	1288.71	785.63
	K	921.98	1731.73	1188.78	1558.75
	L	374.83	2270.62	8490.40	
	M	3300.63	8720.19	2478.31	3466.42
	Q	1291.14	1141.59	1705.81	1134.92
	R	1471.66	1221.25	795.39	903.38
	T	3091.87	1849.01	3098.59	3880.51
	E				
Control	F	1106.71	3183.56	3456.66	3868.38
	N		2231.52	1892.04	
	P	951.66	1178.61	2754.46	657.90
	S	3078.31	4463.11	592.52	
	X	5261.07	2251.80	16866.83	
	Y	4629.15	4508.87	1433.70	2336.01

Table 4.25: Total Individual [35S] Incorporation within Back Peak

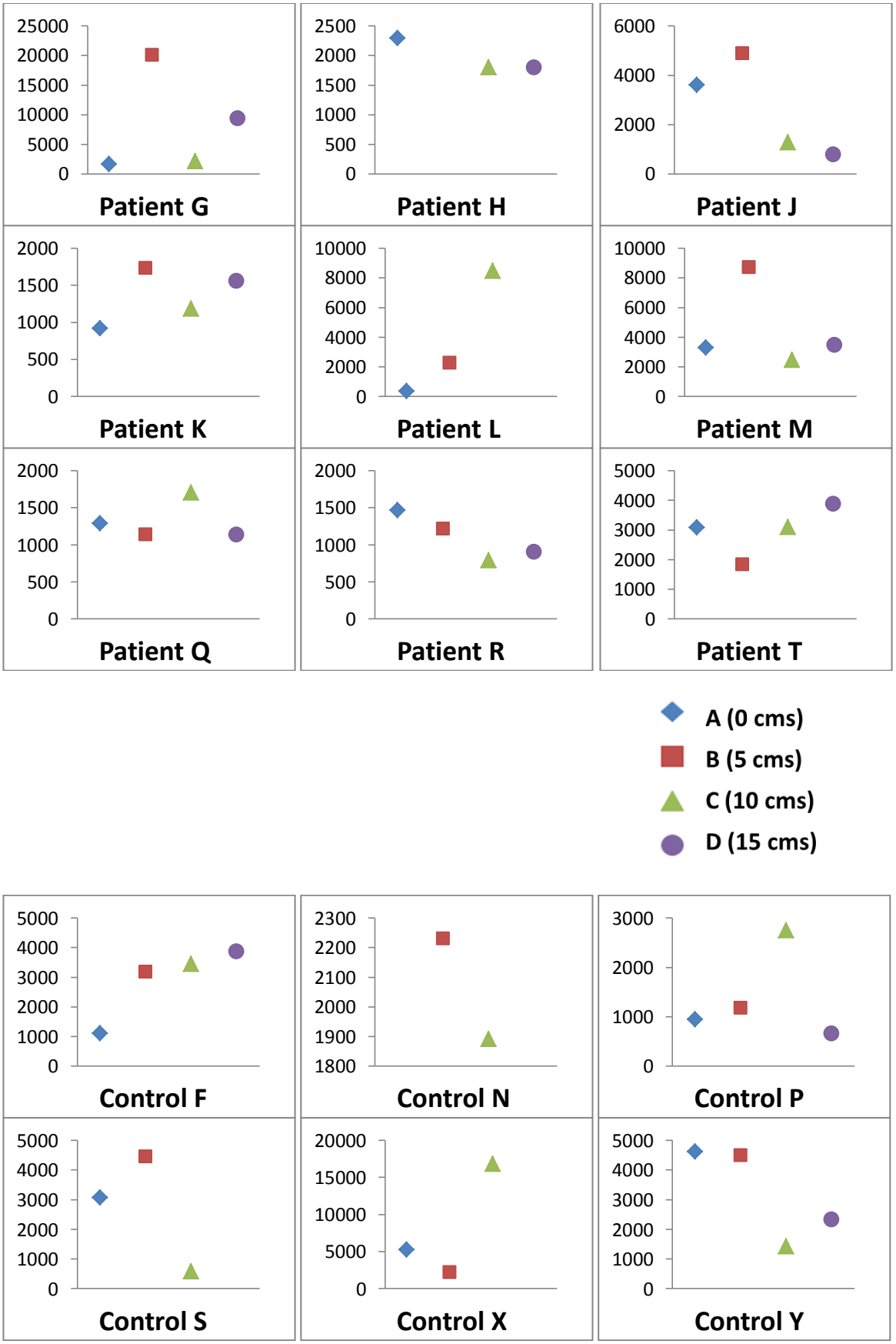


Figure 4.11: Individual subject graphs of total back peak [35S]-sulphate incorporation (corrected disintegrations per minute/ngDNA) at colonic points A to D

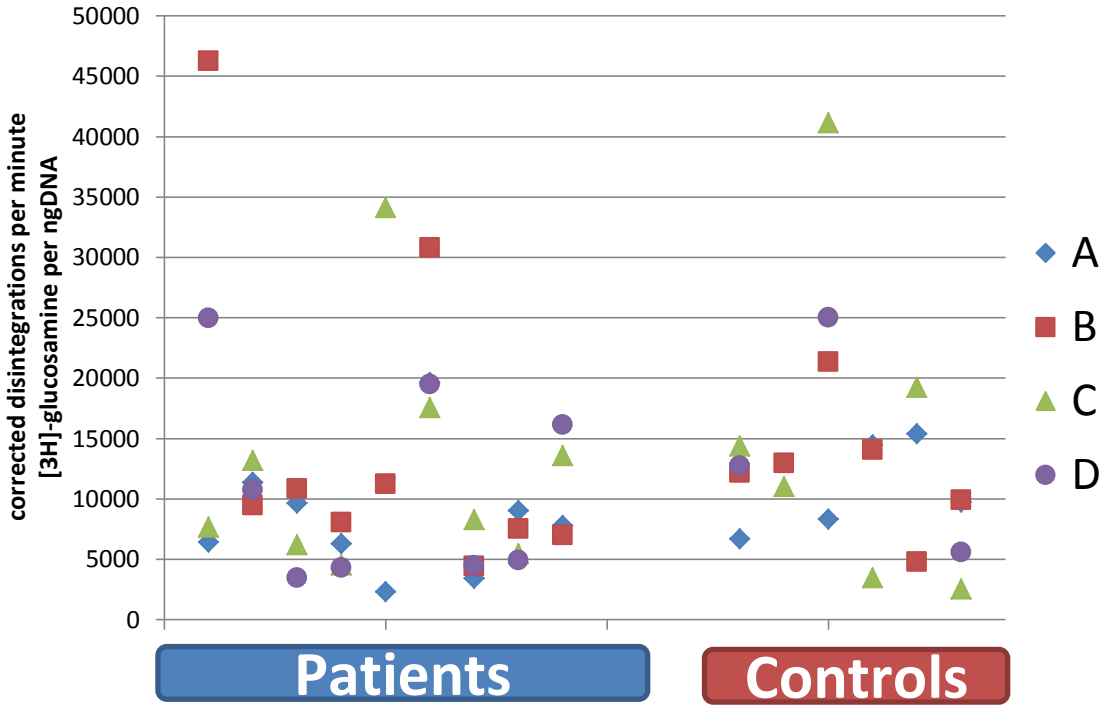


Figure 4.12: Scatter graph of Back Peak [3H]-glucosamine incorporation

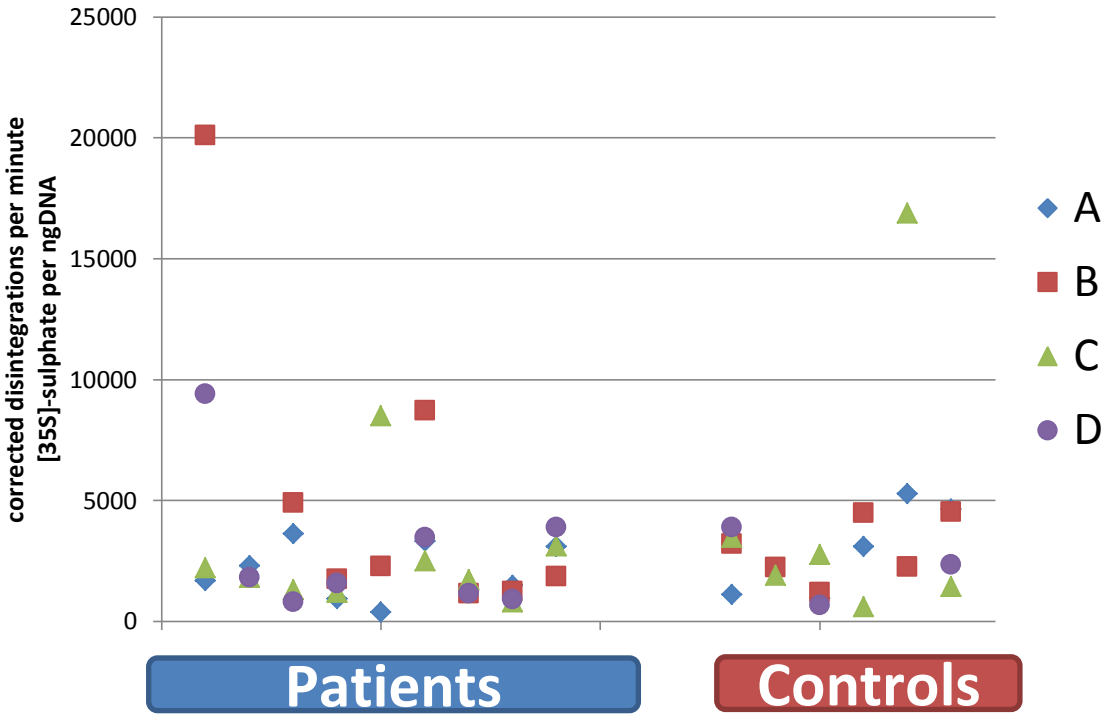


Figure 4.13: Scatter graph of Back Peak [35S]-sulphate incorporation

4.4.2 Mean Back Peak Incorporation at the Different Colonic Points

Mean Back Peak [3H]-glucosamine Incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	8429	±5093	10904	±3821	0.36
B	15065	±13996	12532	±5438	0.63
C	12272	±9273	15289	±14188	0.62
D	11062	±8226	14450	±9832	0.58

Table 4.26: Mean [3H] Incorporation within Back Peak at Colonic Points

Mean Back Peak [35S]-sulphate Incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	2002	±1133	3005	±1972	0.24
B	5241	±6532	2970	±1335	0.37
C	2560	±2332	4499	±6141	0.49
D	2866	±2882	2287	±1606	0.75

Table 4.27: Mean [35S] Incorporation within Back Peak at Colonic Points

4.5 Incorporation within the Sub-Fractions of the Back Peak

The following tables show the incorporation results in dpm/ngDNA obtained from the three sub-fractions within the back peak of both patients and controls.

4.5.1 Secreted Soluble Fraction - [3H]-glucosamine incorporation

Subject		A	B	C	D
Patients	G	1163.30	3447.67	3963.91	4992.64
	H	2942.37	69.42	1518.92	1118.50
	J	1567.00	7400.46	4951.23	2111.40
	K	1615.80	2903.26	1734.63	1451.79
	L	1202.00	2498.91	11713.56	
	M	3550.58	7600.31	4142.52	5990.33
	Q	925.65	1193.26	2852.32	2076.20
	R	2518.04	3123.31	2008.43	1854.98
	T	1552.09	1123.96	3033.01	2471.00
	E				
Controls	F	615.59	1256.73	2214.07	1203.31
	N		3067.79	6500.23	
	P	2111.60	17816.81	32630.06	23199.01
	S	2881.20	10485.94	1631.43	
	X	3239.38	1969.24	3918.97	
	Y	4585.40	3539.42	2913.75	2585.64

Table 4.28: Individual [3H] Incorporation within SSF fraction of the Back Peak

Mucin [3H]-glucosamine incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	1893	±900	2687	±1464	0.23
B	3262	±2540	6356	±6518	0.31
C	3991	±3121	8301	±12041	0.43
D	2758	±1757	8996	±12320	0.47

Table 4.29: Mean [3H] Incorporation within SSF fraction of the Back Peak

4.5.2 Cellular Soluble Fraction - [3H]-glucosamine incorporation

Subject		A	B	C	D
Patients	G	5878.40	44720.69	7018.35	23975.67
	H	9670.87	8346.52	10639.45	9294.72
	J	8156.44	8994.29	4153.43	2316.91
	K	5161.90	7120.29	4000.41	3860.47
	L	1652.28	8303.59	24416.50	
	M	17173.53	32609.90	15696.18	16166.94
	Q	2662.80	3158.56	4942.91	2868.45
	R	8238.62	5415.50	4487.33	2848.77
	T	7158.26	6422.46	11869.46	13896.44
	E	3812.12	1769.94		
Controls	F	6376.51	10996.98	12113.09	11810.46
	N		12031.01	10026.94	
	P	7636.90	8807.93	15357.96	4865.35
	S	13067.69	11815.99	1393.43	
	X	11526.02	4472.57	18250.78	
	Y	9208.61	8195.53	1994.98	5095.87

Table 4.30: Individual [3H] Incorporation within CSF fraction of Back Peak

Mucin [3H]-glucosamine incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	6957	±4419	9563	±2744	0.25
B	12686	±14173	9387	±2877	0.49
C	9692	±6877	9856	±6918	0.96
D	9404	±7972	7257	±3945	0.67

Table 4.31: Mean [3H] Incorporation within CSF fraction of Back Peak

4.5.3 Cellular Insoluble Fraction - [3H]-glucosamine incorporation

Subject		A	B	C	D
Patients	G	591.70	2037.28	897.93	1263.90
	H	699.44	1090.54	1531.92	1211.43
	J	1439.57	2304.12	2067.55	873.27
	K	668.01	1029.67	292.59	469.04
	L	188.38	1311.68	2122.75	
	M	1718.79	1822.69	1519.01	2410.74
	Q	398.07	1052.10	994.42	914.16
	R	706.67	1120.01	715.32	613.14
	T	787.63	1060.43	1288.98	1479.11
	E				
Controls	F	976.96	995.93	1597.64	1112.51
	N		1492.64	975.23	
	P	977.53	957.17	2105.79	700.42
	S	1627.12	1606.11	135.32	
	X	1094.24	372.85	747.71	
	Y	586.98	572.10	86.61	325.39

Table 4.32: Individual [3H] Incorporation within CIF fraction of Back Peak

Mucin [3H]-glucosamine incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	800	±483	1053	±374	0.33
B	1425	±494	999	±488	0.12
C	1270	±609	941	±801	0.38
D	1154	±609	713	±394	0.28

Table 4.33: Mean [3H] Incorporation within CIF fraction of Back Peak

4.5.4 Secreted Soluble Fraction - [35S]-sulphate incorporation

Subject		A	B	C	D
Patients	G	39.18	238.35	154.50	234.27
	H	555.60		83.30	34.60
	J	23.54	74.36	116.15	37.60
	K	44.65	88.20	16.43	33.84
	L	21.42	26.60	222.25	
	M	166.93	246.84	117.53	81.56
	Q	16.04	45.22	87.37	10.74
	R	43.58	0.00	16.36	36.51
	T	76.53	36.12	121.43	80.12
	E				
Controls	F	45.96	88.52	178.69	95.58
	N		38.85	126.29	
	P	32.61	81.61	269.19	88.03
	S	58.82	319.28	49.02	
	X	226.33	89.32	98.68	
	Y	128.47	134.00	61.01	67.83

Table 4.34: Individual [35S] Incorporation within SSF fraction of Back Peak

Mucin [35S]-sulphate incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	110	±173	98	±80	0.89
B	94	±95	125	±100	0.57
C	104	±64	130	±83	0.50
D	69	±71	84	±14	0.58

Table 4.35: Mean [35S] Incorporation within SSF fraction of Back Peak

4.5.5 Cellular Soluble Fraction - [35S]-sulphate incorporation

Subject		A	B	C	D
Patients	G	1717.41	20204.11	2157.14	9708.16
	H	2052.02		1628.89	1627.84
	J	3575.16	4700.07	1252.02	730.46
	K	783.68	1566.20	1080.90	1449.39
	L	366.47	2206.53	8291.40	
	M	3003.91	10086.58	2588.13	3314.59
	Q	964.24	627.14	1534.91	830.51
	R	1838.72	1098.64	714.15	794.57
	T	2974.30	1846.53	2822.55	3130.43
	E	2632.85	1508.68		
Controls	F	1160.19	2934.21	3199.46	3570.33
	N		2187.96	1850.71	
	P	921.00	1144.11	2699.98	628.32
	S	2850.36	4200.59	545.17	
	X	4759.43	2232.26	16270.85	
	Y	4673.30	5071.99	1442.04	2254.65

Table 4.36: Individual [35S] Incorporation within CSF fraction of Back Peak

Mucin [35S]-sulphate incorporation					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	1991	±1063	2873	±1840	0.25
B	4872	±6452	2962	±1444	0.41
C	2452	±2297	4335	±5922	0.74
D	2698	±3009	2151	±1474	0.78

Table 4.37: Mean [35S] Incorporation within CSF fraction of Back Peak

4.5.6 Cellular Insoluble Fraction - [35S]-sulphate incorporation

Subject		A	B	C	D
Patients	G	71.97	601.34	204.25	374.39
	H	152.56		263.23	219.65
	J	142.42	352.08	269.84	106.32
	K	160.70	275.43	110.78	145.48
	L	57.21	220.46	383.59	
	M	314.72	494.83	304.03	299.12
	Q	326.71	670.42	332.98	346.50
	R	103.15	194.41	98.14	92.54
	T	256.83	415.52	337.68	447.59
	E				
Controls	F	231.59	320.64	267.96	479.46
	N		257.16	270.04	
	P	86.84	79.47	110.67	36.24
	S	461.31	253.94	17.04	
	X	316.57	224.55	605.38	
	Y	31.86	182.42	14.34	172.67

Table 4.38: Individual [35S] Incorporation within CIF fraction of Back Peak

Mucin [35S]-sulphate incorporation					
	Patients		Controls		
	Mean	SD	Mean	SD	p value
A	176	±100	226	±174	0.51
B	403	±175	220	±82	**0.04**
C	256	±100	214	±223	0.68
D	254	±133	229	±227	0.82

Table 4.39: Mean [35S] Incorporation within CIF fraction of Back Peak

4.5.7 Ratios of [35S]/[3H] within Back Peak Sub-Fractions

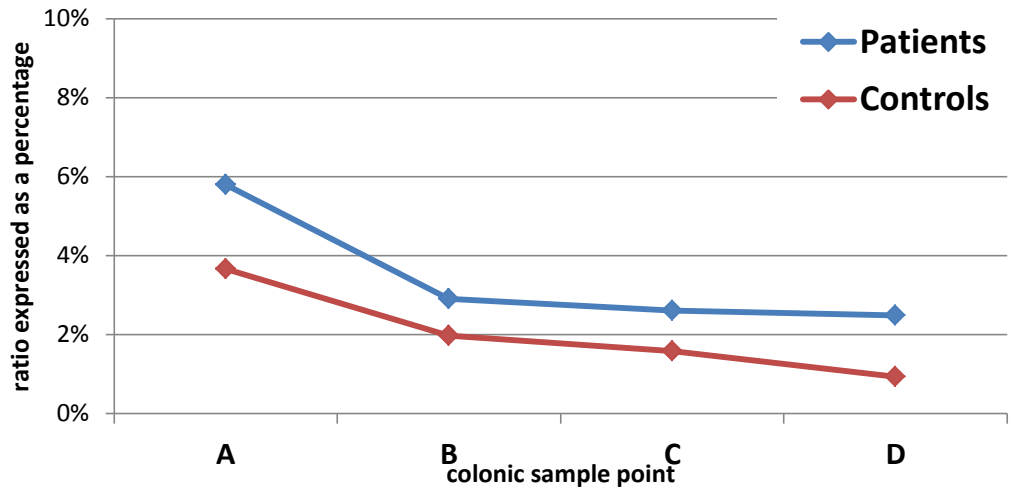


Figure 4.14: Mean Ratio [35S]/[3H] Incorporation within SSF

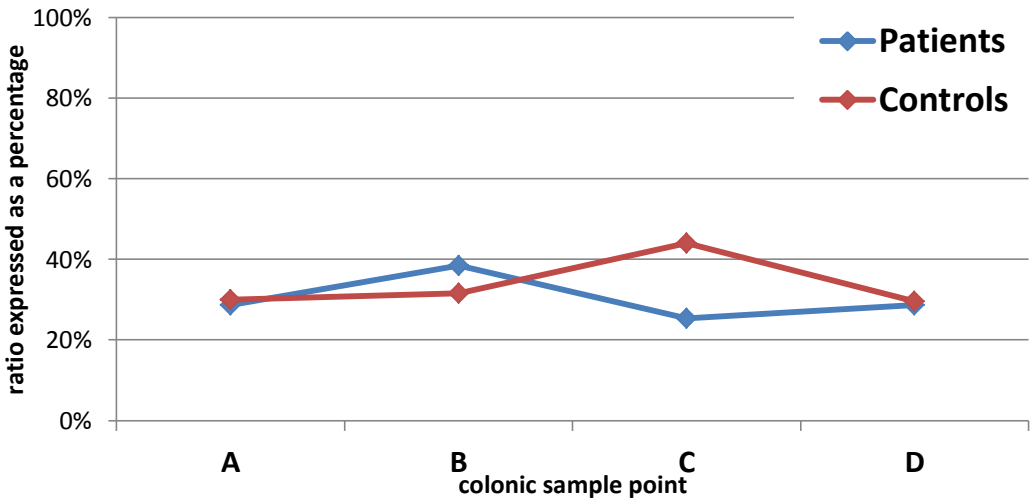


Figure 4.15: Mean Ratio [35S]/[3H] Incorporation within CSF

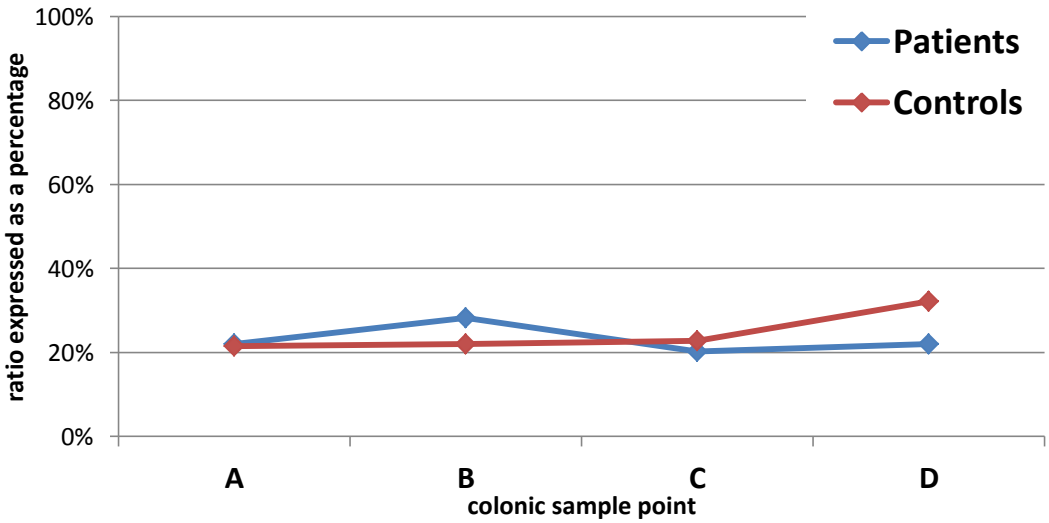


Figure 4.16: Mean Ratio [35S]/[3H] Incorporation within CIF

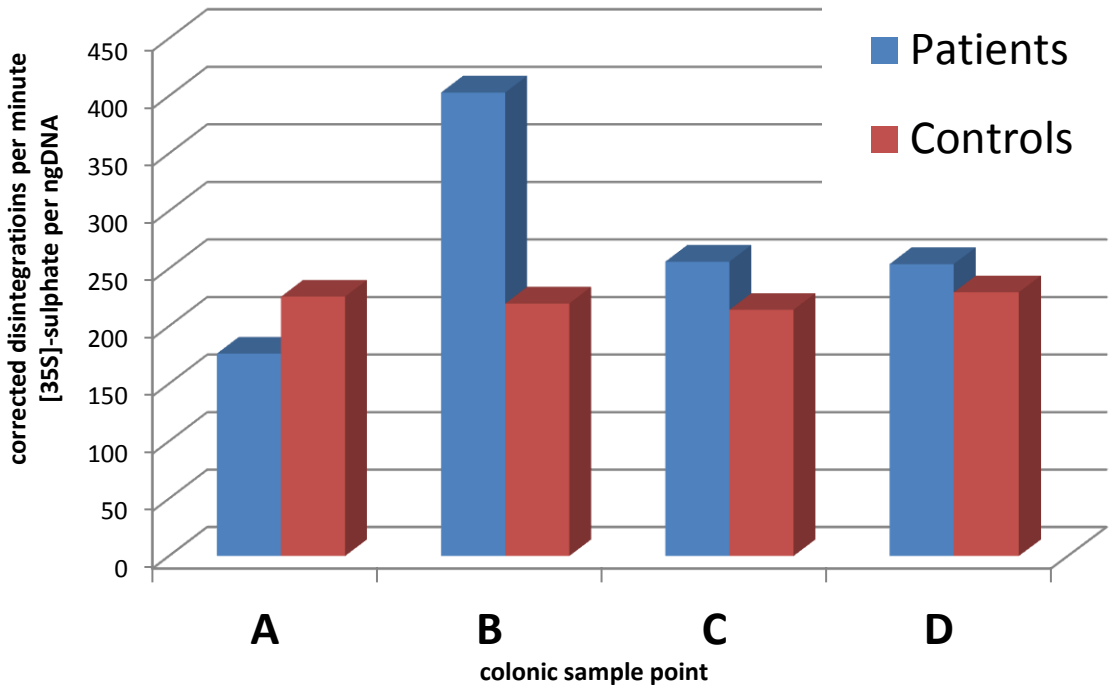


Figure 4.17: Mean [35S] Incorporation within CIF of Back peak

4.6 Lectin Binding

Results were expressed in corrected densitometry per 100ngDNA. Slot blots performed with the lectins wheat germ agglutinin and *Maackia amurensis* agglutinin bound to the mucin samples. Slot blots performed with the lectin *Ulex europaeus* agglutinin (UEA) did not show any binding in any control or patient samples. Slot blots performed with WGA showed binding at the same fractions as mucin peak and back peak incorporation.

4.6.1 Wheat Germ Agglutinin Mucin Binding

Overall WGA binding within mucin peak		
	Mean	**Standard Deviation**
Patients	537.88	± 752.14
Controls	18.13	±33.99
		(p value 0.06)

Table 4.40: Overall mean WGA Mucin binding

Subject		A	B	C	D
Patients	G	789.42	2661.13	3539.55	224.80
	H	2215.85	825.32	2813.37	574.82
	J	350.95	2082.46	1410.56	1759.80
	K	0.15	61.22	112.22	35.18
	L	513.28	556.10	198.43	
	M	0.75	4.79	1.24	0.10
	Q	94.57	37.68	18.03	3.44
	R	2.20	3.45	3.55	1.33
	T	75.06	68.28	0.24	37.28
	E	7.42	0.49		
Controls	F	43.43	55.52	146.85	98.33
	N		2.47	0.01	
	P	0.05	2.39	0.13	0.04
	S	1.10	2.68	0.00	
	X	4.80	16.86	33.79	
	Y	0.19	4.17	0.00	0.01

Table 4.41: Total Individual WGA Mucin Binding Results

Mean Total WGA Mucin Binding					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	404.97	±690.77	9.91	±18.83	0.10
B	630.09	±968.87	14.02	±21.09	0.08
C	899.69	±1378.43	30.13	±58.76	0.10
D	329.59	±610.79	32.79	±56.75	0.22

Table 4.42: Mean Total WGA Mucin Binding Results

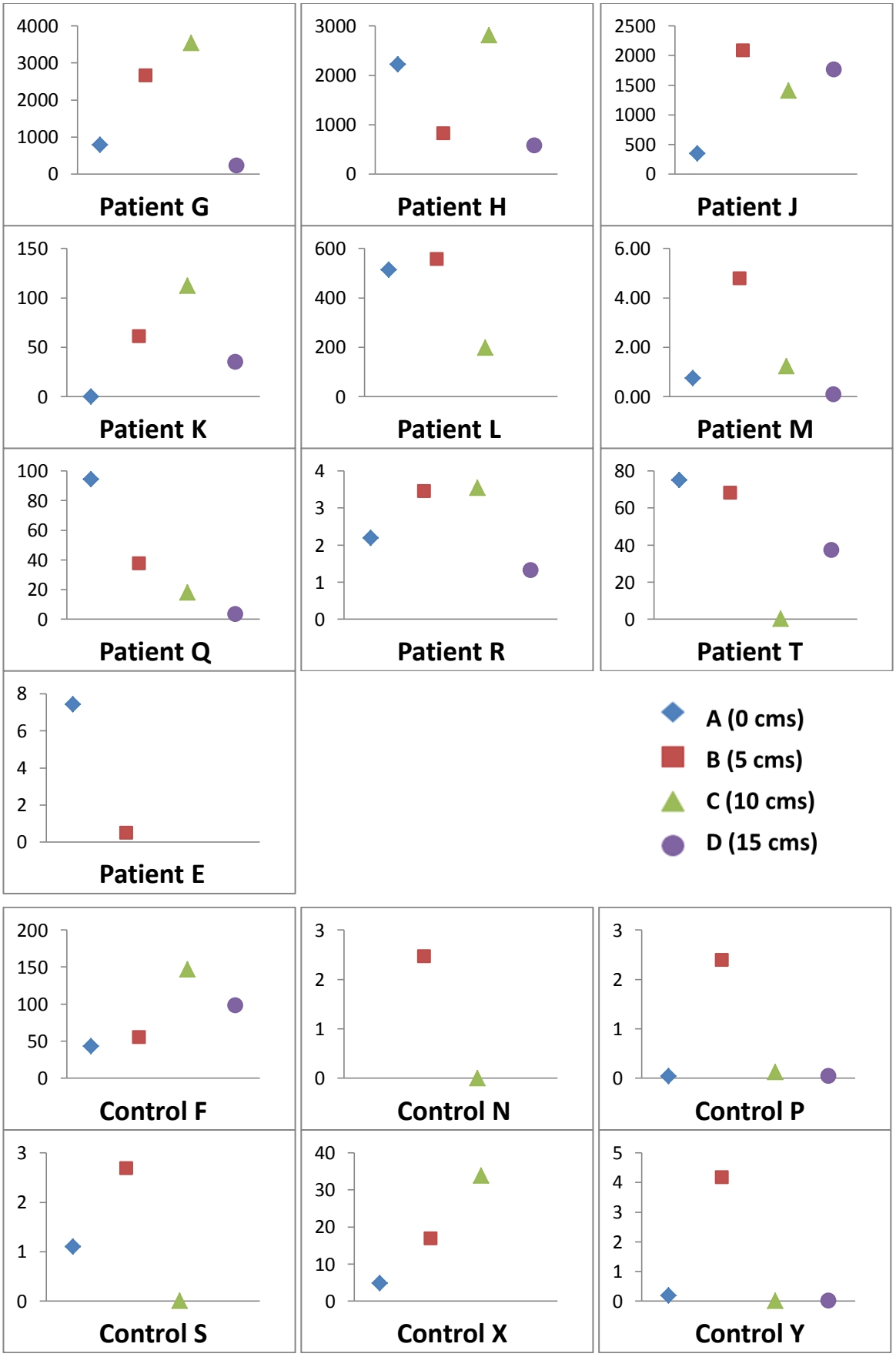


Figure 4.18: Individual subject graphs of total mucin peak WGA binding (densitometry per 100ngDNA) at colonic points A to D

4.6.2 Wheat Germ Agglutinin Binding within Mucin Sub-Fractions

Secreted Soluble Fraction					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	3.22	±9.95	0.14	±0.31	0.35
B	6.11	±19.24	0.16	±0.36	0.35
C	27.18	±80.38	0.39	±0.94	0.35
D	4.13	±10.18	2.20	±3.79	0.76
Overall	10.03	±40.89	0.53	±1.52	0.10

Table 4.43: Mean WGA Mucin Binding Results within SSF Fraction

Cellular Soluble Fraction					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	29.47	±43.33	2.15	±2.75	0.08
B	23.02	±39.86	7.01	±6.76	0.25
C	3.34	±5.10	6.90	±13.42	0.56
D	8.82	±14.60	1.24	±2.13	0.20
Overall	16.90	±32.07	4.90	±8.26	**0.04**

Table 4.44: Mean WGA Mucin Binding Results within CSF Fraction

Cellular Insoluble Fraction					
	Patients		**Controls**		
	Mean	**SD**	**Mean**	**SD**	**p value**
A	372.28	±660.62	7.62	±16.55	0.12
B	600.97	±973.76	6.85	±16.76	0.86
C	869.17	±1338.96	22.84	±55.83	0.95
D	316.65	±610.63	29.36	±50.84	0.23
Overall	542.92	±929.56	15.21	±36.21	**0.002**

Table 4.45: Mean WGA Mucin Binding Results within CIF Fraction

4.6.3 Maackia Amurensis Agglutinin Mucin Binding

Cellular Soluble Fraction					
	Patients		Controls		
	Median	Range	Median	Range	p value
A	2.51	0 – 140.49	0.00	0 – 2.22	0.24
B	3.67	0 – 32.39	0.92	0 – 16.34	0.36
C	4.48	0 – 143.61	0.08	0 – 19.83	0.17
D	1.91	0 – 130.90	0.38	0 – 0.44	0.76
Overall	3.83	0 – 143.61	0.08	0 – 19.83	**0.03**

Table 4.46: Median MAA Mucin Binding Results within CSF Fraction

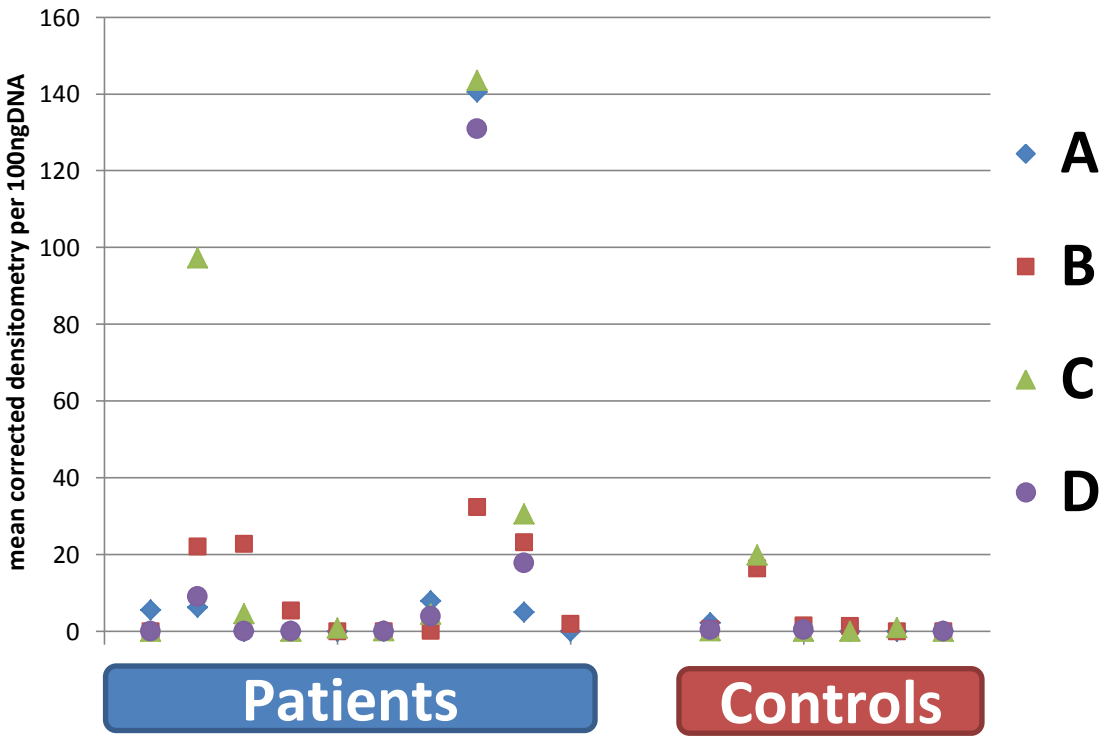


Figure 4.19: Scatter Graph for MAA Binding

Chapter 5 - Discussion

The aim of the study was to investigate the biosynthetic activity of the distal ganglionic colon in patients with Hirschsprung's disease and compare this to controls and to determine whether previously found deficiencies in mucin production in the ganglionic colon, close to the transition zone, persisted more proximally or were confined to a limited region.

5.0 Gel Filtration Graphs

The mucin peak elucidated at a similar fraction number and demonstrated a consistent pattern along the colon and in patients and controls. (Figures 5.1 & 5.2) The peaks for both [3H]-glucosamine and [35S]-sulphate incorporation occurred at approximately the same fractions with the former consistently having higher peak values than the latter. This was to be expected as [3H]-glucosamine would be present in all newly synthesized mucins but not all would be sulphated.

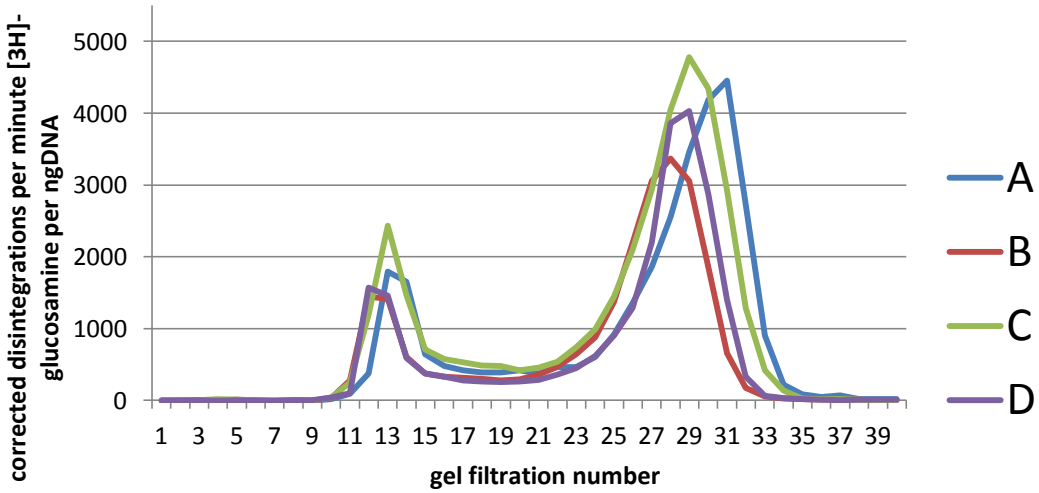


Figure 5.1 Example of [3H]-Glucosamine Incorporation Gel Filtration Graph

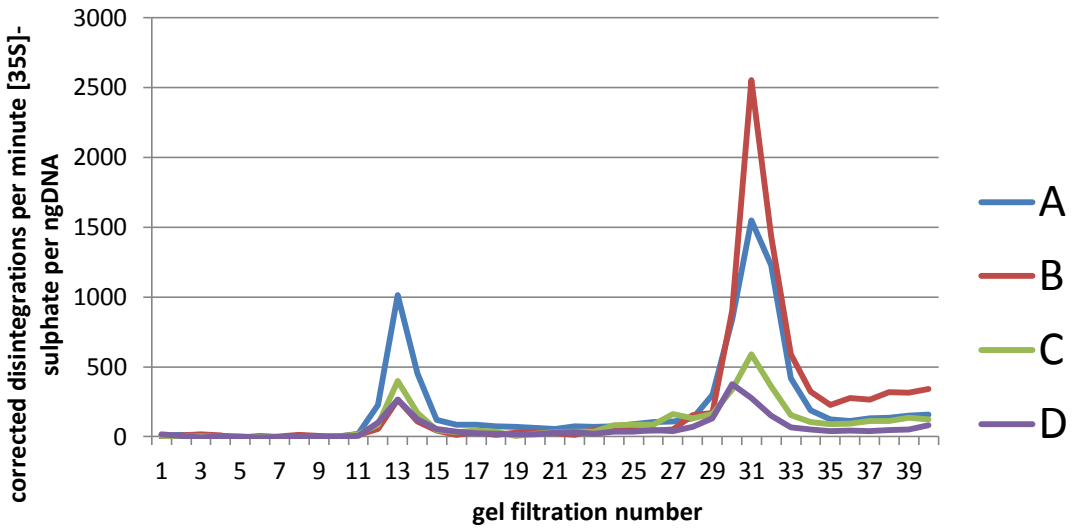


Figure 5.2 Example of [35S]-Sulphate Incorporation Gel Filtration Graph

The precursors [3H]-glucosamine and [35S]-sulphate are not only incorporated into fully formed mucin molecules but also into many other different cellular glycoconjugates formed during the incubation period, including monosaccharides or oligosaccharides which would eventually form part of de novo mucin molecules. All of these are of a lower molecular weight than mucins so will enter the gel beads and are elucidated later during gel filtration forming the back peak. (Figures 5.1 & 5.2)

The back peak is unlikely to consist of mucin degradation products, as the addition of broad spectrum antibiotics to the culture medium would reduce any bacterial activity on the newly produced mucin molecules.

The back peaks of [3H]-glucosamine incorporation were more varied than the mucin peaks in their configuration and in comparison to back peaks of [35S]-sulphate incorporation, which had a narrower base. (Figures 5.3 & 5.4) This suggests that the [35S]-sulphate is incorporated into molecules of more defined molecular weight and that this is unaffected by colonic site, whereas [3H]-glucosamine is incorporated in a broader range of molecules, covering a greater variance in molecular weight and that

this range varies along the colon. In particular the back peaks for the cellular insoluble fraction were very broad, covering a greater range of molecular weights.

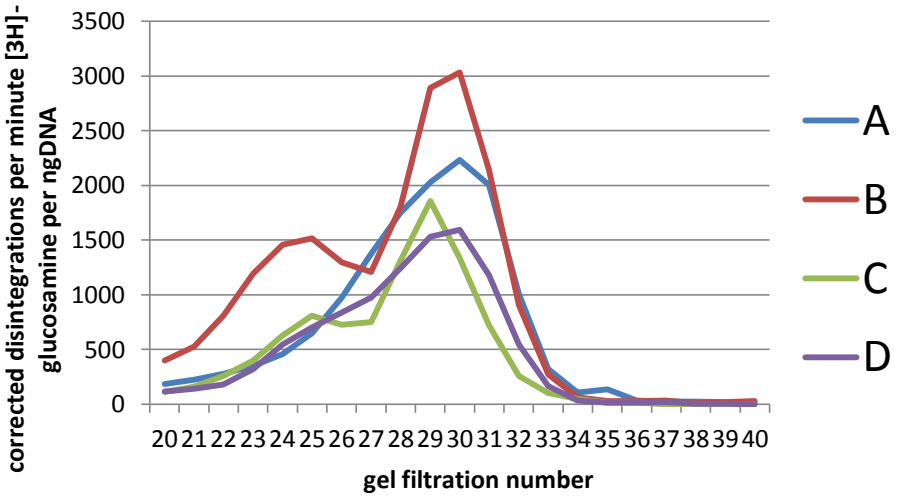


Figure 5.3: Example of Patient [3H]-Glucosamine Incorporation Gel Filtration Back Peak Graph

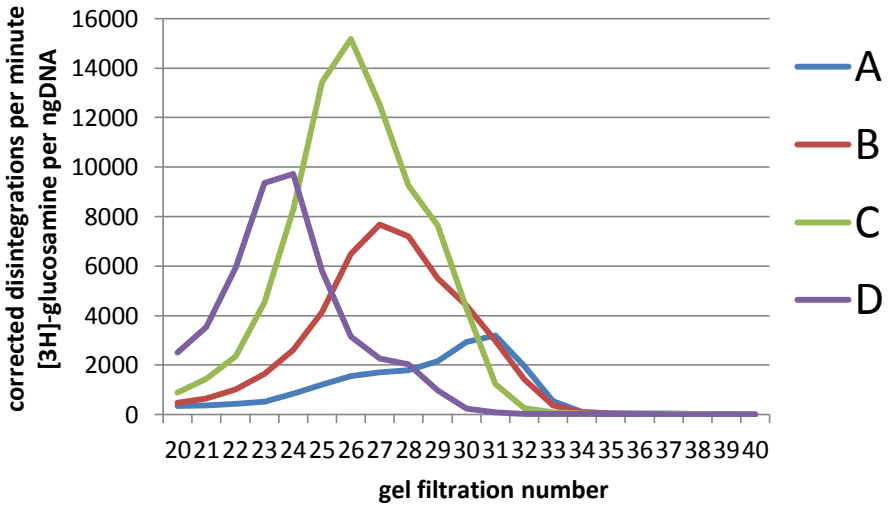


Figure 5.4: Example of Control [3H]-Glucosamine Incorporation Gel Filtration Back Peak Graph

Comparing the graphs obtained within individual subjects, there was not an obvious progression pattern identified moving along the colon from samples A to D for patients or controls. However, within an individual subject, especially within the cellular soluble fraction, the graphs for [3H]-glucosamine and [35S]-sulphate incorporation demonstrated the same relative variances within the mucin and back peak along the colon. This was found to be the case in both patients and control subjects. This suggests that mucin production is closely allied to the production of the back peak of and that uptake of both precursors is altered in a similar way.

5.1 Incorporation within the Mucin Peak

Glucosamine-6-phosphate is the precursor for *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid (sialic acid).[126, 127] The first two in particular are common to all mucins so the amount of [3H]-glucosamine incorporation within the front peak reflects the amount of newly produced glycosylated mucin synthesized within the time of incubation.[44]

Within the mucus barrier there is a constant dynamic equilibrium between mucin synthesis and mucin degradation. The normal enteric bacterial flora secrete a range of enzymes which act to continually break down the mucin gel as the mucosa produces newly formed and secreted mucins.[100] A balance between these conflicting processes allows the host to maintain a functional defensive barrier at the mucosal surface.[90] Sulphation of mucins is particularly characteristic of colonic mucins and is one of the mechanisms which helps reduce bacterial degradation.[113, 168]

Sulphate esters may also regulate the biosynthesis, half-life and biological roles of glycoproteins so sulphation is also an important regulatory feature in colonic mucins.[112,

[169] The amount of incorporation of [35S]-sulphate within the mucin peak and the ratio of [35S]-sulphate to [3H]-glucosamine incorporation reflects the degree of modification of mucins and may reflect their robustness.[100, 111]

Previous research has shown that mucin production in the colon, close to the transition zone, is reduced in patients.[345] One of the aims of this research was to determine whether this deficiency extends to a greater region of colon. Therefore the overall mucin incorporation for the fifteen centimeters of colon studied was calculated as a mean for each study group. Patient values for both precursors were higher than control although this was not statistically significant, $p = 0.53$ [3H] and $p = 0.66$ [35S]. This suggests that the overall ganglionic mucin production is not impaired in Hirschsprung's disease.

The thickness of the mucus layer is known to increase along the colon towards the rectum[9] and under normal circumstances colonic mucins should become more sulphated distally.[104, 112, 172, 177, 178] Therefore an increasing amount of mucin production, using both precursors, from D to A would be expected in the normal bowel.

Mean values for the control subjects were calculated for [3H]-glucosamine incorporation and [35S]-sulphate incorporation for each colonic level. Levels of incorporation in the mucin peak of both precursors were greatest most distally and lowest most proximally and showed a similar pattern for both precursors. (Figures 5.5 & 5.6) These results agree with what would be expected with an increase of mucin production D to A.

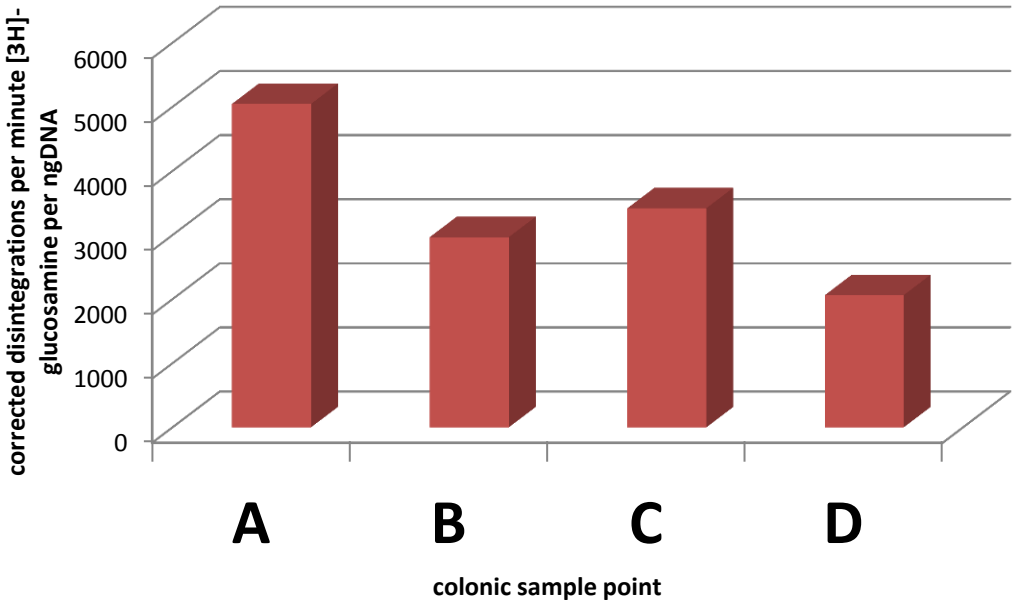


Figure 5.5 : Control Mean Mucin [3H] Incorporation A-D

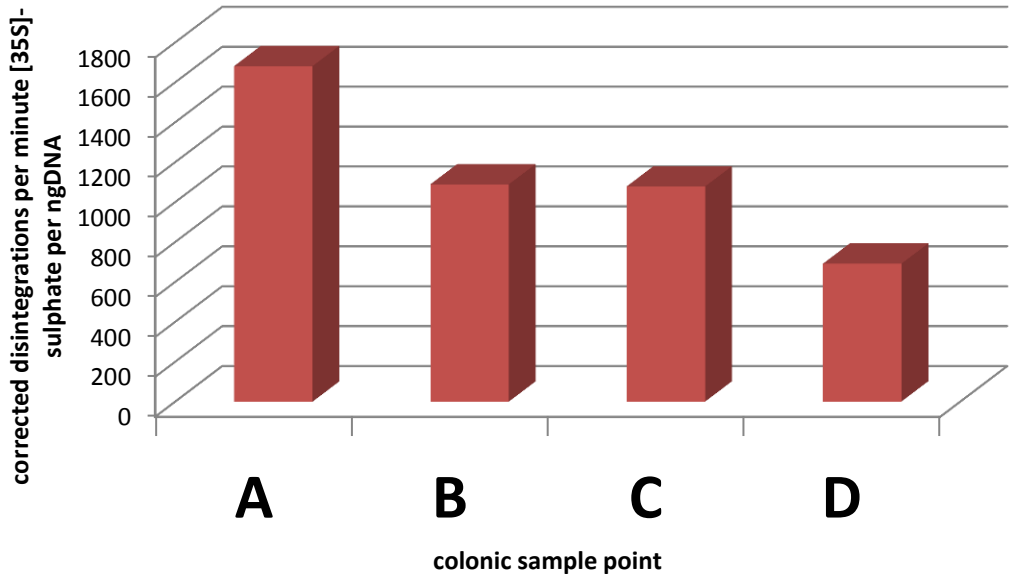


Figure 5.6 : Control Mean Mucin[35S] Incorporation A-D

An increase in the sulphation of colonic mucins, proximal to distal, would show in the ratio of [35S]-sulphate to [3H]-glucosamine incorporation in controls. However, there was no obvious increase in the percentage of sulphated mucins moving distally. (Figure 5.7)

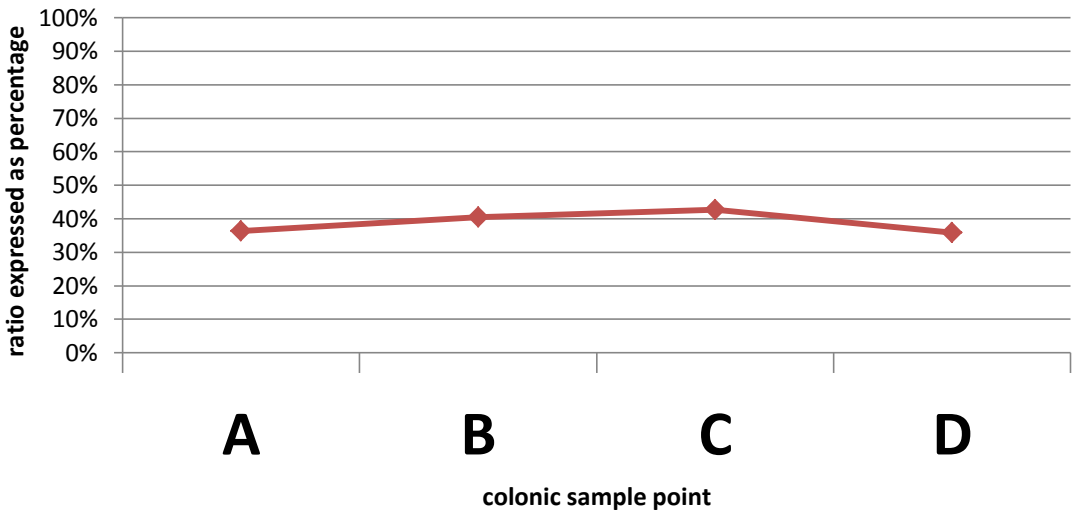


Figure 5.7 : Ratio of Control Mean [35S]/[3H] Incorporation

Patients, in contrast, demonstrated low incorporation values distally, closest to the transition zone, compared to control subjects (Figure 5.8). This is in agreement with the previous research.[345] This low mucin productivity did not persist further away from the transition zone as the patient values were only lower than controls at point A. The patient values at the other points were greater than controls, with the lowest mucin productivity occurring most proximally at D. (Figure 5.9)

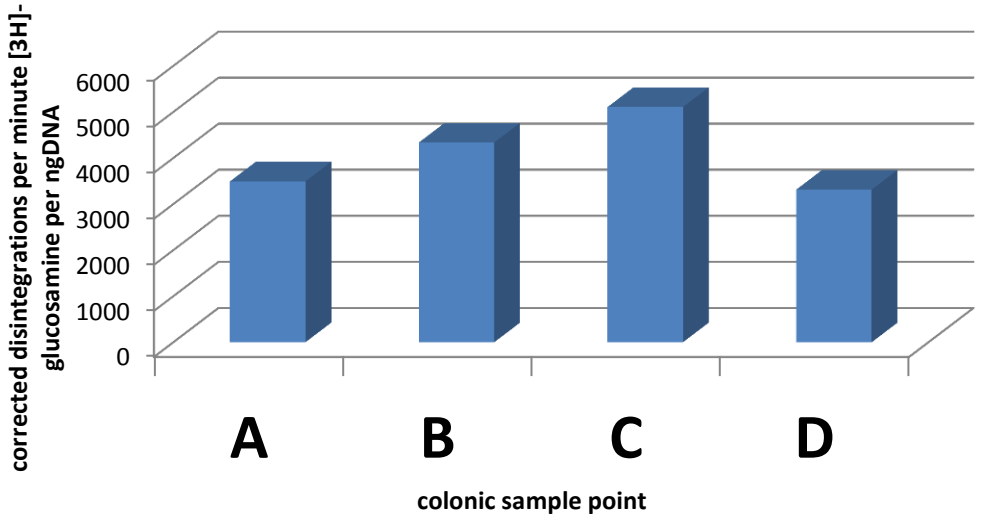


Figure 5.8 : Patient Mean Mucin [3H] Incorporation A-D

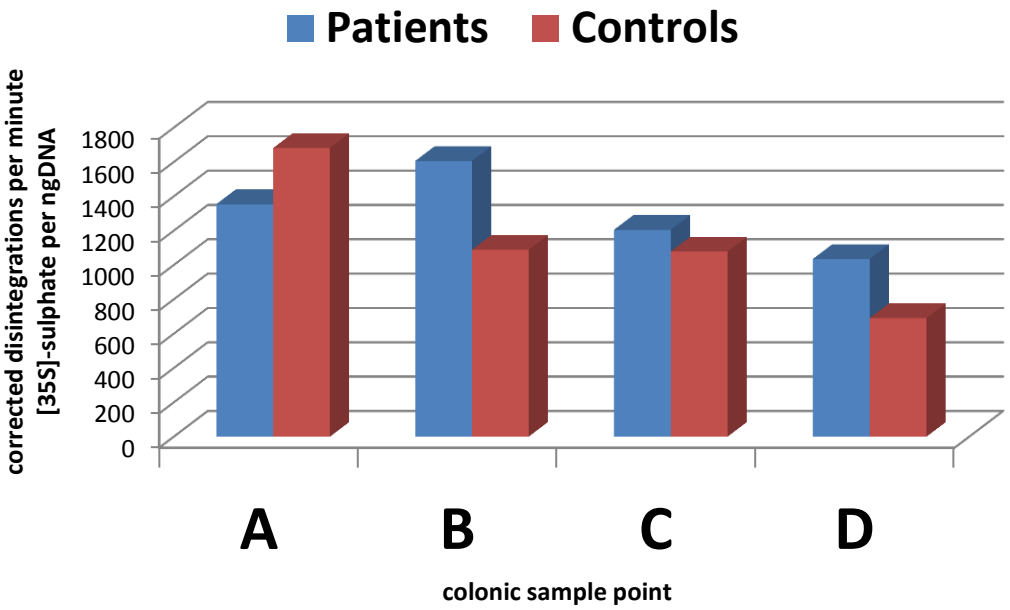


Figure 5.9 : Patient and Control Mean[35S] Incorporation A-D

These differences distally in the two groups were also reflected in the individual gel filtration graphs, as in the majority of controls the mucin peak at A was higher than at B whereas in patients the reverse was true. Ratios of incorporation were similar in the two groups showing that the overall proportion of newly formed mucins which are sulphated was not markedly altered in Hirschsprung's disease (Figure 5.10)

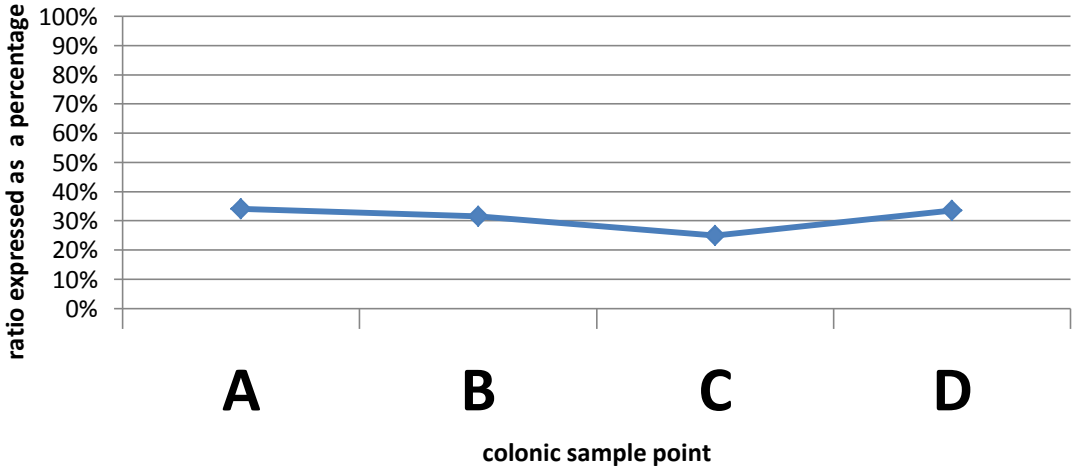


Figure 5.10: Ratio of Patient Mean [35S]/[3H] Incorporation

These results show that in HD mucin production is low in the ganglionic colon closest to the transition zone when compared to normal controls. Mucus secretion is partly under the control of the autonomic nervous system, of which the enteric nervous system is a component.[242] In HD the enteric nervous system of the aganglionic colon is abnormal [239] and the region of the transition zone is characterized by diminished numbers of ganglion cells as well as other reported ENS abnormalities.[209, 227, 339] The margin of the transition zone is known to be asymmetrical and it is possible that some of the biopsies taken at A could be in a region of hypoganglionosis where the abnormal ENS causes lower than normal mucin production.[342]

Neural crest cells migrate to the upper end of the alimentary tract and proceed in a distal direction.[274] The more proximal the colonic biopsy the further it is from the abnormal aganglionic colon and therefore should have an ENS closer to normality and levels of mucin production similar to control values. Instead, moving proximally, the patient levels increased to above what is expected in controls.

It may be possible that poor mucin production distally stimulates a compensatory elevation in the adjacent colon. It is known that membrane bound mucins are likely to be able to provide signals to epithelial cells in response to alterations in the mucin layer through changes in conformation or ligand status of their extracellular domains.[77] Other factors known to influence mucin secretion are prostaglandins, cytokines and intestinal microbes.[156, 160, 161] Bioactive factors released by mucosal immune cells also regulate mucin.[19] For example TNFα increases α-2, 3-sialyltransferase expression in some cell lines potentially causing an increased synthesis of sialomucins.[159] One or more of these mechanisms may be able to influence mucin production in the adjacent regions of the colon.

The low mucin production at A demonstrated in patients could therefore be a result of persisting ENS abnormalities within the distal ganglionic colon. Low mucin levels at this point may trigger compensatory higher mucin production in the adjacent colon, resulting in patient values at this point being greater than controls.

Patient incorporation values can also be considered within smaller subgroups with certain clinical features which could potentially affect mucin production. Intestinal microbes can lead to increased mucus secretion[156] so those patients who had episodes of pre-operative enterocolitis were compared to those who did not. Mean incorporation for both precursors was similar in these groups along the colon with the exception of point B where incorporation was higher in the subgroup of patients who had had at least one episode of EC. (Figure 5.11)

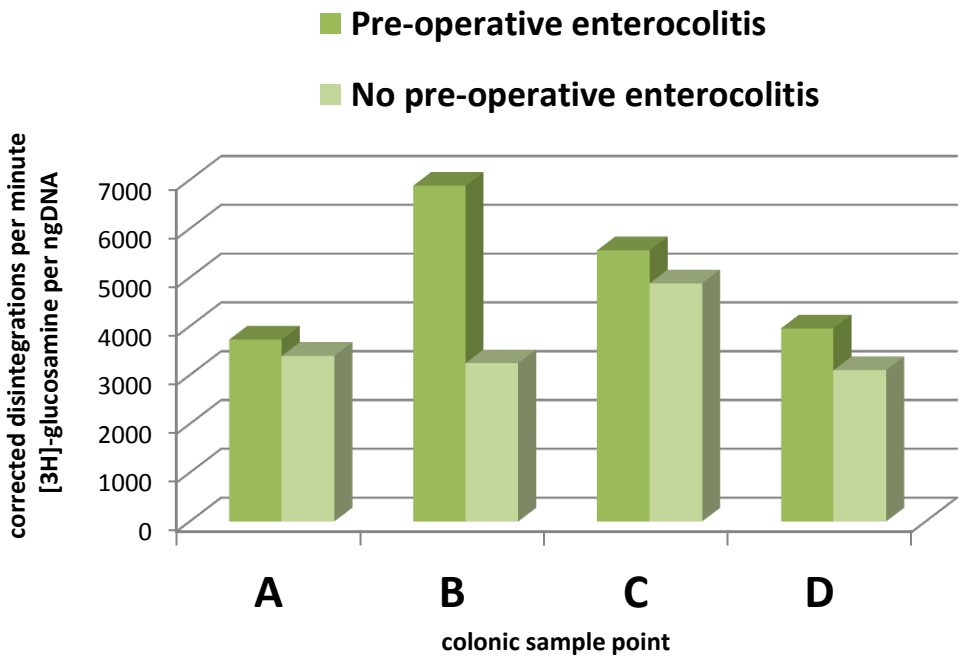


Figure 5.11: Patient Subgroup Mean [3H] Incorporation A-D

Comparing these two patient subgroups to the control group revealed that incorporation at point A was still greater in the control group than either patient group with the converse pattern occurring at point B, but incorporation was much higher than controls in those patients with pre-operative EC at point B. (Figures 5.12 & 5.13)

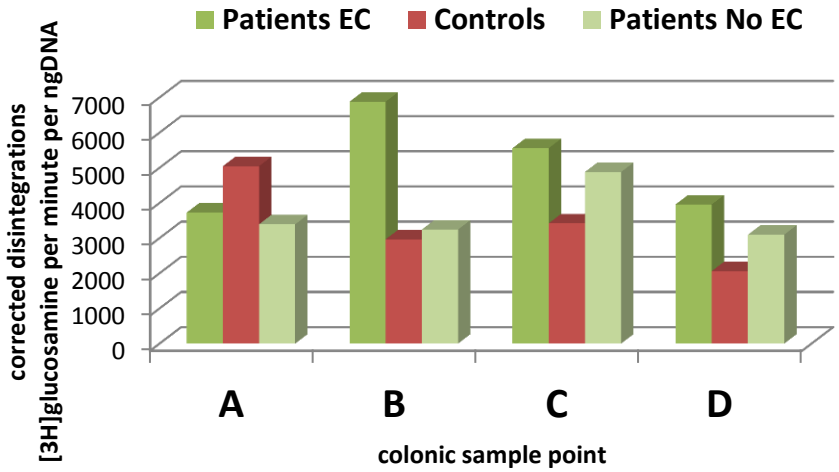


Figure 5.12: Controls and Patient Subgroups Mean [3H] Incorporation

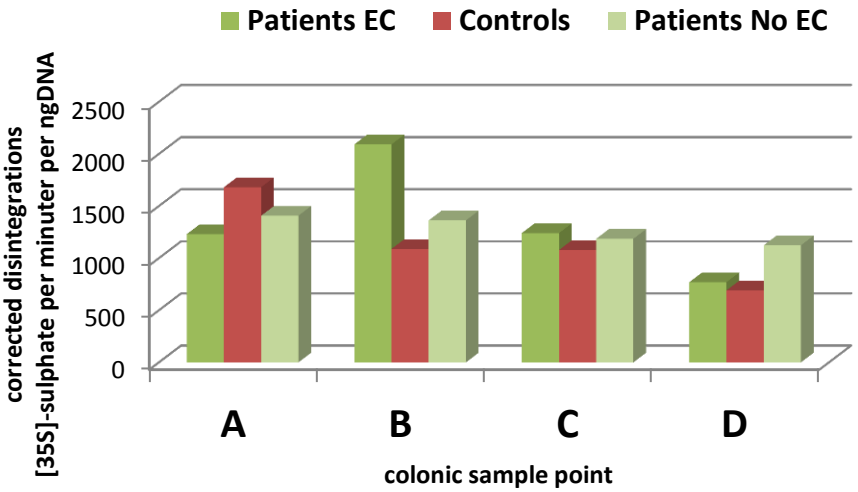


Figure 5.13: Controls and Patient Subgroups Mean [35S] Incorporation

It does appear that those patients who had an episode of EC did skew the data at point B. Despite there only being three patients within this subgroup, they accouted for two of the three highest [3H]-glucosamine incorporation results but only one of the highest [35S]-sulphate incorporation results. (Figure 5.14) The small numbers in the study make it difficult to draw any firm conclusions from this, especially any link between susceptibility to EC and greater mucin production at point B.

If overall mean values (A-D) are compared between these patient groups and controls, both patient subgroups have higher incorporation of [35S]-sulphate than controls but only those patients with pre-operative EC demonstrated greater [3H]-glucosamine incorporation, the other patient subgroup having very similar mean incorporation to controls. EC may persistently stimulate an increase in mucin productivity but why this should be more pronouced at one colonic sample point is difficult to explain. Sampling difficulties (see page 189) may have contributed to this result.

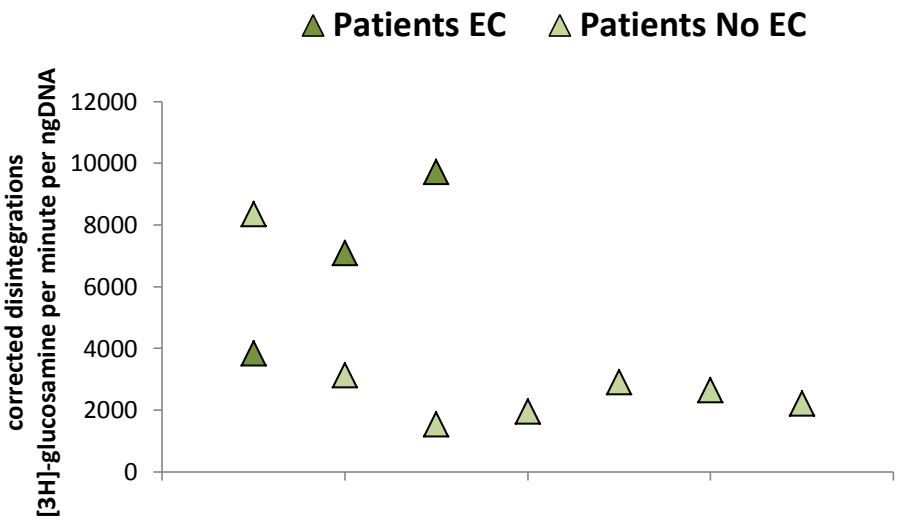


Figure 5.14: Scatter Graph of Individual Patients [3H]-glucosamine Incorporation at Colonic Point B

Colonic washouts were given to those patients who developed enterocolitis as part of their treatment. The effect, if any, of washouts on mucin levels is not known and could be the cause of differences in incorporation levels rather than EC persay. In addition to those patients who developed EC, patients who had a single stage corrective procedure underwent regular colonic washouts pre-operatively in order to decompress the bowel. Unfortunately due to the small size of this study the separate effect of washouts could not be established as the only children who developed EC were also having elective washouts, only one child of the four with a delayed primary pull-through did not have a single EC episode.

Only two patients did not have HD restricted to the rectosigmoid colon, but extending into the descending colon. These small numbers again make it difficult to analyse the data separately but at points A and B, control incorporation values were very similar to patients with descending colonic HD but at points C and D these patients had much higher values. (Figure 5.15) Mucin production should increase proximally to distally. [9] Those with descending colonic HD are likely to have more proximal biopsies compared to those with rectosigmoid HD. Control samples may also be more proximal when compared to patients with rectosigmoid disease as their colostomy may have been sited higher up the sigmoid colon. This could explain why patients with descending colon Hirschsprung's disease incorporation levels are similar to controls at A and B and greater than rectosigmoid disease patients at B, but does not explain the greater incorporation levels more proximally at C and D. Larger study samples would be needed to further investigate any differences.

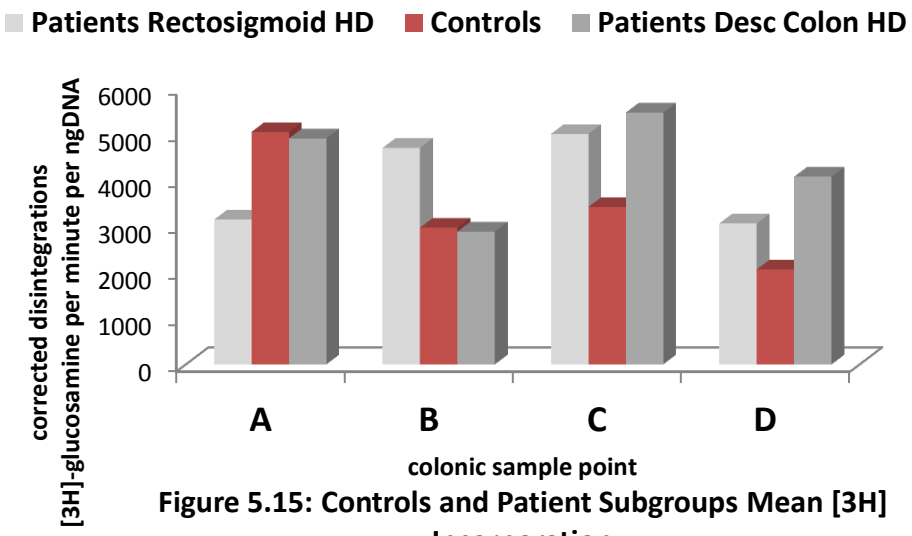


Figure 5.15: Controls and Patient Subgroups Mean [3H] Incorporation

5.2 Incorporation within the Mucin Peak Sub-Fractions

The biosynthetic products, including mucins, created during the incubation time were extracted in three phases, namely a secreted soluble fraction, a cellular soluble fraction and a cellular insoluble fraction.

The soluble secreted fraction contained those mucins within the culture medium which were readily soluble. This would not have included the secreted non-gel forming mucins MUC7, 8 or 9 as these are not found in the intestine.[52, 54, 56] This fraction may include some membrane bound mucins which can exist in secreted and soluble forms [20, 21, 22] and also some MUC2 molecules which have yet to form the intermolecular bonds which would render it insoluble.

The cellular soluble fraction was obtained after homogenization of the mucosal tissue and contained those mucins with an intra-membrane component, e.g. MUC1.[37] In addition contained within this fraction would be those mucins soluble in PBS which had

not entered into the culture medium and were still intracellular. These could include immature secreted and gel-forming mucins or different forms of MUC2 which can be present in the cell layer.[138]

The cellular insoluble fraction contained only those mucins which had formed into an insoluble gel which require the disruption of disulphide bonds in order to become soluble. In particular this would include mature MUC2 and may also contain some membrane-bound mucins which are able to link with gel-forming mucins.[89]

These individual mucin fractions were therefore studied to ascertain whether a particular form of mucin production was altered in HD. The vast majority of mucin incorporation occurred in the cellular soluble fraction. This was true for both patients and controls and for all points A-D. (Figures 5.16 & 5.17) The reason for this is likely to be the length of the incubation period. Although high molecular weight mucin multimers are created from dimers early in biosynthesis, [133] within 24 hours only some of the newly formed mucins produced in the mucosal cells would have reached the stage where they would be secreted or formed into an insoluble gel.[205]

In both study groups the least amount of incorporation of either precursor was found within the secreted soluble mucin. (Figures 5.16 & 5.17) The main mucins expressed in the colorectum in humans from birth onwards are MUC2 and MUC4, along with lower levels of MUC1 and MUC3.[17, 243] Three of these are membrane-bound and one in its mature form is gel-forming so a predominance of cellular and cellular insoluble mucins would be expected.[19, 37]

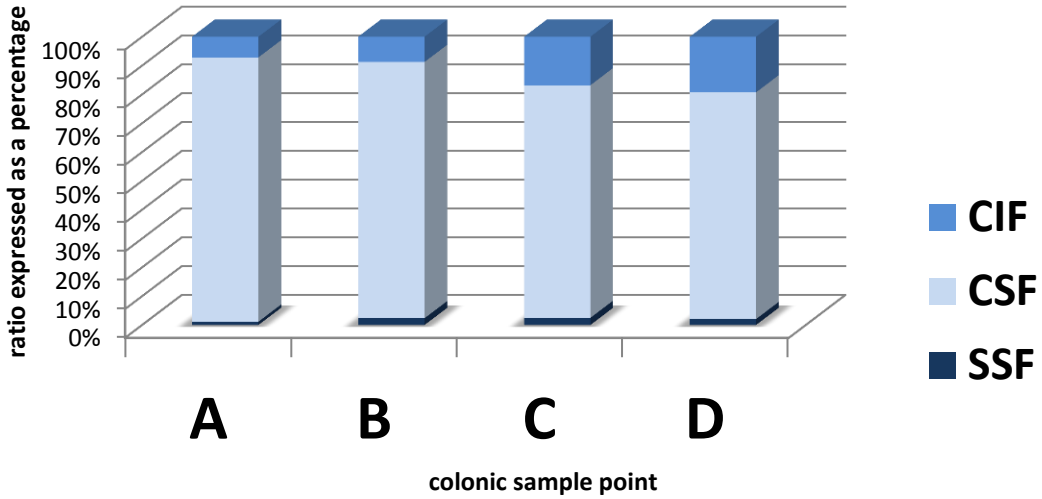


Figure 5.16 : Mean Control [3H] Incorporation A-D within the three mucin containing fractions

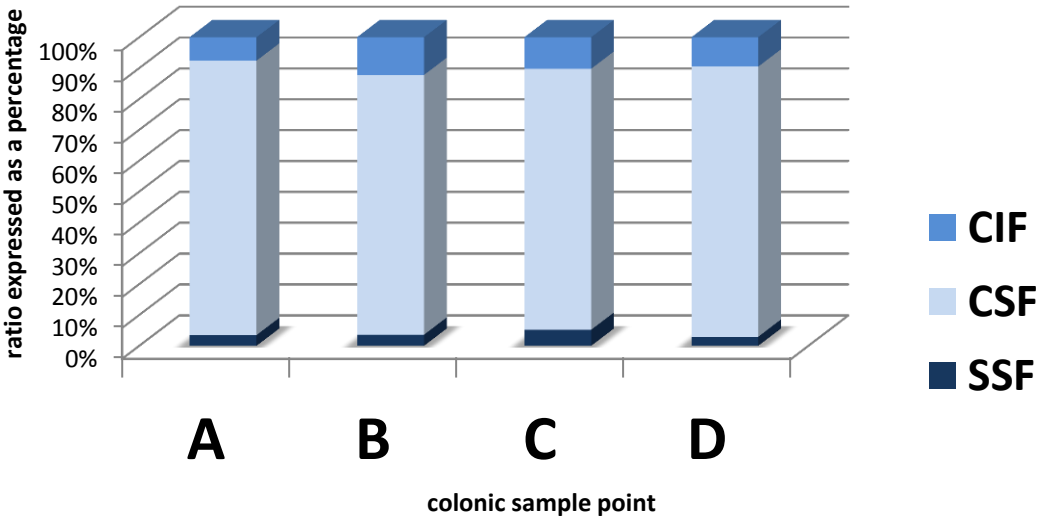


Figure 5.17 : Mean Patient [3H] Incorporation A-D within the three mucin containing fractions

In patients [3H]-glucosamine incorporation within both cellular fractions showed a similar pattern to total mucin incorporation, with lower levels compared to controls at A and greater levels than controls at B, which was a statistically significantly result within the cellular insoluble fraction, $p = 0.04$, unpaired t-test. (Figure 5.18) Control values for the cellular soluble mucin were greatest distally and lowest proximally (Figures 5.16 & 5.17)

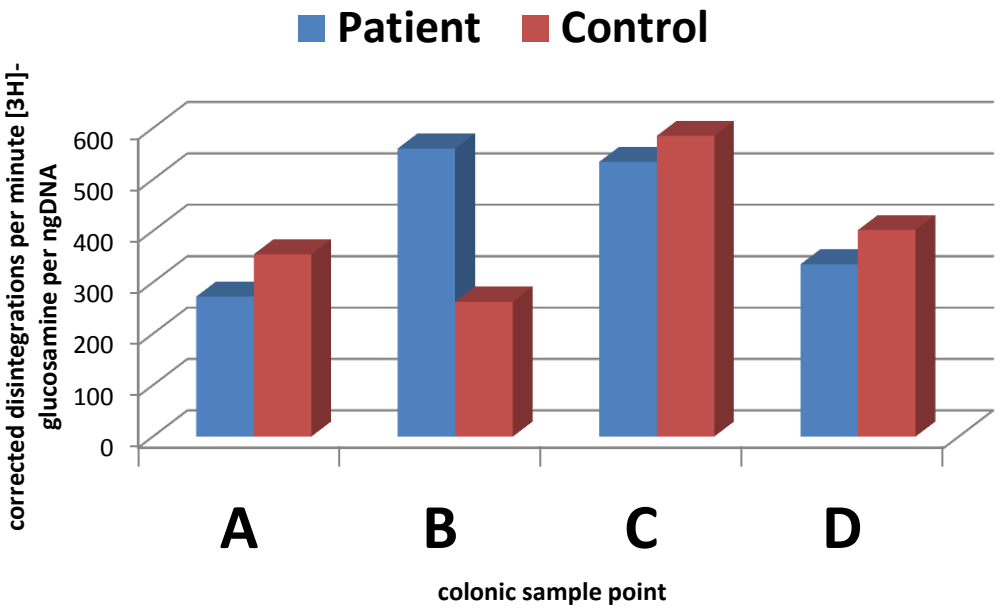


Figure 5.18: Mean [3H] incorporation A-D within CIF mucin fraction

[35S]-sulphate incorporation within the cellular soluble fraction was lower at A in patients than controls and greater at B than controls.

Different glycosylation patterns are known to exist along the length of the intestine. A gradient has been demonstrated from ileum to rectum, with a pattern of decreasing levels of fucose and ABH blood group expression and increasing levels of sialic acids and acidity.[24] Increasing distal values of glucosamine incorporation found in controls overall and in the cellular soluble mucins could reflect greater numbers of newly formed mucins but could also indicate greater levels of sialylation within individual molecules.[126, 127]

Levels of *O*-acetylation of sialic acids in colonic mucins have been shown to be unaltered in HD and sialic acid is largely limited to the secretory mucins of the large intestine.[16, 102] As the secreted soluble and cellular insoluble fractions do not show this

pattern of high control values at A compared to patients, it is more likely that it is a reflection of greater mucin production rather than greater sialylation.

It appears that the deficiency in total mucin production demonstrated by patients distally (Point A) is due to a lack of cellular soluble and insoluble mucins. Secreted soluble mucin production showed some interesting variation between the two groups. Patients had much higher levels of [3H]-glucosamine incorporation than controls at all points within the secreted soluble fraction, although these differences were not statistically significant. (Figure 5.19) [35S]-sulphate incorporation within the secreted soluble fraction was highest distally, being greater than controls, and lowest proximally.

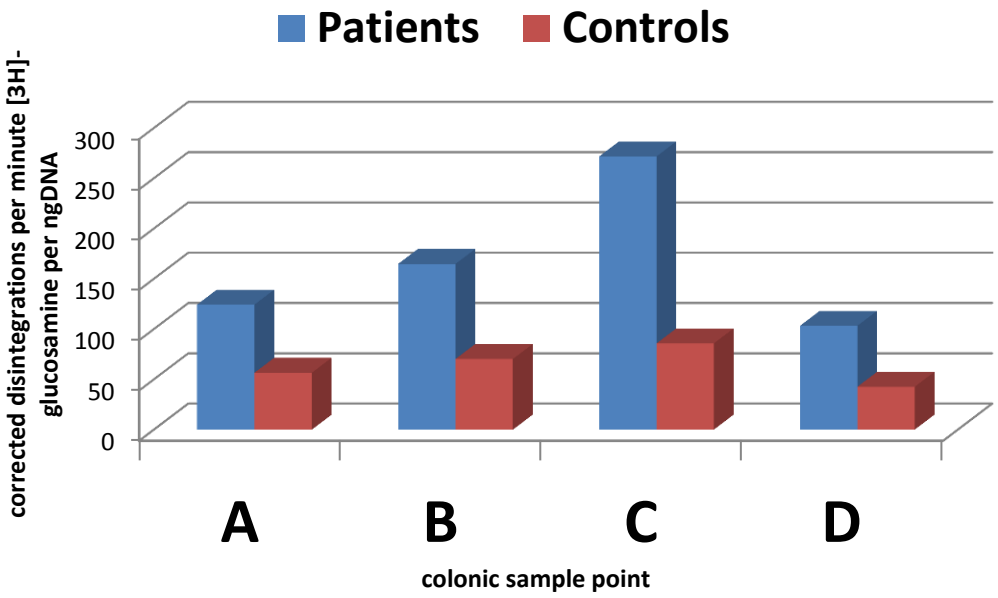


Figure 5.19: Mean [3H] Incorporation A-D within SSF mucin fraction

Although total mucin incorporation in patients was not deficient compared to controls at the more proximal colonic levels, the relative proportion of the different mucins were altered in patients. In controls the relative proportion of cellular insoluble mucins,

incorporating both precursors, increased distally to proximally at the expense of the cellular soluble proportion. (Figure 5.16)

The proportion of [35S]-sulphate incorporation within the secreted soluble fraction in patients also decreased higher up the colon and the ratio of [35S]-sulphate to [3H]-glucosamine was much lower than controls, demonstrating a reduction in the proportion of these mucins which were sulphated. This was in direct opposition to controls and could result in mucins produced proximally in patients being more susceptible to bacterial degradation. (Figures 5.20 & 5.21)

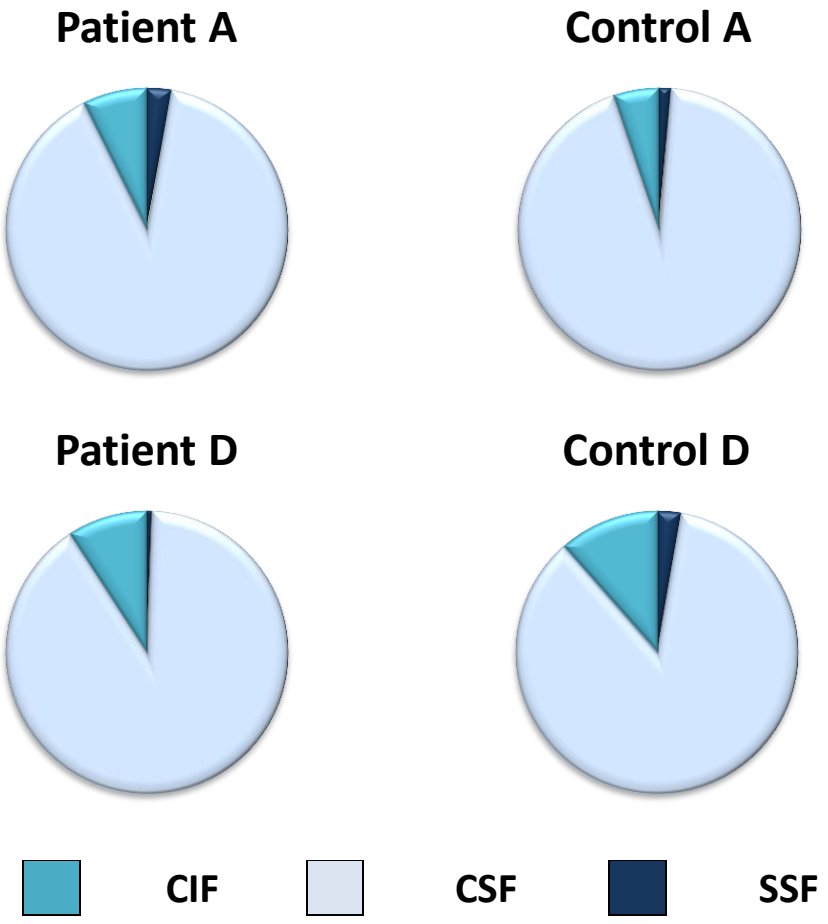


Figure 5.20: Proportion of [35S] incorporation within the mucin fractions at A and D

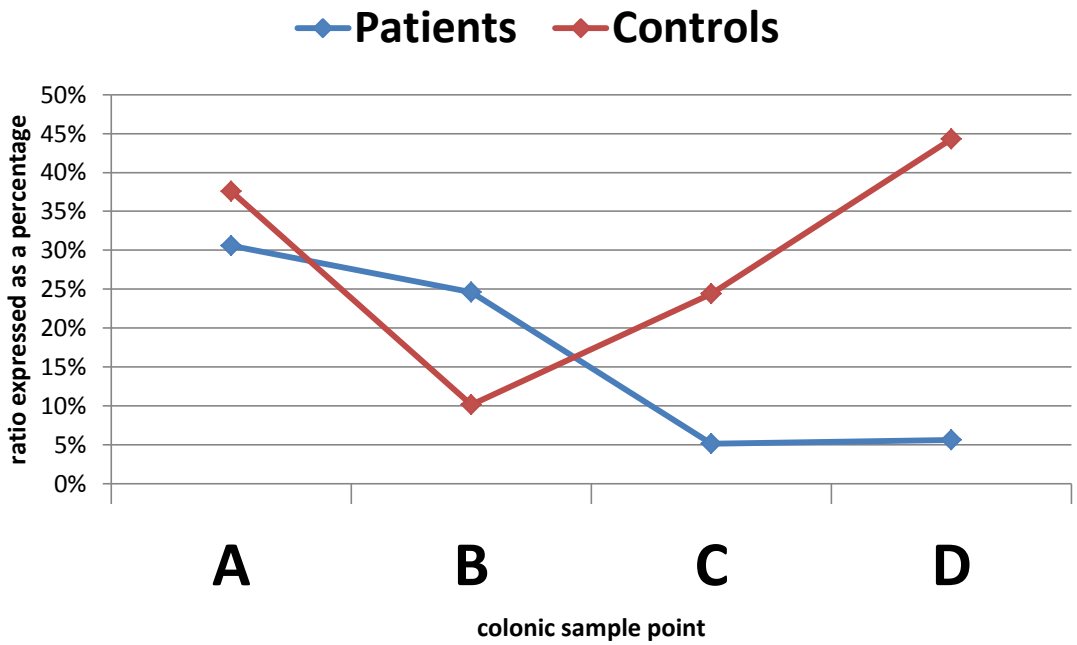


Figure 5.21: Mean Ratio of [35S]/[3H] in SSF fraction

One theory to explain the different mucin fractions found in the two groups involves the main gel-forming mucin in the colon, MUC2.[77, 352] Levels of MUC-2 protein in stool have been shown to be much lower in children with HD despite mRNA levels for MUC2 being found at similar levels to controls.[16, 368] MUC2 is known to exist in different forms, commonly gel-forming, due to a complex, branching network of covalently linked mucin molecules. This makes it almost the sole component of the insoluble mucin in the human colon.[36] It can also be found as partially glycosylated oligomerized intermediates or in the fully glycosylated oligomerized form within the cell layer.[138]

The soluble fraction may include MUC2 prior to forming its gel state and the greater levels in patients may indicate an abnormal expression of this glycoprotein resulting in a problem forming the completed complex mucin gel within the incubation period.[138] Mucosal turnover could be more efficient higher up the colon in controls, meaning more of the mucins produced being of a sufficient maturity to form into an insoluble gel. This

would be reflected by the increase in cellular insoluble mucins and secreted mucins at the expense of cellular soluble mucins.

Abnormalities of mucin production seem to persist in HD at least up to 15 centimetres of proximal ganglionic colon, with a reduction in mucin production being most consistently found just above the transition zone. These changes are unlikely to be simply due to differences in mucin gene expression as these have been found to be unaltered in HD, with MUC1, 2, 3, 4 and 5B being detected in similar quantities in all age groups and in both ganglionic and aganglionic colon.[16] Abnormalities of the ENS have been found in the ganglionic colon in HD including reduced immunoreactivity for general neuropeptide markers and disordered arrangement of neuropeptide nerves in the proximal ganglionic colon in HD.[264, 344] These could affect mucin secretion further up the colon.

Alterations in mucin production could be due to abnormalities within mucin specific biosynthetic pathways or could reflect more widespread changes. The biosynthetic activity of the tissue is reflected by the mucin and back peak totals. Analysis of back peak incorporation in comparison to mucin peak incorporation could indicate whether alterations in mucin synthesis reflect more global mucosal productivity changes.

5.3 Incorporation within the Back Peak

Overall back peak incorporation for the colon samples studied was therefore calculated as a mean for each study group. The incorporation of both precursors was slightly higher in controls but this was not statistically significant, for [3H]-glucosamine incorporation $p = 0.72$ and [35S]-sulphate incorporation $p = 0.81$ and the spread of values and the ratio of incorporation of the two precursors were similar. This suggests

that overall mucosal synthetic activity within the distal colon is unlikely to be diminished in HD.

Mean values for the control subjects were calculated for [3H]-glucosamine incorporation and [35S]-sulphate incorporation for each colonic level. Back peak glucosamine incorporation showed a completely reversed pattern to mucin incorporation with lowest levels distally, higher levels proximally. (Figure 5.22 & 5.5)

Conversely patient back peak values of [3H]-glucosamine incorporation demonstrated a similar pattern to that in the mucin peak in patients, with low levels at A, higher levels at B and C and then lower levels again at D. Control values for incorporation of both precursors were greater than patients at point A with patient values being higher at point B, as with the mucin peak results. None of these differences were statistically significance. Back peak incorporation of [35S]-sulphate was lower at A in patients compared to controls with patients having higher values at B than controls.

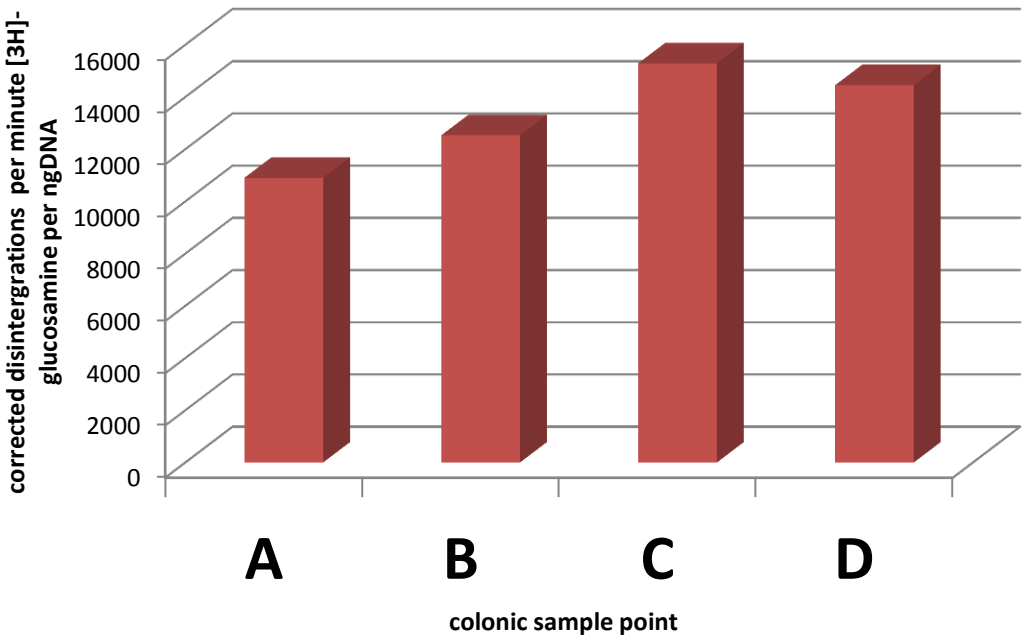
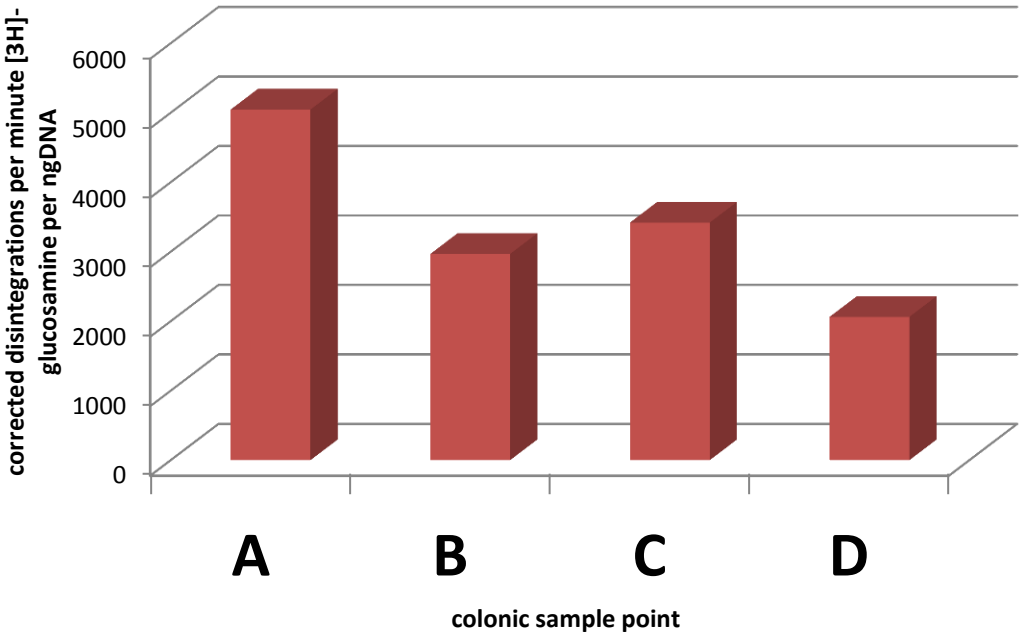


Figure 5.22: Mean Control [3H] Incorporation A-D in Back peak



Copy of Figure 5.5 : Control [3H] Incorporation A-D in Mucin Peak

These results suggest a relationship between [3H]-glucosamine incorporation in the mucin peak and back peak in the normal colon. As discussed above, part of the contents of the back peak are likely to include the mucin precursors. Therefore, at point A, the low back peak and the corresponding high mucin peak could be explained by an increased efficiency of the conversion of mucin precursors to completed mucin molecules.

In HD patients the situation appears different, with both mucin and back peak levels of [3H]-glucosamine being low distally. This suggests that all mucosal productivity involving glucosamine subunits is reduced. If a large proportion of the back peak consists of mucin precursors this could reflect globally diminished mucin synthesis of both precursors and fully formed mucin molecules. Alternatively, if the back peak is more varied in its constituents, low levels of incorporation may reflect a more widespread deficiency in glyco-conjugate synthesis in patients.

5.4 Incorporation within the Back Peak Sub-Fractions

As with the mucin peak, the biosynthetic products were extracted in the three phases. The data from the individual fractions was examined. Most of the back peak incorporation was contained within the cellular soluble fraction, as found in the mucin peak. In controls [3H]-glucosamine incorporation within the cellular soluble fraction followed the pattern shown in the mucin peak, namely the amount increasing distally. This suggests increasing levels of membrane bound mucins are matched by increasing levels of smaller intracellular or membrane bound molecules.

The cellular soluble fraction may contain mucin precursors or mucin related molecules, for example, during intra-cellular post-translational processing of membrane-bound mucins a cleavage occurs at the SEA domain to yield two subunits.[84] As greater numbers of fully-formed mucin molecules are produced within the incubation period more of these smaller subunits will be generated.

In controls [3H]-glucosamine incorporation within the secreted soluble fraction increased from A to D. (Figure 5.23) In both patients and controls the secreted soluble and cellular soluble fractions appeared to be inter-related, the proportion of one increasing at the expense of the other. (Figure 5.24) This is different to the mucin peak where the cellular insoluble and cellular soluble fractions altered in tandem.

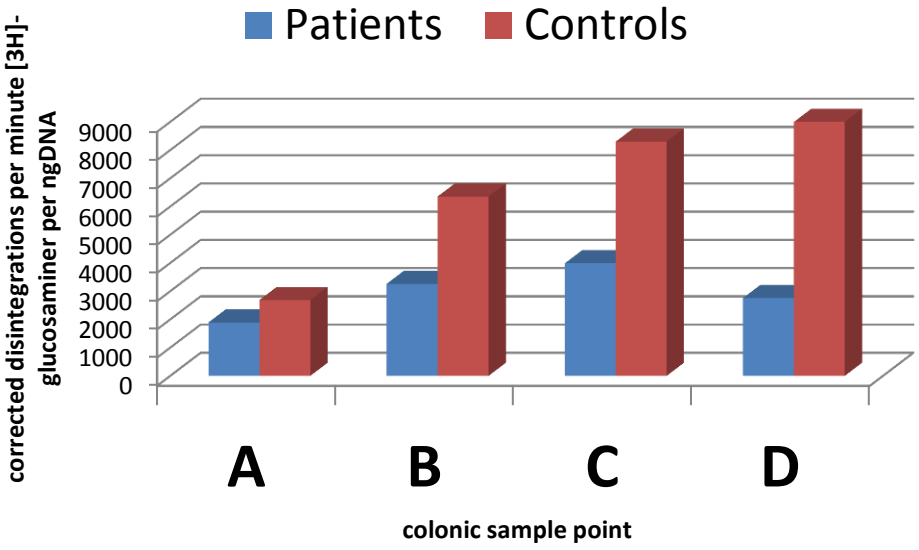


Figure 5.23: Mean [3H] Incorporation within SSF fraction of Back peak

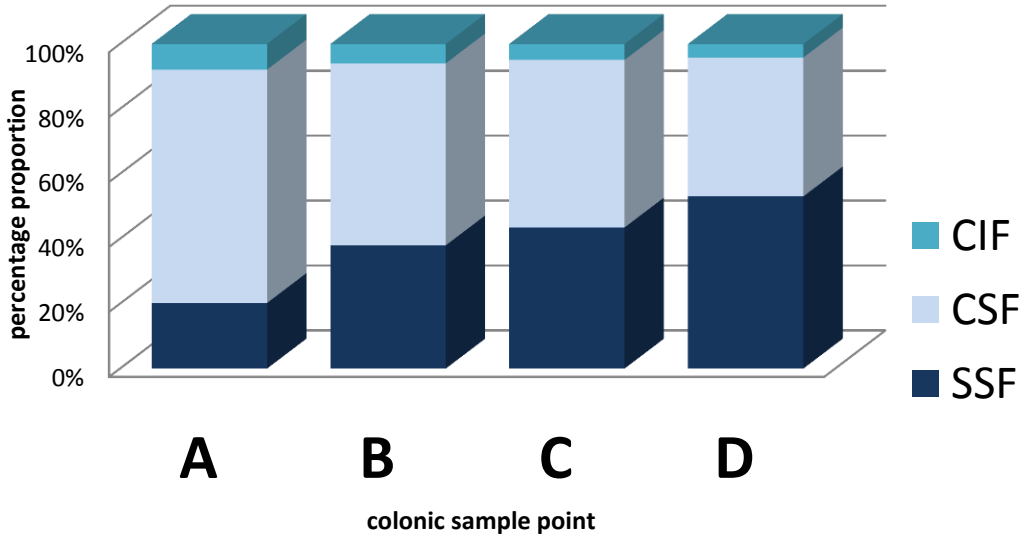


Figure 5.24: Proportion of [3H] Incorporation within Control Back peak fractions

The cellular soluble fraction of the back peak includes newly formed smaller molecules which have not been secreted into the culture medium, so are either membrane-bound or intracellular. Decreases in the proportion of cellular soluble fraction with a concurrent rise in secreted soluble fraction may be due to greater subunit shedding into the

glycocalyx from the membrane-bound structures, or secretion of different isoforms which are soluble.[19, 23]

The proportion of [35S]-sulphate incorporation within the back peak fractions remained similar along the colon and ratios of [35S]-sulphate to [3H]-glucosamine incorporation were similar in the two groups and shown less variation along the colon. However, patients did show statistically higher levels of [35S]-sulphate incorporation in the cellular insoluble fraction at B.

5.5 Lectin Binding

Oligosaccharides of both secreted and cell surface glycoproteins, are classified by the nature of their linkage to the protein core.[92] In mucins, the majority of the oligosaccharides are *O*-linked [91] and can be described in terms of their core, backbone and terminal regions.[91, 95] Terminal residues in mucins are often sialic acid residues, with secretory colonic mucins being particularly rich in sialic acids.[102] Sialic acid is most commonly found as a single terminal residue joined to an underlying galactose or GalNAc by an α-2,3- or a α-2,6-glycosidic linkage.[99, 100] Exceptions to this usual pattern are α-2,8-linked sialic acids which can be attached to an underlying α-2,3 sialic acid residue.[99]

Lectins are proteins isolated from animal, plant and microorganisms which bind to specific non-reducing terminal monosaccharides determinants and can be used to identify terminal residues.[103, 104, 105] Lectins used to study mucins include wheat germ agglutinin (WGA) which binds to sialic acids and *N*-acetylglucosamine residues, *Maackia amurensis* agglutinin (MAA), which is more specific, only binding to α-2,3

linked sialic acids and *Ulex europaeus* agglutinin (UEA) which binds to fucosyl residues.[103, 106, 107]

5.5.1 Wheat Germ Agglutinin

Earlier work with lectins and mucins shows that colonic mucin oligosaccharides contain either a terminal sialic acid residue or an *N*-acetylglucosamine residue accessible for WGA binding.[96, 97] Measurement of WGA binding gives an indication of total mucin present, both the newly synthesized and therefore radioactively labeled, as well as mucin already present prior to incubation.[103]

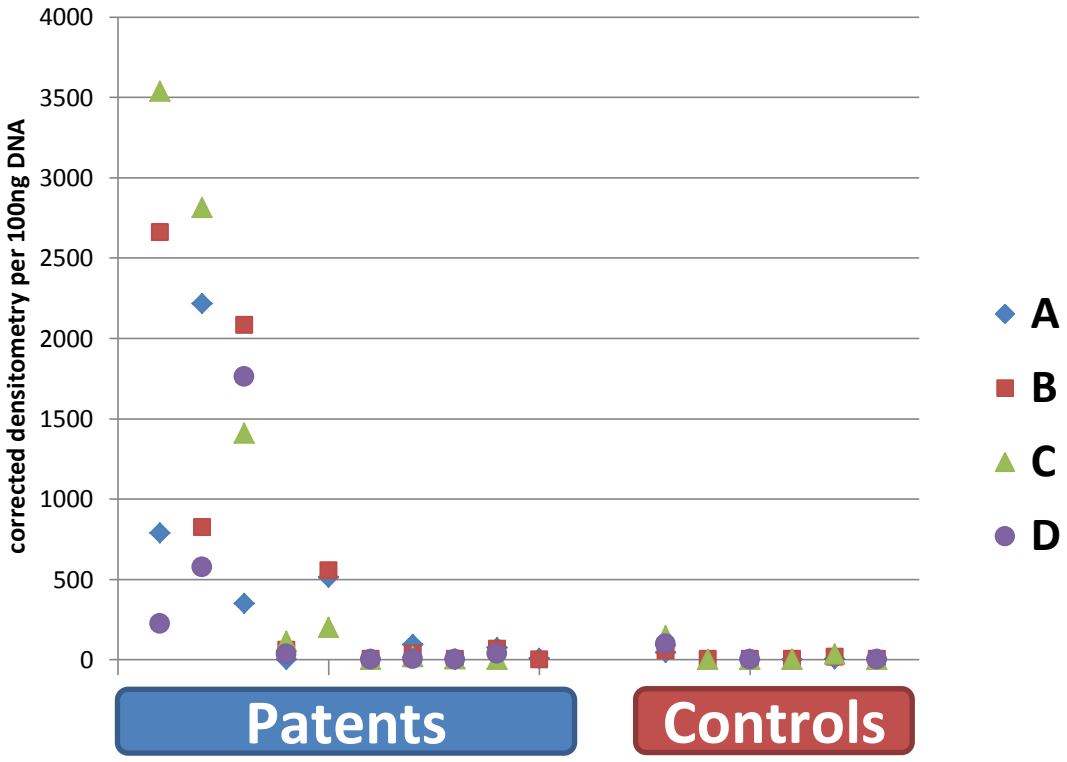


Figure 5.25 : Scatter Graph of WGA Mucin Binding

WGA binding at all points A to D of the mucin peak was much lower in controls compared to patients but within the patient group there appeared to be two subgroups, one with low binding levels similar to controls and another showing much higher levels of WGA binding (>100 densitometry per 100ngDNA). (Figure 5.25) These patients did not have correspondingly higher or lower amounts of either [3H] or [35S] incorporation.

At each individual colonic biopsy level WGA binding was much higher in patients although these differences were not statistically significant. (Figure 5.26) Control values increased A to D, whereas patient values were low at A and D, a similar pattern to mucin incorporation results. Dividing the patient group into these two subgroups still resulted in mean control values being lower than both patient subgroups at all colonic points. (Figure 5.27)

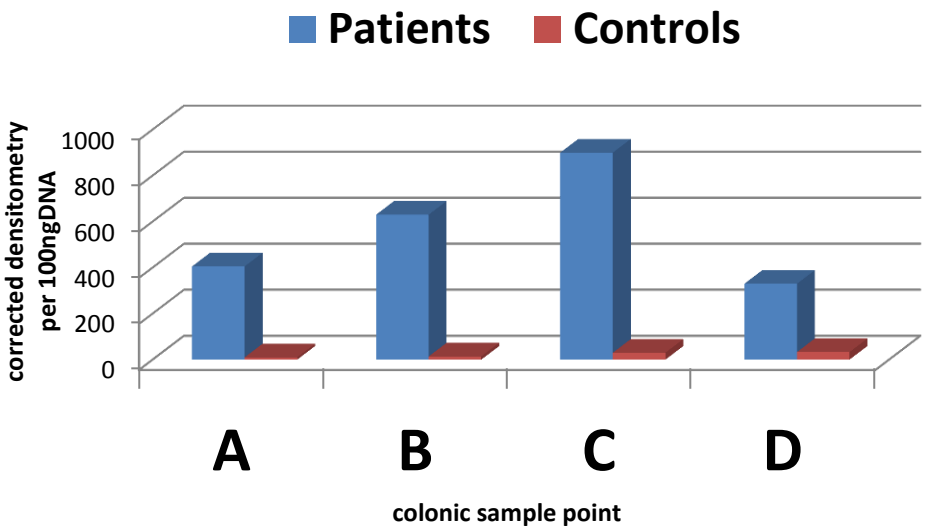


Figure 5.26: Mean WGA binding within Mucin Peak

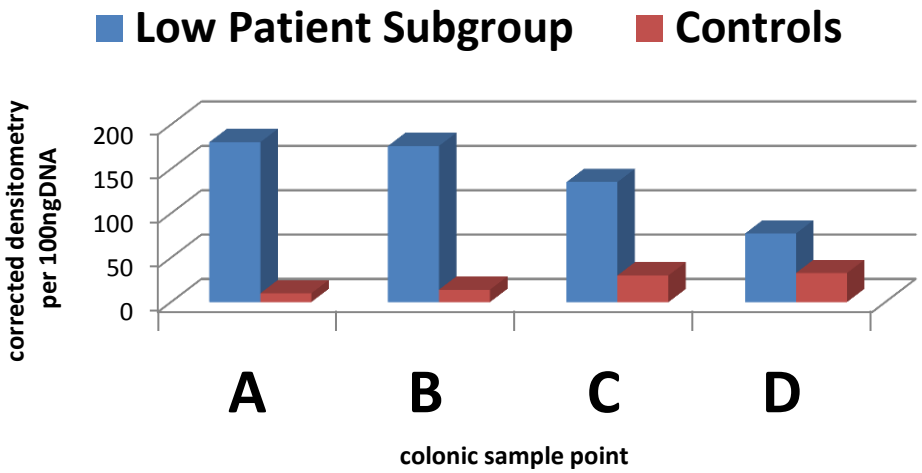


Figure 5.27: Mean WGA Binding within Low Patient Subgroup

The greatest amount of binding was found within the cellular insoluble mucin fraction and overall binding levels were statistically higher in patients in this fraction, p = 0.002, and in the cellular soluble fraction, p = 0.04. This is different to incorporation which showed greatest results in the cellular soluble fraction. The lowest level of binding was found within the secreted soluble fraction. Controls had a higher proportion of binding within the cellular soluble compared to patients.

Newly formed mucins, which would have incorporated the precursors, would not all have reached sufficient maturity to be in the form of a gel within the twenty four hours of incubation.[205] WGA binds to all mucins, including any mucins present prior to the incubation period which would not be radioactively labeled, i.e. those mucins produced by the mucosa *in vivo* which had not degraded. Any such mucins would have had more time to form the cross-linking bonds to create the gel-forming mucins and would therefore remain in the cellular insoluble fraction.

Patient levels of binding within the secreted soluble fraction showed a similar pattern to the cellular insoluble fraction, with highest values at point C and low values at A and D.

In contrast binding levels within the cellular soluble fraction were lowest at C. At all points patients showed greater binding than controls. (Figures 5.28 & 5.29)

Previous work has shown very different results with controls having higher levels of WGA binding than patients in both the secreted and cellular fractions.[345] This could be partly due to differences in methodology. The gel-forming insoluble mucins, which demonstrated the greatest amount of WGA binding, resist extraction with guanidinium chloride unless the disulfide bonds are cleaved.[35,36,] The method used with this research included disruption of these disulphide bridges by DTT, allowing them to be extracted into a further fraction. This additional step was not undertaken by the previous work, and hence any cellular insoluble mucins would not have been included in those lectin studies.

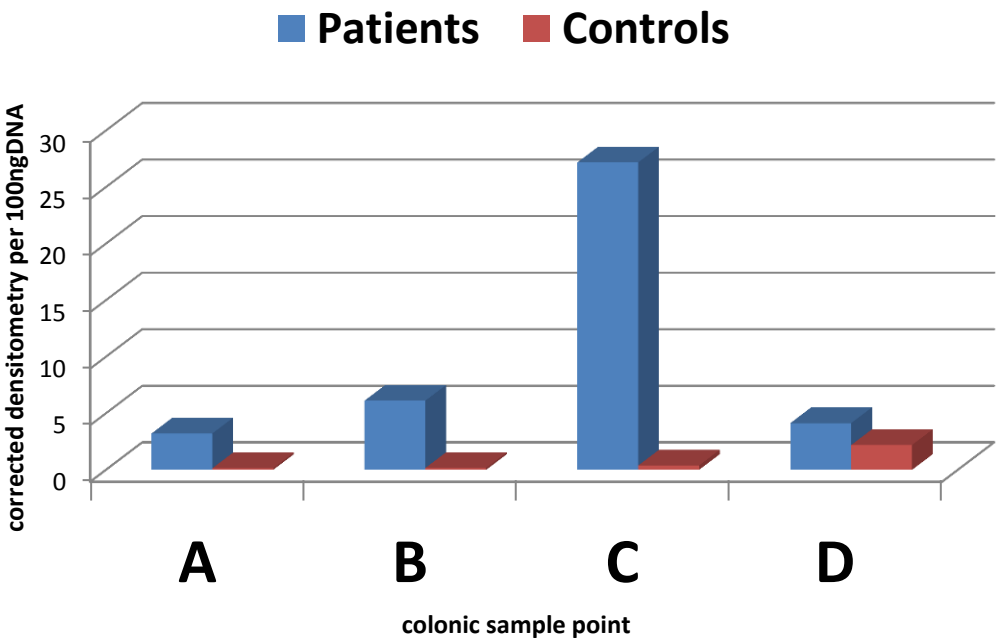


Figure 5.28 : Mean WGA Binding within SSF Fraction

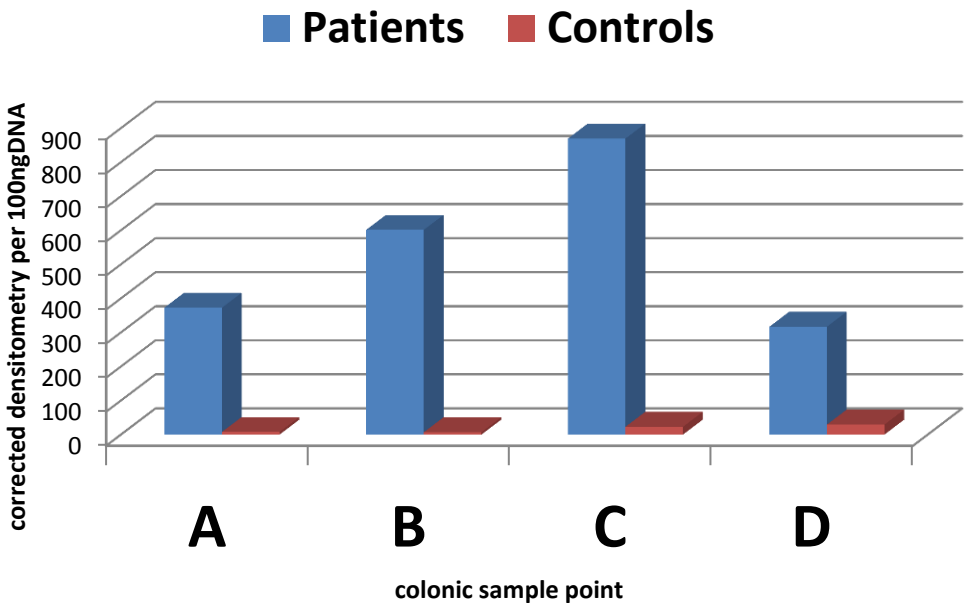


Figure 5.29: Mean WGA Binding with CIF Fraction

5.5.2 Correlation between Incorporation and Wheat Germ Agglutinin Binding

It is interesting that whereas patients had much higher levels of WGA binding compared to controls, corresponding incorporation levels were much more similar between these two groups. WGA binding and incorporation data were compared.

Comparing an individual subject's incorporation data with their WGA binding data did not reveal any obvious pattern with the exception of control F whose scatter graphs for mucin incorporation and WGA binding were similar. (Figures 4.3, 4.4 & 4.18) However when patient and control mean values for incorporation and WGA binding were studied some interesting patterns emerged.

Patient levels of incorporation appeared to mirror those of WGA binding with lower values at A and D and higher values at B and C. This was more obvious when comparing [3H]-glucosamine incorporation to WGA binding than with [35S]-sulphate incorporation. (Figures 5.30 – 5.32)

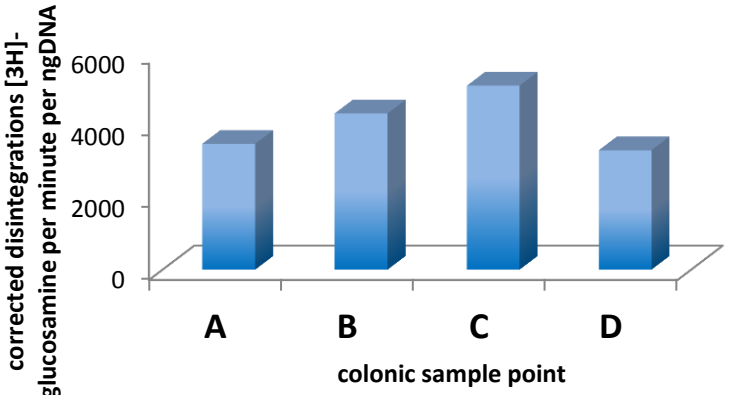


Figure 5.30: Mean Patient [3H] Incorporation A-D

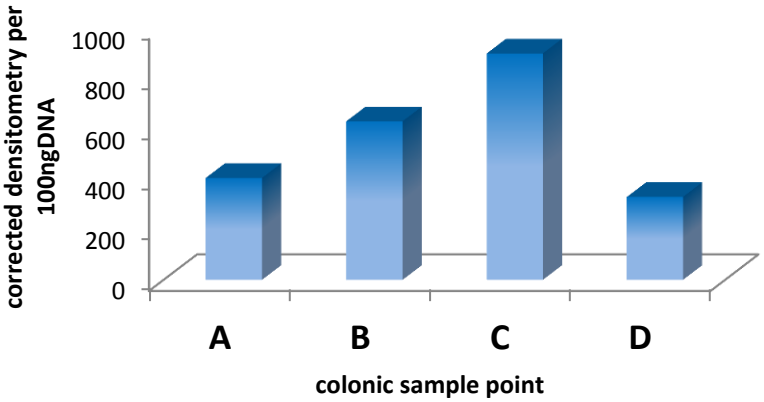


Figure 5.31: Mean Patient WGA Binding A-D

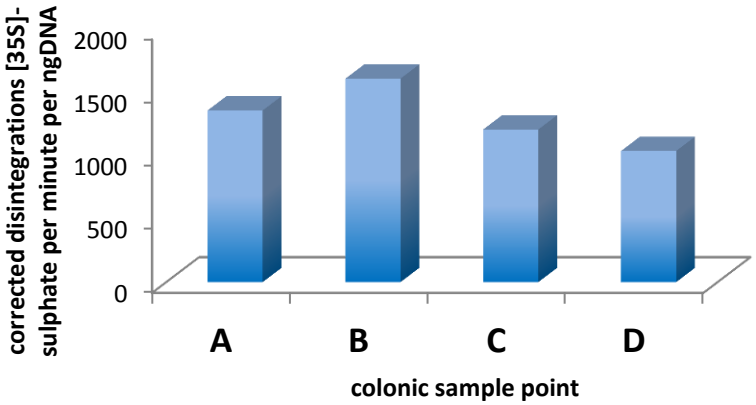


Figure 5.32: Mean Patient [35S] Incorporation A-D

In contrast, in controls, WGA binding levels altered in opposition to incorporation, incorporation being greatest most distally at A and lowering proximally at D whereas WGA binding was lowest distally, rising to its highest level proximally. (Figures 5.33 - 5.35) As WGA binding gives an indication of total mucin present and incorporation the amount of new mucin produced it could be that in controls, in the normal colon, mucin production is under feedback control so that a thick mucin barrier results in lower mucin production.

It appears that in Hirschsprung's disease the amount of new mucin produced is either unaffected by the presence of existing total mucin or new mucin formation is not under the same kind of negative feedback it appears to be in controls. There may be other reasons why WGA binding levels were far higher in Hirschsprung's disease patients than in controls.

Bioactive factors released by mucosal immune cells can enhance mucin secretion and some may increase the synthesis of sialomucins.[19, 159, 160, 161] Release of these factors *in vivo* due to the presence of mucosal damage or infection, could lead to greater WGA binding levels in patients due to increased mucin production and/or increased sialylation of mucins.

Mucus secretion is typically enhanced in response to intestinal microbes.[156] Children with HD are known to develop enterocolitis, the exact aetiology of this condition being unknown but it is thought to have an infective component.[353, 358] These children have also been found to have abnormal pathogen carriage when compared with age-matched controls.[358, 359]

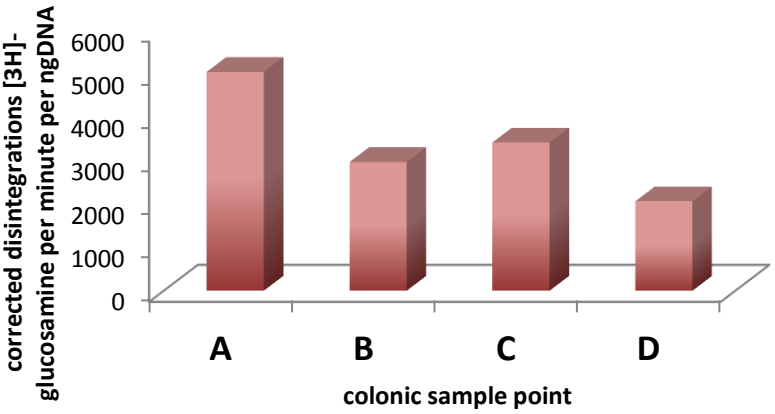


Figure 5.33: Mean Control [3H] Incorporation A-D

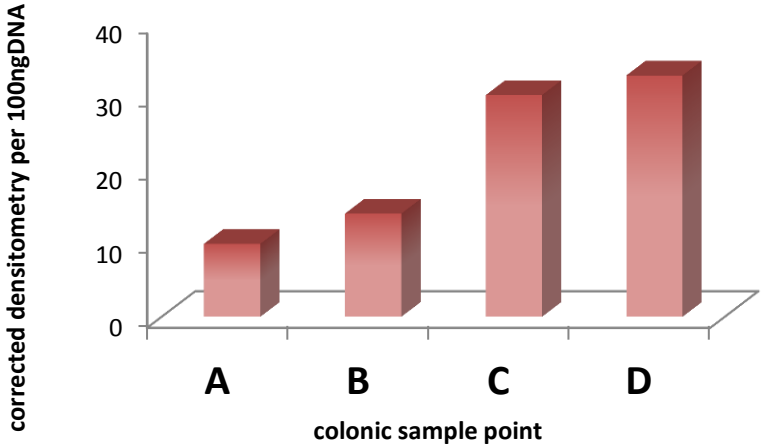


Figure 5.34: Mean Control WGA Binding A-D

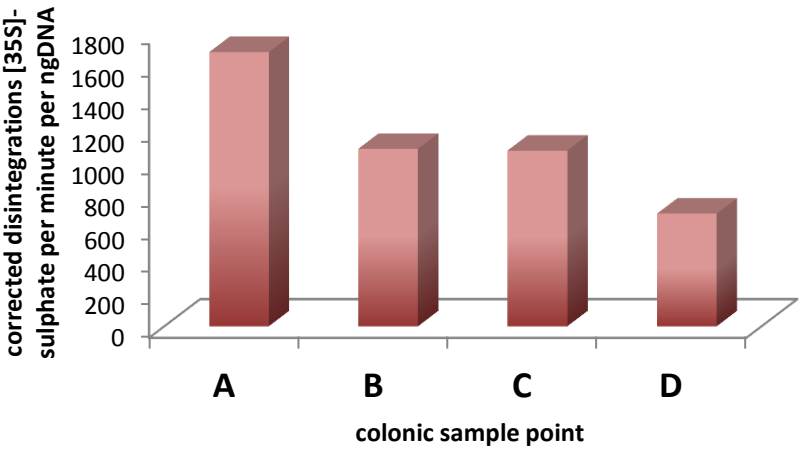


Figure 5.35 : Mean Control [35S] Incorporation A-D

Although all children under going corrective surgery were asymptomatic of enterocolitis, and did not have diarrhoea, some may have had a recent or subclinical infection, or an abnormal pathogen carriage, resulting in an enhanced mucin production prior to surgery. Mucin retention in enterocolitis has been previously noted as a characteristic histopathological change.[355]

In order to reduce any impact of an infectious agent on new mucin synthesis organ culture was performed in the presence of broad spectrum antibiotics. These antibiotics should effectively prevent any additional mucin synthesis due to any ongoing bacterial infection but would not affect mucin already produced *in vivo*. Pre-operative exposure to an infectious agent could therefore explain higher levels of mucin already present, indicated by higher WGA binding, without corresponding higher incorporation levels.

It would be unreasonable to assume all patients were suffering from a subclinical infection at the time of biopsy collection. However, there are two distinct sub-groups within the patient WGA binding results. (Figure 5.26) Four patients showed a greatly elevated total WGA binding, indicating higher amounts of mucin present. If these four patients had been suffering from a subclinical infection, this could go towards explaining this division within the patients.

The control samples were taken under the same conditions and if an infection is the reason for the increased WGA binding, then any subclinical infection would show similar results for a control subject. Statistically four out of ten patients potentially having a subclinical infection, when compared to zero out of six control subjects may seem unlikely. However, as discussed above, HD patients have a greater susceptibility to infection.

Within the patient group were those who had developed at least one episode of EC prior to sample collection. It is possible that after a patient has EC colonic mucin levels are persistently altered for some time, either elevated in response to the microbial threat or alternatively depleted, and this period may have fallen within the dates of sample collection. Alternatively children who develop EC may have different, perhaps lower levels of WGA binding to those who do not. Therefore those patients without EC were compared to those with. (Figure 5.36)

Both patient subgroups had greater levels of WGA binding than controls at all points with the exception of point D where controls demonstrated higher WGA binding levels than patients with a previous episode of EC. Those who had not developed EC had much greater WGA binding than those who had. The reason for this difference could be that an episode of EC results in persistently depleted levels of mucin. Another explanation is that the reason some patients did not succumb to EC was because they had a thicker mucin barrier, indicated by higher WGA binding levels, so were more resistant. The subgroups of low and high patient values mentioned earlier were not explained by the presence of confirmed EC, as the individual patients did not fall into the same groups.

There was no pattern discernable when comparing those patients with rectosigmoid HD with those with descending colon HD or those who had washouts and those who did not.

A possible confounding factor influencing the WGA binding levels is that the age range for patients and controls were slightly different; controls having a median age of 199 days compared to patients which had a median age of 137.5 days. Children under 3 months are known to produce the greatest amount of mucin per unit DNA and those children between 3 months to 3 years the lowest.[205] Although both groups average over

3 months of age if there were a gradual reduction in mucin production from 3 months to 3 years occurs this would affect the comparability of the two groups.

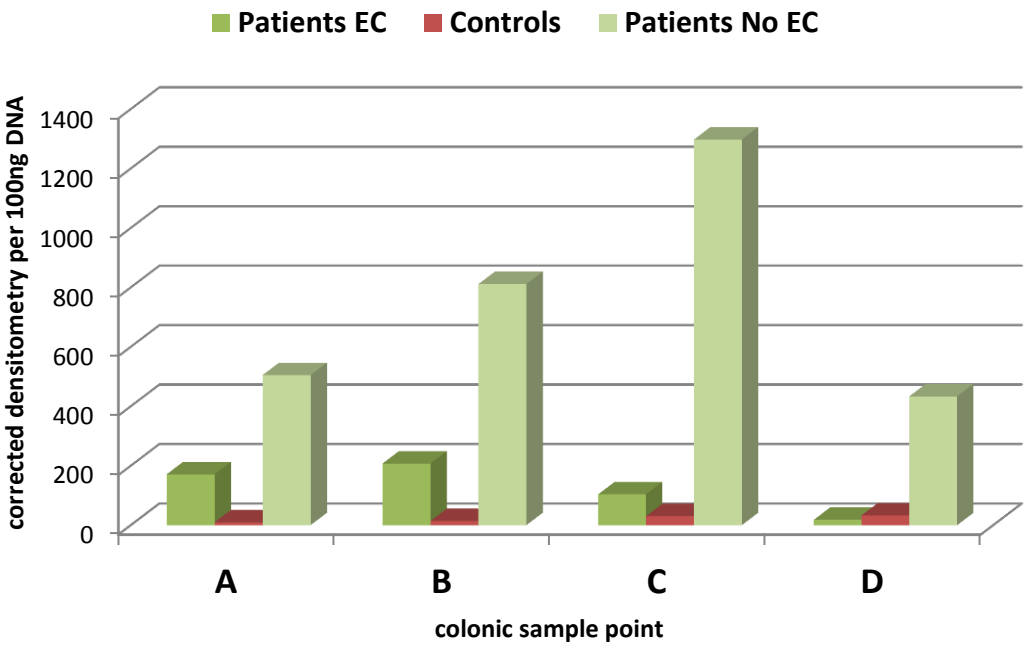


Figure 5.36: Mean Control and Patient Subgroup WGA Binding

As described above, WGA binding is reported to be a good indication of total mucin levels and it can bind to either terminal sialic acid or *N*-acetylglucosamine residues.[103] Although WGA does show specificity towards sialic acids, studies have shown that it preferentially binds to *N*-acetylglucosamine and its ß-1,4 linked oligomers.[375] Other studies using different lectins have shown that the presence of an *O*-acetyl group can block their recognition of sialic acid.[376] Alterations in terminal residues could therefore in influence the level of WGA binding achieved in some patients.

Although patients appear to have greater mucin quantity, this may not correlate with a better defense barrier. Pathogens bind to the outer mucus layer initially before penetrating through to the mucosal cells.[146] Pathogenic bacteria are eliminated from the colon when mucus shedding and degradation rates exceed colonization rates.[146] A thicker mucin barrier may actually represent a poor balance between the conflicting processes of mucin synthesis and mucin degradation, which are necessary to maintain a functional defensive barrier. This would allow bacterial colonization to a level where mucosal damage could result.[90]

5.5.3 *Maackia Amurensis* Agglutinin Binding

MAA binds to sialic acids, specifically to α-2,3 linked sialic acids. Sialic acids in mucins are usually joined to an underlying galactose or GalNAc by an α-2,3- or a α-2,6-glycosidic linkage.[99, 100] Therefore, in addition to the WGA binding study, MAA binding was performed for the cellular soluble mucin fraction. Patients demonstrated higher levels of MAA binding than controls at all points A to D, peaking at point C, but these results were not statistically significant, although overall binding levels had a p value of 0.03. (Figure 5.37) In controls the amount of MAA binding altered in tandem with control levels of WGA binding within the cellular soluble fraction. (Figure 5.38) If it is assumed that a large proportion of WGA binding in controls is to sialic acids, there appears to be a consistent proportion of α-2,3 linked sialic acids found at points A to D in the normal colon.

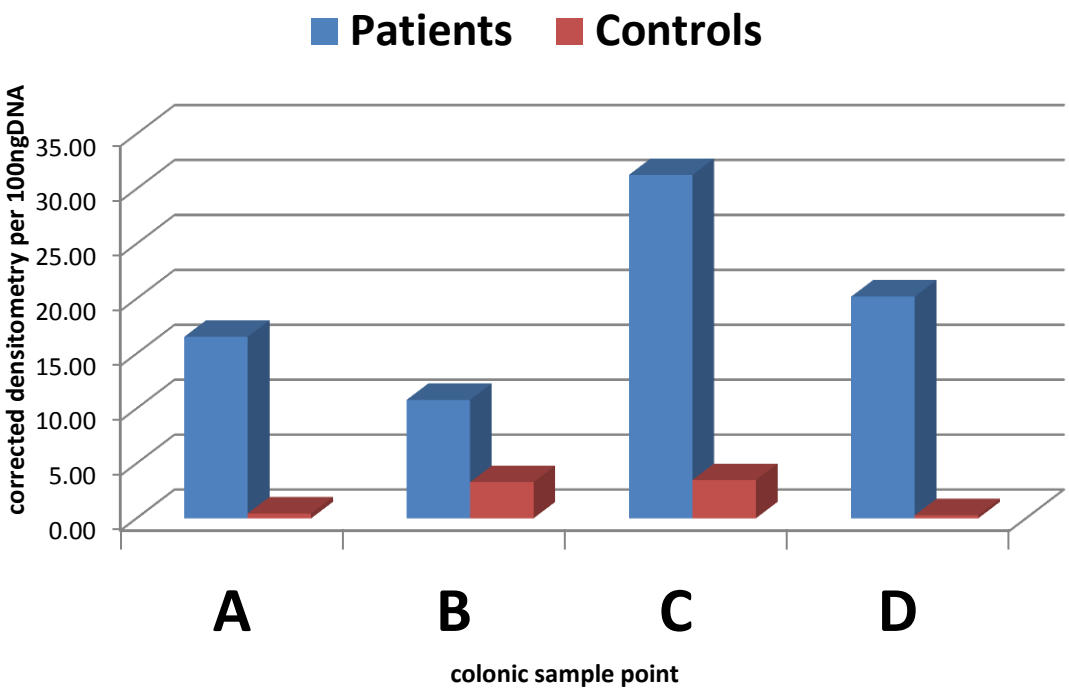


Figure 5.37: Median MAA Binding

In patients the relative binding of MAA to WGA is found to increase proximally at points C and D. (Figure 5.39) This suggests that there is a greater proportion of α-2,3 linked sialic acids at these points in HD. Other studies have demonstrated 50-70% mucin binding using MAA, with no significant differences seen between aganglionic bowel, ganglionic bowel near the transition zone and normal controls, however several of the samples analysed in that study were rectal so are not necessarily comparable.[16]

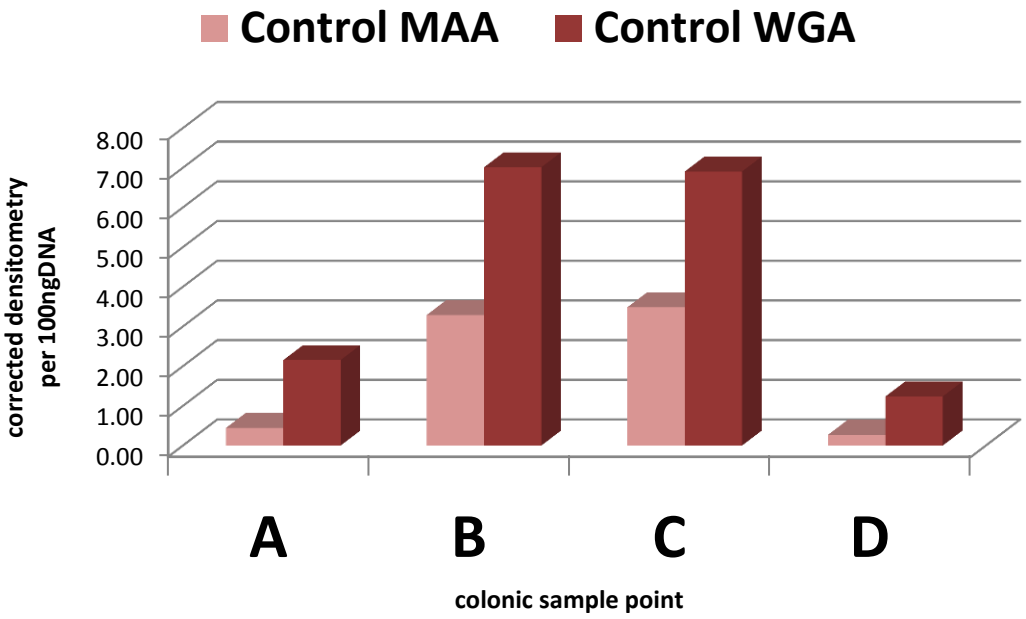


Figure 5.38: Control MAA Binding and WGA Binding within CSF Fraction

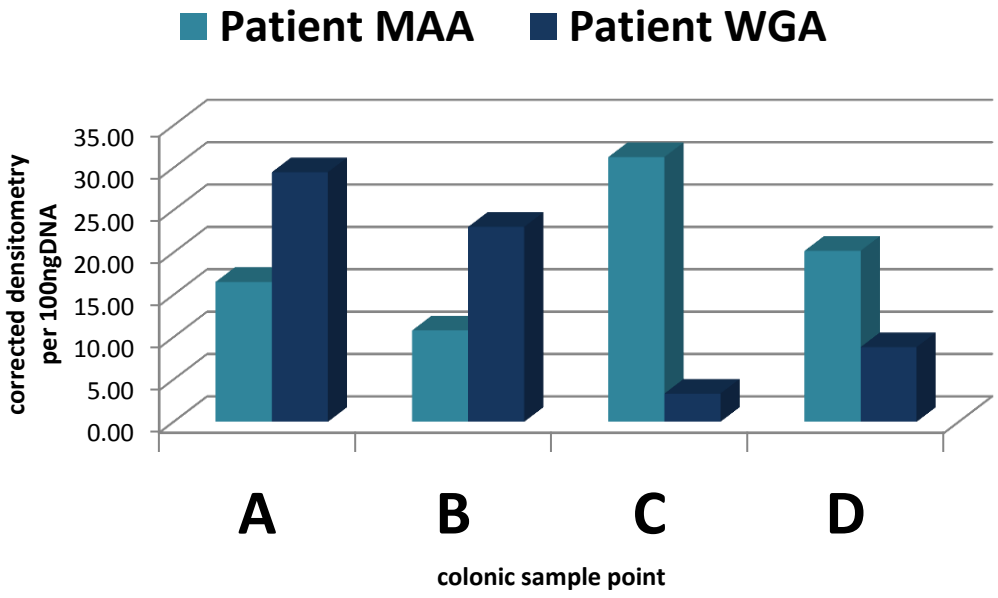


Figure 5.39: Patient MAA Binding and WGA Binding within CSF Fraction

Higher levels of MAA binding could be simply due to patient having greater amounts of α-2,3 linked sialic acids compared to controls. As mentioned above sialic acids are usually joined to the oligosaccharide chain by either an α-2,3- or a α-2,6-glycosidic linkage. [99, 100] In HD, mucin oligosaccharide synthesis may be altered, patients producing lower levels of α-2,6 linked sialic acids compared to controls with corresponding greater levels of α-2,3 linked sialic acids. Alternatively higher MAA binding may be due to lower levels of terminal α-2,8-linked sialic acids in patients. These residues can be attached to an α-2,3 sialic acid residue,[99] and if missing would expose the underlying α-2,3 residues and allow greater MAA binding.

Terminal residues are important in mucosal immunity. They act as decoy ligands for microbes trying to attach to the mucosa [19] and can limit the rate of bacterial degradation of the mucin oligosaccharide chains.[90, 100] Alterations in HD could have implications for the robustness of the mucin defense barrier.

Studies using *Ulex europaeus* agglutinin (UEA) did not demonstrate binding in any of the samples. As UEA binds to fucosyl residues and decreasing levels of fucose have been found from ileum to rectum this was not unexpected.[24]

5.6 Limitations of the Study

Although the control subjects were not known to have a mucosal abnormality these children cannot truly be considered 'normal' controls by virtue of the fact that they were born with a bowel anomaly. It is possible, though not reported, that mucosa mucin production is altered in anorectal malformations. Ideally comparison samples should have been taken from children with no history of any condition affecting the colon but it would be impossible, ethically, to justify removal of biopsies in this situation. A

possible alternative would be to seek consent from parents whose children were undergoing a diagnostic sigmoidoscopy or colonoscopy but there are very few clinical indications for this in paediatric practice, especially in those children under a year of age.

When removing the biopsies there was potential for error in determining the transition zone in patients. In the cases of primary pull-through the colon was viewed and the region of the transition zone was identified by the assessing where the distended, ganglionic bowel met the more contracted and collapsed aganglionic bowel and also by changes in blood vessel configuration. Histological specimens sent at operation for immediate frozen section analysis helped to more accurately delineate the position of the transition zone.

In those patients who had a diverting colostomy performed prior to definitive surgery, a frozen section was sent to confirm that the proximal end of the stoma was ganglionic. The stoma was formed in the region of the distal sigmoid colon and as the majority of transition zones occur in the rectosigmoid, the level of the stoma would be likely to be very close to the transition zone. However, this was less well established in these cases.

Determination of the sampling sites was open to variation between subjects. The uneven and unsymmetrical nature of the transition zone [341,342] meant that its exact location was difficult to ascertain with precision. As measurements and samples were taken proximally from this point there is a limitation on how accurately samples supposedly taken at the same biopsy level in different patients can be compared like for like. In addition, during the process of measuring distance along the bowel, error could have occurred. In particular when taking control samples, the bowel needed to be telescoped to some degree on to the biopsy forceps meaning accurate measurement was

difficult. All of these factors could result in one subject's sample B, for example, being the equivalent of another subject's sample C.

There was a change in practice during the recruitment period from the more traditional two or three stage surgical approach to the single stage procedure.[363, 328] This meant that colonic washouts rather than a colostomy were used to deflate the colon prior to corrective surgery, resulting in the patient group being more mixed than intended. The small sample size meant it could not be determined whether these different methods of surgical correction affected the results.

There are also some reservations on how accurately a control sample can be compared to a patient sample. Controls had a colostomy sited in the lower sigmoid colon and biopsies were taken from the proximal end of this, the part of the colon in contact with faeces, as studies have shown that the mucin production in a defunctioned colon is abnormal.[377] As not all the transition zones in patients were in the sigmoid, the comparison biopsies may have been taken at a different colonic level introducing a certain amount of potential sampling error.

HD is not common and therefore recruitment of sufficient subjects was difficult. Control numbers and the number of samples obtained from each were lower than ideal. It proved more difficult to recruit control subjects as parents were reluctant to agree to an additional procedure when any potential results would not benefit children suffering from a similar condition.

Retrieving biopsies from controls was also more difficult as during the stoma closure the proximal colon is not as easily accessible. Some surgeons were reluctant to biopsy at 15 cms from the colostomy for this reason and hence some controls only had three

samples taken. In addition one sample was inadvertently placed in formalin before access to the theatre was gained, effectively destroying the sample.

All the controls had a colostomy and despite the first sample being taken from inside the stoma, it is possible that the distal mucin production may be falsely elevated because of the closer proximity to the outside.

Patients were generally of a slightly younger age than controls. Timing of corrective surgery for HD and anorectal anomalies is similar [331] but as control samples were removed at colostomy closure, at a period of time after definitive surgery, these children were slightly older when specimens were collected. A further contributory factor is the increased use of the single stage pull-through procedure in Hirschsprung's surgery which is performed earlier than the two or three stage procedure.[327, 328, 331]

Several results showed wide variability between different subjects. The relatively small number of subjects and samples meant that it was difficult to determine whether some of these results were abnormal and represent some error in the methodology or if these were a true reflection of the spread of results.

Chapter 6 - Conclusions and Suggestions for Further Work

The aim of this study was to further investigate the distal ganglionic colon in Hirschsprung's disease to try to determine whether previously noted abnormalities were present and if they were limited to the region close to the transition zone.

This study confirmed the previous work, where the most distal ganglionic colon in HD patients shows abnormal mucin levels.. Newly produced mucin, as assessed by incorporation of two precursors, was lower than control levels closest to the transition zone and this was due to a lack of cellular mucins. In contrast incorporation levels within the secreted mucin fraction were greater at this point than controls.

The rest of the colon studied also showed changes in mucin production. The colon at point B, 5 centimetres proximal, produced much higher levels of new mucins compared to controls, possible demonstrating some form of compensatory reaction to the low distal mucin levels. More proximally in HD patients there was also a lack of production of gel-forming mucins as well as a relative lack of mucin sulphation and sulphated secreted mucins suggesting that certain aspects of the mucin defensive barrier remains abnormal up to 15 centimetres from the transition zone.

In contrast existing *in vivo* mucin production seemed to be much greater in patients, as indicated by WGA binding levels, and there appeared to be two different groups of patients with either very high binding levels or lower levels, similar to controls. The terminal residues found in Hirschsprung's also appear different to controls with a greater proportion of α-2,3 sialic acids as demonstrated by greater levels of MAA binding.

Although Hirschsprung's disease is a rare condition,[207] the associated problem of enterocolitis still causes significant morbidity and mortality [331, 348] and the fact that the ability to produce new mucins is diminished in the distal ganglionic colon suggests that this could be a contributing factor. Extended surgical resection of the distal 5 centimetres of the ganglionic colon at pull-through surgery would remove this region. However, the next 15 centimetres of ganglionic colon would retain these identified mucin abnormalities which may affect the defensive capacity of the colon and still render patients susceptible to infective complications including enterocolitis.

Suggestions for further work

Due to the uncommon nature of Hirschsprung's disease [207] and the wide spread of results obtained, a large multicentre study is needed in order to recruit enough patients and controls to generate more definitive results. This would also allow patient subgroups to be studied; for example, those who developed pre and/or post operative enterocolitis, to determine whether they had different mucin profiles, in order to better establish a link between aspects of mucin production and risk.

Better correlation between the site of patient and control samples is also needed. This may require more stringent inclusion and exclusion criteria. In laparoscopic assisted pull-through surgery, which is becoming the more common form of corrective surgery, intra-operative colon mapping is performed, where biopsies are removed from different sites and sent for immediate frozen section analysis. This allows a more exact placement of the transition zone and could be utilized in further studies with only those children undergoing this form of procedure included.

Controls were children who had a sigmoid colostomy placed and it is difficult to find a more comparable group from which biopsies could be easily and ethically removed. Placing a stoma in this group is essentially a blind procedure and it would be difficult to determine exactly where along the sigmoid the proximal end was. Before stoma closure, these children often have a distal contrast study performed where radio opaque fluid is instilled from either the distal colostomy loop or from the rectum and radiological imaging taken. It may be possible to estimate the length of colon distally to the stoma as well as estimating this distance intra-operatively in patients and aim to match controls and patients better.

A further way of establishing the site of the colostomy in controls would be to perform the addition procedure of an on table sigmoidoscopy/colonoscopy and estimate the distance from a definable landmark such as the splenic flexure in order to better match biopsy samples. However, it may be difficult to justify this on ethical grounds.

Concurrent analysis on biopsies looking at the levels of neuropeptide markers and the arrangement of neuropeptide nerves within the specimens could help determine a correlation between poor mucin production and ENS abnormalities.

Previous studies on the distal ganglionic bowel and aganglionic bowel have not shown differences in MUC mRNA levels but this has not been established at higher levels. It would also be useful to try to establish exactly which mucins are found in the secreted soluble, cellular soluble and cellular insoluble fractions to see if mucin expression is altered in HD.

The results from the slot blots were very interesting. A larger study would help to establish whether there are two subgroups of patients with very high or low WGA

binding levels. Due to the time constraints of establishing a working methodology, the cellular insoluble and secreted soluble fractions were not analysed for MAA binding. This could provide useful information.

The mucin peak samples could be further analysed to ascertain the length of oligosaccharide chains and to determine the nature of the terminal residues. Blood group probes could be used and levels of *O*-acetylation of sialic acids could be assessed to see if this varies higher up the colon. This would also help to clarify whether differences in WGA binding levels are due to greater amounts of mucin present or greater sialylation. It may also be possible to determine levels of α-2,8, linked sialic acids, to see if these are deficient in Hirschsprung's disease and whether this accounts for greater MAA binding.[378]

The theory mentioned previously that greater WGA binding may be due to subclinical infection in some patients could be further researched by collecting stool samples in subjects to see if they showed evidence of pathogen carriage or by carrying out further studies on part of the biopsy specimen, in tandem with organ culture, to determine whether pathogens are present.

Analysis of the constituents of the back peak would also yield useful information to ascertain whether low mucin production is associated with more widespread mucosal synthesis problems or if the back peak is mainly composed of mucin precursors.

The lower relative sulphation in the proximal Hirschsprung's colon could be further investigated by assessing the expression of sulphotransferases to see if these are also altered in HD.

The interesting findings of much high new mucin production in the colon next to the area of low production is worthy of further study but it is difficult to suggest a strategy for this. Whether colonic conditions can be simulated in the laboratory, with a section of colon placed in organ culture and mucin production in one area actively suppressed and the mucin production in response to this determined, would need careful consideration.

This research has resulted in some very interesting results but much more work is required to fully understand the changes in mucin production in the ganglionic colon in Hirschsprung's disease patients.

.

References

1 Allen A. Structure and function of gastrointestinal mucus. In Johnson LR(ed) Physiology of the gastrointestinal tract. (1981) New York. Raven Press:617-639

2 Allen A. The structure of colonic mucus. In Fernandez B (ed) The colon: structure and function. (1983) New York. Plenum Press:45-63

3 Forstner JF, Forstner GG. Gastrointestinal mucus. In Johnson LR (ed.). Physiology of the gastrointestinal tract. (1994) Raven Press, New York:1255-1283

4 Kim YS, Gum JR, Byrd JC, Toribara NW. The structure of human intestinal apomucins. (1991) Am Rev Respir Dis. 144:S10-S14

5 Lamont JT. Mucus: The front line of intestinal mucosal defence. Neuroimmunology of gastrointestinal mucosa. Implications for inflammatory diseases. (1992) ANNYAS. 664: 190-201

6 Smith AC, Podolsky DK. Colonic mucin glycoproteins in health and disease. (1986) Clin Gastroentero. 15(4):815-837

7 Strous GJ, Dekker J. Mucin-type glycoproteins. (1992) Crit Rev Biochem Mol Biol. 27: 57-92.

8 Creeth JM. Constituents of mucus and their separation. (1978) Br Med Bull. 34:17-24

10 Moniaux N, Escande F, Porchet N, Aubert J et al. Structural organization and classification of the human mucin genes. (2001) Front Biosci. 6:1192-1206

11 Gum JR. Mucins: their structure and biology. (1995) Biochem Soc Trans. 23: 795-799

12 Perez-Vilar J, Hill RL. The structure and assembly of secreted mucins. (1999) J Biol Chem. 274(45):31751-31754

13 Reid L, Clamp JR. The biochemical and histochemical nomenclature of mucus. (1978) Br Med Bull. 34:5-8

13 Forstner JF, Oliver MG, Sylvester FA. Production, structure, and biologic relevance of gastrointestinal mucins. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL, eds. Infections of the gastrointestinal tract. (1995) New York: Raven Press:71–88.

14 Van Klinken BJ, Tytgat KM, Buller HA, Einerhand AW et al. Biosynthesis of intestinal mucins: MUC1, MUC2, MUC3 and more. (1995) Biochem Soc Trans. 23:814-818

15 Brockhausen I. Intestinal candyfloss. (2004) Biochem J. 1:384(Pt2): E3

16 Aslam A, Spicer RD, Corfield AP. Histochemical and genetic analysis of colonic mucin glycoproteins in Hirschsprung's disease. (1999) J Pediatr Surg. 34:330-333

17 Gum JR, Hicks JW, Toribara NW, Siddiki B et al. Molecular cloning of human intestinal mucin (MUC2) cDNA. (1994) J. Biol. Chem. 269(4):2440-2446

18 Spurr-Michaud S, Argüeso P, Gipson I. Assay of mucins in human tear fluid. (2007) Exp Eye Res.84(5):939-50

19 Linden SK, Sutton P, Karlsson NG, Korolik V et al. Mucins in the mucosal barrier to infection. (2008) Mucosal Immunol.1(3):183-97. Epub 2008

20 Moniaux N, Escande F, Batra SK, Porchet N et al. Alternative splicing generates a family of putative secreted and membrane-associated MUC4 mucins. (2000) Eur J Biochem. 267:4536-4544

21 Williams SJ, Munster DJ, Quin RJ, Gotley DC et al. The MUC3 gene encodes a transmembrane mucin and is alternatively spliced. (1999) Biochem Biophys Res Commun. 261:83-89

22 Choudhury A, Moniaux N, Winpenny JP, Hollingsworth MA et al. Human MUC4 mucin cDNA and its variants in pancreatic carcinoma. (2000) J Biochem. 128:233-243

23 Williams SJ, Wreschner DH, Tran M, Eyre HJ et al. MUC13- a novel human cell surface mucin expressed by epithelial and hemopoietic cells. (2001) J Biol Chem. 276(21):18327-36

24 Robbe C, Capon C, Maes E, Rousset M et al. Evidence of regio-specific glycosylation in human intestinal mucins. (2003) J Biol chem. 278(47):46337-46348

25 Majuri ML, Mattila P, Renkonen R. Recombinant E-selectin protein mediates tumor cell adhesion via sialyl-Le(a) and sialyl-Le(x). (1992) Biochem Biophys Res Commun. 182:1376-1382

26 Ligtenberg MJ, Buijs F, Vos HL, Hilkens J. Suppression of cellular aggregation by high levels of episalin. (1992) Cancer Res. 52:2318-2324

27 Hulsken J, Birchmeier W, Behrens J. E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. (1994) J Cell Biol. 127:2061-2069

28 Hulsken J, Behrens J, Birchmeier W. Tumor-suppressor gene products in cell contacts: the cadherin-APC-armadillo connection. (1994) Curr Opin Cell Biol. 6:P711-716

29 Pallesen LT, Berglund L, Rasmussen LK, Petersen TE et al. Isolation and characterization of MUC15, a novel cell membrane-associated mucin. (2002) Eur J Biochem. 269(11):2755-63

30 Cohen RE, Auguirre A, Neiders ME, Levine MJ et al. Immunochemistry and immunogenicity of low molecular weight human salivary mucin. (1991) Arch Oral Biol. 36:347-356

31 Bobek LA, Tsai H, Biesbrock AR, Levine MJ. Molecular cloning, sequence and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). (1993) J Biol Chem. 268:20563-20569

32 Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS et al. Human mucin genes assigned to 11p15.5; identification and organization of a cluster of genes. (1996) Genomics. 38:340-352

33 Tytgat KM, Klomp AJ, Bovelander FJ, Opdam FJ et al. Preparation of anti-mucin polypeptide antisera to study mucin biosynthesis. (1995) Anal Biochem. 226:331-341

34 Guyonnet-Duperat V, Audie JP, Debailleul V, Laine A et al. Characterization of the human mucin gene MUC5AC: a consensus cysteine-rich domain for 11p15 mucin genes? (1995) Biochem J. 305:211-219

35 Carlstedt I, Hermann A, Hovenbery H, Lindell G et al. 'Soluble' and 'Insoluble' mucins – identification of distinct populations. (1995) Biochem Soc Trans. 23(4):845-51

36 Herrmann A, Davies JR, Lindell G, Martensson S et al. Studies on the 'insoluble' glycoprotein complex from human colon. (1999) J. Biol. Chem. 274(22):15828-15836

37 Ho SB, Shekels LL, Toribara NW, Kim YS et al. Mucin gene expression in normal preneoplastic and neonplastic human gastric epithelium. (1995) Cancer Res. 55:2681-90

38 Gendler SJ, Lancaster CA, Talyor-Papadimitriou J, Duhig T et al. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. (1990) J. Biol. Chem. 265(25):15286-15293

39 Pratt WS, Crawley S, Hicks J, Ho J et al. Multiple transcripts of MUC3: evidence for two genes MUC3A and MUC3B. (2000) Biochem Biophys Res Commun. 275:916-923

40 Gum JR, Hicks JW, Swallow DM, Lagace RL et al. Molecular cloining of cDNAs derived from a novel human intestinal mucin gene. (1990). Biochem Biophys Res Commun. 171:407-415

41 Moniaux N, Nollet S, Porchet N, Degand P et al. Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. (1999) Biochem J. 338:325-333

42 Williams SJ, McGuckin MA, Gotley DC, Eyre HJ et al. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. (1999) Cancer Res. 59:4082-4089

43 Yin BW, Lloyd KO. Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. (2001) J Biol Chem. 276(29):27371-5. Epub 2001 May 21.

44 Gum JR Jr, Crawley SC, Hicks JW, Szymkowski DE et al. MUC17, a novel membrane-tethered mucin. (2002) Biochem Biophys Res Commun. 291(3):466-75

45 Lehmann JM, Riethmüller G, Johnson JP. MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. (1989) Proc Natl Acad Sci U S A. 86: 9891-5

46 Higuchi T, Orita T, Nakanishi S, Katsuya K et al. (2004) Molecular cloning, genomic structure, and expression analysis of MUC20, a novel mucin protein, up-regulated in injured kidney. J Biol Chem. 279(3):1968-79. Epub 2003 Oct 17.

47 Sheehan JK, Thornton DJ, Howard M, Carlstedt I et al. Biosynthesis of the MUC2 mucin: evidence for a slow assembly of fully glycosylated units. (1996) Biochem J. 315:105-1060

48 Willem van Klinken BJ, Einerhand AWC, Buller HA, Dekker J. The oligomerization of a family of four genetically clustered human gastrointestinal mucins. (1998) Glycobiology. 8 (1):67-75

49 Ho SB, Roberton AM, Shekels LL, Lyftogt CT et al Expression cloning of gastric mucin complementary DNA and localization of mucin gene expression. (1995) Gastroenterol. 109:735-747

50 Desseyn JL, Guyonnet-Duperat V, Porchet N, Aubert JP et al. Human mucin gene MUC5B, the 10.7-kb large central exon encodes various alternate subdomains resulting in a super-repeat. Structural evidence for a 11p15.5 gene family. (1997) J. Biol Chem. 272:3168-317

51 Audie JP, Janin A, Porchet N, Copin MC et al Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. (1993) J Histochem Cytochem. 41:1479-1485

52 Chen Y, Zhao YH, Kalaslavadi TB, Hamati E et al. Genome-wide search and identification of a novel gel-forming mucin MUC19/Muc19 in glandular tissues. (2004) Am J Respir Cell Mol Biol. 2004 30(2):155-65. Epub 2003 Jul 25.

53 Murray PA, Prakobphol A, Lee T, Hoover CI et al. Adherence of oral streptococci to salivary glycoproteins. (1992) Infect Immun. 60:31-38

54 Liu B, Rayment S, Oppenheim FG, Troxler RF. Isolation of human salivary mucin MG2 by a novel method and characterisation of its interactions with oral bacteria. (1999) Arch Biochem Biophys. 364:286-293

55 Shankar V, Pichan P. Eddy R, Tonk V et al.Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxy terminus. (1997) Am J Resp Cell and Mol Biol. 16:232-241

56 Lapensée L, Paquette Y, Bleau G. Allelic polymorphism and chromosomal localization of the human oviductin gene (MUC9). (1997) Fertil Steril. 68(4):702-8.

57 Corfield AP, Myerscough N, Longman R, Sylvester P et al. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. (2000) Gut 47 (4): 589-594

58 Shirazi T, Longman RJ, Corfield AP, Probert CS. Mucins and inflammatory bowel disease. (2000) Postgrad Med. 76:473-478

59 Lan MS, Batra SK, Qi W, Metzgar RS et al. Cloning and sequencing of a human pancreatic tumour mucin cDNA. (1990) J. Biol. Chem. 265(25):15294-15299

60 Gum JR, Byrd JC, Hicks JW, Toribara NW et al. Molecular cloning of human intestinal mucin cDNAs. Sequence analysis and evidence for genetic polymorphism. (1989) J Biol Chem. 264:6480-6487

61 Briand JP, Andrews SP, Cahill E, Conway NA et al. Investigation of the requirements for O-glycosylation by bovine submaxillary gland UDP-N-Acetylgalactosamine: Polypeptide N-acetylgalactosamine transferase using synthetic peptide substrates. (1981) J Biol Chem. 256(23):12205-1207

62 Toribara NW, Roberton AM, Ho SB, Kuo WL et al. Human gastric mucin. Identification of a unique species by expression cloning. (1993) J. Biol Chem. 268:5879-5885

63 Gum JR, Hicks JW, Toribara NW, Rothe E et al. The human MUC2 intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. (1992) J. Biol. Chem. 267(30):21375-21383

64 Swallow DM, Gendler S, Griffiths B, Kearney A et al. The hypervariable gene locus PUM, which codes for the tumour associated epithelial mucins, is located on chromosome 1, within the region 1q21-24. (1987) Ann. Hum. Genet. 51:289-294

64 Toribara NW, Gum JR, Culhane PJ, Lagace RE et al. MUC2 human small intestinal mucin gene structure. Repeated arrays and polymorphism. (1991) J. Clin Invest. 88:1005-1013

65 Griffiths B, Matthews DJ, West L, Attwood J et al. Assignment of the polymorphic intestinal mucin gene (MUC2) to chromosome 11p15. (1990) 54:277-283

67 Gururaja TL, Ramasubbu LN, Venugopalan P, Reddy MS et al. Structural features of the human salivary mucin, MUC7. (1998) Glycoconj J. 15:457-467

68 Bobek LA, Liu J, Sait SN, Shows TB et al. Structure and chromosomal localization of the human salivary mucin gene, MUC7. (1996) Genomics. 31:277-282

69 Nollet S, Moniaux N, Maury J, Petitprez D et al. Human mucin gene MUC4; organisation of its 5'region and polymorphism of its central tandem repeat array. (1998) Biochem J. 332:739-748

70 Escande F, Aubert JP, Porchet N, Buisine MP. Human mucin gene MUC5AC: organization of its 5'-and central repetitive regions. (2001) Biochem J. 15:763-72

71 Cohen-Salmon M, El-Amraoui A, Leibovici M, Petit C et al. A glycoprotein specific to the acellular membranes of the inner ear. (1997) Proc. Natl. Acad. Sci. USA. 94:14450-14455

72 Legan PK, Rau A, Keen JN, Richardson GP. The mouse tectorins. (1997) J. Biol. Chem. 272(13):8791-8801

73 Hoffman W, Joba W. Biosynthesis and molecular architecture of gel-forming mucins: implications from an amphibian system. (1995) Biochem Soc Trans. 23(4):805-10.

74 Desseyn JL, Buisine MP, Porchet N, Aubert JP et al. Genomic organization of the human mucin gene MUC5B. cDNA and genomic sequence upstream of the large central exon. (1998) J Biol Chem. 273:30157-30164

75 Offner GD, Nunes DP, Keates AC, Afdhal NH et al. The amino-terminal sequence of MUC5B contains conserved multifunctional D domains; implications for tissue-specific mucin functions. (1998) Biochem Biophys Res Commun. 251:350-355

76 Porchet N, Pigny P, Buisine MP, Debailleul V et al.Human mucin genes:genomic organisation expression of MUC4, MUC5AC and MUC5B. (1995) Biochem Soc Trans. 23:800-804

77 Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. (2004) 4:45-60

78 Godl K, Johansson ME, Lidell ME, Morgelin M et al. The N terminus of the MUC2 mucin forms trimers that are held together within a trypsin-resistant core fragment.(2002) J Biol Chem. 277:47248-47256

79 Asker N, Axelsson MA, Olofsson S, Hansson GC. Dimerization of the human MUC2 mucin in the endoplasmic reticulum is followed by a N-glycosylation-dependent transfer of the mono-and dimers to the golgi apparatus. (1998) J. Biol. Chem. 273(30):18857-18863

80 Bell SL, Xu G, Khatri IA, Wang R et al. N-linked oligosaccharides play a role in disulphide-dependent dimerization of intestinal mucin Muc2. (2003) Biochem. J. 373:893–900

81 Lidell ME, Johansson ME, Hansson GC. An autocatalytic cleavage in the C-terminus of the human MUC2 mucin occurs at the low pH of the late secretory pathway. (2003) J Biol Chem. 278:13944-13951

82 Gendler S, Taylor-Papadimitriou J, Duhig T, Rothbard J et al. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. (1988) J. Biol Chem. 263:12820-12823

83 Desseyn J, Tetaert D, Gouyer V. Architecture of the large membrane-bound mucins. (2008) Gene.410:215-222

84 Wreschner DH, McGuckin MA, Williams SJ, Baruch A et al. Generation of a ligand-receptor alliances by 'SEA' module-mediated cleavage of membrane-associated mucin proteins. (2002) Protein Sci. 11:698-706

85 Thathiah A, Blobel C, Carson D. Tumor necrosis factor-α converting enzyme (TACE)/ADAM 17 mediates MUC1 shedding. (2002) J Biol Chem.278.3386-3394

85 Gum JR, Ho JJ, Pratt WS, Hicks JW et al. MUC3 human intestinal mucin. Analysis of gene structure, the carboxyl terminus and a novel upstream repetitive region. (1997) J Biol Chem. 272:26678-26686

87 Carraway KL, Price-Schiavi SA, Komatsu M, Idris N et al. Multiple facets of sialomucin complex/MUC4; a membrane mucin and erbb2 ligandin tumors and tissues. (2000) Front Biosci. 5:D95-D107

88 Hauser F, Hoffmann W. P-domains as shuffled cysteine-rich modules in integumentary mucin C.1(FIM-C.1) from *Xenopus laevis*. (1992) J. Biol. Chem. 267(34):24620-24624

89 Crawley SC, Gum JR, Hicks JW, Pratt WS et al. Genomic organization and structure of the 3' region of human MUC3: alternative splicing predicts membrane-bound and soluble forms of the mucin. (1999) Biochem Biophys Res Commun. 263:728-736

90 Corfield AP, Warren BF. Mucus glycoproteins and their role in colorectal disease. (1996). J Path.180:8-17

91 Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin. (1998). Int J Biochem Cell Biol. 30:797-801

92 Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. (1985) Annu Rev Biochem. 54:631-64

93 Morris ER, Rees DA. Principles of biopolymer gelation. Possible models for mucus gel structure. (1978) Br Med Bull. 34:49-53

94 Dennis JW, Granovsky M, Warren CE. Protein glycosylation in development and disease. (1999) Bioessays. 21:412-421

95 Carraway KL, Hull SR. O-glycosylation pathway for mucin-type glycoproteins. (1989) Bioessays. 10(4):117-121

96 Podolsky DK. Oligosaccharide structures of human colonic mucin. (1985) J Biol Chem. 260:8262-8271

97 Podolsky DK. Oligosaccharide structures of isolated human colonic mucin species. (1985) J Biol Chem. 260:15510-15515

98 Marcauelle LA, Bertozzi CR. Recent advances in the chemical synthesis of mucin-like glycoproteins. (2002). Glycobiol. 12(6):69R-77R

99 Robbe C, Capon C, Coddeville B, Michalski JC. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. (2004) Biochem J. 384:307-316

100 Corfield AP, Myerscough N, Gough M, Brockhausen I et al.Glycosylation patterns of mucins in colonic disease. (1995) Biochem Soc Trans. 23:840-844

101 Lamblin G, Lhermitte M, Klein A, Houdret N et al. The carbohydrate diversity of human respiratory mucins: a protection of the underlying mucosa? (1991) Am Rev Respir Dis. 144:S19-S24

102 Jass JR, Walsh MD. Altered mucin expression in the gastrointestinal tract; a review. (2001) J Cell Mol Med. 5:327-351

103 Etzler ME. Lectins as probes in studies of intestinal glycoproteins and glycolipids. (1979) Am J Clinc Nut. 32:133-138

104 Hounsell EF, Feizi T. Gastrointestinal mucins:structures and antigenicities of the carbohydrate chains in health and disease. (1982) Med Biol. 60:227-236

105 Blaszyck M, Pak KY, Herlyn M, Sears HF, Steplewski Z. Characterization of Lewis antigens in normal colon and gastrointestinal adenocarcinomas. (1985) Proceedings Nat Aca Sci USA. 82:3552-3556

106 Jacobs LR, Huber PW. Regional distribution and alterations of lectin binding to colorectal mucin in mucosal biopsies from controls and subjects with inflammatory bowel disease. (1985) J Clin Invest. 75:112-118

107 Bresalier RS, Boland CR, Kim YS. Regional differences in normal and cancer-associated glycoconjugates of the human colon. (1985) J National Can Institute. 75:249-260

108 Sheahan DG, Jervis HR. Comparative histochemistry of gastrointestinal mucosubstances. (1976) Am J Anat.146:103–31

109 Varki A. Diversity in the sialic acids. (1992) Glycobiology. 2(1):25-40

110 Culling CF, Reid PE, Dunn WL. A new histochemical method for the identification and visualisation of both side chain acylated and non-acylated sialic acids. (1976) J Histochem Cytochem. 24:1225-30

111 Corfield AP, Do Amaral Corfield C, Wagner SA, Warren BF et al. Loss of sulphate in human colonic mucins during ulcerative colitis. (1992) Biochem Soc Trans. 20:95S

112 Brockhausen I. Sulphotransferases acting on mucin-type oligosaccharides. (2003) Biochem Soc Trans. 31:318-325

113 Brockhausen I, Kuhns W. Role and metabolism of glycoconjugate sulfation (1997) Trends Glycosci. Glycotechnol. 9:379-398

114 Bergwerff A, Van Oostrum J, Kamerling J, Vliegenthart J. The major N-linked carbohydrate chains from human urokinase. The occurrence of 4-O-sulfated, (alpha 2-6)-sialylated or (alpha 1-3)-fucosylated N-acetylgalactosamine(beta 1-4)-N-acetylglucosamine elements. (1995) Eur J Biochem. 228:1009-1019

115 Hemmerich S, Leffler H, Rosen S. Structure of the O-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. (1995) J. Biol. Chem.270:12035-12047

116 Perez-Vilar J, Eckhardt AE, DeLuca A, Hill RL. Porcine submaxillary mucin forms disulfide-linked multimers through its amino-terminal D-domains. (1998) J. Biol.Chem. 273(23):14442-14449

117 Perez-Vilar J, Hill RL. The carboxyl-terminal 90 residues of porcine submaxillary mucin are sufficient for forming disulfide-bonded dimers. (1998) J. Biol. Chem. 273(12):6982-6988

118 Dong Z, Thoma RS, Crimmins DL, McCourt DW et al. Disulfide bonds required to assemble functional von Willebrand factor multimers. (1994) J. Biol Chem. 269:6753-6758

119 Voorberg J, Fontijn R, Calafat J, Janssen H et al. Assembly and routing of von Willebrand factor variants; the requirements for disulfide-linked dimerization reside within the carboxy-terminal 151 amino acids. (1991) J Cell Biol. 113:195-205

120 Roth J. Cytochemical localization of terminal *N*-acetyl-D-galactosamine residues in cellular compartments of intestinal goblet cells: implications for the topology of O-glycosylation. (1984) J Cell Biol. 98:39-406

121 Paulson JC, Colley KJ. Glycosyltransferases: Structure, localization and control of cell type-specific glycosylation. (1989) J Bio Chem. 264: 17615-8

122 Hill HD, Schwyzer M, Steinman HM, Hill RL. Ovine submaxillary mucin. Primary structure and peptide substrates of UDP-N-acetyl galactosamine:mucin transferase. (1977) Biol Chem. 252:3799-3804

123 Wilson-Storey D, Scobie WG, Raeburn JA. Defective white blood cell function in Hirschsprung's disease: A possible predisposing factor to enterocolitis. (1988) J R Coll Surg Edinb. 33:195-188

124 Hanisch F, Reis CA, Clausen H, Paulsen. Evidence for glycosylation-dependent activities of polypeptide N-acetlygalactosaminyltransferases rGalNAc-T2 and –T4 on mucin glycopeptides. (2001) Glycobiology.11:731-740

125 Corfield AP, Wember M, Schauer R, Rott R.,The specificity of viral sialidases. The use of oligosaccharide substrates to probe enzymic characteristics and strain-specific differences. (1982) Eur J Biochem. 124(3):521-5.

126 Corfield AP, Wagner SA, Paraskeva C, Clamp JR et al. Loss of sialic acid O-acetylation in human colorectal cancer cells. (1992) Biochem Soc Trans. 20:94S

127 Varki A, Freeze HH. The major glycosylation pathways of mammalian membranes. A summary. (1994) Subcell Biochem.22:71-100

128 Roth J. Subcellular organization of glycosylation in mammalian cells. (1987) Biochim Biophys Acta. 906: 405-436

128 Seko A, Hara-Kuge S, Yamashita K. Molecular cloning and characterization of a novel human galactose 3-O-sulfotransferase that transfers sulfate to gal beta 1--
>3galNAc residue in O-glycans. (2001) J Biol Chem.276:25697-25704

129 Midura RJ, Hascall VC. Bone sialoprotein--a mucin in disguise? (1996) Glycobiology. 6:677-681

131 Brockhausen I, Schutzbach J, Kuhns W. Glycoproteins and their relationship to human disease. (1998) Acta Anat (Basel). 161:36–78.

132 Kuhns W, Jain R, Matta K, Paulsen H et al. Characterization of a novel mucin sulphotransferase activity synthesizing sulphated O-glycan core 1,3-sulphate-Gal beta 1-3GalNAc alpha-R. (1995) Glycobiology. 5:689-697

133 Axelsson MA, Asker N, Hansson GC. O-glycosylated MUC2 monomer and dimmer from LS 174T cells are water-soluble, whereas larger MUC2 species formed early during biosynthesis are insoluble and contain nonreducible intermolecular bonds. (1998) J. Biol. Chem. 273(30):18864-18870

134 Verdugo P. Mucin Exocytosis. (1991). Am Rev Respir Dis. 144:S33-S37

135 Laboisse C, Jarry A, Branka E, Merlin D et al. Regulation of mucin exocytosis from intestinal goblet cells. (1995) Biochem Soc Trans.23:810-813

136 Wickstrom C, Carlstedt I. N-terminal Cleavage of the Salivary MUC5B Mucin: analogy with the von Willebrand propolypeptide? (2001) J. Biol. Chem. 276: 47116-47121

137 MacAdam, A. The effects of gastro-intestinal mucus on drug adsorption. (1993) Adv Drug Deliv Rev. 11:201– 220

138 Aksoy N, Thornton J, Corfield AP, Paraskeva C et al. A study of the intracellular and secreted forms of the MUC2 mucin from the PC/AA intestinal cell line. (1999) Glycobiology. 9 (7):739-746

139 Matsuo K, Ota H, Akamatsu T, Sugiyama A et al. Histochemistry of the surface mucous gel layer of the human colon. (1997) Gut. 40:782–9

140 Bou-Hanna C, Berthon B, Combettes L, Claret M et al. Role of calcium in carbachol- and neurotensin-induced mucin exocytosis in a human colonic goblet cell line and cross-talk with the cyclic AMP pathway. (1994) Biochem J. 299:579-585

141 Merlin D, Augeron C, Tien XY, Guo X et al. ATP-stimulated electrolyte and mucin secretion in the human intestinal goblet cell line HT29-Cl.16E. (1994) J Membrane Biol. 137:137-149

142 Peppas NA, Huang Y. Nanoscale technology of mucoadhesive interactions. (2004) Advanced Drug Delivery Reviews. 56: 1675– 1687

143 Bourlioux P, Koletzko B, Guarner F, Braesco V. The intestine and its microflora are partners for the protection ofthe host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002. (2003) Am J Clin Nutr. 78:675–83

144 Lichtenberger, LM. The hydrophobic barrier properties of gastrointestinal mucus. (1995) Annu Rev Physiol. 57:565-583

145 Rintala RJ, Lindahl H. Sodium cromoglycate in the management of chronic or recurrent enterocolitis in patients with Hirschsprung's disease. (2001) J Pediatr Surg. 36:1032-1035

146 Moncada DM, Kammanadiminti SJ, Chadee K. Mucin and Toll-like receptors in host defense against intestinal parasites. (2003) Trends in Parasitology. 19(7):305-11

147 Bhaskar KR, Garik P, Turner BS, Bradley JD et al. Viscous fingering of HCL through gastric mucin. (1992) Nature. 360:458-461

148 Sands BE, Podolsky DK. The trefoil peptide family. (1996) Ann Rev Physiol. 58:253-273

149 Tomasetto C, Masson R, Linares JL, Wendling C et al. pS2/TFF1 interacts directly with the VWF C cysteine-rich domains of mucins. (2000) Gastroenterol. 118:70-80

150 Tran Cp, Cook GA, Yeomans ND, Thim L et al. Trefoil peptide TFF2 potently accelerates healing and reduces inflammation in a rat model of colitis. (1999) Gut. 44:636-642

151 Wright NA, Hoffmann W, Otto WR, Rio MC et al. Rolling in the clover; trefoil factor family domain peptides, cell migration and cancer. (1997) FEBS Lett. 408:121-123

152 Kawakubo M, Ito Y, Okimura Y, Kobayashi M et al . Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. (2004) Science. 305:1003-1006

153 Bruno LS, Li X, Wang L, Soares RV et al. Two-hybrid analysis of human salivary mucin MUC7 interactions. (2005) Biochem Biophys Acta. 1746:65-72

154 Vinall LE, King M, Novelli M, Green CA et al. Altered expression and allelic association of the hypervariable membrane mucin MUC1 in Heliocbacter pylori gastritis. (2002) Gastroenterology. 123:41-49

155 Alexander DA, Dimock K. Sialic acid functions in enterovirus 70 binding and infection. (2002) J. Virol. 76:11265-11272

156 Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA. Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. (1999) Am J Physiol. 276:G941–50

157 Lencer WI, Reinhart FD, Neutra MR. Interaction of cholera toxin with cloned human goblet cells in monolayer culture. (1990) Am J Physiol. 258:G96–102

158 Branka JE, Vallette G, Jarry A, Bou-Hanna C et al. Early functional effects of *Clostridium difficile* toxin A on human colonocytes. (1997)Gastroenterol. 112:1887–94.

159 Majuri ML, Niemela R, Tiisala S, Renkonen O et al. Expression and function of alpha 2,3-sialyl and alpha 1,3/1,4-fucosyltransferases in colon adenocarcinoma cell lines: role in synthesis of E-selectin counter receptors. (1995) Int J Cancer. 63: 551-9

160 Enss ML, Cornbery M, Wagner S, Gebert A et al. Proinflammatory cytokines trigger MUC gene expression and mucin release in the intestinal cancer cell line LS180. (2000) Inflamm Res. 49: 162-9

161 Belley A, Chadee K. Prostaglandin E2 stimulates rat and human colonic mucin exocytosis via the EP(4) receptor. (1999) Gastroenterology. 117: 1352-62

162 Quigley ME, Kelly SM. Structure, function, and metabolism of host mucus glycoproteins. In: Gibson GR, Macfarlane GT, eds. Human colonic bacteria. (1995) Boca Raton, FL: CRC Press:175–201

163 Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. (2001) Am J Clin Nutr. 73(suppl):1131S-41S

164 Corfield AP, Wagner SA, O'Donnell LJD, Durdey P et al. The roles of enteric bacterial sialidase, sialate O-acetyl esterase and glycosulfatase in the degradation of human colonic mucin (1993) Glycoconj J. 10:72-81

165 Soeda J, O'Briain S, Puri P. Regional reduction in intestinal neuroendocrine cell populations in entercolitis complicating Hirschsprung's disease. (1993) J Paed Surg. 28(8):1063-1068

166 Corfield AP, Wagner SA, Clamp JR, Kriaris MS et al. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. (1992) Infect Immun. 60(10):3971-8.

167 Lui VC, Sham MH, Tam PK. CDX-1 and CDX-2 are expressed in human coloini mucosa and are down-regulated in patients with Hirschsprung's disease associated enterocolitis. (2001) Biochim Biophys Acta. 1537(2):89-100

168 Fontaine N, Meslin JC, Lory S, Andrieux C. Intestinal mucin distribution in the germ-free rat and in the heteroxenic rat harbouring a human bacterial flora: effect of inulin in the diet. (1996) Br J Nutr. 75:881–92

169 Jass JR, Roberton AM Colorectal mucin histochemistry in health and disease: a critical review (1994) Pathol. Int. 44:487-504

170 Roberton Am, McKenzie C, Scharfe N, Stubbs L. A glycosulphatase that removes sulphate from mucus glycoprotein. (1993) Biochem J. 293 (Pt 3):683-9

171 Delcommenne M, Kannagi R, Johnson P. TNF-alpha increases the carbohydrate sulfation of CD44: induction of 6-sulfo N-acetyl lactosamine on N- and O-linked glycans. (2002) Glycobiology. 12:613-622

172 Vimal DB, Khullar M, Gupta S, Ganguly NK. Intestinal mucins: the binding sites for *Salmonella typhimurium*. (2000) Mol Cell Biochem. 204:107–17

173 Hoyer LL, Hamilton AC, Steenbergen SM, Vimr ER. Cloning, sequencing and distribution of the *Salmonella typhimurium* LT2 sialidase gene, *nanH*, provides evidence for interspecies gene transfer. (1992) Mol Microbiol. 6:873–84

174 Savage DC. Factors involved in colonization of the gut epithelial surface. (1978) Am J Clin Nutr. 31(suppl):S131–5.

175 Hoskins LC, Augustines M, McKee WB, Boulding ET et al. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. (1985) J Clin Invest. 75:944–53

176 Hoskins LC. Mucin degradation in the human gastrointestinal tract and its significance to enteric microbial ecology. (1992) Eur J Gastroenterol Hepatol. 5:205–12

177 Culling CF, Reid PE, Worth AW. Carbohydrate markers in the lower gastrointestinal tract. (1981) . Meth Achievements Exp Path. 10:73-100

178 Filipe MI, Branfoot AC. Mucin histochemistry of the colon. (1976) Current Topics Path. 63:143-178

179 Nieuw Amerongen AV, Bolscher JGM, Bloemena E, Veerman ECI. Sulfomucins in the human body. (1998) Biol Chem. 379:1–18

180 Boat TF, Welsh MJ, Beaudet AL. Cystic fibrosis. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds. The Metabolic Basis of Inherited Disease. (1989) New York, McGraw Hill:2649–2680

181 McCormick DA, Horton LW, Mee AS. Mucin depletion in inflammatory bowel disease. (1990) J Clin Pathol. 43:143-6

182 Rhodes JM. Colonic mucus and ulcerative colitis [comment]. (1997) Gut. 40:807–808

183 Bolande RP. The neurocristopathies. A unifying concept of disease arising in neural crest maldevelopment. (1974) Hum Pathol. 5:409-429

184 Tygat KM, Van der Wal TW, Einerhand AW, Büller HA et al. Quantitative analysis of MUC2 synthesis in ulcerative colitis. (1996) Biochem Biophys Res Commun. 224:397-405

185 Ho SB, Neihans GA, Lyftogt C, Yan PS et al. Heterogeneity of mucin gene expression in normal and neoplastic tissues. (1993) Cancer Res. 53:641-51

186 Byrd JC, Yunder CK, Xu QS, Sternbery LR et al. Inhibition of gastric mucin synthesis by Helicobacter pylori. (2000) Gastroenterology. 118: 1072-9

187 Buisine MP, Desreumaux P, Debailleul V et al. Abnormalities in mucin gene expression in Crohn's disease. (1999) Inflamm Bowel Dis.5;24-32

188 Boland CR, Deshmukh DH. The carbohydrate composition of mucin in colonic cancer. (1990) Gastroenterol. 98:1170-1177

189 Irzkowitz SH, Bloom EJ, Kokai WA, Modin G et al. Sialosyl-Tn. A novel mucin antigen associated with prognosis in colorectal cancer patients. (1990) Cancer. 66(9):1960-6

190 Orntoft TF, Harving N, Langkilde NC. O-linked mucin-type glycoproteins in normal and malignant colon mucosa: lack of T-antigen expression and accumulation of Tn and sialosyl-Tn antigens in carcinomas. (1990) Int J Cancer. 45:666-72

191 Mann B, Klussmann E, Vandamme-Feldhaus V, Iwersen M et al. Low acetylation of sialyl-Le(x) contributes to its overexpression in colon carcinoma metastases. (1997) Int J Cancer. 72:258–64.

192 Vavasseur F, Dole K, Yang J, Matta K et al. O-glycan biosynthesis in human colorectal adenoma cells during progression to cancer. (1994) Eur J Biochem.222:415-424

193 Hanski C, Stolze B, Rieken EO. Tumorigenicity, mucin production and AM-3 epitope expression in clones selected from the HT-29 colon carcinoma cell line (1992) Int J Cancer. 50:924-929

194 Parkkinen J, Rogers GN, Korhonen T, Dahr W et al. Identification of the O-linked sialyloligosaccharides of glycophorin A as the erythrocyte receptors for S-fimbriated Escherichia coli. (1986) Infec Immun. 54:37-42.

195 Yang J, Byrd J, Siddiki B, Chung Y et al. Alterations of O-glycan biosynthesis in human colon cancer tissues. (1994). Glycobiol. 4(6):873-884

196 Ito H, Hiraiwa N, Sawada-Kasugai M, Akamatsu S et al. Altered mRNA expression of specific molecular species of fucosyl- and sialyl-transferases in human colorectal cancer tissues. (1997) Int J Cancer. 71:556-64

197 Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. (2001) Science. 292:1115–8

198 Chambers JA, Hollingsworth MA, Trezise AEO, Harris A. Developmental expression of mucin genes MUC1 and MUC2. (1994) J Cell Sci. 107:413-424

199 Reid CJ, Harris A. Developmental expression of mucin genes in the human gastrointestinal system. (1998) Gut. 42: 220-226

200 Filipe MI, Sandey A, Carapeti EA. Goblet cell mucin in human foetal colon, its composition and susceptibility to enzyme degradation: a histochemical study. (1989) Symp Soc Exp Biol. 43: 249-58

201 Filipe MI, Sandey A. Mucin in the developing foetal gut; an insight into cell differentiation and maturation in the adult. (1988) J. Pathol. 154: 73A

202 Brunsgaard G. Morphological characteristics, epithelial cell proliferation, and crypt fission in caecum and colon of growing pigs. (1997) Diges Dis Sci. 42: 2384-2393.

203 King TP, Begbie R, Slater D, McFadyen M et al. Sialylation of intestinal microvillar membranes in newborn suckling and weaned pigs. (1995) Glycobiology. 5:525-534.

204 Robbe-Masselot C, Maes E, Rousset M, Michalski JC et al. Glycosylation of human fetal mucins: a similar repertoire of O-glycans along the intestinal tract. (2009) Glycoconji J. 26(4):397-413. Epub 2008 Sep 20

205 Aslam A. The formation and development of the colonic mucus defensive barrier in normal children and in those with Hirschsprung's disease. (1998) Bristol thesis. P147-160

206 Hirschsprung H. Stuhltragheit Neugeborener in Folge von Dilatation und Hypertrophie des Colons. (1888) Jb Kinderheilk. 27:1

207 Ehrenpreis T. Hirschsprung's disease. (1971) Digest Dis. 16:1032-1052

208 Philipart AI. Hirschsprung's disease. In Holder KW and Ashcroft TM (eds.). Pediatric Surgery. (1993) London, WB Saunders Company: 358-371

209 Whitehouse FR, Kernohan JW. Myenteric plexus in congenital megacolon. Study of eleven cases. (1948) Arch Intern Med. 82:75-111

210 Zuelzer WW, Wilson JL. Functional intestinal obstruction on a congenital neurogenic basis in infancy. (1948) Am J Dis Child. 75:40-64

211 Swenson O. How the cause and cure of Hirschsprung's disease were discovered. (1989) J Paed Surg. 34(10):1580-1587

212 Swenson O, Rheinlander HF, Diamond L. Hirschsprung's disease: a new concept of the etiology. (1949) N Engl J Med. 241:551-556

213 Swenson O, Fisher JH, MacMahon HE. Rectal biopsy as an aid in the diagnosis of Hirschsprung's disease. (1955) N Engl J Med. 253:632-635

214 Swenson O, Bill AH. Resection of rectum and rectosigmoid with preservation of the sphincter for benign spastic lesions producing megacolon: an experimental study. (1948) Surgery. 24:212-20

215 Swenson O. A new surgical treatment for Hirschsprung's disease. (1950) Surgery. 28:371-83

216 Duhamel B. A new operation for the treatment of Hirschsprung's disease. (1960) Arch Dis Child. 35:38-9

217 Soave F. Hirschsprung's disease; a new surgical technique. (1964) Arch Dis Child. 39:116-24

218 Martin LW, Altemeier WA. Clinical experience with a new operation (modified Duhamel procedure) for Hirschsprung's disease. (1962) Ann Surg. 156:678-81

219 Boley SJ. New modifications of the surgical treatment of Hirschsprung's diseae. (1964) Surgery. 56:1015-7

220 Badner JA, Sieber WK, Garver KL, Chakravarti A. A genetic study of Hirschsprung's disease. (1990) Am J Hum Genet.46:568-580

221 Jung PM. Hirschsprung's disease: One surgeon's experience at one institution. (1995) J Pediatr Surg. 30: 646-651

222 Klein MD, Philipart AI. Hirschsprung's disease: Three decades' experience at a single institution. (1993) J Pediatr Surg. 10:1291-1294

223 Orr J, Scobie W. Presentation and incidence of Hirschsprung's disease. (1983) BMJ. 287:1671

224 Ikeda K, Goto S. Diagnosis and treatment of Hirschsprung's disease in Japan: an analysis of 1628 patients. (1984) Ann Surg. 199:400-405

225 Swenson O,Sherman JO, Fisher JH. Diagnosis of congenital megacolon: an analysis of 501 patients. (1973) J Paed Surg.8:587-594

226 Romeo G, Ronchetto P, Luo Y , Barone V et al. Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. (1994) Nature.27;367(6461):377-8

227 Tam PKH, Boyd GP. Origin, course and endings of abnormal enteric nerve fibres in Hirschsprung's disease defined by whole mount immunohistochemistry. (1990) J Pediatr Surg. 25:457-461

228 Polley T Jr, Coran A, Wesley J. A ten-year experience with ninety-two cases of Hirschsprung's disease; including sixty-seven consecutive endorectal pull-through procedures. (1985) Ann Surg. 202:349-355

229 Kusafuka T, Puri P. Genetic aspects of Hirschsprung's disease. (1998) Semin Pediatr Surg. 7:140

230 Puri P, Ohshiro K, Wester T. Hirschsprung's disease: a search for etiology. (1998) Semin Pediatr Surg. 7(3):140-7

231 Passarge E. The genetics of Hirschsprung's disease. Evidence for heterogenous etiology and a study of sixty-three families. (1967) N Engl J Med.276:138

232 Reding R, de Ville de Goyet J, Gosseye S, Clapuyt P et al. Hirschsprung's disease: A 20-year experience. (1997) J Pediatr Surg. 32:1221-1225

233 Klein MD, Coran AG, Wesley JR, Drongoski RA. Hirschsprung's disease in the newborn. (1984) J Pediatr Surg. 19:370-374

234 Goldberg E. An epidemiological study of Hirschsprung's disease. (1984) Int J Epidemiol. 13:479-485

235 Baltogiannis N, Mavridis G, Soutis M, Keramidas D. Currarino triad associated with Hirschsprung's disease. (2003) 38:1086-1089

236 Elhalaby E, Coran A. Hirschsprung's disease associated with Ondine's curse: report of three cases and review of the literature. (1994) J Pediatr Surg. 29:530-535

237 Kim E, Boutwell W. Smith-Lemli-Opitz syndrome associated with Hirschsprung's disease, 46, XY female karotype and total anomalous pulmonary venous drainage. (letter 1985) J Pediatr Surg. 106:861

238 Hyde G Jr, DeLorimier A. Colonic atresia and Hirschsprung's disease. (1968) Surgery. 64:976-978

239 Boley SJ, Dinari G, Cohen MI. Hirschsprung's disease in the newborn. (1978). Clinc Perinatol. 5(1):45-60

240 Clark D. Times of first void and first stool in 500 newborns. (1977) Pediatrics. 60:457-459

241 Teitelbaum DH, Coran AG, Weitzman JJ, Ziegler MM et al. Hirschsprung's disease and related neuromuscular disorders of the intestine. In Pediatric Surgery, Fifth Edition, Eds O' Neill JA et al. (1998) Mosby: 1381-1424

242 Newgreen D, Young H. Enteric nervous system: development and developmental disturbances--part 1. (2002) Pediatr Dev Pathol. 5(3):224-47

243 Furness JB. Types of neurons in the enteric nervous system. (2000) J Auton Nerv Syst;81:87–96.

244 Galligan JJ, LePard KJ, Schneider DA, Zhou X. Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. (2000) J Auton Nerv Syst;81:97–103.

245 Newgreen D, Young H. Enteric nervous system: development and developmental disturbances--part 2. (2002) Pediatr Dev Pathol. 5(4):329-49

246 Vanner S, Surprenant A. Neural reflexes controlling intestinal microcirculation. (1996) Am J Physiol. 271:G223-G230

247 Lichtenstein JE, Schotz FJ. Diseases of The Abdomen and Pelvis. (2006). Milan. Springer:52 - 59

248 Furness John. Structure of the Enteric Nervous System and The Constituents of the Neurons of the Enteric Nervous System. In: The Enteric Nervous System. (2006) Oxford. Blackwell Publishing Ltd:1 - 78

249 Natarajan D, Pachnis V. Development of the enteric nervous system. In Development of the gastrointestinal tract. Sanderson IR, Walker WA (eds). (1999) Blackwell Science Ltd. Oxford:197-210

250 Sanders KM. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. (1996) Gastroenterol. 111:492-515

251 Torihashi S, Nishi K, Tokutomi Y, Nishi T et al. Blockade of kit signalling induces transdifferentiation of interstitial cells of cajal to a smooth muscle phenotype. (1999) Gastroenterol. 117:140-148

252 Sanders KM, Ordog T, Koh SD, Torihashi S et al. Development and plasticity of interstitial cells of Cajal. (1999) Neurogastroenterol Motil. 11:311-338

253 Horisawa M, Watanabe Y, Torihashi S. Distribution of c-Kit immunopositive cells in normal human colon and in Hirschsprung's disease. (1998) J Paed Surg. 33:1209-1214

254 Nemeth L, Yoneda A, Kader M, Devancy D et al. Three-dimension morphology of gut innervation in total intestinal aganglionosis using whole-mount preparation. (2001) J Paed Surg. 36:291-295

255 Yamataka A, Kato Y, Tibboel D, Murata Y et al. A lack of intestinal pacemaker (c-kit) in aganglionic bowel of patients with Hirschsprung's disease. (1995) J Pediatr Surg 30:441–444

256 Sandgren K, Larsson L, Ekblad E. Widespread changes in neurotransmitter expression and number of enteric neurons and interstitial cells of cajal in lethal spotted mice. (2002) Dig Diseases and Sciences. 47 (5):1049-64

257 Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH et al. Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. (1990) Nature. 345:346-7

258 Takahashi T. Pathophysiological significance of neuronal nitric oxide synthase in the gastrointestinal tract. (2003) J Gastroenterol. 38:421-430

259 McConalogue K, Furness JB. Gastrointestinal neurotransmitters. (1994) Baillieres Clin Endocrinol Metab. Jan;8(1):51-76

260 Kusafuka T, Puri P. Altered RET gene mRNA expression in Hirschsprung's disease. (1997) J Pediatr Surg. 32(4):600-4

261 Patric WJA, Beaslehy GTN, Smith II. Histochemical diagnosis of Hirschsprung's disease and a comparsion of the histochemical and biochemical activity of acetylcholine esterase in rectal mucosal biopsies. (1980) J Clin Pathol. 33:336-343

262 Bennet A, Garret J, Howard E. Adrenergic myenteric nerves in Hirschsprung's disease. (1968) Br Med J. 1:487-489

263 Larsson LT, Malmfors G, Sundler F. Peptidergic innervation in Hirschsprung's disease. (1983) Z Kinderchir. 38:301-304

264 Tam PK, Gill PB. New insights into peptidergic abnormalities in Hirschsprung's disease by wholemount immunohistochemistry. (1991) J Pediatr Surg. 26(5):595-597

265 Green T, Dockray GJ. Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea pig. (1988) Neuroscience. 25:181-193

266 Larsson LT, Malmrofs G, Sundler F. Neuropeptide Y (NYP), calcitonin gene-related peptide (CGRP) and galanin in Hirschsprung's disease – an immunocytochemical study. (1988) J Pediatr Surg. 23:342-345

267 Soeda J, O'Briain DS, Puri P. Mucosal neuroendocrine cell abnormalities in the colon of patients with Hirschsprung's disease. (1992) J Pediatr Surg. 27:323-7

268 Oue T, Yoneda A, Shima H, Puri P. Muscarinic acetylcholine receptor expression in aganglionic bowel. (2000) Pediatr Surg. 16:267-71

269 Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk visera from vagal neural crest in the chick embryo. (1954) J Comp Neurol. 101:515-541

270 Yntema CL, Hammond WS. Experiments on the origin and development of the sacral autonomic nerves in the chick embryo. (1955) J Exp Zool. 129:375-413

271 LeDouarin NM, Teillet MA. The migration of neural crest cells to the wall of the digestive tract in avian embryo. (1973) Embryol Exp Morphol. 30:31-48

272 Burns AJ, LeDouarin NM. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. (1998) Development 125:4335-4347

273 Durbec PL, Larsson-Blomberg LB, Schuchardt A, Costantini F et al. Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. (1996) Development. 122:349-358

274 Kapur RP, Yost C, Palmiter RD. A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice. (1992) Development. 116:167

275 Gershon MD, Chalazonitis A, Rothman TP. From neural crest to bowel: development of the enteric nervous system. (1993) J Neurobiol.24:199

276 Fujimoto T, Hata J, Yokoyama S, Mitomi T. A study of the extracellular matrix protein as the migration pathway of neural crest cells in the gut: analysis in human embryos with special reference to the pathogenesis of Hirschsprung's disease. (1989) J Pediatr Surg.24:550-556

277 Pawson T, Bernstein A. Receptor tyrosine kinases: genetic evidence for their role in *Drosophilia* and mouse development. (1990) Trends Genet. 6:350-6

277 Bates MD. Development of the enteric nervous system. (2002) Clinc Perinatol. 29(1):97-111

278 Pasini B, Ceccherini I, Romeo G. RET mutations in human disease. (1996) Trends Genet. 12:138

280 Tsuzuki T, Takahashi M, Asai N, Iwashita T et al. Spatial and temporal expression of the ret proto-oncogene product in embryonic infant and adult rat tissues. (1995) Oncogene. 10:191

281 Watanabe Y, Harada T, Ito T, Ishiguro Y et al. ret proto-oncogene product is a useful marker of lineage determination in the development of the enteric nervous system in rats. (1997) J Pediatr Surg. 32:28

282 Durbec P, Marcos-Guiterrez CV, Kilkenny C, Grigoriou M et al. GDNF signalling through the Ret receptor tyrosine kinase. (1996) Nature. 381:789

283 Ebendal T, Tomac A, Hoffer BJ, Olson L. Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. (1995) J Neurosci Res. 40:276-284

284 Heuckeroth RO, Enomoto H, Grider JR, Golden JP et al. Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory and parasympathetic neurons. (1999) Neuron. 22:253-63

285 Newgreen DF, Powell ME, Moser B. Spatiotemporal changes in HNK-1/L2 glycoconjugates on avian embryo somite and neural crest cells. (1990) Dev Biol. 139:100-120

286 Young HM, Hearn CJ, Farlie PG, Canty AJ et al. GDNF is a chemoattractant for enteric neural cells. (2001) Dev Biol. 229:503-516

287 Southard-Smith EM, Kos L, Pavan WJ. Sox-10 mutation disrupts neural crest development in *Dom* Hirschsprung mouse model. (1998) Nat Genet. 18:60-64

288 Pusch C, Hustert E, Pfeifer D, Südbeck P et al. The SOX10/Sox10 gene from human and mouse: sequence, expression and transactivation by the encoded HMG domain transcription factor. (1998) Hum Genet. 103:115

289 Lang D, Chen F, Milewski R, Li J et al. Pax-3 is required for enteric ganglia formation and functions with Sox-10 to modulate expression of c-ret. (2000) J Clin Invest. 106:963-971

290 Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathophysiology. (1994) Pharmacol Rev. 46:325

291 Martucciello G, Ceccherini I, Lerone M, Jasonni V. Pathogensis of Hirschsprung's disease. (2000). J Paed Surg. 35(7):1017-1025

292 Woodward MN, Kenny SE, Vaillant C, Lloyd DA et al. Time-dependent effects of endothelin-3 on enteric nervous system development in an organ culture model of Hirschsprung's disease. (2003) J Paed Surg. 35:25-29

293 Wu JJ, Chen JX, Rothman TP, Gershon MD. Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. (1999) Development. 126:1161-1173

294 Maxwell GD. Cell cycle changes during neural crest cell differentiation in vitro. (1976) Dev Biol. 49:66-79

295 Guillemot F, Lo LC, Johnson JE, Auerbach A et al. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. (1993) Cell.75:463

296 Lo LC, Johnson JE, Wuenschell CW, Saito T et al. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. (1991) Genes Dev.5:1524

297 Hoehner JC, Wester T, Pahlman S, Olsen L. Localization of neurotrophins and their high affinity receptors during human enteric nervous system development. (1996) Gastroenterol. 110: 756-767

298 Chalazonitis A, Pham TD, Rothman TP, DiStefano PS et al. Neurotrophin-3 is required for the survival-differentiation of subsets of developing enteric neurons. (2001) J Neurosci. 21:5620-5636

299 Golstein AM, Brewer KC, Doyle AM, Nagy N et al. BMP signalling is necessary for neural crest cell migration and ganglion formation in the enteric nervous system. (2005) Mech Dev. 122:821-33

300 Vanderwinden JM, Rumessen JJ. Interstitial cells of Cajal in human gut and gastrointestinal disease. (1999) Microsc Res Tech. 47:344-360

301 Bronner-Fraser M. Segregation of cell lineage in the neural crest. (1993) Curr Opin Genet Dev. 3:641-647

302 Robertson K, Mason I, Hall S. Hirschsprung's disease:genetic mutations in mice and men. (1997) Gut. 41:436–441

303 Treanor JS, Goodman L, de Sauvage F, Stone DM et al. Characterization of a multicomponent receptor for GDNF. (1996) Nature. 382:80-83

304 Lane PW. Association of megacolon with two recessive spotting genes in the mouse. (1966) J Hered. 57:29-31

305 Dembowski C, Hofmann P, Koch T, Kamrowski-Kruck H et al. Phenotype intestinal morphology and survival of homozygous and heterozygous endothelin B receptor-deficient (spotting lethal) rats. (2000) J Paed Surg. 35:480-488

306 Lane PW, Lui HM. Assocation of megacolon with a new dominant spotting gene (*Dom*) in the mouse. (1984) J Hered. 75:435-439

307 Cacalano G, Farinas I, Wang LC, Hagler K et al. GFRα1 is an essential receptor component for GDNF in the developing nervous system and kidney. (1998) Neuron. 21:53-62

308 Amiel J, Attic T, Jan D, Pelet A et al. Heterozygous endothelin receptor B (EDNRB) mutations in isolated Hirschsprung's disease. (1996) Hum Mol Genet. 5:355-357

309 Leibl MA, Ota T, Woodward MN, Kenny SE et al. Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum. (1999) Gut. 44:246-252

310 Svensson PJ, Von Tell D, Molander ML, Anvret M et al. A heterozygous frameshift mutation in the endothelin-3 (EDN-3) gene in isolated Hirschsprung's disease. (1999) Pediatr Res. 45:714-717

311 Yanagisawa H, Yanagisawa M, Kapur RP, Richardson JA et al. Dual genetic pathways of endothelin-mediated intercellular signalling revealed by targeted disruption of endothelin converting enzyme-1 gene. (1998) Development. 125:825-836

312 Hearn CJ, Murphy M, Newgreen D. GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. (1998) Dev Biol. 197:93-105

313 Shin MK, Levorse JM, Ingram RS, Tilghman SM. The temporal requirement for endothelin receptor-B signaling during neural crest development. (1999) Nature. 402:496-501

314 Pattyn A, Morin X, Cremer H, Goridis C et al. Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis. (1997) Development. 124:4065-4075

315 Pattyn A, Morin X, Cremer H, Goridis C et al. The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. (1999) Nature. 399:366-370

316 Morin X, Cremer H, Hirsch MR, Kapur RP et al. Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homoeobox gene *Phox2a*. (1997) Neuron. 18:411-423

317 Garcia-Barcelo M, Sham MH, Lui VC, Chen BL et al. Association study of PHOX2B as a candidate gene for Hirschsprung's disease. (2003) Gut. 52(4):563-7

318 Kuhlbrodt K, Schmidt C, Sock E, Pingault V et al. Functional analysis of Sox 10 mutations found in human Waardenburg-Hirschsprung's patients. (1998) J Biol Chem. 273:23033

319 Tylzanowski P, Verschueren K, Huylebroeck D, Luyten FP. SIP1 is a repressor of liver/bone/kidney alkaline phosphatise transcription in BMP-induced osteogenic differentiation of C2C12 cells.(2001) J Biol Chem .27:27.

320 Facer P, Knowles CH, Thomas PK, Tam PK et al. Decreased tyrosine kinase C expression may reflect developmental abnormalities in Hirschsprung's disease and idiopathic slow-transit constipation. (2001) Br J Surg. 88:545-552

321 Benailly H, Lapierre J, Laudier B, Amiel J et al. PMX2B, a new candidate gene for Hirschsprung's disease. (2003) Clin Genet. 64(3):204-209

322 Parikh DH, Tam PK, Van Velzen D, Edgar D. Abnormalities in the distribution of laminin and collagen type IV in Hirschsprung's disease. (1992) Gastroenterology. 102:1236-1241

323 Parikh DH, Tam PK, Van Velzen D, Edgar D. The extracellular matrix components, tenascin and fibronectin, in Hirschsprung's disease: an immunohistochemical study. (1994) J Pediatr Surg.29:1302-1306

324 Kobayashi H, O'Briain D, Puri P. Lack of expression of NADPH-diaphorase and neural cell adhesion molecule (NCAM) in colonic muscle of patients with Hirschsprung's disease. (1994) J Pediatr Surg.29:301-304

325 Taguchi T, Tanaka K, Ikeda K. Fibromuscular dysplasia of arteries in Hirschsprung's disease. (1985) Gastro. 88:1099-103

326 Newby DE, Strachan FE, Webb DJ. Abnormal endothelin B receptor vasomotor responses in patients with Hirschsprung's disease. (2002) QJM. 95(3):159-63

327 McCready R, Beart R Jr. Classic articles in colonic and rectal surgery: constipation in the newborn as a result of dilation and hypertrophy of the colon: Harald Hirschsprung, Jahrbuch fur Kinderheilkunde, 1888 adult Hirschsprung's disease: results of surgical treatment at Mayo clinic. (1981) Dis Colon Rectum.24:408-410

328 Carcassonne M, Delarue A. Management of Hirschsprung's disease. The definitive operation: which, when and how? (1984) Aust N ZJI Surg. 54: 435-8

329 Georgeson KE, Fuenfer MM, Hardin WD. Primary laparoscopic pull-through for Hirschsprung's disease in infants and children.(1995) J Pediatr Surg. 30: 1–7

330 Carcassonne M, Morisson-Lacombe G, Letourneau JN. Primary corrective operation without decompression in infants less than three months of age with Hirschsprung's disease. (1982) J Pediatr Surg. 17: 241-3.

331 Teitelbaum DH. Hirschsprung's disease. In Principles of Pediatric Surgery, second edition. O'Neill JA, Grosfeld JL, Fonkalsrud, Coram AG et al (eds) (2003) Mosby. Missouri:580

332 Kleinhaus S, Boley SJ, Sheran M, Sieber WK. Hirschsprung's disease – a survey of the members of the surgical section of the American academy of Pediatrics. (1979) J Paed Surg. 14:588-597

333 Swenson O. Hirschsprung's disease:a review. (2002) Pediatric. 109(5):914-917

334 Yanchar NL, Soucy P. Long-term outcome after Hirschsprung's disease: patient's perspectives. (1999) J Pediatr Surg. 34(7):1152-60

335 Rescorla FJ, Morrison AM, Engles D, West KW et al. Hirschsprung's disease. Evaluation of mortality and long-term function in 260 cases. (1992) Arch Surg. 127(8):934-41

336 Gendler, SJ; Spicer, AP. Epithelial mucin genes. (1995) Annu Rev Physiol. 57:607–634.

337 Soave F. Endorectal pull-through 20 years experience. (1985) J Pediatr Surg20:568–579

338 Quinn FMJ, Fitzgerald RJ, Guiney EJ, O'Donnell B et al. Hirschsprung's disease: a follow-up of three surgical techniques 1979–1988. In: Hadziselmovic F, Herzog B, eds. Pediatric Gastroenterology: InflammatoryBowel Disease and Morbus Hirschsprund. (1992) Dordrecht, the Netherlands:Kluwer Academic Publisher:297–301

339 Harrison MW, Deitz DM, Campbell JR, Campbell TJ. Diagnosis and management of Hirschsprung's disease; a 25 year perspective. (1986) Am J Surg. 152:49-54

340 Miura H, Ohi R, Tseng SW, Takahashi T. The structure of the transitional and aganglionic zone of Auerbach's plexus in patients with Hirschsprung's disease: a computer-assisted three-dimensional reconstruction study. (1996) J Pediatr Surg. 31:420-426

341 Ghose SI, Squire BR, Stringer MD, Batcup G et al. Hirschsprung's disease: problems with transition-zone pull-through. (2000) J Pediatr Surg. 35(12):1805-9

342 White FV, Langer JC. Circumferential distribution of ganglion cells in the transition zone of children with Hirschsprung's disease. (2000) Pediatr Dev Pathol. 3:216-222

343 Farrugia M, Alexander N, Clarke S, Nash R et al. Does transitional zone pull-through in Hirschsprung's disease imply a poor prognosis? (2003) J Paed Surg. 38(12):1766-1769

344 Romanska HM, Bishop RJ, Brereton RJ, Spitz L et al. Immunocytochemistry for neuronal markers shows deficiencies in conventional histology in the treatment of Hirschsprung's disease. (1993) J Pediatr Surg. 28:1059-1062

345 Aslam A, Spicer RD, Corfield AP. Children with Hirschsprung's disease have an abnormal colonic mucus defensive barrier independent of the bowel innervation status. (1997) J Paed Surg. 32(8):1206-1210

346 Bill JH, Chapman ND. The enterocolitis of Hirschsprung's disease: its natural history and treatment. (1995) Am J Surg. 103:70-74

347 Surana R, Quinn F, Puri P. Evaluation of risk factors in the development of enterocolitis complicating Hirschsprung's disease. (1994) Pediatr Surg Int. 9:234-236

348 Foster P, Cowan G, Wrenn E. Twenty-five year's experience with Hirschsprung's disease. (1990) J Paed Surg. 25:531-534

349 Elhalaby EA, Coran AG, Blane CE, Hirschl RB et al. Enterocolitis associated with Hirschsprung's disease: a clinical-radiological characterization based on 168 patients. (1995) J Paed Surg. 30:76-83

350 Teitelbaum DH, Qualman SJ, Caniano DA. Hirschsprung's disease. Identification of risk factors for enterocolitis. (1988) Ann Surg.207:240-244

351 Vieten D, Spicer R. Enterocolitis complicating Hirschsprung's disease. (2004) Semin Pediatr Surg. 13:263-272

352 Murthi GS, Raine PM. Preoperative enterocolitis is associated with poorer long-term bowel function after Soave-Boley endorectal pull-through for Hirschsprung's disease. (2003) J Paed Surg. 38:69-72

353 Teitelbaum DH, Coran AG. Enterocolitis. (1998) Sem Paed Surg. 7(3):162-169

354 Swenson O, Fisher JH. Hirschsprung's disease during infancy. (1956) Surg Clin North Am. 36:115-122

355 Teitelbaum DH, Caniano DA, Qualman SJ. The pathophysiology of Hirschsprung's associated enterocolitis: importance of histologic correlates.(1989) J Paed Surg. 24(12):1271-1277

356 Carneiro PR, Brereton RJ, Drake DP et al. Entercolitis in Hirschsprung's disease. (1992) Pediatr Surg Int. 7:356-360

357 Lifschitz CH, Bloss R. Persistence of colitis in Hirschsprung's disease. (1985) J Pediatr Gastroenterol Nutr.4:291-293

358 Thomas DF, Fernie DS, Bayston R, Spitz l et al. Enterocolitis in Hirschsprung's disease: a controlled study of the etiologic role of clostridium difficile. (1986). J Paed Surg. 21(1):22-25

359 Hardy SP, Bayston R, Spitz L. Prolonged carriage of Clostridium difficile in Hirschsprung' s disease. (1993) Arch Dis child. 69:221-224

360 Wilson-Storey D, Scobie WG, McGenity KG.Microbiological studies of the enterocolitis of Hirschsprung' s disease. (1990) Arch Dis Child. 65:1338-1339

361 Rietra PM, Slaterus KW, Zanen HC, Meuwissen SG. Clostridial toxin in faeces of healthy infants. (1978) Lancet. 2(8084):319

362 Levin S. The immune system and susceptibility to infections in Down's syndrome. In McCoy E, Epstein C (eds): Oncology and Immunology in Down's syndrome. (1987) New York, NY,Liss:143

363 Wilson-Storey D, Scobie WG. Impaired gastrointestinal mucosal defense in Hirschsprung's disease: a clue to the pathogenesis of enterocolitis. (1989) J Paed Surg. 24(5):462-464

364 Imamura A, Puri P, O'Briain DS, Reen DJ. Mucosal immune defence mechanisms in enterocolitis complication Hirschsprung's disease. (1992) Gut. 33:801-806

365 Turnock RR, Spitz L, Strobel S. A study of mucosal gut immunity in infants who develop Hirschsprung's-associated enterocolitis. (1992) J Paed Surg. 27:828-829

366 Fujimoto T. Natural history and pathophysiology of entercolitis in the piebald lethal mouse model of Hirschsprung's disease. (1988) J Paed Surg. 23:237-242

367 Akkary S, Sahwy E, Kandil W, Hamdy MH. A histochemical study of the mucosubstances of the colon in cases of Hirschsprung's disease with and without enterocolitis. (1981) J Paed Surg. 16(5):664-668

368 Mattar AF, Coran AG, Teitelbaum DH. MUC-2 mucin production in Hirschsprung's disease: Possible association with enterocolitis development. (2003) J Paed Surg. 38(3):417-421

369 Swenson O, Sherman J, Fisher J, Cohen E. The treatment and postoperative complications of congenital megacolon: a 25 follow-up. (1975) Ann Surg.182:266-273

370 Marty T, Seo T, Matlak M, Sullivan JJ et al. Gastrointestinal function after surgical correction of Hirschsprung's disease: long-term follow-up in 135 patients. (1995) J Paed Surg. 30:655-658

371 Fortuna RS, Weber TR, Tracy TF Silen ML, Cradock T. Critical analysis of the operative treatment of Hirschsprung's disease. (1996) Arch Surg. 131:520-524

372 Aslam A, Spicer RD, Corfield AP. Turnover of radioactive mucin precursors in the colon of patients with Hirschsprung's disease correlates with the development of entercolitis. (1998) J Paed Surg. 33:103-105

373 Corfield AP, Warren BF, Bartolo DC, Wagner SA et al. Mucin changes in ileoanal pouches monitored by metabolic labelling and histochemistry. (1992) Br J Surg. 79**:**1209–1212.

374 Corfield AP, Paraskeva C. Secreted mucus glycoproteins in cell and organ culture. In Hounsell EF (ed):Glycoprotein Analysis in Biomedicine. Vol 14. (1993) Totowa, NJ Humana Press Inc:211-213

375 Goldstein, I. J., and R. D. Porez. Isolation, physicochemical characterization,and carbohydrate-binding specificity of lectins. In I,E Liener, N Sharon, I,J Goldstein (ed). The Lectins. Properties, Functions and applications in biology and medicine. (1986) Academic Pree, London:35

376 Shi W, Chammas R, Varki N, Powell L et al. Sialic acid 9-O-Aectylation on murine erythroleukemai cells affects complement activation, binding to I-type lectins and tissue homing. (1996) Am Soc Biochem Mole Biol. 271: 31526-31532

377 Shimamoto C, Hirata I, Takao Y, Takiuchi H et al. Alteration of colonic mucin after ureterosigmoidostomy. (2000) Dis Colon Rectum. 43(4):526-31

378 Liu T Y, Gotschlich E C , Egan W , Robbins, J B. Sialic acid-containing polysaccharides of Neisseria meningitidis and Escherichia coli strain Bos-12: structure and immunology.(1977) J Infect Dis. 136 Suppl:S71-7

Appendix 1

PARENT INFORMATION SHEET Version 03/121

A Study of the Large Bowel's Defence against Inflammation in Hirschsprung's Disease

What is Hirschsprung's Disease?
Hirschprung's disease is a congenital condition where nerve cells in the bowel are absent. This means that the bowel does not work properly and becomes blocked. The 'pull through' operation removes the part of the bowel without nerve cells and joins normal bowel to normal bowel.

What is this project about?
Unfortunately some children develop inflammation of the bowel (enterocolitis)after this operation which can prolong their recovery and in some cases make them very unwell. Previous studies have shown that the so-called 'normal' bowel in these children, has a defective barrier to infection and that this correlates with their chance of developing enterocolitis. What we currently do not know is whether this defective barrier extends throughout the whole bowel or whether it only occurs near where the abnormal and 'normal'bowel meet.

What is the aim of this study?
The aim of this study is to find this out. If we can tell how much bowel is affected in this way we could remove it during the operation and therefore reduce the number of children suffering with enterocolitis in the future. We are asking you, as the parent of a child with Hirschsprung's disease, whether you would like to participate in this research. If you decide to decline this will NOT affect the treatment your child receives in any way.

Who is organising the study?
The research project is being carried out within the Paediatric surgery department. The principal research investigator is Miss Ruth Groves, a clinical research fellow working within the department under the close supervision of Mr Aslam, consultant paediatric surgeon.

What is involved?
During the 'pull through' operation 4 small ($4mm^2$) samples of tissue (biopsies) would be taken from the large bowel. These would be removed by the surgeon performing the operation. The extra time taken would be a matter of a couple of minutes. The samples are then sent to the laboratory for analysis. The samples will NOT be used for any other research. All you have to do in order to take part is to sign a consent form giving your permission for the samples to be taken.

Appendix 1

Are there any risks involved?
At the time of taking a biopsy there is a small chance that a hole could be made in the bowel. As the surgeon is able to see the bowel as s/he takes the samples, this is made very unlikely, but if this did occur this would be noticed at the time and the hole repaired with a stitch. There can also be a small amount of bleeding from the biopsy site but this should be minimal within the context of the operation as a whole.

Are there any compensation arrangements if anything did go wrong?
In the unlikely event of something going wrong, if this is due to medical negligence this will be covered by the hospital trust. There are no arrangements for non-negligent compensation.

Who will have access to my child's notes?
Only the principal investigator will use medical records for the purpose of the study. Data collected will be anonyomised. Your GP will be informed of your participation.

What happens at the end of the study?
The results will be processed and analysed and the data will be sent for publication. Depending on the results current surgical practice in dealing with these children may be altered to reduce complications for future children.

Thank you for agreeing to take part in this valuable piece of research. Remember that you can withdraw from this study at anytime, WITHOUT explanation and WITHOUT affecting your child's current or future treatment.

Contact person: Ruth Groves
Department of Paediatric Surgery,
Box 201.
01223 245151 ext 4455 (Mr Aslam's secretary)

Appendix 1

PARENT INFORMATION SHEET:CONTROL SUBJECT Version 1

A Study of the Large Bowel's Defence against Inflammation in Hirschsprung's Disease

What is Hirschsprung's Disease?
Hirschprung's disease is a congenital condition where nerve cells in the bowel are absent. This means that the bowel does not work properly and becomes blocked. The 'pull through' operation removes the part of the bowel without nerve cells and joins normal bowel to normal bowel.

What is this project about?
Unfortunately some children develop inflammation of the bowel (enterocolitis)after this operation which can prolong their recovery and in some cases make them very unwell. Previous studies have shown that the so-called 'normal' bowel in these children, has a defective barrier to infection and that this correlates with their chance of developing enterocolitis. What we currently do not know is whether this defective barrier extends throughout the whole bowel or whether it only occurs near where the abnormal and 'normal'bowel meet.

What is the aim of this study?
The aim of this study is to find this out. If we can tell how much bowel is affected in this way we could remove it during the operation and therefore reduce the number of children suffering with enterocolitis in the future. We are asking you, as the parent of a child who has a colostomy which is to be reversed, whether you would like to participate in this research. The information we gather from your child is extremely important in order for us to draw meaningful conclusions from the data we collect from children with Hirschsprung's disease. Your child will be acting as a control subject. If you decide to decline this will NOT affect the treatment your child receives in any way.

Who is organising the study?
The research project is being carried out within the Paediatric surgery department. The principal research investigator is Miss Ruth Groves, a clinical research fellow working within the department under the close supervision of Mr Aslam, consultant paediatric surgeon.

What is involved?
During the closure of colostomy operation, 4 small ($4mm^2$) samples of tissue (biopsies) would be taken from the large bowel. These would be removed by the surgeon performing the operation. The extra time taken would be a matter of a couple of minutes. The samples are then sent to the laboratory for analysis. The samples will NOT be used for any other research. All you have to do in order to take part is to sign a consent form giving your permission for the samples to be taken.

Are there any risks involved?
At the time of taking a biopsy there is a small chance that a hole could be made in the bowel. As the surgeon is able to see the bowel as s/he takes the samples, this is made very unlikely, but if this did occur this would be noticed at the time and the hole repaired with a stitch. There can also be a small amount of bleeding from the biopsy site but this should be minimal within the context of the operation as a whole.

Are there any compensation arrangements if anything did go wrong?
In the unlikely event of something going wrong, if this is due to medical negligence this will be covered by the hospital trust. There are no arrangements for non-negligent compensation.

Who will have access to my child's notes?
Only the principal investigator will use medical records for the purpose of the study. Data collected will be anonymised. Your GP will be informed of your participation.

What happens at the end of the study?
The results will be processed and analysed and the data will be sent for publication. Depending on the results current surgical practice in dealing with these children may be altered to reduce complications for future children.

Thank you for agreeing to take part in this valuable piece of research. Remember that you can withdraw from this study at anytime, WITHOUT explanation and WITHOUT affecting your child's current or future treatment.

Contact person: Ruth Groves
Department of Paediatric Surgery,
Box 201.
01223 245151 ext 4455 (Mr Aslam's secretary)

Appendix 1

CONSENT FORM

A Study of the Large Bowel's Defence against Inflammation in Hirschsprung's Disease.

LREC Reference Number: 03/121

Lead investigator Ruth Groves.

Please read the following statements, initial the boxes accordingly and sign at the bottom.

1. I confirm that I have read and understood the information sheet (version 1) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of my child's notes may be looked at by responsible individuals employed by the hospital trust, where it is relevant to my taking part in research. I give permission for these individuals to have access to my child's records. ☐
4. I am willing that my general practitioner is notified of my participation in this research. ☐
5. I agree that my child is to take part in the above study. ☐

Name of child	Date	Signature

Relationship to child:_____

Name of Witness to Signature	Date	Signature

Name of Research Team Member	Date	Signature

(3 copies, one for parent, one for researcher, one for medical notes)

Appendix 1

LETTER TO GENERAL PRACTITIONER

Department of Paediatric Surgery,
Box 267,
Addenbrookes Hospital,
Hills Road,
Cambridge.
CB2 2QQ

Re: A Study of the Mucus Defensive Barrier in the Pulled-through Bowel in Hirschsprung's disease

_____(Child's name)

Dear Dr_____,

I am writing to inform you that _____(child's name) parents have kindly agreed to participate in a research project run by the Paediatric Surgery department.

Previous research in this field has demonstrated that the ganglionic bowel in Hirschsprung's disease has a defective mucus defence barrier and that this correlates with the risk of these children developing enterocolitis after 'pull through' surgery. What is not known is whether this deficiency extends throughout the bowel or is limited to an area close to the aganglionic bowel which could, therefore, potentially be resected at surgery, thus reducing the post-operative incidence of enterocolitis.

The current research project involves taking 4 small biopsies at different sites from the colon at the time of 'pull-through' surgery, or, in the case of controls, at the time of closure of colostomy, and analysing these for mucin turnover.

This should make no different to the usual post-operative course for these children and there are no anticipated problems.

Please feel free to contact me if you have any questions or concerns.

Yours Sincerely,

Miss Ruth Groves
Clinical Research Fellow
Department of Paediatric Surgery
01223 245 151 bleep 152-681