ALTERED GANGLIOSIDE METABOLISM IN INFLAMMATORY BOWEL DISEASE AND THE IMPACT OF DIETARY GANGLIOSIDE INTAKE

by

John Miklavcic

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Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Gangliosides are integral to the structure and function of the plasma membrane. Ganglioside composition of the small intestinal brush border membrane and apical surface of the colon influence numerous cell processes including microbial attachment, cell division, differentiation, and signaling. Accelerated catabolism of ganglioside in intestinal disease results in increased pro-inflammatory signaling. Restoring proper structure and function to the diseased intestine can resolve inflammation, increase resistance to infection and improve gut integrity to induce remission of conditions like necrotizing enterocolitis and Crohn disease. Pre-clinical studies indicate that the amount of ganglioside GD3 in intestinal mucosa is decreased with inflammation, low level of ganglioside is associated with higher production of pro-inflammatory signals, and ganglioside content of intestinal mucosa can be increased by intake of dietary ganglioside. Inducing inactive state of inflammatory bowel disease may be achieved by reducing the rate that gangliosides are degraded or by increasing the intake of specific dietary gangliosides. Sections of intestinal mucosa from terminal ileum or colon were obtained from patients with ulcerative colitis or active inflammatory Crohn disease undergoing surgical bowel resection. Control samples of normal intestine were obtained from participants with benign colon polyps and from participants with colorectal cancer. Gangliosides and phospholipids of intestinal mucosa were characterized using reverse-phase liquid chromatography-QQQ mass spectrometry. Ganglioside catabolism enzymes beta-hexosaminidase A and sialidase-3 were measured in intestinal mucosa by western blot. A cohort of healthy participants and patients with inflammatory bowel disease completed an eight-week feeding study to determine the safety and efficacy of ganglioside consumption. Participants consumed a milk fat fraction containing 43 mg ganglioside daily or the equivalent milk fat fraction without ganglioside. Plasma gangliosides

were characterized using reverse-phase liquid chromatography-QQQ mass spectrometry, quality of life was assessed by quality of life inflammatory bowel disease questionnaire, intestinal permeability was assessed by oral lactulose/mannitol challenge and inflammatory markers LTB_4, PGE_2 and TNF-α were measured by ELISA in participants in the intervention study. Ganglioside GM3 was 2-fold higher ($P<0.05$) in inflammatory bowel disease intestine compared to control intestine. Control intestine exhibited 3-fold higher ($P<0.001$) ganglioside GD1a content than intestine from patients with inflammatory bowel disease. Intestine from patients with inflammatory bowel disease exhibited 1.7-fold increase ($P<0.05$) in beta-hexosaminidase A content in comparison to control intestine. The level of sialidase-3 in intestine from patients with inflammatory bowel disease was increased 8.3-fold ($P<0.001$) compared to normal intestine. The level of gangliosides GD3 and GD1a with two and three unsaturated bonds in the ceramide component was lower ($P<0.001$) in intestine from patients with inflammatory bowel disease than control intestine. In addition, polyunsaturated constituents of phosphatidylcholine were significantly reduced ($P<0.05$) in intestine from patients with inflammatory bowel disease versus control intestine. There were no serious or other adverse events associated with dietary ganglioside intake. Ganglioside consumption increased ($P<0.05$) plasma content of total GD3 by 35% over eight weeks. Consumption of ganglioside increased ($P<0.01$) emotional health by 39% and improved ($P<0.02$) systemic symptoms in patients with inflammatory bowel disease by 36% over eight weeks. Participants consuming ganglioside exhibited 19% decrease in intestinal permeability ($P=0.04$). Consuming ganglioside did not change the plasma concentration of acute systemic inflammatory mediators LTB_4, PGE_2 or TNF-α. This study suggests a new paradigm by proposing that inflammatory bowel disease occurs as a consequence of increased catabolism of specific gangliosides. Impaired intestinal integrity characteristic of inflammatory bowel disease

may be overcome by dietary treatment with specific species of ganglioside shown to be deficient in inflammatory bowel disease. Ganglioside is a bioavailable dietary compound that can be consumed to improve quality of life in patients with inflammatory bowel disease and potentially treat other disorders involving altered ganglioside metabolism.

PREFACE

This thesis is an original work by John Miklavcic. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Health Research Ethics Board – Biomedical Panel; project name "MILK-DERIVED GANGLIOSIDE AS NOVEL ANTI-INFLAMMATORY THERAPY FOR INFLAMMATORY BOWEL DISEASE," Pro00001371 (Legacy #5932) on May 29, 2009.

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a. IBD intestine is characterized by gangliosides with fewer unsaturated bonds. ISSFAL 2014. M1.03

b. Dietary ganglioside consumption increases monounsaturated gangliosides GM3 and GD3 in human plasma. ESPGHAN 2013. PO-N-0301

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a. IBD intestine is characterized by gangliosides with fewer unsaturated bonds. ISSFAL 2014. M1.03

b. Inflammatory bowel disease is a disorder of ganglioside metabolism. EB 2014. 14-1732-EB

c. Monounsaturated C40 GM3 and C34 GM3 constitute the most abundant gangliosides in bowel from Crohn's disease. ESPGHAN 2013. PO-G-0106

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AA	arachidonic acid
ANOVA	analysis of variance
ATG16L1	autophagy related 16-like 1
BAP	benign adenomatous polyposis
BSA	bovine serum albumin
CCR9	chemokine receptor type 9
CD	Crohn's disease
Cer	ceramide
CID	collision-induced dissociation
COX	cyclo-oxygenase
$cPLA_2$	cytosolic phospholipase A_2
CRC	colorectal cancer
DSS	dextran sodium sulfate
ELISA	enzyme-linked immunosorbent assay
GalNac	N-acetylgalactosamine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBP-1	guanylate-binding protein-1
GG	ganglioside

GlcCer	glucosylceramide
HEXA	beta-hexosaminidase A
IBD	inflammatory bowel disease
IBDQ	inflammatory bowel disease questionnaire
IL	interleukin
L/M	lactulose/mannitol
LacCer	lactosylceramide
LC	liquid chromatography
LTB_4	leukotriene B_4
LOX	lipoxygenase
LPS	lipopolysaccharide
m/z	mass/charge
MRM	multiple reaction monitoring
MS	mass spectrometer
NEU3	sialidase-3
nd	not detected
NFKB	nuclear transcription factor kappaB
NOD	nucleotide-binding oligomerization domain-containing protein
NS	not significant

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PGE_2	prostaglandin E_2
PL	phospholipid
PLAP	phospholipase A2 activating protein
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
ROS	reactive oxygen species
SEM	standard error of the mean
TBST	tris-buffered saline with 0.1% tween 20
TLR4	toll-like receptor-4
TNF-α	tumour necrosis factor-alpha
UC	ulcerative colitis

CHAPTER 1. DIETARY GANGLIOSIDE REDUCES PRO-INFLAMMATORY SIGNALING IN THE INTESTINE

1.10 Review

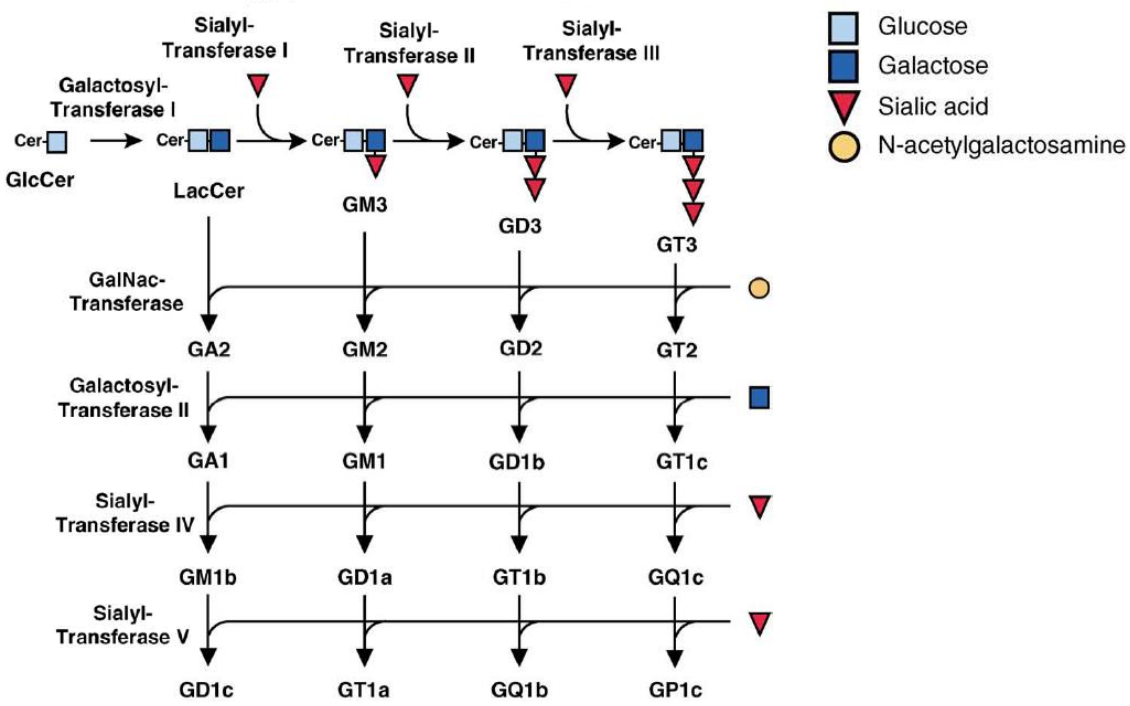
Ganglioside refers to a network of sialylated glycosphingolipids, each with independent biologic properties (Figure 1-1)[1]. Gangliosides are found mainly in the lipid rafts of cells including the intestinal mucosa[2]. Gangliosides consist of a charged, hydrophilic region that protrudes from the membrane surface, and a hydrophobic ceramide anchored in the plasma membrane[3]. Gangliosides have been shown to influence apoptosis[4], protein localization[5], endocytosis[6], membrane fluidity[7] and ligand binding[8].

1.11 Ganglioside background

After initial discovery in bovine and human brains[9], the variable carbohydrate configuration of gangliosides were derived by chromatographic methods[10]. Ganglioside contributes to about 25% of total lipid content of outer membrane in brain tissue[11]. Inhibition of ganglioside synthesis causes severe neural defects and death shortly after birth[12]. Altered ganglioside content and metabolism play roles in neurological disorders like Parkinson[13] and Alzheimer[14] disease. Gangliosides also influence binding and/or pathogenicity of microorganisms including *Helicobacter pylori*[15], *Giardia Muris*[16] and *Vibrio Cholerae*[17,18]. Enrichment of adipocyte plasma membrane with ganglioside GM3 suppresses insulin receptor phosphorylation leading to decreased glucose uptake and an insulin resistant phenotype[19]. The level of plasma membrane ganglioside GD3 influences apolipoprotein secretion and may play a role in atherosclerosis[20]. Provision of GD3 has cytotoxic effects on Lewis lung carcinoma[21], SKBR3 breast cancer[4] and U-1242 glioma[22]. Depletion of cellular ganglioside also appears to decrease metastasis of melanoma and breast cancer cells in experimental animal models[23,24]. Gangliosides are crucial to

essential and basic functions in the body and play critical roles in human health and in the underlying pathology of several chronic diseases.

1-1 General scheme for ganglioside synthesis



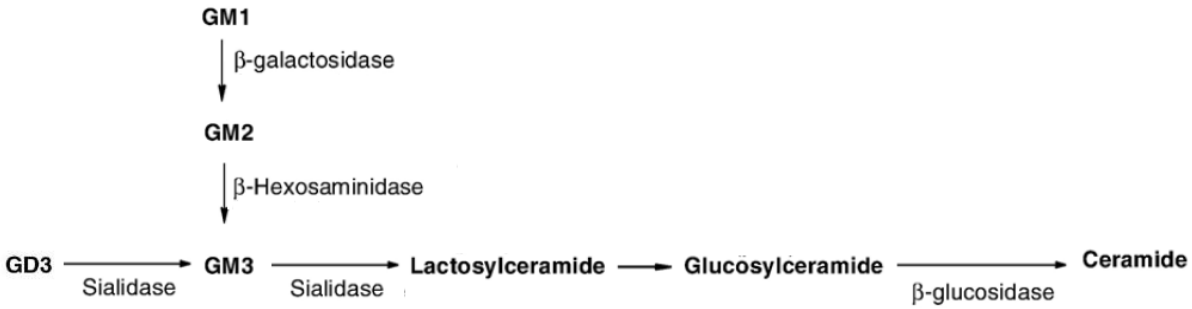
Network of ganglioside synthesis; steps are also reversible. "G" denotes "ganglioside;" "A" denotes "asialo" or lacking sialic acid, "M" denotes "monosialo," "D" denotes "disialo;" numbers denote carbohydrate sequence. Cer = ceramide; GlcCer = glucosylceramide; LacCer = lactosylceramide; GalNac = N-acetylgalactosamine.

1.12 Ganglioside synthesis and degradation

In mammalian cells, ganglioside synthesis begins with ceramide synthesis in the endoplasmic reticulum[25,26]. Ceramide is transported to the cytosolic Golgi face for addition of glucose[27]. From this point, sugar moities and sialic acid(s) are added to form one of several gangliosides (Figure 1-1). These reactions are accomplished by sialyltransferases, galactosaminyltransferases and galactosyltransferases on the luminal face of the Golgi complex at controlled rates[28]. Like

ganglioside synthesis, regulation of ganglioside catabolism (Figure 1-2) varies among cells as shown in the comparison of neuronal and non-neuronal tissue[29]. Deficiency in the enzymes regulating ganglioside catabolism cause disorders of gangliosidoses such as Tay-Sachs, Niemann-Pick and Sandhoff disease[30].

1-2 Ganglioside catabolism



Enzyme responsible for catabolic processing step is shown adjacent to metabolic conversion.

1.13 Ganglioside content and composition

The amount and content of ganglioside varies among species and in tissues within species[31]. For instance, the major brain gangliosides consist of GM1, GD1a, GD1b and GT1b[32] but GM3 and GD3 are reported as major gangliosides in human intestinal mucosa[33]. Transcriptional and post-translational events regulate the amount and content of ganglioside in cells[34-36]. Ganglioside content is particularly high in the central nervous system, relative to other tissues of the body[37]. The ceramide component of ganglioside varies in the number of carbon atoms and in the number of unsaturated bonds[38] as demonstrated in A2780 ovarian carcinoma cells[39]. Variability in sialic acid configuration, oligosaccharide size and ceramide composition may have consequences that alter ganglioside localization and functionality[40-42]. Phospholipid classes have also been shown to vary in fatty acid composition[43]. Furthermore, each phospholipid class allocates predominantly to specific organelles to carry out specific functions like those involved in endocytosis and

pinocytosis[44] and the composition of phospholipids influences cell growth and cell signaling[45]. It is unknown whether many of the health benefits attributed to gangliosides are due to specific ganglioside classes like GM3 or GD3, or whether the fatty acid and sphingosine components of ceramide alters the molecular role of the ganglioside.

1.20 Ganglioside in diet

In addition to endogenous ganglioside biosynthesis, ganglioside can also be obtained exogenously from diet[46]. The milk fat globule membrane is a biological membrane enriched in ganglioside that protects and stabilizes milk fat in the aqueous phase[47]. Average dietary intake of ganglioside is less than 100 µg/1000 kcal/day and rarely exceeds 200 µg/1000 kcal/day and is well below levels shown to have therapeutic benefit[48]. Dietary ganglioside intake is very low unless consuming whole organ foods (ie. brain), whole milk, buttermilk, or colostrum in high quantities. Several tissues have been shown to incorporate dietary gangliosides. Gangliosides may be taken up in micellar or monomeric forms by cell *in vitro*: by adsorption, insertion, or receptor binding[49]. Model intestinal epithelial Caco-2 cells incorporate GD3[50] when provided with ganglioside *in vitro*[51]. Ganglioside uptake also occurs in several tissues *in vivo*. Absorptive mechanisms include uptake by prosaposin, glycolipid transfer protein[52] and Niemann-Pick C1-like 1 protein[53]. Providing GM3 and GD3 in the diet increases total ganglioside content of epithelial cells within intestine and retina in rats[46,54].

1.21 Fates of dietary ganglioside

GD3 is specifically localized to the basolateral membrane surface, while GM3 is localized at the brush border membrane of the enterocyte[55]. According to Pagano's vesicle sorting theory[56], absorbed gangliosides have three fates: transport back to the plasma membrane immediately after being endocytosed; endocytosis to the Golgi apparatus for glycosylation to form more complex

ganglioside species; and transport by the endosome to the lysosome for degradation. Metabolic kinetics of GD3 has been described in depth in Caco-2 cells[50]. GD3 taken up by the brush border membrane is mainly metabolized into new ganglioside species, with smaller portions being retained or transferred, whereas GD3 taken up by the basolateral membrane is not retained or transferred to any significant degree[50]. These observations suggest that particular species of ganglioside localizes to particular regions of the enterocyte to carry out specific functions, depending on the site of uptake in the enterocyte (apical vs. lateral/basolateral). There is a gap in understanding how ganglioside uptake by different regions of the gut is regulated and the corresponding functions of ganglioside in specific organelles.

1.22 Ganglioside in intestinal health

Uptake of ganglioside may result in altered physiologic changes. For example, provision of dietary ganglioside enhances uptake of glucose, and stearic acid and linoleic acid irrespective of levels of intestinal glucose and lipid transporters in weanling rats[57,58]. Important observation from study in rats has shown that inflamed intestinal mucosa has less total ganglioside content than healthy intestinal mucosa[59]. Dietary ganglioside is able to replace mucosal gangliosides that are continually degraded in inflammatory states. Moreover, increasing ganglioside content through diet decreases pro-inflammatory cytokine production in intestinal mucosa[54,59], and prevents hypoxia-induced bowel necrosis and cell injury in cultured infant bowel[60]. Ganglioside is a bioavailable component of diet which may alter fundamental cellular processes in the intestine (Section 1.10). The following section summarizes the different modes of action by which specific dietary gangliosides promote intestinal health.

1.30 Mechanisms of action of ganglioside

1.31 Gut integrity

Previous studies indicate that ganglioside prevents pro-inflammatory stimuli from disrupting integrity of tight junctions between enterocytes. Feeding ganglioside to rats prevented lipopolysaccharide (LPS)-stimulated decrease in cellular tight junction protein occludin[61]. This work indicates that low levels of GD3 in the intestinal mucosa are associated with degradation of tight junction proteins. Improving intestinal integrity may be important for management of diarrhea, penetration of allergens, malnutrition and infection leading to mortality and IBD[62]. Guanylate-binding protein-1 (GBP-1) has been recently identified as a marker of intestinal integrity. Downregulation of GBP-1 has been reported to increase permeability and apoptosis of intestinal cells[63]. The effect of ganglioside on GBP-1 stability is currently unknown and is of interest as a potential therapeutic target. The functional effect of improving tight junction integrity via modulation of tight junction proteins by intake of dietary ganglioside in humans remains to be investigated.

1.32 Immune cell differentiation

Gangliosides play an important role in immune cell differentiation. Inhibition of plasma membrane-localized enzyme sialidase-3 (NEU3) prevents the LPS-mediated differentiation of monocytes to dendritic cells and decreases production of interleukin (IL)-6 and tumour necrosis factor (TNF)-α[64]. Sialidase catalyzes degradation of several ganglioside by removal of sialic acid (Figure 1-2). This study also showed that sialic acid content is about two-fold greater in monocytes than dendritic cells when standardized to protein content, and sialic acid content is about four-fold greater in dendritic cells than monocytes when standardized on a per cell basis. These observations indicate that the content of individual gangliosides are specifically regulated

for particular immune cell functions. Furthermore, increased levels of three distinct forms of sialidase are detected in the differentiation of monocytes to macrophages[65]. When monocytes are provided with a mix of exogenous ganglioside, the expression of one or more sialidase enzymes is induced suggesting that provision of ganglioside can influence immune cell maturation[65]. Maturation of naïve T cells into T effector cells and production of IL-12 induced by pertussis and cholera toxins is inhibited by co-incubation with ganglioside GD1a in culture[66]. This evidence indicates that cellular ganglioside content, ganglioside metabolism and provision of exogenous gangliosides influence the maturation and function of monocytes or naïve immunocytes into mature immune cells.

1.33 Immune cell targeting

Chemokine receptor type 9 (CCR9) enables immune cells to target to the gut[67]. While CCR9-positive immune cells are found mainly in small intestine, integrin $\alpha_4\beta_7$-positive cells tend to home to both small intestine and colon[68]. Integrin-mediated binding may be indirectly influenced by ganglioside. In the plasma membrane, gangliosides are known to localize with proteins which bear specific amino acid sequences[69]. GD3 has been shown to cluster with β_1 integrin and affect properties controlled by integrin-mediated signaling[70]. The interactions between gangliosides and integrins have not received much attention, but may provide important insights into homing of immune cells to gut in conditions like inflammatory bowel disease (IBD).

1.34 Immune cell signaling

Gangliosides are organized into microdomains termed lipid rafts that float freely in the lipid bilayer[71] and serve as organizing centers for assembly of signaling molecules and receptor trafficking[72,73]. Organization of signaling molecules into lipid rafts is vital for regulation of T-lymphocyte activation pathways that play a major role in pathology of IBD[74,75]. Disruption of

lipid rafts displaces cellular signaling molecules and alters immunoreceptor signal transduction[76-78]. Specifically, sphingolipid depletion inhibits glycophosphatidylinositol-anchored protein trafficking in microdomains[79]. Absence or increased catabolism of ganglioside adversely affects lipid raft trafficking and signaling functions, and promotes a pro-inflammatory environment. Gangliosides are imperative for proper structure and function of lipid rafts and dietary ganglioside may disrupt constitutive activation of pro-inflammatory pathways that are hallmark in intestinal diseases characterized by chronic inflammation.

1.35 Pro-inflammatory mediators

Inflammation characterizes several chronic diseases including cardiovascular disease, cancer, necrotizing enterocolitis and IBD. Changes in ganglioside content and composition also occur in the oncogenic transformation of tissue and may contribute to tumour-associated inflammation. Specifically, undifferentiated CaCo-2 cells have lower total GD3 and polar ganglioside content than differentiated CaCo-2 intestinal epithelial cells[80]. In *in vitro* study, inflamed intestinal mucosa has significantly decreased total ganglioside content[59]. Enrichment of intestinal mucosa with ganglioside GD3 causes a reduction in cholesterol content[59]. Cholesterol depletion disrupts membrane microdomain structure and inhibits generation of pro-inflammatory mediators[81,82]. In pre-clinical studies, ganglioside treatment increased ganglioside content of intestine and inhibited signals caused by pro-inflammatory stimuli TNF-α and IL-1β in rats[59]. Similarly, ganglioside reduced IL-6 and IL-8 production in cultured infant bowel when exposed to LPS under hypoxic conditions[60]. This data suggests that increased ganglioside catabolism precedes pro-inflammatory signals and the subsequent inflamed state. Accordingly, replacing ganglioside that is degraded is proposed to protect the gut by attenuating pro-inflammatory signals.

1.36 Anti-inflammatory mediators

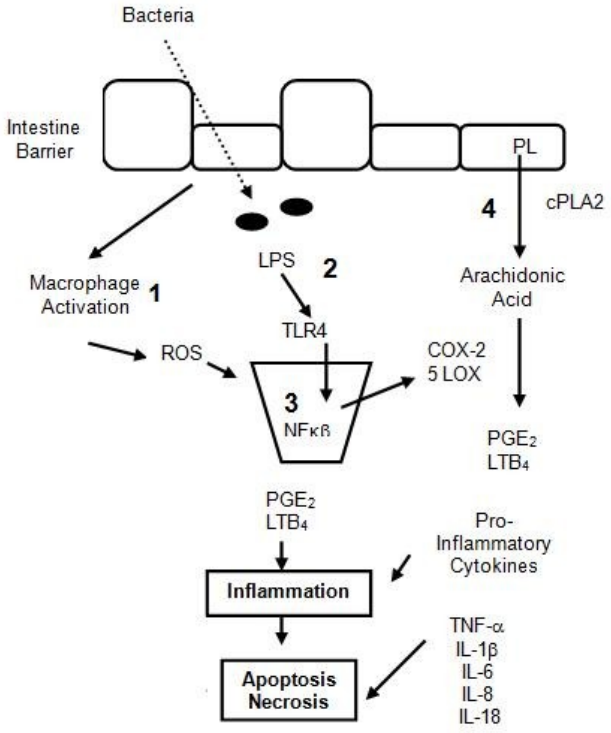
Previous study has shown enhanced production of IL-10 with dietary ganglioside treatment[61]. IL-10 is an anti-inflammatory cytokine that is involved in resolution of inflammation. Polyunsaturated docosahexaenoic acid-derived resolvins and protectins have recently been discovered as having anti-inflammatory properties[83]. Production of resolvin D3 and protectin 1/D1 blocks dextran sodium sulfate (DSS)-induced colitis in mice[84]. Therefore, resolvins and protectins have been suggested as novel candidates for IBD therapy[85]. Ganglioside in the diet increases the amount of polyunsaturated fat relative to saturated fat in weanling rat intestine[86]; and thus, may enable enhanced production of resolvins and protectins ultimately interrupting the perpetual pro-inflammatory cascade inherent to IBD.

1.37 Prevention of infection

Provision of dietary ganglioside known to have anti-bacterial properties increases the resistance of an individual to negative effects of microbial pathogens like diarrhea. Evidence suggests that patients with IBD may be more prone to infection than healthy individuals[87]. In a Spanish population, mutation in authophagy related 16-like 1 (ATG16L1) is associated with prevalence of Crohn's disease (CD)[88]. ATG16L1 is part of a group of proteins involved in autophagy[89]. Defects in ATG16L1 may allow for infectious organisms to persist, triggering an exacerbated immune response in the gut. Toll-like receptor-4 (TLR4) was found to be higher in intestinal mucosa of children with IBD than healthy control participants[90]. Upon stimulation of TLR4 by pathogens or enterotoxins (Figure 1-3)[1], immune cells produce reactive oxygen species that lead to activation of nuclear transcription factor-kappaB (NFκB) pathway and production of pro-inflammatory mediators. Ganglioside inhibits binding, toxin production, and infectivity of several intestinal pathogens (Section 1.11)[91,92], thereby attenuating NFκB inflammatory signaling

pathways. Ganglioside may play a critical role in supporting gut health by preventing diarrhea cause by secondary infection and the associated inflammatory signaling cascade.

1-3 Inflammatory signaling cascade and mechanisms by which ganglioside protects the intestine from inflammation and injury



There are at least four possible mechanisms by which ganglioside protects intestine from injury: (1) gangliosides prevent proliferation, maturation and targeting of immune cells; (2) gangliosides bind enterotoxic LPS and prevent interaction with TLR4; (3) gangliosides inhibit NFκB activation; and (4) gangliosides prevent production of LTB_4 and PGE_2. COX-2 = cyclooxygenase-2; cPLA2 = cytosolic phospholipase A2; LTB_4 = leukotriene B_4; LPS = lipopolysaccharide; 5 LOX = 5 lipoxygenase; PGE_2 = prostaglandin E_2; PL = phospholipid; ROS = reactive oxygen species; 1, 2, 3, 4 = steps inhibited by ganglioside; TLR4 = toll-like receptor-4.

1.38 NFκB pathway

CARD15 [nucleotide-oligomerization domain-containing protein (NOD) 2] polymorphism has most consistently arisen as a genetic risk factor for CD[93,94]. NOD2 is involved in coding for a pattern recognition receptor involved in the immune response. On of the normal functions of NOD2 is to suppress NFκB stimulation[95]. Defects in NOD2 may allow constitutive activation of NFκB, resulting in chronic inflammation and injury to intestinal mucosa. NOD1 is an activator of NFκB and wildtype NOD1 is associated with increased risk of CD[96]. Ganglioside may attenuate NFκB signaling as a previous study showed that GD3 prevented activation of NFκB in mitogen-stimulated T-cells[97]. This is particularly important since cyclo-oxygenase (COX) and lipoxygenase (LOX) enzyme production and activity is increased by stimulation of NFκB pathway (Figure 1-3)[98]. COX and LOX metabolize arachidonic acid (AA) into pro-inflammatory mediators leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2). It has been shown that ganglioside prevents production of LTB_4 and PGE_2 in infant bowel when cultured with LPS[60]. Ganglioside appears to inhibit production of LTB_4 and PGE_2 in intestine by blocking nuclear translocation of NFκB (1-3)[99].

1.39 Polyunsaturated fatty acids in ganglioside and phospholipid

The balance of omega-3 and -6 fatty acids appears to be important in the inflamed intestine. Patients with ulcerative colitis (UC) have been shown to have an increase in saturated fatty acids as a component of phosphatidylcholine (PC)[100]. The corresponding decrease in relative content of n-3 polyunsaturated fatty acid (PUFA) may impede production of pro-resolving mediators (Section 1.36) and subsequent resolution of inflammation[85]. Experimental induction of colitis in mice using DSS results in an increase of phospholipase A_2 activating protein (PLAP)[101]. Increased levels of PLAP may result in increased liberation of AA from phospholipid for

conversion to pro-inflammatory mediators (Figure 1-3, Section 1.38). Moreover, experimental UC can be alleviated in mice by consumption of n-3 PUFA[102]. Content of essential fatty acids and elongated products have not been thoroughly characterized in ganglioside and phospholipids of human intestinal mucosa. Altered metabolism of ganglioside and phospholipid in intestinal disease liberates specific dietary fatty acids which either promote or disrupt the perpetual inflammatory signaling cascade in IBD.

1.40 IBD background

Ganglioside has shown therapeutic benefit in models of pro-inflammatory diseases that have common features with IBD. IBD is diagnosed by direct endoscopic visualization of intestinal mucosa, which can be considerably invasive and unpleasant for patients being screened. Histologic assessment is used to confirm mucosal inflammation[103]. Collectively known as IBD, CD and UC severely impede quality of life in afflicted individuals. IBD presents with abdominal pain, gastrointestinal bleeding, diarrhea, weight loss, malnutrition; all of which negatively impact social and emotional welfare. IBD can be associated with development of joint, liver and kidney diseases, and an elevated risk of intestinal lymphoma and colorectal cancer (CRC). Disease management is difficult, and may consist of costly drug treatment including corticosteroids, immunosuppressants[104], antibiotics[105] or biologics such as anti-TNF therapy. Some individuals with IBD do not respond to standard drug treatment, while others experience negative or toxic adverse effects[106]. Administration of prednisolone, commonly used to treat IBD, has been shown as a risk factor for development osteoporosis in older patients with IBD[107]. Severe cases require surgery to remove the affected bowel, and psychological factors including stress may trigger disease flares[108]. The etiologies of CD and UC are poorly understood and there is no cure for IBD.

1.41 IBD epidemiology

At a rate of 0.60% of the population[109], prevalence of IBD is particularly high in Canada[110] compared to other areas of the world[111-113]. Prevalence of IBD is also high in the United States; where reported incidence is greater than 25,000 people per year[111]. IBD constitutes a considerable economic burden. In 2008, economic cost per patient with IBD was estimated above $9,000/year in Canada[109]. Another study reported direct healthcare costs greater than $18,000/patient-year in the United States[114]. There is a clear need for knowledge of disease mechanisms to develop novel, cost-effective treatment strategies for sustained remission of intestinal disease.

1.42 IBD pathology

CD is chronic inflammatory condition that can occur at any site along the gastrointestinal tract, but most commonly affects the distal small intestine, colon and perianal region[115]. Initial lesions are characterized by tiny mucosal defects termed aphthous ulcers[115]. There is an infiltration of macrophages locally in the gut that release pro-inflammatory mediators and perpetuate the inflammatory process. This process contributes to development of fibrosis and granulomas. Ulcers grow in size and as submusoca thickens, fistulae may develop. While inflammation and intestinal injury associated with CD occurs in a transmural fashion in the colonic wall, UC-associated inflammation is present superficially at the level of mucosa only in large intestine[116]. With respect to immune system involvement, UC is characterized by IL-27-driven inflammation and T_h2 cells; and CD by IL-12, IL-23, T_h1, T_h17, and cells involved in innate immunity. There is a genetic component that contributes to IBD risk (Section 1.43), particularly CD[117]. While a number of genes have been linked to aspects of IBD, environment also plays a large role in active disease. IBD rates are very high in industrialized countries like Canada and the USA.

Studies have linked urban environment, smoking in CD, diet high in sugar or total fat, antibiotic use in childhood, non-steroidal anti-inflammatory use and other factors to IBD risk[118]. IBD is a multifactorial disorder of complex origin that appears to stem from changes initially occurring at the plasma membrane of intestinal mucosa.

1.43 Genes in IBD

The relationship between diet and gene expression has been established in several investigations of IBD[60-62]. Several genetic markers have been identified that relate to prevalence, severity, site of disease in intestine and post-operative recurrence of IBD. NOD2 has most consistently arisen as a genetic risk factor for CD (Section 1.38). Polymorphisms in ATG16L1 and NOD1 have been associated with aspects of IBD (Section 1.37). ATG16L1 is part of a group of proteins involved in autophagy. In a Spanish population, Thr300Ala mutation in ATG16L1 is associated with prevalence of CD but not UC[63]. A defect in ATG16L1 may allow for infectious organisms to persist, triggering an exacerbated immune response in the gut. A variant of the IL-23 receptor gene has been associated with CD in a Hungarian cohort[119]. Several variants of NOD1 modify risk of IBD in a New Zealand cohort[64]. There is strong contribution of genetic polymorphisms in specific genes to the risk of IBD, particularly in CD. Given the importance of ganglioside in gut health, future studies may establish evidence suggesting the regulatory role of ganglioside in expression of individual genes that predispose individuals to IBD.

1.44 Intestinal immune system

Biopsy of colon from patients with active IBD reveals large numbers of polymorphonuclear leukocytes, lymphocytes and monocytes. Intravascular naïve T cells migrate to mesenteric lymph nodes via high endothelial venules where antigen-presenting dendritic cells are encountered. T cells then expand into T_h1, T_h2, T_h17 or T_h1/T_h17 effector cells. Effector T cells enter systemic

circulation through efferent nodal lymphatics and home to gut (Section 1.33). In the gut interstitium, effector T cells encounter antigen-presenting dendritic cells, B cells and macrophages; and produce pro-inflammatory mediators which promote expansion of T cells, enhance helper functions of T and B cells and recruit and activate granulocytes leading to chronic gut inflammation[120].

Identifying factors which propagate a chronic inflammatory phenotype in IBD is important in identifying effective treatment. Th1 cells produce IL-21 which promotes expansion of Th17 cells[121]. Th17 cells then also produce IL-21 which sustains Th17 cell population in an autocrine fashion[122]. Production of IL-17 is enhanced in mononuclear cells from lamina propria in IBD intestine versus intestine from healthy subjects and has been shown to sustain chronic inflammation[123]. Stimulated dendritic cells produce IL-23 which promotes IL-17 production by Th17 cells and activates myeloid cells to perpetually produce pro-inflammatory mediators[124]. Macrophages produce acute inflammatory mediators IL-1, nitric oxide and TNF-α and production of these cytokines is inhibited by provision of ganglioside[59-61]. Dietary ganglioside may be of therapeutic value in disrupting the production of acute inflammatory markers which propagate and feed the cycle of chronic inflammation.

1.45 Ganglioside in IBD pathology

Ganglioside species composition differs among several disease states (Section 1.11). For example, Sandhoff, Gaucher, and Tay Sach diseases are characterized by abnormal glycosphingolipid metabolism due to gene deficiencies for catabolic enzymes and accumulation of glucosylceramide or GM2 ganglioside[125]. One study has demonstrated the relationship between a gene that regulates ganglioside metabolism and IBD. A genetic variant of lysosomal sialidase is associated with CD[126], but this study did not assess whether ganglioside content

correlates with sialidase genotype. Another study showed there was no difference in β-galactosidase (Figure 1-2) enzyme activity between LPS-stimulated mononuclear cells from IBD patients and healthy controls[127]. In the same study however, β-hexosaminidase A (HEXA) enzyme activity was higher in peripheral blood monocytes of patients with IBD than in healthy control subjects when incubated with LPS. HEXA generates GM3 from GM2 and thus accelerated ganglioside catabolism is believed to contribute to pathogenesis of IBD. The role of dietary ganglioside in attenuating burdensome disease processes that stem from genetic polymorphism in ganglioside metabolism enzymes is of interest in future study.

1.5 Conclusions

IBD is a disorder influenced by many environmental and genetic factors. Signs regularly present in individuals with IBD include chronic inflammation, overactive immune response, and impaired integrity of gut (Section 1.40). While signs and symptoms may subside for short periods of time, recurrence of IBD-related episodes is regular. There is appreciable cost associated with treating IBD. As surgical intervention or drug administration does not result in a cure, there is demand for new treatment initiatives. Emerging evidence shows the critical role of ganglioside in supporting intestinal health (Section 1.30). Ganglioside metabolism in the intestinal mucosa is fundamental to the etiology of IBD. Studies show that low levels of ganglioside in the intestinal mucosa are associated with increased levels of inflammatory markers, susceptibility to pathogens, and poor gut integrity. Dietary ganglioside constitutes an exciting new therapeutic agent which targets intestinal cells and associated immune surveillance by interrupting the pro-inflammatory signaling cascade and subsequently alleviating signs of inflamed intestine. Dietary ganglioside consumption alleviates many of the burdensome

processes in models of intestinal disease that are also characteristic of IBD and thus, may provide therapeutic benefit to afflicted individuals.

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CHAPTER 2. STUDY RATIONALE

2.1 Rationale

CD and UC constitute IBD; chronic and potentially debilitating disorders primarily affecting the gastrointestinal tract (Section 1.40). Manifestations of IBD extend to bone and joint disorders, eye lesions, hepatic and renal diseases as well as hematologic and skin abnormalities[1]. The cause of IBD is not clearly known but is believed to be influenced by a combination of genetic and environmental factors including dietary intake, urbanized habitation and geographical latitude[2]. Treatment of IBD consists of lifestyle counselling, dietary management and drug therapy. Complications like stricture or fistula that may develop usually require surgical intervention[3]. Burden of disease substantially impedes quality of life in sufferers. New diagnostic tools and treatment measures are sorely needed.

Treatment and associated diagnostic tests for IBD incur a large financial burden. The Crohn's and Colitis Foundation of Canada reported that the cost associated with care for over 200,000 patients living with IBD was conservatively estimated at about $12,000 per person per year in Canada in 2012[4]. Physician visits accounted for about $100 million, hospitalization for about $400 million and cost of medication exceeded $500 million[4]. In 2012, indirect health care costs associated with IBD exceeded $1.5 billion in Canada[4]. Costs associated with IBD care in the USA are about 1.5 times higher than in Canada[5]. Thus, the need for a low cost, safe, effective treatment with high compliance is of utmost importance.

Diagnosis of disease presents with several challenges[6]. Diagnosis of CD may be mistaken as UC or even as another disorder of the intestine[7,8]. Accurately diagnosing and classifying disease is necessary for optimal treatment regimens and thus, there is a strong need for novel biomarkers that correlate to severity of disease, are easily accessible and can be assayed quickly

and cost-effectively. Some novel diagnostic and prognostic tools include serologic markers C-reactive protein and anti-Saccharomyces cerevisiae antibody, fecal biomarkers calprotectin and lactoferrin and endoscopy and imaging techniques like capsule endoscopy, double-balloon endoscopy, magnetic resonance imaging and computerized tomography[9,10]. These tools can be hampered by sensitivity and specificity issues in addition to high costs and high demand resulting in long patient wait times. Advances in lipid analysis technology have only recently allowed for highly sensitive and specific characterization of ganglioside content and composition. The integral involvement of ganglioside in the inflammatory process (Section 1.22, Section 1.30, Section 1.45) of the intestine highlights glycosphingolipid content and composition as a promising biomarker candidate for IBD.

Inherited genetic disorders of both ganglioside metabolism[11] and IBD[12] are found to occur at a much higher frequency in Ashkenazi Jews than in other populations. However, commonalities of the two disorders have yet to be discerned. Disorders of ganglioside metabolism that do not manifest as gangliosidoses (Section 1.12, Section 1.45) may influence development of IBD in the Ashkenazi Jews and in other populations. Colitis-associated carcinogenesis is also linked to aberration in ganglioside metabolism[13]. As individuals with IBD are at higher risk of developing colorectal cancer, further elucidating the role of ganglioside in gut health is of interest. Epidemiologic evidence points to a link between altered ganglioside metabolism and intestinal inflammation and further investigation is warranted.

Ganglioside species are located in specific areas of the enterocyte cell membrane and lipid rafts[14]. GM3 is predominantly located on the brush border membrane and GD3 is predominantly found in lateral and basolateral membranes, indicating that ganglioside species localize to particular regions of the enterocyte to interact with neighbouring molecules (Section

1.33, Section 1.34) and send signals to the intracellular environment affecting many cellular functions (Section 1.10). Providing ganglioside in the diet increases ganglioside content in intestinal mucosa[14]. Important observations from animal studies show that inflamed intestinal mucosa has less ganglioside than healthy intestinal mucosa[15]. Moreover, increasing ganglioside content through diet decreases pro-inflammatory cytokine production in intestinal mucosa[15], and prevents hypoxia-induced bowel necrosis and cell injury in cultured infant bowel[16] affecting the same pro-inflammatory signals basic to IBD (Section 1.35, Section 1.38). Collectively, these studies of animal and infant intestinal mucosa indicate that the amount of specific gangliosides is decreased with inflammation. Levels of particular ganglioside metabolites in the intestinal mucosa can be associated with pro-inflammatory signals (Figure 1-3).

The protective role of ganglioside in inflammatory disorders and in promotion of intestinal health has been described (Section 1.30). Exposure to dietary ganglioside is shown to be safe in several *in vitro*[16,17] and animal feeding studies[14,18,19]. Ganglioside as a component of foods like breast milk, beef and dairy eaten regularly by humans for generations has not been linked to any specific adverse effects. However, investigating the effects of ganglioside supplementation on health and disease in human adults has not been directly assessed in clinical study. In some disease models, therapeutic dose of ganglioside (10 – 30 μg/ml) can ameliorate specific acute and chronic inflammatory disease processes[15,20,21]. These observations stress the importance of establishing the safety of dietary ganglioside consumption and determining potential utility of ganglioside to treat IBD: to improve gut-barrier function, ameliorate adverse inflammatory signalling and enhance quality of life.

2.2 Hypotheses and objectives

The overall hypothesis of this thesis research is that altered ganglioside metabolism is fundamental to the etiology of IBD and that supplemental ganglioside consumption can attenuate altered physiologic processes characteristic of IBD. This hypothesis is explored in two studies.

Study 1: Safety, bioavailability and metabolism of ganglioside in human participants

Hypothesis: Ganglioside is safe and bioavailable for consumption and potential treatment of IBD.

Objectives:

i. To determine the safety of supplemental ganglioside by analyzing adverse event reports and blood chemistry and immune cell measures in participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period. This objective will show whether ganglioside consumption is associated with serious or other adverse events. This objective will also show whether supplemental ganglioside intake is associated with abnormalities blood chemistry: white blood cells, red blood cells, platelets, hemoglobin; and thus, whether supplemental ganglioside is safe and tolerable for daily consumption.

ii. To determine the bioavailability of dietary ganglioside by measuring plasma ganglioside content (GM3, GD3) in participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period. This objective will show whether ganglioside consumption leads to an increase in ganglioside content in blood and the associated time course by which ganglioside affects blood ganglioside concentration;

and thus, whether ganglioside is available systemically for circulation to tissues affected by disease to exert beneficial effects.

iii. To determine the effect of supplemental ganglioside intake on ganglioside metabolism by measuring plasma HEXA activity in participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period. This objective will show whether catabolism of GM2 ganglioside to GM3 is increased when plasma ganglioside concentration (Study 1: ii) is increased by diet.

iv. To determine whether patients with IBD may benefit from ganglioside supplementation, a quality of life in IBD questionnaire (IBDQ) is administered to participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period. This objective will show whether bowel symptoms, emotional health, systemic symptoms and social function are improved by supplemental ganglioside ingestion in patients with IBD.

Study 2: Ganglioside catabolism is elevated in IBD: importance of dietary ganglioside intake

Hypothesis: Content and composition of ganglioside and phospholipid classes differ among IBD and healthy intestine and ganglioside intake increases intestinal integrity.

Objectives:

i. To determine whether the class of ganglioside generated by HEXA (GM3) is elevated and substrates of NEU3 (GD3, GD1a) are decreased in active IBD, the content of gangliosides is measured in intestine from participants with IBD and compared to healthy intestine from non-IBD participants. This objective will inform whether ganglioside metabolism is altered in IBD and whether altered level of

specific gangliosides (GM3, GD3, GD1a) may be implicated in disease processes characteristic of IBD.

ii. To determine whether ganglioside catabolism is increased in active IBD, the content of ganglioside catabolism enzymes (HEXA, NEU3) is measured in the intestine of patients with IBD and compared to healthy intestine from non-IBD participants. This objective will inform whether increases in ganglioside catabolism enzymes corroborate the differences in intestinal ganglioside content (Study 2: i).

iii. To determine whether ganglioside (GM3, GD3, GD1a) and phospholipid [PC, phosphatidylethanolamine (PE)] classes have decreased PUFA content in active IBD, the number of unsaturated bonds in ganglioside and phospholipid classes is assessed in intestine from patients with IBD and compared to healthy intestine from non-IBD participants. This objective will inform whether ganglioside and phospholipid composition is altered in IBD and inform whether essential fatty acid status may be implicated in disease processes characteristic of IBD.

iv. To determine whether ganglioside intake improves intestinal integrity in healthy human participants, an oral lactulose/mannitol (L/M) challenge is administered to participants consuming ganglioside at baseline (week 0) and study conclusion (week 8). This will inform whether dietary ganglioside decreases intestinal permeability and whether supplemental ganglioside may be of benefit to patients with IBD that have impaired intestinal integrity.

v. To determine whether ganglioside intake decreases levels of acute systemic inflammatory mediators in healthy human participants, cytokine (PGE_2, LTB_4, TNF-α) assays are performed in participants consuming ganglioside at baseline and week 6

of the supplementation period. This objective will inform whether dietary ganglioside decreases some of the acute pro-inflammatory signals in IBD and whether improved intestinal integrity (Study 2: iv) occurs independently of changes in inflammatory markers.

2.3 Chapter organization

Chapter 3 "Safety, bioavailability and metabolism of ganglioside in human participants" investigates the hypothesis that **ganglioside is safe and bioavailable for consumption and potential treatment of IBD**. This chapter outlines outcomes for a cohort of healthy participants and patients with IBD that consumed ganglioside supplement for eight weeks (Study 1: i). The bioavailability of ganglioside is measured in plasma (Study 1: ii) as well as the activity of ganglioside catabolism enzyme HEXA in plasma of participants consuming ganglioside (Study 1: iii). Finally, quality of life in IBD is assessed in participants consuming ganglioside (Study 1: iv). Results of each objective are discussed in relation to the hypothesis proposed.

Chapter 4 "Ganglioside catabolism is elevated in IBD: importance of dietary ganglioside intake" investigates the hypothesis that **content and composition of ganglioside and phospholipid classes differ among IBD and healthy intestine and ganglioside intake increases intestinal integrity**. The chapter outlines the differences in content of major ganglioside classes (Study 2: i) and enzymes that catabolize ganglioside (Study 2: ii) in the intestine of patients with IBD and in healthy non-IBD intestine. In addition, composition of ganglioside and phospholipid classes are described and contrasted among study groups (Study 2: iii). The impact of ganglioside intake on functional intestinal permeability (Study 2: iv) and acute systemic inflammatory marker (Study 2: v) outcomes is also assessed. Results of each objective are discussed in relation to the hypothesis proposed.

Chapter 5 is a general summary and discussion regarding the safety and bioavailability of ganglioside consumption, the metabolism of ganglioside in plasma and in the intestinal mucosa, and the effects of dietary ganglioside on inflammatory markers, intestinal permeability and quality of life in IBD. The outcomes of Study 1 and 2 are discussed in relation to the overall hypothesis **that altered ganglioside metabolism is fundamental to the etiology of IBD and that supplemental ganglioside consumption can attenuate altered physiologic processes characteristic of IBD.**

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CHAPTER 3. SAFETY, BIOAVAILABILITY AND METABOLISM OF GANGLIOSIDE IN HUMAN PARTICIPANTS

3.1 Introduction

Safety and bioavailability of ganglioside has been demonstrated in several *in vitro* models and in various tissues in many animal models (Section 1.20). The uptake of ganglioside has been shown in Caco-2 cells[1], breast[2] and prostate cancer cells[3]; and in rat brain, plasma and intestine[4]. Safety of ganglioside for human consumption is presumed as ganglioside is a component of food like meat and milk that is a staple of many diets. Safety and bioavailability of ganglioside however has not been demonstrated in a human clinical study. As a potential novel therapeutic agent for IBD (Section 1.22, Section 1.30) and other disorders (Section 1.11), it is of interest to discern whether the concentration of specific gangliosides in plasma can be increased by supplemental consumption of ganglioside in humans.

Activity of HEXA is reduced or absent in patients with Tay-Sachs disease[5] which is assessed by a fluorometric plasma or serum activity assay using a HEXA-specific substrate (Section 3.25). Mononuclear cells from patients with IBD have increased HEXA activity compared to control participants[6]. The impact of supplemental or dietary ganglioside on the activity level of ganglioside catabolism enzymes has not been investigated. Since ganglioside may prove effective for clinical treatment or management of IBD, the influence of ganglioside on HEXA activity or other ganglioside metabolism activity may yield insight into optimal dosing or consumption regimen. Patients living with IBD are also hindered with poor quality of life (Section 1.40). So, the impact of supplemental ganglioside intake on bowel symptoms, systemic symptoms, emotional health and social function was assessed in this study.

This study demonstrates the safety and bioavailability of ganglioside consumption in human participants. Furthermore, this study shows that plasma HEXA activity is not affected by daily consumption of ganglioside. Finally, intake of dietary ganglioside is associated with improved quality of life in patients with IBD.

3.20 Methods

Ethics approval for this study was obtained from the Biomedical Panel of the University of Alberta Health Research Ethics Board.

3.21 Participant enrollment

Healthy control participants (N=18), 22 to 56 y of age were recruited for an 8-wk double-blind, randomized, placebo-controlled study to demonstrate the safety of dietary ganglioside. Patients with IBD (N=4 CD, N=1 UC) were blinded to treatment allocation and non-randomly allocated to ganglioside arm to demonstrate efficacy of dietary ganglioside for potential treatment of IBD. Participants (non-pregnant) with mildly active CD of the terminal ileum or the terminal ileum plus the right colon were recruited from the University of Alberta Hospital's gastroenterology clinic under the direction of Dr. Alan Thomson. Diagnosis of CD was based on established radiologic, endoscopic and histologic criteria. Only stable oral doses (up to 3 g/d for 8 wk prior to the study) of 5-acetylsalicylic acid were allowed as concomitant and prior therapy. Individuals taking glucocorticosteroids, immunosuppressants, antibiotics, or infliximab were not eligible for inclusion. Patients with planned or emergency surgery, inadequate liver or renal function, cancer, active infectious disease, history of alcohol/drug abuse, serious complications of CD, or other serious medical conditions were not permitted to participate.

3.22 Assessment of ganglioside intake

Participants were allocated to receive either a milk fat fraction with or without ganglioside. Individuals in the ganglioside group consumed 1.0 g of milk fat fraction (ZETA dairy lipid, Fonterra) containing approximately 43 mg of ganglioside [80% GD3; 20% GM3 (w/w)] daily for 8 wk. The equivalent milk fat fraction void of ganglioside constituted the placebo. Participants maintained written records throughout the 8-wk study to document ganglioside supplement and medication use and adverse events. Adverse events were characterized as per clinical trial registry (NCT02139709, clinicaltrials.gov). Compliance was assessed by totalling the number of full, unused supplement packs at successive follow-ups.

3.23 Ganglioside safety and inflammatory bowel disease questionnaire

A fasting blood panel was completed in all participants enrolled at baseline and week 2, 4, 6 and 8 of supplementation study to measure mean white blood cell count, red blood cells, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, red blood cell distribution width %, platelets, mean platelet volume, neutrophils, lymphocytes, monocytes, eosinophils, basophils, % neutrophils, % lymphocytes, % monocytes and % basophils. The IBDQ (Section 7.1) was completed by all study participants enrolled at baseline and wk 2, 4, 6 and 8 to assess social function, emotional health, and systemic and bowel symptoms. In the IBDQ (Section 7.1), bowel symptoms were assessed by responses to questions 1, 5, 9, 13, 17, 20, 22, 24, 26 and 29, systemic symptoms were assessed by responses to questions 2, 6, 10, 14 and 18, emotional health was assessed by responses to questions 3, 7, 11, 15, 19, 21, 23, 25, 27, 30, 31 and 32, and social function was assessed by responses to questions 4, 8, 12, 16 and 28. Subjects responded to questions on a 7-point graded scale in which the

lowest score of 1 indicated poor quality of life and the highest score of 7 indicated high quality of life.

3.24 Ganglioside bioavailability

Blood samples were centrifuged to obtain plasma. Gangliosides were isolated from plasma using a modified Folch extraction[7]. The final supernatant was used for ganglioside profiling. Aqueous extracts were injected onto a Poroshell 120 EC-C18 column. Gangliosides were separated using reverse-phase liquid chromatography and the eluent was directed to the inlet of an Agilent QQQ 6430 mass spectrometer (MS) operated in negative ion mode. Electrospray ionization generated deprotonated gas-phase ions from the various ganglioside species. The MS was operated in multiple reaction monitoring (MRM) mode to provide selective and sensitive ganglioside detection by allowing only select precursor ions and characteristic gas-phase fragments to be detected. The mass spectra were screened against a library of theoretical precursor ions from over 600 gangliosides with variable ceramide and carbohydrate compositions. The relative percent of ganglioside classes GM3, GD1a, GD3 and GT1 was determined using Agilent Mass Hunter Qualitative Analysis software.

3.25 Beta-hexosaminidase A activity

Plasma samples (100 µL) from a healthy control participant (N=1) and patients with IBD (N=4) who consumed ganglioside were diluted in 900 µL of 0.04 M citrate phosphate buffer (pH 4.4). Samples were incubated on ice (10 min). Diluted sample (50 µL) was placed in water (50°C) for 4 hr to inactivate heat-labile HEXA or placed in ice water for four hr. After incubation, 4-methylumbelliferyl-N-acetyl beta-D-glucosaminide (1.0mmol/L) was made by adding 9.9 mg of 4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide (M-2133, Sigma) to 25 mL of 0.04 M citrate phosphate buffer and 100 µL of this substrate was added to samples before incubation for

1 hr (37°C). Reactions were stopped by adding 5 mL of cold 0.25 M glycine carbonate buffer (pH 9.9) to samples. Enzyme activity was measured using a LS50B fluorescence spectrometer using FL WinLab software and quantified by interpolating from a standard curve. HEXA activity was computed by subtracting beta-hexosaminidase B (samples incubated at 50°C for 4 hr) activity from total hexosaminidase activity (samples incubated on ice for 4 hr).

3.26 Statistics

As a pilot study, no formal sample size calculation was calculated. Counts, means, totals and standard errors were computed for participant characteristics including sex, age and weight; as well as adverse events and compliance. Means and standard errors were computed for counts, concentrations, volumes and relative amounts of blood panel measures (Section 3.23) and compared among study groups by ANOVA. Mean plasma concentrations of total GD3, total GM3, C34:1 GD3 and C34:1 GM3 and associated standard errors were computed. C34:1 indicates 34 carbons and 1 unsaturated bond within the ceramide component of ganglioside. Repeated measures ANOVA was used to assess bioavailability of ganglioside. Healthy participants and patients with IBD were pooled for statistical analysis of ganglioside bioavailability for adequate power (β=0.20). Means were computed for IBDQ: social function, emotional health, systemic and bowel symptoms. Responses to questions in the IBDQ were averaged at each study time point (wk 0, 2, 4, 6, 8) for each of quality of life parameter: bowel symptoms, systemic symptoms, emotional health, social function (Section 3.23, Section 7.1). Repeated measures ANOVA was used to compare IBDQ outcomes for healthy participants in the supplementation study. Linear regression was used to assess IBDQ outcomes in patients with IBD and HEXA activity over time in the supplementation study. Significance was defined as $\alpha<0.05$.

3.30 Results

3.31 Study participants

The GD3 fraction of the ganglioside treatment was composed mainly of C41:1 (equating to 41 carbons and 1 unsaturated bond within the ceramide component), followed in abundance by C40:1 and C34:1. The GM3 fraction of the ganglioside treatment was composed of C34:1 > C40:1 > C41:1 (Table 3-1). Average dietary intake of ganglioside is less than 100 μg/1000 kcal/day and rarely exceeds 200 μg/1000 kcal/day[8]. The dose of ganglioside consumed in this study exceeds average dietary intake in human adults by about 200-fold depending on average daily total caloric consumption. Control participant randomization to placebo and ganglioside groups was similar for females and males. Patients with IBD were not randomized, and allocated specifically to ganglioside treatment arm. There were no serious adverse events reported in any of the study groups. In control participants, ganglioside and placebo groups were similar with respect to age and the number of other adverse events. Mean weight, but not median weight tended to be higher in participants allocated to the ganglioside arm than the placebo group. Mean weight, but not median weight tended to be higher in patients with IBD than control participants allocated to the ganglioside arm. Compliance was measured by counting the number of full, unused supplement packets returned at each follow-up week: 2, 4, 6, 8. Compliance was generally good as patients consumed supplement according to study protocol on about 90% of the days in the 8-wk intervention study. The groups that consumed ganglioside tended to have better compliance than participants allocated to placebo arm but there was no significant difference among study groups (Table 3-2).

3-1 Composition of Ganglioside Treatment

	Ceramide	Relative abundance (%)
GD3	C41:1	12.32
	C40:1	11.84
	C34:1	10.23
	C42:1	7.85
	C39:1	5.88
	C38:1	4.41
	C40:0	3.07
	C41:0	2.89
	C39:0	2.45
	other	15.91
GM3	C34:1	6.46
	C40:1	4.33
	C41:1	2.59
	other	8.78
other	-	< 1.00

Relative abundance of ganglioside species (as % of total ganglioside) that constitute the supplemental ganglioside fraction used in this study.

3.32 Ganglioside safety

A blood panel consisting of mean white blood cell count, red blood cells, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, red blood cell distribution width %, platelets, mean platelet volume, neutrophils, lymphocytes, monocytes, eosinophils, basophils, % neutrophils, % lymphocytes, % monocytes and % basophils was completed in all participants at baseline and weeks 2, 4, 6 and 8 to assess safety throughout the study period. At baseline and week eight, patients with IBD had greater mean corpuscular volume, red blood cell distribution width percentage, more platelets, more

neutrophils and relative percent of neutrophils than healthy control participants. At baseline and study conclusion, control participants had more lymphocytes and higher relative percent of lymphocytes than patients with IBD. Neutrophils and lymphocytes were outside the normal standard clinical range in patients with IBD. The relative percent of monocytes, but not the number of monocytes was outside normal clinical range in healthy participants in the ganglioside arm at study conclusion. In the case where mean corpuscular hemoglobin concentration was outside the normal clinical range at study conclusion in healthy participants allocated to the ganglioside treatment arm, the respective measure was also outside normal clinical range at baseline and thus not attributed to consumption of ganglioside (Table 3-3). Blood panel measures did not change between baseline and study conclusion in healthy control participants allocated to the placebo arm (data not shown).

3-2 Participant Enrollment

	Control		IBD
	Placebo	Ganglioside	Ganglioside
Female (#)	8	6	2
Male (#)	2	2	3
Age (y)	33 (±3)	34 (±4)	35 (±6)
Weight (kg)	62 (±5.5)	72 (±3.7)	89 (±11.5)
Subjects experiencing Serious Adverse Event (#)	0	0	0
Subjects experiencing Other Adverse Event (#)	7	4	4
Other Adverse Events (total)	10	8	5
Compliance (mean # unused packets returned)	6.0 (±1.0)	5.0 (±2.0)	5.2 (±1.0)

Characteristics and other outcomes of participants enrolled in the supplementation study.

3-3 Blood panel of participants consuming ganglioside

	Normal Range	Control		IBD		Pooled SEM	
		Week 0	Week 8	Week 0	Week 8	Week 0	Week 8
Whole Blood Count (10^9/L)	4.0 - 11.0	5.7	6.17	6.23	7	0.31	0.38
Red Blood Cells (10^{12}/L)	3.8 - 6.5	4.23	4.33	4.56	4.19	0.12	0.13
Hemoglobin (g/L)	120 - 180	135	132.71	140.75	130.67	2.15	2.83
Hematocrit	0.36 - 0.54	0.36	0.37	0.42	0.39	0.01	0.01
Mean Corpuscular Volume (fL)	76 - 96	84.29	86.51	91	92.67	1.03	1.15
Mean Corpuscular Hemoglobin (pg)	27 - 32	33.06	30.74	.	.	1.31	1.25
Mean Corpuscular Hemoglobin Concentration (g/L)	310 - 350	393.29	355.29	339.5	338	12.67	12.19
Red Blood Cell Distribution Width %	11 - 16	12.81	12.91	14.53	15	0.32	0.32
Platelets (10^9/L)	150 - 400	261.57	273.14	313	330.33	19	20.03
Mean Platelet Volume (fL)	6.0 - 10.0	8.3	8.26	.	.	0.24	0.24
Neutrophils (10^9/L)	2.0 - 7.5	2.66	2.87	5.17	5.7	0.3	0.39
Neutrophils %	45 - 70	51.2	50.1	72.3	85.5	2.39	3.17
Lymphocytes (10^9/L)	1.5 - 4.0	1.94	2.43	1.2	0.83	1.1	0.17
Lymphocytes %	20 - 40	36.7	40.4	18.0	8.5	1.94	2.73
Monocytes (10^9/L)	0.2 - 0.8	0.45	0.67	0.53	0.43	0.03	0.07
Monocytes %	3.0 - 10.0	8.5	12.2	8.0	5.5	0.41	1.63
Eosinophils (10^9/L)	0.04 - 0.40	0.17	0.22	0.1	0.1	0.03	0.03
Eosinophils %	1.0 - 5.0	3.0	3.6	1.0	1.0	0.5	0.62
Basophils (10^9/L)	0.02 - 0.10	0.17	0.05	.	.	0.04	0.04
Basophils %	0 - 0.5	0.7	0.9	0.7	.	0.08	0.13

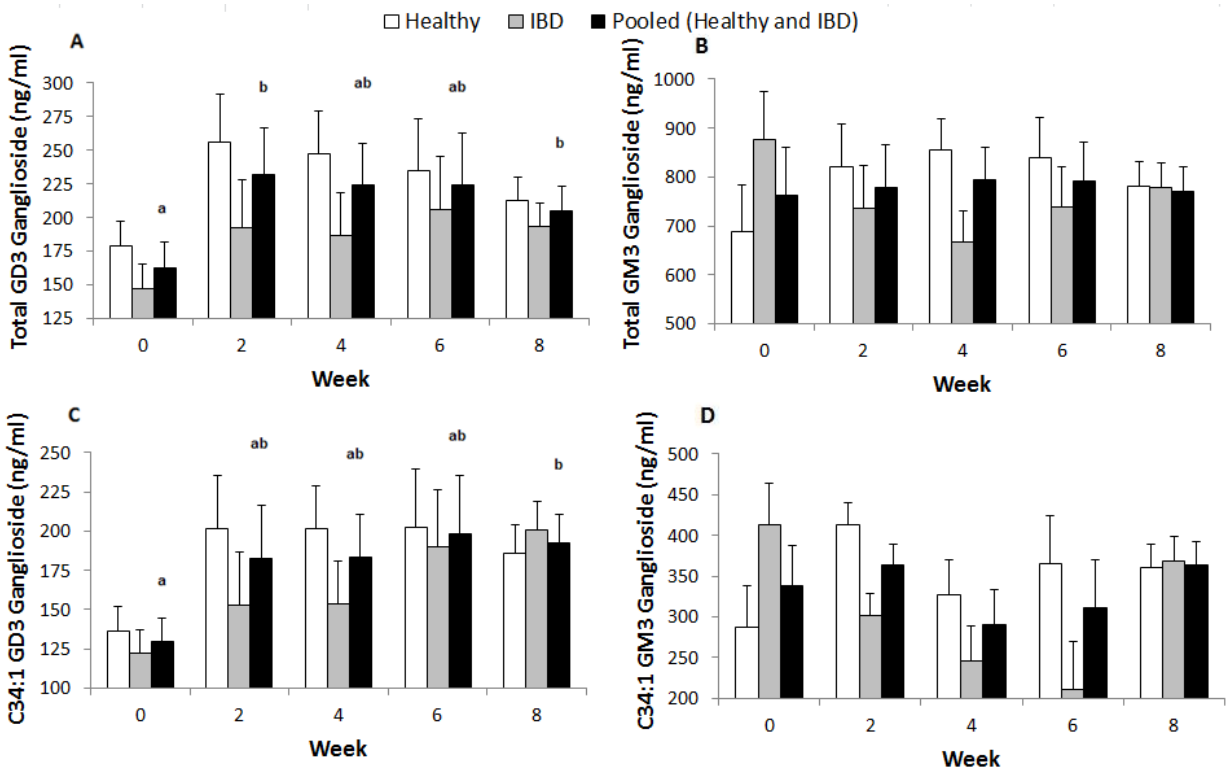
Consumption of ganglioside did not significantly change blood chemistry measures in healthy control participants (N=8) or patients with IBD (N=5) over eight weeks. Blank cells indicate insufficient data. IBD = inflammatory bowel disease; SEM = standard error of the mean

3.33 Ganglioside bioavailability

Ganglioside content of plasma was measured at baseline and weeks 2, 4, 6 and 8 to assess bioavailability. Monounsaturated C34 GD3 was the most abundant species of GD3 in plasma, accounting for 50 - 80% of total GD3. Consuming ganglioside caused plasma content of C34:1 GD3 and total GD3 to increase about 40% from baseline to week 2 in pooled analysis of healthy participants and patients with IBD. In the group consuming ganglioside, monounsaturated C34

GD3 ultimately reached 193 ng/ml at week eight from baseline level of 129 ng/ml ($P<0.05$). Monounsaturated C34 GM3 was the most abundant species of GM3 in plasma, accounting for 50 - 60% of total GM3. Neither total GM3 nor C34:1 GM3 significantly increased at any time point after baseline in the group consuming ganglioside (Figure 3-4). In the placebo group, the level of C34:1 GD3 and C34:1 GM3 did not change from baseline to study conclusion (Figure 3-5).

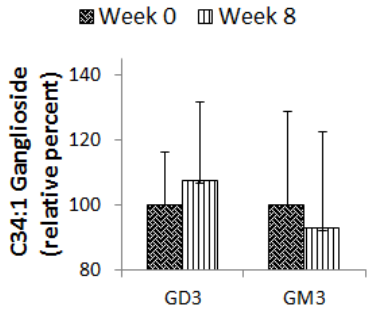
3-4 Plasma ganglioside concentration in participants consuming ganglioside



Bars represent mean ganglioside content of plasma (+SEM) in participants consuming ganglioside (N=7). Ganglioside C34:1 GD3 increased in participants consuming ganglioside at weeks 2, 4, 6 and 8 from baseline. When healthy control participants and patients with IBD were pooled, the total level of total GD3 was significantly greater at weeks 2 and 8 than week 0 ($P<0.05$). Similarly, the level of ganglioside C34:1 GD3 was significantly greater at week 8 than

week 0 (P<0.05). In pooled analysis of control participants and patients with IBD, consuming ganglioside does not lead to changes in total or C34:1 plasma GM3. Lowercase letters (a, b) denote statistically significant differences (P<0.05) among time points in the analysis of pooled healthy participants and patients with IBD. IBD = inflammatory bowel disease; SEM = standard error of the mean

3-5 Plasma ganglioside concentration in participants consuming placebo

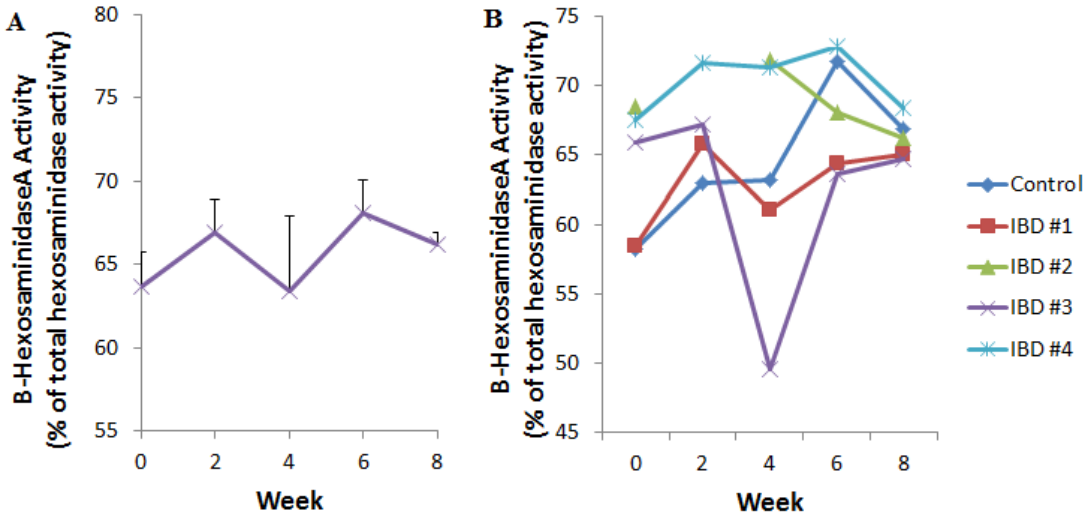


Bars represent mean relative ganglioside content of plasma (+SEM) in healthy control participants in the placebo arm (N=5). Monounsaturated C34 gangliosides GD3 and GM3 did not increase from baseline to study conclusion. SEM = standard error of the mean

3.34 Ganglioside metabolism

Plasma HEXA activity was measured in five participants (N=4 IBD, N=1 control) that consumed ganglioside for eight weeks. Relative HEXA activity was (computed as a percent of total hexosaminidase activity and) compared across time points. Plasma HEXA activity was between 62 – 72% of total hexosaminidase activity which is within normal clinical range (150 – 620 μmol/hr/L or 50 – 70% of total hexosaminidase activity), and did not significantly change between baseline and study conclusion in participants consuming ganglioside (Figure 3-6).

3-6 Plasma HEXA activity in participants consuming ganglioside



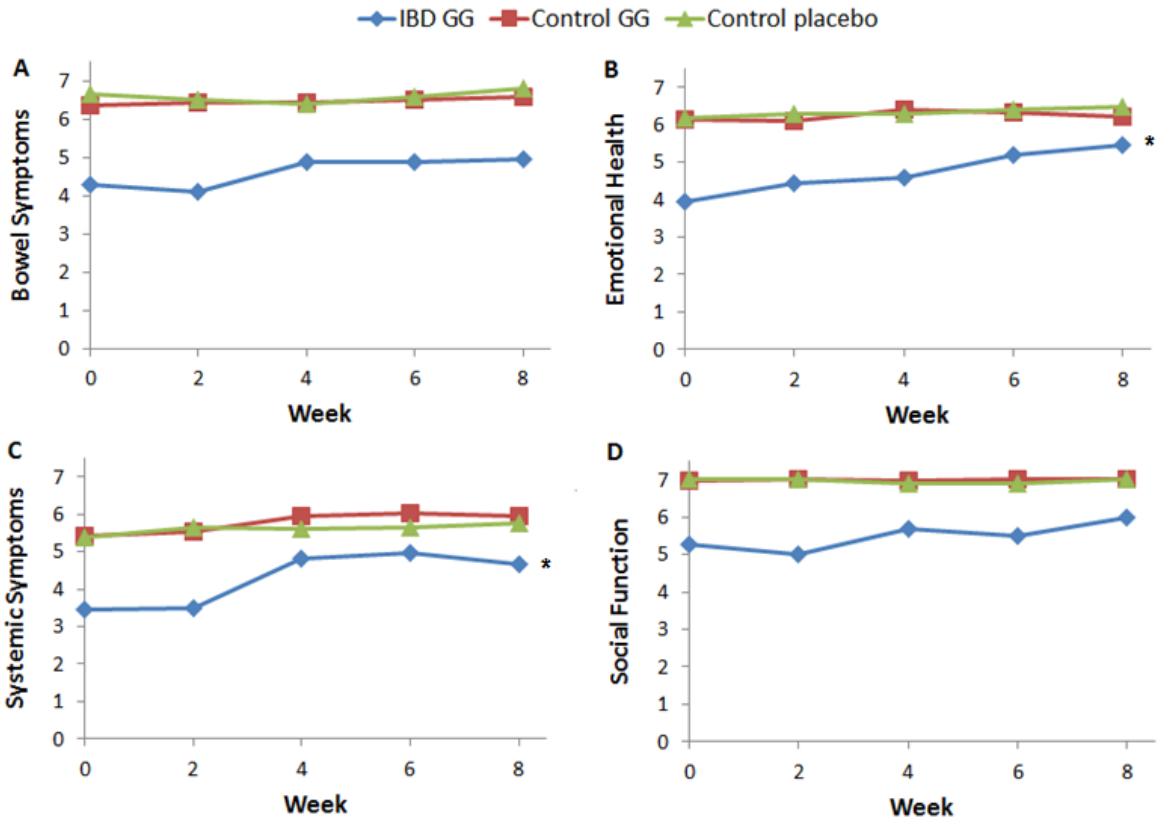
Points in Panel A represent mean HEXA activity (+SEM) as a percent of total hexosaminidase activity (N=5).Points in Panel B represent individual subject plasma HEXA activity (N=4 IBD, N=1 control). Mean HEXA activity did not change in participants consuming ganglioside over eight weeks. HEXA = beta-hexosaminidase A; SEM = standard error of the mean

3.35 Quality of life IBD

IBDQ was completed at baseline and weeks 2, 4, 6 and 8 to assess effect of ganglioside on bowel symptoms, emotional health, systemic symptoms and social function. In healthy control participants, there was no difference between placebo and ganglioside groups and no change over time with respect to bowel symptoms, emotional health, systemic symptom and social function outcomes. Patients with IBD had lower absolute scores for bowel symptoms, emotional health, systemic symptoms and social function than control participants at baseline. Consuming ganglioside increased ($P<0.01$) emotional health by 39% over eight weeks in patients with IBD. Consuming ganglioside also significantly ($P<0.02$) improved systemic symptoms in

patients with IBD by 36% over eight weeks. Consuming ganglioside did not have a significant effect on bowel symptoms or social function in patients with IBD over eight weeks (Figure 3-7).

3-7 Inflammatory bowel disease questionnaire scores



Data points represent mean IBDQ score. There was no difference among healthy participants in the ganglioside (N=8) and placebo (N=10) arms over time. Emotional health improved 39% (P<0.01) and systemic symptoms improved 36% (P<0.02) over the course of study in patients with IBD (N=5) consuming ganglioside. Standard error at each time point was small (<0.5) and is not shown. Asterisk (*) indicates significant effect of time (P<0.02) in participants with IBD consuming ganglioside. IBD = inflammatory bowel disease; GG = ganglioside

3.4 Discussion

It is necessary to establish the safety and bioavailability of potential new treatments for disease. Blood chemistry and immune cells measures (Section 3.23) are potentially indicative of anemia[9], potential for clot formation[10], inflammation and resistance to or presence of infection[11]. Consumption of 43 mg/day of ganglioside is considered safe as ganglioside treatment did not affect occurrence of adverse events (Table 3-2), blood chemistry or immune cell (Figure 3-3) measures compared to consuming a milk fat fraction void of ganglioside. The dose was selected by estimating intake of ganglioside from breast milk[12] in neonates and emulating the corresponding concentration of ganglioside for intake in human adults. Increase in plasma level of ganglioside is observed after two weeks of daily ganglioside consumption and elevated plasma ganglioside concentration is sustained up to eight weeks of supplementation (Figure 3-4). Furthermore, consuming milk fat fraction containing ganglioside is associated with improved emotional health and systemic symptoms in patients with IBD (Figure 3-7).

This is one of the first studies to characterize the composition of ganglioside species in plasma. Previous studies have reported relative blood levels of ganglioside classes[13-16] but not the composition of ceramide. Monounsaturated C34 constituted the most abundant GM3 and GD3 species in plasma. Although C40:1 and C41:1 GD3 are more abundant than C34:1 GD3 in the ganglioside supplement (Table 3-1), C40:1 constituted a very small proportion of GD3 in plasma and C41:1 GD3 was not detected in plasma. It is possible that monounsaturated C40 and C41 GD3 pools are absent in plasma due to intestinal modification; for instance, metabolism to GD2 or GT3. On the other hand, C40:1 and C41:1 gangliosides may circulate to other tissues for uptake from blood which may be directed by the respective ceramide constituent. The fates of GM3 and GD3 ganglioside classes appear to be specifically regulated within plasma. The

ceramide composition of plasma GM3 and GD3 is primarily C34 monounsaturated, despite intake of other GM3 and GD3 ganglioside species in higher abundance.

Subjects in the placebo arm would be consuming some trace level of ganglioside if their diet included beef or dairy products. However, the vastly greater amount (~200 times) of supplemental ganglioside used in this study as compared to usual dietary intake (Section 3.31) was sufficient to avoid potential confounding of participant allocation to ganglioside or placebo arm. Uptake of ganglioside *in vitro* and in the intestine occurs by micelle absorption, monomer insertion, or receptor-mediated uptake (Section 1.20)[17]. Absorption, metabolism, transfer and retention of gangliosides GM3 and GD3 have been detailed in human intestinal cells (Section 1.21)[1]. This study is among the first to show that GD3 taken up by intestine from diet is found at elevated concentrations in plasma during supplementation (Figure 3-4). Total GD3 and C34:1 GD3 levels increased about 40% after 2 weeks when participants consumed ganglioside and elevated plasma concentration of ganglioside lasted for the duration of the eight-week study.

Despite the presence of GM3 in the supplement (about 20% of total ganglioside), no increase in plasma GM3 was detected in participants consuming ganglioside. This may be due to the much lower abundance of GM3 in the supplement relative to GD3 or due to strict regulation GM3 concentration in blood. Alternatively, a statistically and biologically significant increase in plasma GM3 may be more difficult to achieve as the plasma pool of GM3 is estimated to be about 4 – 5 times higher than the plasma pool of GD3 (Figure 3-4). The presence of glycosphingolipid metabolizing enzymes in the brush border membrane[18] and in plasma[19] suggests that dietary ganglioside may undergo conversion to other ganglioside species in the gut before absorption. Dietary GM3 may be used by the intestinal mucosa for cellular processes or metabolized to lactosylceramide or GD3 before entry into systemic circulation. The regulation of

dietary gangliosides by intestine and in circulation is not yet fully understood in human participants.

Prolonged administration of drug or functional food may lead to compensatory actions taken by the body. Physiologically functional compounds in the form of drug or food may decrease in efficacy over time of regular administration or consumption due to decreased absorption, increased excretion or increased catabolism of parent compound[20-22]. The ganglioside supplement consumed in this study was a mixture of GD3 and GM3 (Table 3-1). To ascertain whether intake of dietary ganglioside induces ganglioside catabolism, a HEXA activity assay was performed. Consumption of ganglioside did not lead to an increase in the activity of ganglioside catabolic enzyme HEXA in plasma (Figure 3-6). Plasma GD3 remained elevated for the entire duration of study and the level of plasma GM3 did not increase in the cohort consuming ganglioside (Figure 3-4). These observations suggest that daily consumption of ganglioside (43 mg) is sufficient to maintain a physiologically therapeutic concentration of plasma ganglioside in lieu of endogenous regulation/metabolism of dietary ganglioside.

Supplementation with ganglioside was associated with improved quality of life in patients with IBD (Figure 3-7). Specifically, ganglioside improved emotional health and systemic symptoms. At study conclusion, emotional health and systemic symptoms improved to about 80 – 90% of the quality of life scores reported in healthy participants. At baseline, the respective emotional health and systemic symptoms scores in patients with IBD were only about 60% of the quality of life scores reported in health participants. Ganglioside is proposed to improve gut health by ameliorating inflammation (Section 1.35) and promoting gut-barrier integrity (Section 1.31), but there was no corresponding improvement reported in bowel symptoms in patients with IBD allocated to supplemental ganglioside in this study. Correlating

quality of life measures with inflammatory markers (Section 4.34, Figure 4-6) and functional intestinal permeability testing (Section 4.33, Figure 4-5) is of interest in future study to determine the mode of action by which ganglioside is associated with improved aspects of quality of life.

Daily consumption of ganglioside at 43 mg/day safely increases and sustains plasma ganglioside concentration and constitutes a viable agent for treatment of IBD or for potential treatment of other disorders for which altered ganglioside content and metabolism have been implicated (Section 1.11).

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CHAPTER 4. GANGLIOSIDE CATABOLISM IS ELEVATED IN IBD: IMPORTANCE OF DIETARY GANGLIOSIDE INTAKE

4.1 Introduction

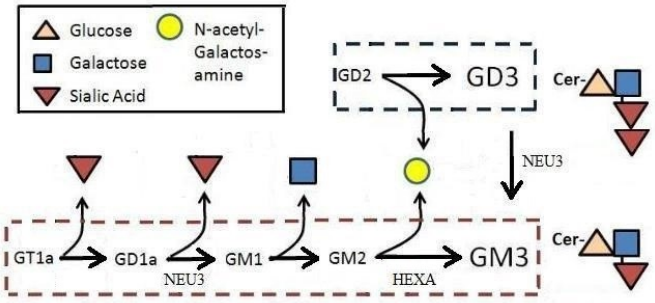
IBD is a global problem[1] and is particularly prevalent in the USA[2] and Canada[3]. IBD is associated with elevated standardized all-cause mortality ratio[4] and there is an alarming 38% increase in all-cause mortality for CD. IBD can present with abdominal pain, gastrointestinal bleeding, increased stool frequency, diarrhea, weight loss and malnutrition (Section 1.40, Section 2.1). CD can be associated with development of joint, liver and kidney diseases and a much elevated risk of small intestinal lymphoma and colorectal cancer. Some individuals with IBD do not respond to drug treatment, while others experience serious adverse effects[5] like hepatotoxicity and renal dysfunction[6]. The cause of IBD is unknown and there is a clear need for innovative knowledge of disease mechanisms to enable novel treatment strategies.

Gangliosides are complex molecules of many molecular species varying in sialic acid and ceramide composition (Figure 4-1). Certain species of ganglioside are involved in regulation of cell signaling, apoptosis and receptor-ligand interactions (Section 1.10). Dietary ganglioside intake is very low unless a person is consuming whole organ foods (ex. brain), whole milk or buttermilk in excessively high quantities that is not practical on a daily basis[7]. Infants normally obtain[8] and incorporate gangliosides into brain[9] and other tissue from human milk[10].

Providing supplemental ganglioside in the diet increases the ganglioside content in intestinal mucosa in rat[11]. Important observations from animal studies reveal that inflamed intestinal mucosa has less GD3 ganglioside than healthy intestinal mucosa[12]. Moreover, increasing ganglioside content of intestinal cells through diet decreases pro-inflammatory cytokine signaling in intestinal mucosa[12], and prevents hypoxia-induced bowel necrosis and cell

injury in cultured infant bowel[10] affecting the same signals underlying IBD. Caco-2 cells incorporate GD3[13] when provided with ganglioside at physiological concentrations *in vitro*[14]. Localization of GD3 in the basolateral membrane surface and GM3 to the brush border membrane of enterocytes[11] suggests specific mechanisms by which ganglioside supports intestinal cell functions like barrier integrity. Intake of GD3 prevents degradation of tight junction protein occludin in rat intestine when challenged by enterotoxic LPS[15]. Collectively, these studies of animal and infant intestinal mucosa indicate that content of GD3 is decreased with inflammation and higher levels of GM3 can be associated with increased intestinal permeability, cell injury and pro-inflammatory signaling[16].

4-1 Biochemical pathway of ganglioside catabolism



Pathway of ganglioside catabolism adapted from Miklavcic et al.[14] A-series gangliosides are highlighted in red-dash boxed area and b-series gangliosides are highlighted in black-dash boxed area. Enzyme catalyzing specific metabolic processes are adjacent to respective arrows. Cer = ceramide; NEU3 = sialidase; HEXA = beta-hexosaminidase A.

The present study demonstrates an elevation in ganglioside catabolism and corresponding alteration in ganglioside content of intestinal mucosa in IBD. Moreover, ganglioside and phospholipid in healthy intestine is characterized by a higher proportion of polyunsaturated

constituents than IBD intestine. Finally, consumption of ganglioside improves intestinal integrity independently of effects on acute systemic inflammatory mediators.

4.20 Methods

Ethics approval for this study was obtained from the Biomedical Panel of the University of Alberta Health Research Ethics Board. The intervention study is registered as a clinical trial (NCT02139709, clinicaltrials.gov).

4.21 Analysis of ganglioside metabolism in intestine

Patients with IBD (N=11) requiring surgical resection of bowel were recruited from the University of Alberta Hospital surgical program. Surgical referral was based upon usual and accepted standards of care and included patients with indications such as hemorrhage, obstruction and perforation. Male and non-pregnant female adults (>17 years of age) were eligible for study. Patients with inadequate liver or renal function, active infectious disease, previous bowel resection, history of alcohol/drug abuse or other serious medical conditions were excluded from study. Diagnosis of IBD was based on established radiologic, endoscopic, and histologic criteria. Patients were taking a variety of medications preoperatively for their IBD, such as melamine, corticosteroids, immunosuppressants, anti-TNF-α biological agents or antibiotics. A first control group (N=6) consisted of participants undergoing bowel resection for benign adenomatous polyposis (BAP). The BAP group (non-familial, non-malignant) was characterized by >10 polypoid lesions (maximum diameter: 10–20 mm) localized in the descending colon. A second age- and sex-matched control group (N=12) consisted of intestine supplied from the Canada Breast Cancer Foundation Tumour Bank (Calgary, Canada). This control tissue consisted of non-cancerous regions of large bowel from participants with low-to-moderate grade CRC with no known metastases, chemo- or radiotherapy treatment.

Intestine from terminal ileum or colon was obtained from surgical sections of viable tissue in the vicinity of the most prominent ulcerative lesion in patients with IBD. Visually normal regions of intestine were excised from surgical sections of control tissue at least 10 cm from the closest polyp. Mucosa was separated from the bowel wall by scraping with a glass slide. The sample was collected in a sterile cryovial before being snap-frozen in liquid nitrogen within 20 min of devascularisation and stored at -80°C until analysis.

4.22 Ganglioside profiling

Gangliosides were isolated from tissues using a modified Folch extraction[17]. The final supernatant was used for ganglioside profiling (as in Section 3.24). Aqueous extracts were injected onto a Poroshell 120 EC-C18 column (Agilent). Gangliosides were separated using reverse-phase liquid chromatography and the eluent was directed to the inlet of a QQQ 6430 MS (Agilent) operated in negative ion mode. Electrospray ionization generated deprotonated gas-phase ions from the various ganglioside species. The MS was operated in MRM mode to provide selective and sensitive ganglioside detection by allowing only select precursor ions and characteristic gas-phase fragments to be detected. The mass spectra were screened against a library of theoretical precursor ions from over 600 gangliosides with variable ceramide and carbohydrate compositions. The relative percent of ganglioside subspecies GM1, GT3, GM3, GD1a, GD3 and GT1 was determined using Mass Hunter Qualitative Analysis software.

4.23 Phospholipid profiling

Phospholipids were isolated from tissues using a modified Folch extraction[17]. Organic extracts were frozen at -20°C until analysis. Samples were thawed at room temperature, dried under nitrogen and resuspended in 75% acetonitrile, 25 % water. Samples were subjected to normal phase chromatography with an Agilent Zorbax RX-Sil column (3.0 x 100 mm, 1.8 µm particle

size) using a 1260 Infinity LC system (Agilent). The mobile phase was composed of 75% acetonitrile, 25% water/methanol (50/50) with 5 mM ammonium acetate and 0.01% acetic acid. The total LC run time was 10 min at a flow rate of 0.5 µl/min. All MS measurements were obtained using a 6430 QQQ LC/MS system (Agilent) operating in positive ion mode. Protonated gas-phase ions of the various phospholipid species were obtained using electrospray ionization, with the electrospray needle held at 4500 V. The MS was operated in MRM. A library of theoretical precursor ions was generated for PC and PE with various predicted fatty acid compositions. The first quadruple mass filter was set to scan for these specific precursor ions, allowing each to sequentially pass into the hexapole collision cell where ions were fragmented using collision induced dissociation (CID). PC species readily undergo head group specific fragmentation, so the second mass filter was set to monitor m/z = 184. For PE species, which fragment with the neutral loss of 141 mass units, the second mass filter monitored the pre-cursor m/z minus 141. The CID and ion source voltages for each phospholipid class were optimized using the Agilent Optimizer software. Data acquisition and analysis was carried out using the Agilent Mass Hunter software package.

4.24 Ganglioside catabolism

Western blotting was performed to analyze HEXA and NEU3 content in intestinal samples. Total protein was extracted from intestinal mucosa using T-PER Tissue Protein Extraction Reagent (Fisher Scientific) in the presence of protease inhibitor (Fisher Scientific). The RC DC Protein Assay Kit II (Bio-rad) small volume method was used to quantify total protein using a SPECTRAmax 190 (Molecular devices) against a known concentration of bovine serum albumin (BSA) standard. The supernatant was collected and stored at -80˚C until analysis. Standard ladder (Bio-rad, #161-0305) and 10 µg of protein were loaded into 10% (w/v) sodium dodecyl

sulphate-polyacrylamide gel (Sigma) after boiling samples (2 min). Protein electrophoresis was conducted at 200 V using the SE260 mini-vertical electrophoresis unit (Hoefer).

Gel was transferred onto nitrocellulose membrane using the TE22 Mighty Small Tank Transfer unit (Hoefer). Following transfer, membrane blots were blocked using 5% BSA in tris-buffered saline with 0.1% tween 20 (TBST) (pH 7.6). Blots were incubated (1 hr) with primary antibodies diluted in 5% BSA in TBST (w/v): 1:250 rabbit polyclonal IgG anti-HEXA, 1:7000 rabbit polyclonal IgG anti-NEU3, 1:2500 rabbit polyclonal IgG anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam). After incubation, blots were rinsed twice with TBST. Blots were incubated with 1:2000 horseradish peroxidise-conjugated goat anti-rabbit IgG secondary antibody (Cell Signalling Technology) at room temperature (1 hr). Blots were rinsed as before, then incubated with enhanced chemiluminescence prime reagent (GE Healthcare) as per manufacturer instructions. The blot was imaged using a Typhoon 8600 Variable Imager and Scanner (Amersham Biosciences GE) and the image was analyzed with ImageQuant software. Protein bands were manually selected to determine band density.

4.25 Impact of ganglioside intake

Healthy control participants, 22 to 56 years of age were recruited for an 8-wk double-blind, randomized, placebo-controlled study to demonstrate the efficacy of ganglioside for potential treatment of IBD (as per Section 3.21, Section 3.22). Participants were allocated to receive either a milk fat fraction with or without ganglioside. Individuals in the ganglioside group consumed 1.0 g of milk fat fraction (ZETA dairy lipid, Fonterra) containing approximately 43 mg of ganglioside [80% GD3; 20% GM3 (w/w)] daily for 8 wk. Participants maintained written records throughout the 8-wk study to document ganglioside supplement and medication use.

4.26 Intestinal permeability

Permeability was assessed according to Meddings *et al*.[18]. Briefly, participants consumed a beverage containing sucrose, lactulose and mannitol probes. All voided urine was collected for 5 hr post-ingestion into bottles containing a thymol (10% v/v isopropanol) solution. Urine was kept at 4˚C until total volume was measured and stored at -80˚C. Sugar probes were measured by high-performance LC[18]. The ratio of L/M was computed as an index of intestinal permeability. Sucrose concentration was measured to assess gastric permeability.

4.27 Inflammatory mediators

Study participants had blood drawn at baseline and at 2-wk intervals throughout the intervention study. Plasma LTB_4, PGE_2 and TNF-α (R&D Systems) was measured as per manufacturer protocol at baseline and at wk 6 of the supplementation study.

4.28 Statistics

As a pilot study, no formal sample size calculation was calculated. Mean and standard error of the mean was computed for relative ganglioside and phospholipid content of intestine. Average relative content of ganglioside classes (as a proportion of total ganglioside) and phospholipid classes (as a proportion of total phospholipid) was compared among study groups using an ANOVA. Content of HEXA and NEU3 was standardized to content of GAPDH with intestinal specimens as a method of quantitation for comparison among study groups using an ANOVA. Mean and standard error of the mean was computed for relative ganglioside and phospholipid content of intestine containing 0, 1, 2 and 3 (or more) unsaturated bonds. Relative content of saturated, monounsaturated and polyunsaturated constituents within ganglioside and phospholipid classes was compared among study groups using an ANOVA. Change in intestinal permeability was compared between ganglioside and placebo groups using a one-tail Mann-

Whitney U-test. Plasma LTB_4, PGE_2 and TNF-α was compared among study time points and between treatment groups using a repeated measures ANOVA. Significance was defined as $\alpha<0.05$. Patients with IBD were not stratified by drug intake as corticosteroids[19] have not been observed to influence glycosphingolipid metabolism and the small number of participants taking immunosuppressants and biological agents stopped therapy at least 30 d prior to surgery.

4.30 Results

4.31 Ganglioside and phospholipid profiles of intestine

The average relative ganglioside content of normal intestine from participants with BAP was: GM1 not detected, 0.7±0.2 GT3, 7.7±2.1 GT1a, 27.5±3.9 GM3, 26.3±3.0 GD3, 38.0±4.0 GD1a. The average relative ganglioside content of normal intestine from participants with CRC was: GM1 not detected, 0.3±0.1 GT3, 4.6±2.6 GT1a, 24.4±5.5 GM3, 39.4±4.0 GD3, 31.7±3.1 GD1a. No difference was observed in content of gangliosides between BAP and CRC control groups. The average relative ganglioside content of intestinal mucosa from participants with IBD was: 0.1±0.1 GM1, 0.8±0.3 GT3, 7.2±1.6 GT1a, 48.6±7.0 GM3, 31.4±5.0 GD3, 11.6±2.7 GD1a. Average relative GM3 content was increased by 2-fold ($P<0.01$) in IBD intestine compared to BAP and CRC control groups. Control intestine groups exhibited about 3-fold higher GD1a content ($P<0.001$) than IBD intestine (Table 4-2A).

Control participants had about 5-fold greater GD3 ($P<0.001$) with 2 unsaturated bonds and about 3.5-fold greater GD1a ($P<0.001$) with 2 unsaturated bonds than patients with IBD. IBD group exhibited less GD3 and GD1a ($P<0.001$) with 3 unsaturated bonds in the fatty acid ceramide component than BAP and CRC control groups. Ganglioside GD3 and GD1a with a ceramide constituent composed of 3 unsaturated bonds was not detected in IBD intestine (Table 4-2B).

4-2 Content and proportion of unsaturated bonds in GM3, GD3 and GD1a gangliosides in control and IBD intestine

A. Content of ganglioside classes in intestine

	Control (BAP) (N=6)	Control (CRC) (N=12)	IBD (N=11)	Pooled SEM
GM3	27.50[a]	24.42[a]	48.55[b]	3.78
GD3	26.33	39.42	31.36	2.43
GD1a	38.00[A]	31.67[a**]	11.55[b**]	2.62

B. Composition of ganglioside classes in intestine

	Unsaturated Bonds	Control (BAP)	Control (CRC)	IBD	Pooled SEM
GM3	0	5.73[a]	2.77[b]	3.3[ab]	0.41
	1	69.96	73.40	68.34	1.30
	2	21.86	21.62	25.24	1.05
	3	2.45	2.22	3.10	0.21
GD3	0	9.28	6.18	5.91	1.18
	1	59.39[a**]	58.67[a**]	87.88[b**]	2.91
	2	28.28[a**]	32.98[a**]	6.21[b**]	2.45
	3	3.09[a**]	2.18[a**]	nd[b**]	0.3
GD1a	0	5.32[ab]	6.8[a]	3.11[b]	0.68
	1	64.95[a**]	59.26[a**]	89.93[b**]	3.05
	2	26.21[a**]	30.51[a**]	8.06[b**]	2.44
	3	3.55[a**]	3.43[a**]	nd[**b**]	0.44

*Total GM3, GD3 and GD1a are reported as average relative percent of total ganglioside within participant groups (Table A). Major classes of gangliosides are stratified by number of unsaturated bonds and reported as average relative percent of total respective ganglioside (Table B). Lowercase superscript letters (a, b) denote differences (P<0.05) and asterisked superscript letters (a**, b**) denote differences (P<0.001) among study groups. Ganglioside*

species constituting less than 8% of total ganglioside are not listed (GT1a, GM1, GT3). BAP = benign adenomatous polyposis; CRC = colorectal cancer; IBD = inflammatory bowel disease; SEM = standard error of the mean; nd = not detected.

IBD group exhibited less (P<0.05) PC with three or more unsaturated bonds than BAP and CRC control groups. Control participants had about 1.5-fold greater PC with at least three unsaturated bonds than patients with IBD. There were no differences among participant group intestine in PC containing zero, one or two unsaturated bonds (Table 4-3A). IBD group exhibited less (NS) PE with three or more unsaturated bonds than BAP and CRC control groups. Control participants had about 1.4-fold greater PE with at least three unsaturated bonds than patients with IBD. In addition, the relative content of PE containing two unsaturated bonds is higher (NS) in IBD intestine as compared to control intestine groups. There were no statistically significant differences among participant group intestine in PE containing zero, one, two or more than two unsaturated bonds (Table 4-3B).

4-3 IBD intestine is characterized by lower PUFA content in phosphatidylcholine and phosphatidylethanolamine

A. Phosphatidylcholine composition of intestine

Unsaturated Bonds	Control (BAP)	Control (CRC)	IBD	Pooled SEM
0	9.82	10.05	12.43	0.76
1	6.14	7.77	8.8	0.59
2	53.78	54.57	58.87	1.69
>2	30.26[a]	27.61[a]	19.9[b]	1.38

B. Phosphatidylethanolamine composition of intestine

Unsaturated Bonds	Control (BAP)	Control (CRC)	IBD	Pooled SEM
0	2.23	4.4	3.73	0.23
1	14.04	13.65	18.65	1.05
2	30.05	33.7	40.3	2.08
>2	53.68	48.25	37.32	2.74

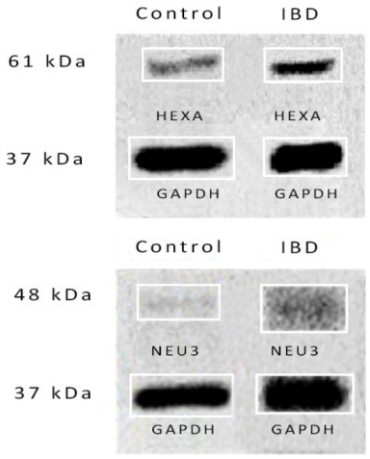
The mean relative content of phospholipid classes stratified by the number of unsaturated bonds is presented for PC (Table A) and PE (Table B). Lowercase superscript letters (a, b) denote differences (P<0.05) among study groups. BAP = benign adenomatous polyposis; CRC = colorectal cancer; IBD = inflammatory bowel disease; SEM = standard error of the mean.

4.32 Ganglioside catabolism in intestine

Ganglioside catabolic enzymes HEXA and NEU3 were measured in normal intestinal mucosa and compared to intestinal mucosa from participants with IBD (Figure 4-4A). The mean relative expression of HEXA in CRC and IBD groups was 0.06±0.01 and 0.09±0.03, respectively. The mean relative expression of NEU3 in CRC and IBD groups was 0.02±0.01 and 0.18±0.03, respectively (Figure 4-4B). The mean relative expression of HEXA in IBD exhibited 1.7-fold (P<0.05) increase in comparison to the control intestine. The mean relative expression of NEU3 in IBD was increased 8.3-fold (P<0.001) versus the control group.

4-4 Ganglioside catabolism enzymes HEXA and NEU3 are elevated in IBD

A. Representative western blot of ganglioside catabolism enzymes in intestine



B. Comparison of ganglioside catabolism enzyme content in intestine

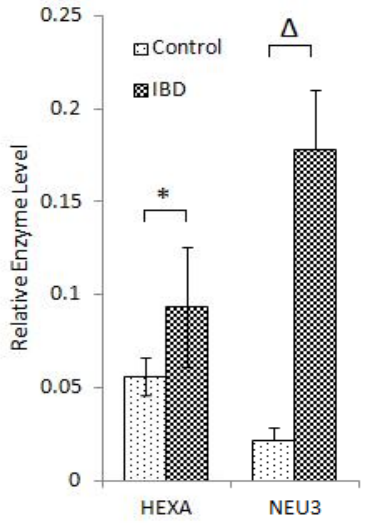


Figure A shows representative western blot of enzymes HEXA and NEU3 which regulate ganglioside catabolism (N=9 CRC Control, N=7 IBD). Bars in Figure B report the mean protein density (with standard error bars) of intestinal HEXA and NEU3 content expressed as a proportion relative to GAPDH (, P<0.05; Δ, P<0.001). HEXA = beta-hexosaminidase A; IBD*

= inflammatory bowel disease; NEU3 = sialidase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

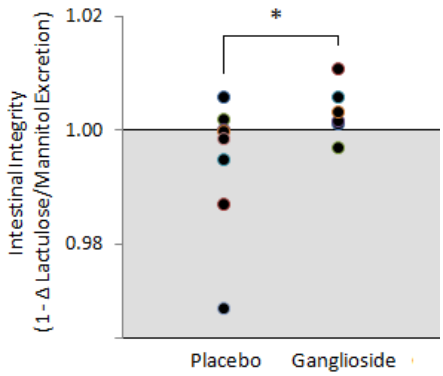
4.33 Intestinal permeability

Healthy control participant characteristics for the intervention study are reported in Table 3-2. Participants consuming ganglioside exhibited a reduction in intestinal permeability and enhanced intestinal integrity compared to those consuming placebo (P=0.04). Seven participants receiving ganglioside exhibited an average 19% decline in intestinal permeability over eight weeks of treatment (Figure 4-5). Six of the seven participants receiving ganglioside treatment exhibited lower permeability over course of the study. The only participant consuming ganglioside that did not demonstrate lower intestinal permeability had a high baseline level of intestinal integrity. The excretion of sucrose did not change among test groups over the study period (data not shown). As sucrose is used to probe permeability of the upper gastrointestinal tract[20], the decrease observed in permeability is attributed to positive effects of ganglioside in intestinal epithelium rather than gastric epithelium.

4.34 Inflammatory markers

Consuming ganglioside did not change plasma concentrations of LTB_4, PGE_2 or TNF-α (Table 4-6) from baseline (31.08 pg/mL ±4.86, 407.13 pg/mL ±74.56, 17.98 pg/mL ±11.79, respectively) to week six (30.74 pg/mL ±3.70, 421.21 pg/mL ±109.05, 26.54 pg/mL ±22.02). LTB_4, PGE_2 and TNF-α did not change from baseline to week six in the placebo group and did not differ from baseline levels in the ganglioside group (data not shown).

4-5 Consumption of ganglioside improves intestinal permeability



Individual data points represent the change in intestinal permeability of a participant over eight weeks (N=8 placebo, N=6 ganglioside). White area represents improved intestinal permeability and shaded area indicates increase in intestinal permeability. Intestinal integrity increased 19% on average (, P=0.04) in the group consuming ganglioside.*

4-6 Ganglioside intake does not alter acute systemic inflammatory mediators in plasma

	LTB4 (pg/ml)	PGE2 (pg/ml)	TNFα (pg/ml)
Week 0	31.1 ±4.9	407.1 ±74.6	18.0 ±11.8
Week 6	30.7 ±3.7	421.2 ±109.1	26.5 ±22.0
	NS	NS	NS

Plasma LTB_4, PGE_2 and TNF-α did not differ at 6 weeks of study between ganglioside or placebo groups (data not shown) and did not differ from baseline measures (N=9 placebo, N=8 ganglioside). LTB_4 = leukotriene B_4; PGE2 = prostaglandin E_2; TNF-α = tumor necrosis factor-alpha; NS = not significant.

4.4 Discussion

The significance of this study resides in novel data showing that ganglioside content and enzymatic regulation of ganglioside metabolism differ between healthy and IBD intestine. Moreover, provision of dietary ganglioside may improve intestinal integrity. These observations

lead to an innovative hypothesis that IBD occurs as a consequence of a difference in ganglioside signaling which impacts intestinal barrier and immunological functions altered in IBD. We propose that increased ganglioside metabolism to produce specific gangliosides in intestinal mucosa is fundamental to the etiology of IBD. This new paradigm also suggests that remission could potentially be achieved by increasing intake of appropriate gangliosides to restore normal ganglioside balance in diseased intestine thus reducing intestinal permeability and enhancing intestinal barrier integrity.

The relative quantity of GM3 is increased and content of GD1a is decreased in inflamed intestinal mucosa from patients with IBD (Table 4-2A). HEXA is a lysosomal enzyme that catalyzes conversion of GM2 to GM3 and NEU3 is primarily located at the cell surface where it catalyzes conversion of GD3 to GM3 and GD1a to GM1 (Figure 4-1). The elevation in GM3 and lower content of GD1a in IBD (Table 4-2) is corroborated by the increase observed in HEXA and NEU3 protein content (Figure 4-4). Knock-down of sialidase with small interfering RNA reduces susceptibility of mice to colitis-associated colon carcinogenesis[21]. Increased NEU3 content in IBD intestine may degrade beneficial ganglioside which promote a pro-inflammatory environment in gut and may explain, in part, the elevated risk for development of colon cancer in IBD.

The relative abundance of GD3 was not different among study groups, however; polyunsaturated (3 unsaturated bonds) GD3 and GD1a species were not detected in IBD (Table 4-2B). NEU3 may preferentially cleave sialic acid from species of GD3 and GD1a that contain ceramide having a higher number of unsaturated fatty acid constituents. Similar modes of enzyme specificity have been observed in the regulation of essential fatty acids by desaturase enzymes[22]. Other observations in previous studies have revealed that the status of long chain n-3

and n-6 PUFA are higher and linoleic and α-linolenic acid precursors are lower in active CD and UC than inactive disease[23-25]. Lower content of GD3 and GD1a with 3 unsaturated bonds in IBD intestine may reflect decreased intake of α-linolenic acid (18:3n-3) in patients IBD or an increased demand of PUFA for production of resolvins used in resolution of inflammation (Section 1.36).

IBD intestine also exhibited lower PUFA content in PC compared to control intestine (Table 4-3). Patients with previous bowel resection were excluded as intestinal resection has been associated with changes in phospholipid class and composition[26]. Evidence suggests that there is low dietary intake of PUFA in patients with IBD[27]. Results from the European Prospective Investigation into Cancer and Nutrition study suggest that about 30% of UC cases could be attributed to very high intakes of n-6 PUFA[28]. Together, this evidence and results from this study (Table 4-2, Table 4-3) suggest that patients with IBD may have higher n-6:n-3 fatty acid consumption. Mice harbouring a mutation that enables production of n-3 fatty acids from n-6 fatty acids have an increase in n-3 fatty acid status in all tissues including colon and have longer colon length and decreased histological score compared to wildtype mice[29]. These observations suggest a possible role for modulating n-3 PUFA content of intestinal ganglioside and phospholipid for improved outcomes in IBD.

Patients with CD have compromised gut-barrier function and decreased intestinal integrity[30]. Ganglioside increased intestinal integrity as indicated by decreased excretion of L/M over eight-week supplementation period. The ratio of L/M which assesses the amount of epithelial cell damage as a proportion of small intestine[31] reported in this study is similar to the range of permeability measures reported in a previous study of CD[32]. Compromised intestinal barrier is associated with systemic inflammation[33] which may be caused by bacterial endotoxin[34].

No change in acute systemic inflammatory markers LTB_4, PGE_2 or TNF-α (Table 4-6) accompanied the increase observed in intestinal integrity. Intake of dietary ganglioside may not have resulted in a decrease in pro-inflammatory mediators as a cohort of healthy participants may not necessarily have elevated plasma levels of LTB_4, PGE_2 and TNF-α. Alternatively, ganglioside may improve intestinal permeability by mediating tight junction integrity rather than by influencing production of inflammatory mediators. The proposed mechanism of action is supported by a study showing that ganglioside GD3 increases intestinal integrity in animals by mediating the tight junction protein occludin[15].

The present study showed that intake of milk fat fraction enriched in ganglioside GD3 has positive effects on intestinal permeability and evidence from previous studies suggest that ganglioside high in PUFA content locates primarily to lipid rafts where interaction with proteins protect the tight junction and support intestinal integrity[15,35-39]. Intestinal permeability is mediated by tight junction proteins[35] which are located primarily in lipid rafts[36]. Treatment with PUFA prevents displacement of tight junction proteins from lipid rafts and attenuates histological score in a rat model of colitis[37]. Furthermore, low level of GD3 in intestinal mucosa is associated with degradation of tight junction proteins[15] and increasing GD3 content of intestine reduces degradation of tight junction proteins, thus improving integrity and reducing intestinal permeability. This study showed a decrease in the relative content of polyunsaturated GD3 in IBD intestine compared to control intestine (Table 4-2B). Membranes containing saturated ceramides form gel domains and are more ordered than membranes with the corresponding unsaturated ceramide[38]. Accordingly, gangliosides containing ceramide with higher PUFA content localize to lipid rafts and form the sealing elements of tight junctions[39].

The role of PUFA, particularly as a component of GD3 ganglioside is important in stabilizing tight junction proteins in the lipid rift and supporting gut-barrier function.

Metabolic kinetics of GD3 has been described in Caco-2 cells[13]. Schnabl *et al.* have shown that GD3 is absorbed by epithelial cells at the intestinal brush border membrane and is mainly metabolized into new ganglioside species, with smaller portions being retained or transferred, whereas GD3 taken up by the basolateral membrane is not retained or transferred to any significant degree[13]. Moreover, ganglioside incorporated in mucosal cells of intestine from diet can be used for further ganglioside biosynthesis[11,40]. These observations suggest that each species of ganglioside has specific functions which depend on site of uptake. These studies suggest that regions of intestine damaged in IBD could receive dietary ganglioside via basolateral uptake and transfer to apical and lateral membranes where protective effects on intestine may be exhibited (Section 1.21). The high bioavailability of ganglioside (Figure 3-4) also suggests that ganglioside can circulate to colon for basolateral uptake to potentially benefit patients with ulcerative colitis.

Collectively, the present study shows that metabolism of specific gangliosides is altered at the level of intestinal mucosa in IBD and that ganglioside is a bioavailable dietary compound (Figure 3-4) having beneficial effects on intestinal permeability (Figure 4-5). This study suggests that providing GD1a and polyunsaturated GD3 shown to be deficient in inflamed intestine (Table 4-2) may correct the altered content and balance of gangliosides in the intestinal mucosa. Dietary provision of GD3 is bioavailable and can improve the defective barrier integrity present in IBD.

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CHAPTER 5. GENERAL SUMMARY AND DISCUSSION

5.1 Summary of hypotheses posed

The overall hypothesis of this thesis research is that altered ganglioside metabolism is fundamental to the etiology of IBD and that supplemental ganglioside consumption can attenuate altered physiologic processes characteristic of IBD. This hypothesis was explored in two studies.

Study 1: Safety, bioavailability and metabolism of ganglioside in human participants

Study 1 hypothesis was investigated in Chapter 3. To show that **ganglioside is safe and bioavailable for consumption and potential treatment of IBD**, the following objective were studied:

i. To determine the safety of supplemental ganglioside by analyzing adverse event reports and blood chemistry and immune cell measures assessed in participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period.

ii. To determine the bioavailability of dietary ganglioside by measuring plasma ganglioside content (GM3, GD3) in participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period.

iii. To determine the effect of supplemental ganglioside on ganglioside metabolism by measuring plasma HEXA activity in participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period.

iv. To determine whether patients with IBD may benefit from ganglioside supplementation, an IBDQ was administered to participants consuming ganglioside at weeks 0, 2, 4, 6 and 8 of the supplementation period.

By investigation of Study 1 (i), it was determined that consuming ganglioside does not incur any adverse effects when compared to a milk fat fraction void of ganglioside (Table 3-2). In addition, there were no clinically significant changes in blood chemistry or immune cell measures associated with supplemental ganglioside consumption of eight weeks (Table 3-3). Therefore, supplemental ganglioside is safe and tolerable for daily consumption. In investigating Study 1 (ii), consuming ganglioside did not affect total or C34:1 plasma GM3 level over eight weeks (Figure 3-5). Total and C34:1 plasma GD3 content increased from baseline to weeks two and eight by 35-40% in a group of healthy control participants and patients with IBD consuming supplemental ganglioside (Figure 3-4). Consumption of GD3 leads to an increase in plasma GD3 content within two weeks and daily ganglioside intake sustains increased plasma GD3 content for eight weeks. By investigating Study 1 (iii), it was shown that consumption of ganglioside did not increase plasma HEXA activity over eight weeks (Figure 3-6). The catabolism of ganglioside is not increased when plasma ganglioside concentration is increased by diet. As shown in investigation of Study 1 (iv), consuming ganglioside is associated with increased quality of life in patients with IBD by improving emotional health and systemic symptoms over eight weeks (Figure 3-7). There was no significant effect of ganglioside on quality of life measures of bowel symptoms or social function assessed by IBDQ. Study 1 hypothesis is supported by results of the four corresponding objectives. Specifically, ganglioside consumption is a safe and bioavailable treatment which has positive effects on quality of life in patients with IBD.

Study 2: Ganglioside catabolism is elevated in IBD: importance of dietary ganglioside intake Study 2 hypothesis was investigated in Chapter 4. To show that **the content and composition of ganglioside and phospholipid classes differ among IBD and healthy intestine and that ganglioside intake increases intestinal integrity**, the following objectives were studied:

i. To determine whether the class of ganglioside generated by HEXA (GM3) is elevated and substrates of NEU3 (GD3, GD1a) are decreased in active IBD, the content of gangliosides were measured in intestine from participants with IBD and compared to healthy intestine from non-IBD participants.

ii. To determine whether ganglioside catabolism is increased in active IBD, the content of ganglioside catabolism enzymes (HEXA, NEU3) were measured in the intestine of patients with IBD and compared to healthy intestine from non-IBD participants.

iii. To determine whether ganglioside (GM3, GD3, GD1a) and phospholipid (PC, PE) have decreased PUFA content in active IBD, the number of unsaturated bonds in ganglioside and phospholipid classes were assessed in intestine from patients with IBD and compared to healthy intestine from non-IBD participants.

iv. To determine whether ganglioside intake improves intestinal integrity in healthy human participants, an oral L/M challenge was administered to participants consuming ganglioside at baseline (week 0) and study conclusion (week 8).

v. To determine whether ganglioside intake decreases levels of acute systemic inflammatory mediators in healthy human participants, cytokine (PGE_2, LTB_4, TNF-α) assays were performed in participants consuming ganglioside at baseline and week 6 of the supplementation period.

In investigation of Study 2 (i), it was determined that GM3 was twice as high in intestine from patients with IBD than control intestine. GD1a was three times higher in control intestine than intestine from patients with IBD (Table 4-2A). Ganglioside metabolism is altered in IBD intestine and altered ganglioside content is fundamental to the etiology of IBD. These observations (Study 2: i) were corroborated in Study 2 (ii) where increased content of HEXA and

NEU3 were observed in IBD intestine (Figure 4-4). HEXA was almost two times higher and NEU3 was about eight times higher in IBD intestine than control intestine. As shown in Study 2 (iii), the proportion of GD3 and GD1a with two or three unsaturated bonds was lower in intestine from patients with IBD than controls (Table 4-2B). Furthermore, the polyunsaturated constituents of phospholipid classes (PC, PE) was lower is IBD intestine than in healthy intestine (Table 4-3). The composition of ganglioside and phospholipids is altered in IBD. Specifically, intestine from participants with IBD has lower PUFA content than control intestine. It was shown in Study 2 (iv) that consumption of ganglioside leads to improvement in intestinal integrity versus consumption of milk fat fraction void of ganglioside (Figure 4-5). Dietary ganglioside may be of benefit in treatment of IBD by reducing intestinal permeability. Lastly, there was no observed effect of ganglioside on systemic markers of acute inflammation LTB_4, PGE_2 or TNF-α (Table 4-6) as investigated in Study 2 (v). This finding suggests that the effect of ganglioside on improving intestinal integrity (Study 2: iv) may occur independently of changes in systemic inflammatory markers. Study 2 hypothesis is supported by results of the five corresponding objectives. Specifically, the content and composition of intestinal ganglioside differs among IBD and control participants and dietary intake of ganglioside improves intestinal integrity irrespective of effects on acute systemic inflammatory markers in human participants. The overall hypothesis **that altered ganglioside metabolism is fundamental to the etiology of IBD and that supplemental ganglioside consumption can attenuate altered physiologic processes characteristic of IBD** was supported by evidence from the current studies. Altered ganglioside metabolism is fundamental to the etiology of IBD and supplemental ganglioside is a safe, bioavailable, functional dietary treatment with potential therapeutic application for treatment of IBD.

5.20 General discussion

The studies conducted as part of this thesis work show that altered ganglioside metabolism is fundamental to the etiology of IBD. Dietary ganglioside was demonstrated to be safe and bioavailable in healthy human participants and patients with IBD. Consumption of ganglioside has beneficial physiologic effects on intestinal integrity in healthy participants and is associated with improved quality of life in patients with IBD.

Study of patients with IBD requires several design considerations due to substantial heterogeneity in presentation. Study variables which require matching or control in design (inclusion/exclusion criteria) or statistical analyses include age at diagnosis, disease activity index (CD activity index, Mayo score), location of disease, behaviour of disease, medication use, failure of response to medication and prior surgery. Due to the plethora of factors which contribute to pathogenesis of IBD, optimizing drug and diet treatment regimens are also a challenge. Generalized recommendations for dietary intake are not usually advised to individuals living with IBD. Patients are usually counselled individually to identify triggers for disease and advised accordingly. Based on the beneficial findings in this study, it is of interest to pursue whether ganglioside supplementation may be universally recommended for patients with IBD.

5.21 Study design

The gold standard of clinical trials includes a placebo-controlled arm of study. In a clinical intervention, a placebo arm is necessary to measure the true treatment effect. The placebo arm measures the effect of intervention while the treatment arm measures the sum net effects of intervention and treatment agent. The effect of treatment in longitudinal analysis cannot necessarily be inferred without the placebo arm. Without a placebo arm, it is unknown whether effects in the treatment arm are due to the therapeutic agent or due simply to

intervention or standard of care. The difference in longitudinal effect between treatment and placebo arms in patient cohort studies helps to discern the true effect of a potential therapeutic agent. The strengths of the ganglioside intervention study lie in inclusion of a placebo arm for healthy participants and in longitudinal analysis of both healthy participants and patients with IBD receiving supplemental ganglioside.

A role for the microbiome in gut health has been proposed[1] but the precise mechanisms and modes of action are largely unknown. The microbiome is comprised of a number of diverse species of microorganism that reside in mucous membranes of the body, particularly in the intestine. Constitutive activation of pro-inflammatory pathways are believed to contribute to pathogenesis of IBD and the presence of specific microbiota in gut may confer tolerance of surveillance immune cells in mesenteric lymph nodes and Peyer's patches to ultimately disrupt the constitutive pro-inflammatory cascade. It has been hypothesized that the presence or consumption of beneficial microbiota may compete with other potentially enteropathogenic microorganisms and prevent or manage episodes related to IBD[1]. However, the microbiome is transient and differs within and between individuals on the basis of age, genetics, geography, dietary intake and medication use[2]. The microbiome is arguably as diverse among healthy individuals as it is between healthy and disease states. Thus, discerning intestinal dysbiosis directly involved in the etiology in IBD from aberration in intestinal microbiota uninvolved in intestinal pathology remains a challenge.

There can be considerable difficulty in controlling for dietary intake statistically in studies of IBD. Attempts however can be made to stratify participant populations by food intolerances to agents like lactose and gluten to limit potential confounding of dietary intake. Furthermore, the ability to utilize certain nutrients may be compromised based on previous

bowel resection or based on the region(s) of intestine affected by disease. To this regard, patients with overt food intolerance or having undergone previous intestinal surgery were excluded from recruitment in the studies described in this thesis. The recruitment of Crohn's colitis and ileitis patients in this study is not believed to be confounded by region of intestine with respect to measures of ganglioside because previous study has shown that the ratio of GD3 to GM3 ganglioside does not differ between colon and small intestine[3]. Thus, the study design adequately addresses potential confounders including food intolerance, previous intestinal surgery and region of intestine affected by disease.

5.22 Participant recruitment

The appropriateness of control group is another challenge in studies investigating the etiology of IBD. As healthy colon tissue may not be readily available in sufficient quantity, the use of tissue excised from surgeries or biopsy constitute a viable alternative to serve for control comparison. Use of healthy regions of tissue from patients with CRC requires unique considerations. Inflammation may be present in malignant tumours of the colon. To properly distinguish the inflammatory phenotype of IBD from control CRC tissue, healthy tissue sampled from CRC needs to be sufficiently distant to the tumour. Accordingly, CRC control tissue assayed in this study was sampled at least 10 cm from tumour. Anterior resection cases were excluded as anterior resection of colorectal tumours is often treated with radiotherapy prior to surgery. Thus, anterior resection cases may be inappropriate for control comparison to IBD intestine for the purposes of ganglioside analysis since ganglioside content and exposure to radiation are interrelated[4]. To measure the ganglioside content of inflamed tissue from participants with IBD and to discern how ganglioside content differs from healthy intestine, healthy control tissue was

sampled sufficiently distant from CRC tumours and cases treated with radiotherapy were excluded.

As patients with IBD are at increased risk of developing CRC, longitudinal analysis of patients with IBD may yield novel insight into the appropriate use of healthy tissue from excised CRC specimens for control comparison. Intestine of patients with IBD that develop CRC later in life may resemble CRC tissue more than healthy tissue as Slaughter's concept of field cancerization proposes that there are underlying genetic changes in tissue that proceed oncogenic transformation[5]. Analysis of intestinal tissue from patients with IBD that later develop neoplasm(s) may inform early changes in metabolism that lead to development of cancer. Findings could inform measures of intervention at an early preventative stage for optimized outcomes or cancer prevention; and consequent decreased mortality and reduced IBD-associated morbidity. Another possible region of control comparison consists of healthy tissue in patients with IBD as a measure of internal control. This strategy was not employed in the thesis study however as fatty acid metabolites generated by LOX, like LTB_4 are similar between inflamed tissue and adjacent-to-inflamed tissue[6].

Apparently normal intestinal mucosa from participant with benign colon polyps may serve as another appropriate control group for comparison to IBD intestine. Normal intestinal tissue from patients with familial BAP however may more closely resemble that of colon cancer than healthy colon[7,8]. Accordingly, this study used normal regions of excised intestine from non-familial cases of benign colon polyps. Comparison to alternative control groups/tissues like healthy intestinal mucosa from participants undergoing screening and biopsy for CRC and found to be negative, or from participants being investigated for symptoms related to irritable bowel syndrome is warranted in future study. This study benefits from comparison of intestinal mucosa

from patients with IBD to two groups of healthy control intestine from BAP and CRC participants and from the high correlation between control intestine groups with respect to relative ganglioside content (Table 4.2A) and composition (Table 4.2B) and phospholipid composition (Table 4-3).

5.23 Ganglioside bioavailability

This is the first study to show an increase in plasma ganglioside GD3 content (Figure 3-4) when participants consume a supplemental milk fat fraction containing ganglioside. Increase in plasma ganglioside content was noted two weeks after study initiation (Figure 3-4). It is of interest in future studies to determine a dose-response relationship to ganglioside intake and to measure plasma ganglioside content closer to study initiation (< two weeks after start of supplementation regime). Determining the length of time required to increase tissue ganglioside content after beginning supplementation regime and other pharmacokinetic parameters may inform optimal therapeutic dosing regimen. It is also of interest to discern which species of tissue ganglioside (in addition to monounsaturated C34 GD3; Figure 3-4) are modifiable by intake of dietary ganglioside. Knowledge of this information could inform formulation of specific ganglioside supplement(s) for optimal health outcomes in the treatment of disease.

The ganglioside supplement in this study was administered in powder form. Participants could consume the supplement in other foods. The study ganglioside could be eaten on its own, or mixed with food or drink. Patients with IBD are individually counselled with respect to diet and nutrition since some foods may be negative triggers in some sufferers, but not in other patients. Food is complex in composition and structure and as such, food components interact in additive, synergistic or antagonistic manners. Little is known regarding the intake and food interactions of ganglioside in the context of diet. In this study, participants consuming

ganglioside showed an increase in plasma ganglioside concentration, improved quality of life and reduced intestinal permeability. In future study of larger IBD cohorts, collecting and analyzing dietary intake data from participants may help to explain how or why study subsets have a higher or lower beneficial response to ganglioside consumption. Specific dietary intakes may help or hinder the beneficial actions of candidate therapeutic drug or food. Thus, food frequency or food recall data may be useful in discerning how individuals may respond to treatment since dietary intakes vary greatly within study participant populations and in patients with IBD.

5.24 Ganglioside analysis

Analysis of ganglioside in bowel tissue by MS revealed a wide array of species of ceramide. Analyses also revealed key differences in the composition of ceramide between IBD and control groups. This study showed a decrease in the relative content of PUFA within GD3 and GD1a in IBD versus control intestine (Figure 4-3A). This may be due to differences in dietary intake of fatty acids or to differential regulation of fatty acids for incorporation into ganglioside biosynthesis among patients with IBD and healthy individuals. The incorporation of (poly)unsaturated fatty acids into ganglioside could be decreased relative to incorporation of saturated fatty acids in patients with IBD. On the other hand, treatment of IBD with aspirin is known to produce resolvins[9] which are derived from PUFA. The PUFA used for resolvin synthesis may be derived in part from ganglioside; and thus, may explain the observed decrease in relative content of ganglioside containing two or more unsaturated bonds in IBD bowel. The difference in PUFA content of ganglioside between IBD and healthy intestine may reflect different dietary intakes, altered regulation of fatty acid or higher demand or specific fatty acid for resolution of inflammation.

MS provides highly sensitive and specific characterization of detailed ganglioside species. Gangliosides are considered a diverse class of sphingolipid as the number and position of sialic acids and sugar units can vary considerably (Figure 1-1). While LC is successful in detecting the (a-, mono-, di-, trisialo) class of ganglioside, retention times do not enable precise discernment of acetylated or glycolylated species or the number of carbon atoms and unsaturated bonds in ceramide. Fragmentation of ganglioside and detection of the m/z by MS provides more detailed information regarding ceramide species present in gangliosides. Fragmentation of ceramide to determine fatty acid constituents by introduction of charge to the carbon chain is difficult due to the highly hydrophobic nature of ceramide. The strength of this study lies not only in the accurate characterization of ganglioside class, but also in ceramide composition. This study also confirms that presence of polyunsaturated ganglioside species as previous study have only ascertained the presence of two unsaturated bonds[10] within the ceramide of gangliosides. Analysis of ganglioside from 29 bowel specimens is another strength in this study as other studies characterizing ganglioside by MS have only analyzed one to four specimens and thus have limited applicability in describing the involvement of glycosphinoglipids in disease[11,12]. Novel species of ganglioside with detailed compositional information from a large study cohort were described in this thesis study.

Compliance in nutritional clinical study is commonly measured by self-reported means or counting returned/unused supplement. Even with this data, barriers to determining the true compliance rate are still apparent. Misreported or misinterpreted compliance may substantially confound interpretation of study results; especially when intention-to-treat is employed as this principle does not exclude participants from analyses on the basis of dropout or non-compliance. Intention-to-treat may limit study bias, but may introduce confounding in the event of different

dropout or compliance rates among study arms. Metabolomics techniques allow for highly sensitive and specific detailed characterization of compounds in biologic tissues. Urine, hair, blood or other tissue samples can be collected and assayed for specific metabolites that could indicate intake of treatment and/or placebo substrates. Sphingolipidomic techniques utilizing MS may thus be utilized in future studies of ganglioside supplementation for detection of specific ganglioside metabolites unique to supplemental intake (ie. not endogenously produced) for an additional compliance measure.

5.25 Conclusions

The dietary intervention study (Section 3.22) showed that daily consumption of milk fat fraction ganglioside increases plasma ganglioside content (Figure 3-4). This finding suggests that dietary ganglioside can be consumed to potentially correct the irregular balance of gangliosides in inflamed intestine (Table 4-2) and in several other disorders like diabetes, cardiovascular disease and cancer where altered ganglioside content plays an integral role in pathogenesis (Section 1.11). Ultimately, the dysregulation of endogenous ganglioside catabolism (Figure 4-4) in states of disease can be influenced and overcome by absorption of specific species of dietary gangliosides like GD3 for circulation to affected tissues where beneficial effects can be exerted.

Several mechanisms have been proposed in the pathogenesis of IBD. The contribution of polymorphisms in genes regulating ganglioside content and composition has been described as it relates to the underlying pathophysiology of IBD (Section 1.45). Many of the treatments for IBD still only address symptoms associated with disease and not the underlying cause. The novelty of this study lies in showing that altered ganglioside content and composition is fundamental to the etiology of IBD as inflamed intestine can be characterized by aberrations in ganglioside metabolism. Dietary ganglioside in an enriched milk fat fraction is bioavailable and results from

this thesis research support the safety of regular supplementation in human diet (Table 3-2, Table 3-3). Finally, dietary ganglioside has beneficial effects on intestinal permeability and on quality of life in patients with IBD. This thesis research shows that ganglioside constitutes a safe and effective dietary component for treatment of IBD.

5.3 Literature cited

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APPENDIX

7.1 QUALITY OF LIFE IN INFLAMMATORY BOWEL DISEASE QUESTIONNAIRE IBDQ

This questionnaire is designed to find out how you have been feeling during the last 2 weeks. You will be asked about symptoms you have been having as a result of your inflammatory bowel disease, the way you have been feeling in general, and how your mood has been.

1. How frequent have your bowel movements been during the last two weeks? Please indicate how frequent your bowel movements have been during the last two weeks by picking one of the options from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

2. How often has the feeling of fatigue or of being tired and worn out been a problem for you during the last 2 weeks? Please indicate how often the feeling of fatigue or tiredness has been a problem for you during the last 2 weeks by picking one of the options from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

3. How often during the last 2 weeks have you felt frustrated, impatient, or restless?

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

4. How often during the last 2 weeks have you been unable to attend school or do your work because of your bowel problem? Please choose an option from IBDQ

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

5. How much of the time during the last 2 weeks have your bowel movements been loose? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

6. How much energy have you had during the last 2 weeks? Please choose an option from

1 NO ENERGY AT ALL
2 VERY LITTLE ENERGY
3 A LITTLE ENERGY
4 SOME ENERGY
5 A MODERATE AMOUNT OF ENERGY
6 A LOT OF ENERGY
7 FULL OF ENERGY

7. How often during the last 2 weeks did you feel worried about the possibility of needing to have surgery because of your bowel problem. Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

8. How often during the last 2 weeks have you had to delay or cancel a social engagement because of your bowel problem? Please choose an option from

IBDQ

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

9. How often during the last 2 weeks have you been troubled by cramps in your abdomen? Please choose an option from

I ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

10. How often during the last 2 weeks have you felt generally Unwell? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

11. How often during the last 2 weeks have you been troubled because of fear of not finding a washroom? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

12. How much difficulty have you had, as a result of your bowel problems, doing leisure or sports activities you would have liked to have done during the last 2 weeks? Please choose an option from IBDQ

1 A GREAT DEAL OF DIFFICULTY; ACTIVITIES MADE IMPOSSIBLE
2 A LOT OF DIFFICULTY
3 A FAIR BIT OF DIFFICULTY
4 SOME DIFFICULTY
5 A LITTLE DIFFICULTY
6 HARDLY ANY DIFFICULTY
7 NO DIFFICULTY; THE BOWEL PROBLEMS DID NOT LIMIT SPORTS OR LEISURE ACTIVITIES

13. How often during the last 2 weeks have you been troubled by pain in the abdomen? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

14. How often during the last 2 weeks have you had problems getting a good night's sleep, or been troubled by waking up during the night? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

15. How often during the last 2 weeks have you felt depressed or discouraged?

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

16. How often during the last 2 weeks have you had to avoid attending events where there was no washroom close at hand? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

17. Overall, in the last 2 weeks, how much of a problem have you had with passing large amounts of gas? Please choose an option from

1 A MAJOR PROBLEM
2 A BIG PROBLEM
3 A SIGNIFICANT PROBLEM
4 SOME TROUBLE
5 A LITTLE TROUBLE
6 HARDLY ANY TROUBLE
7 NO TROUBLE

18. Overall, in the last 2 weeks, how much a problem have you had maintaining or getting to, the weight you would like to be at. Please choose an option from

1 A MAJOR PROBLEM
2 A BIG PROBLEM
3 A SIGNIFICANT PROBLEM
4 SOME TROUBLE
5 A LITTLE TROUBLE
6 HARDLY ANY TROUBLE
7 NO TROUBLE

19. Many patients with bowel problems often have worries and anxieties related to their illness.

These include worries about getting cancer, worries about never feeling any better, and worries about having a relapse. In general, how often during the last 2 weeks have you felt worried or anxious? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

20. How much of the time during the last 2 weeks have you been troubled by a feeling of abdominal bloating? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

21. How often during the last 2 weeks have you felt relaxed and free of tension? Please choose an option from

1 NONE OF THE TIME
2 A LITTLE OF THE TIME
3 SOME OF THE TIME
4 A GOOD BIT OF THE TIME
5 MOST OF THE TIME
6 ALMOST ALL OF THE TIME
7 ALL OF THE TIME

22. How much of the time during the last 2 weeks have you had a problem with rectal bleeding with your bowel movements? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

23. How much of the time during the last 2 weeks have you felt embarrassed as a result of your bowel problem? Please choose an option from

I ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

24. How much of the time during the last 2 weeks have you been troubled by a feeling of having to go to the bathroom even though your bowels were empty? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

25. How much of the time during the last 2 weeks have you felt tearful or upset? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

26. How much of the time during the last 2 weeks have you been troubled by accidental soiling of your underpants? Please choose an option from

IBDQ

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

27. How much of the time during the last 2 weeks have you felt angry as a result of your bowel problem? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

28. To what extent has your bowel problem limited sexual activity during the last 2 weeks?
Please choose an option from

1 NO SEX AS A RESULT OF BOWEL DISEASE
2 MAJOR LIMITATION AS A RESULT OF BOWEL DISEASE
3 MODERATE LIMITATION AS A RESULT OF BOWEL DISEASE
4 SOME LIMITATION AS A RESULT OF BOWEL DISEASE
5 A LITTLE LIMITATION AS A RESULT OF BOWEL DISEASE
6 HARDLY ANY LIMITATION AS A RESULT OF BOWEL DISEASE
7 NO LIMITATION AS A RESULT OF BOWEL DISEASE

29. How much of the time during the last 2 weeks have you been troubled by nausea or feeling sick to your stomach? Please choose an option. from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

30. How much of the time during the last 2 weeks have you felt irritable? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

31. How often during the past 2 weeks have you felt a lack of understanding from others? Please choose an option from

1 ALL OF THE TIME
2 MOST OF THE TIME
3 A GOOD BIT OF THE TIME
4 SOME OF THE TIME
5 A LITTLE OF THE TIME
6 HARDLY ANY OF THE TIME
7 NONE OF THE TIME

32. How satisfied, happy, or pleased have you been with your personal life during the past 2 weeks? Please choose one of the following options from

1 VERY DISSATISFIED, UNHAPPY MOST OF THE TIME
2 GENERALLY DISSATISFIED, UNHAPPY
3 SOMEWHAT DISSATISFIED, UNHAPPY
4 GENERALLY SATISFIED, PLEASED
5 SATISFIED MOST OF THE TIME, HAPPY
6 VERY SATISFIED MOST OF THE TIME, HAPPY
7 EXTREMELY SATISFIED, COULD NOT HAVE BEEN MORE HAPPY OR PLEASED