Dietary Gangliosides Modify *Helicobacter pylori* Adhesion to Human Gastric Epithelial Cells

by

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Abstract

This research determined if alteration of plasma membrane by exogenous gangliosides inhibits *Helicobacter pylori* adhesion to human gastric epithelial cells. The objective was to evaluate the composition of gangliosides from bovine whole raw milk, bovine colostrum and powder buttermilk using Liquid Chromatography/Mass Spectrometry (LC/MS). This research determined if alteration of the GM3 and GD3 content of the apical and basolateral membrane of a human gastric cell line was modified by exogenous gangliosides. The effect of ganglioside treatment on the composition and content of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and on *H. pylori* adhesion was assessed.

A rapid and sensitive LC/MS method was established and used to determine content and composition of gangliosides. Ganglioside composition was analyzed in three different milk products (raw milk, colostrum, buttermilk). GD3 was found to be the most abundant ganglioside species. Human gastric cells fed with exogenous GM3 and GD3 modified ganglioside content, but most abundant ganglioside was GM3. GM3 was found in the apical side and GD3 in the basolateral side of the cell. After ganglioside treatments, composition of ganglioside content of membrane domains was modified. Composition and content of phospholipids was not altered after ganglioside treatment. Gastric cells treated with gangliosides decreased *H. pylori* adhesion, but concentration of these gangliosides was not crucial for reduction. GM3 and GD3 decrease *H. pylori* adhesion. The combination of GM3:GD3 also decreased *H. pylori* adhesion suggesting that human and bovine milk might decrease *H. pylori* adhesion.

This thesis demonstrates that 1) gangliosides are taken up in gastric cells, 2) gangliosides fed from the apical side also affect content of gangliosides in the basolateral side, and 3) GD3 and GM3 reduce *H. pylori* adhesion to human gastric cells. This research suggests that gangliosides may be an anti-adhesion strategy to decrease or prevent *H. pylori* infection.

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I dedicate this thesis to my parents who have given me one of the best gifts anyone could give another person, they believed in me.

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Abbreviations

ANSA	8-Anilino-1-Naphthalene-Sulfonic Acid
BabA	Blood Group Antigen Binding Adhesion
BCA	Bicinchoninic assay
BHI	Brain Heart Infusion
CagA	Cytotoxin-associated gene A
CagPAI	Cag Pathogenecity Island
CEA	Carcinoembryonic Antigen
CFU	Colonies Forming Units
CID	Collision Induced Dissociation
CT	Cholera Toxin
DIGEM	Detergent Insoluble Glhycosphingolipid Enriched Microdomains
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EPEC	Enteropathogenic
ESI	Electrospray Ionization
ETEC	Enterotoxigenic
FA	Fatty Acid
FaaA	Flagella-associated Autotransporter A
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
FISH	Fluorescent In Situ Hybridization
Fuc	Fucose
Fuc	Fucose
Gal	Galactose
GalCer	Galactosylceramide
GalNAc	N-Acetylgalactosamine
GD	Di-sialilated Gangliosides
GERD	Gastroesophageal Reflux Disease
Glc	Glucose

GlcNAc	N-Acetylglucosamine
GlucCer	Glucosylceramide
GM	Mono-sialilated gangliosides
GT	Tri-sialilated gangliosides
HPSPA	*H. pylori* Special Peptone Agar
HPTLC	High Performance Thin Layer Chromatography
HTST	High Temperature, Short Time Pasteurization
IR	Insulin Receptors
IUPAC-IUB	Comission of International Union of Biochemistry
LacCer	Lactosylceramide
LC	Liquid Chromatography
LPS	Lipopolysaccharide
LT	Heat Labile Toxin
LTLT	Low Temperature, Long Time Pasteurization
M	Monounsaturated ceramide
MALT	Mucosa-Associated Lymphoid Tissue
MCP	Methyl-accepting Chemotaxis Proteins
MDCK	Madin-Darby Canine Kidney
MFGM	Milk Fat Globule Membrane
MIC	Minimum Inhibitory Concentration
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NAC	N-acetylcysteine
NANA, Neu5Ac, SIA	N-acetylneuraminic acid
NGNA, Neu5Gc	N-glycoloylneuraminic acid
NSAID	Non-steroidal Anti-inflammatory Drug
OD	Optical Density
Oip	Outer Membrane Inflammatory Protein
OMP	Outer Membrane Proteins

P	Polyunsaturated ceramide.
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PPI	Proton Pump Inhibitor
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
RUT	Rapid Urea Test
S	Saturated ceramide
SabA	Sialic-Acid Binding Adhesin
SOD	Superoxide Dismutase
SPE	Solid Phase Extraction
T-LBSA	Total Lipid Bound Sialic Acid
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
UBT	Urea Breath Test
VacA	Vacioling Cytotoxin A
YE	Yeast Extract
ZO-1	Zonula Occludens-1

CHAPTER I. Introduction

1.1 Introduction

J.L.W. Thudichum has been called the father of brain chemistry or the father of sphingolipid research due to his studies of the brain.[1,2] During the 19th century, Thudichum isolated many lipids such as cerebroside, sphingosine and ceramide from human brain. Hence sphingolipids and glycosphingolipids, the categories to which these lipids belong, were first found and studied in neural tissues.[2]

Ernest Klenk (1941), one of Thudichum's sucessors, isolated a new component of the brain, which was called substance X, in patients with storage disorders such as Niemann-Pick's disease and Tay Sachs disease. This substance had a purple color reaction with the orcinol reagent. Klenk renamed it substance X ganglioside because of its abundance in grey matter and found that neuraminic acid was the ganglioside component responsible for giving the purple color reaction.[2-4]

Lars Svennerholm (1956), a pioneer of glycobiology, discovered different kinds of brain gangliosides. Svennerholm also identified the sequence of ganglio core structure, and developed a well-accepted nomenclature. Methods for ganglioside quantification to analyze human brain gangliosides were proposed by Svennerholm's research group.[5]

1.2 Sphingolipids

Sphingolipids act in vital roles as both messengers in several signaling pathways, and components of membrane domains or lipid rafts.[6] Sphingolipids, called long-chain bases, are amphiphilic molecules distributed in the structure of cellular membranes of eukaryotic and

some prokaryotic cells,[7,8] and are also found in Golgi membranes and lysosomes.[9] Sphingolipids are composed of three main portions (Fig. 1.1): a sphingoid base, composed of a long-chain (12-22 carbon atoms) aliphatic amino alcohol, a polar head group (R), and a fatty acid which is linked to the sphingoid base via an amide bond (-NHCOR').[10]

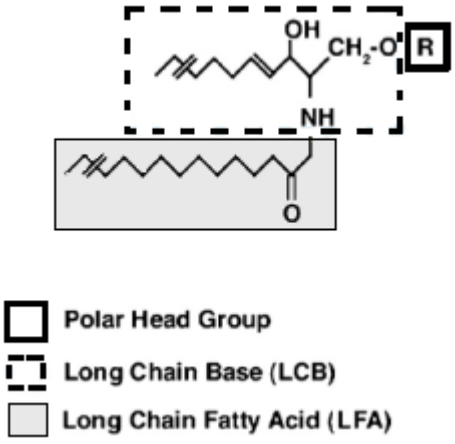
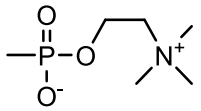
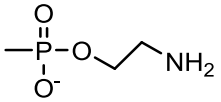
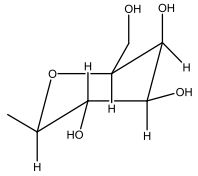
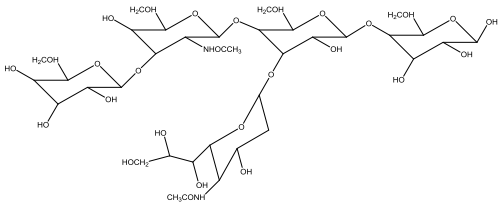
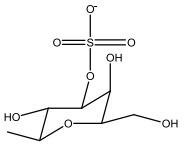


Figure 1.1 Main structure of sphingolipids (abstracted and modified from reference 11).

Sphingolipids are classified, according to head group, into three categories (Table 1.1): simple sphingolipids, phosphosphingolipids, and glycosphingolipids.

Ceramide and sphingoid bases belong to the group of simple sphingolipids. Sphingosine is an animal sphingoid.[10] Sphingolipids contain a phosphocholine head group and a sphingoid backbone. Sphingophospholipids have certain similarities to lecithin (phosphatidylcholine). The most prevalent is cer-phosphocholine in mammalian tissues.[8,10] Glycosphingolipids, the focus of this research, are found in all vertebrate cells such as molluscs, plants, fungi and microorganisms.[22] The distribution varies according to both species and tissue.[8]

Table 1.1 Classification of sphingolipids

Name of sphingolipid	Head group R	Structure of R
SIMPLE SPHINGOLIPIDS		
Sphingosine	Hydrogen atom	-H
Ceramide	Hydrogen atom	-H
PHOSPHOSPHINGOLIPIDS		
Sphingomyelin	Phosphocholine	
Sphingosine-1-phosphate	Phosphate	$-PO_3^{2-}$
Ceramide-1-phosphate	Phosphate	$-PO_3^{2-}$
Ceramide-Phosphoethanolamine	Phosphoethanolamine	
GLYCOSPHINGOLIPIDS		
Cerebroside, e.g. galactosylceramide	Monosaccharide, e.g. galactose	
Ganglioside	Oligo-, polysaccharide + sialic acid	
Sulphatides	Sugar with sulphate residue	

Modified from reference 10.

Glycosphingolipids are composed of a hydrophobic portion and a hydrophilic portion. The hydrophobic portion comprises a sphingoid long-chain base (normally sphingosine, sphinganine or phytosphingosine) which is linked through an amide bond to a fatty acid. The hydrophilic portion consists of hydroxyl groups for the simplest glycosphingolipids or phosphate and sugar residues for the most complex glycosphingolipids.[6,12,13] The minimum carbohydrate portion is a monosaccharide bound to a ceramide unit. In higher mammals, this monosaccharide is glucose or galactose, producing glucosylceramide or galactosylceramide.

Glucosylceramide can be modified by adding a galactose, producing lactosylceramide. Other carbohydrates present, in glycosphingolipids are N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), sialic acid and fucose (Fuc).[7,8,14]

Glycosphingolipids are usually classified with respect to the carbohydrate structure into the following series ganglio-, isoganglio-, lacto-, neolacto-, lactoganglio-, globo-, isoglobo-, muco-, gala-, neogala-, mollu-, arthro-, schisto- and spirometo- series (Table 1.2).

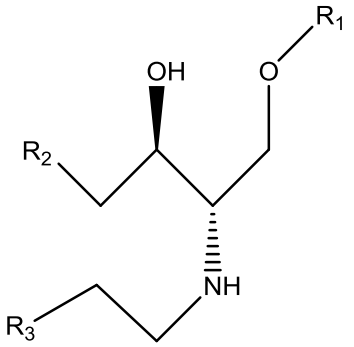
Table 1.2 Glycosphingolipid organization

Glycosphingolipids	**General structure**			
(Abbreviation)	**IV**	**III**	**II**	**I**
Glu (Glc)				Glcβ1-Cer
Gala				Galβ1-Cer
Ganglio (Gg)	Galβ1-	3GalNAcβ1-	4Galβ1-	4Glcβ1-Cer
Lacto(Lc)	Galβ1-	3GlcNAcβ1-	3Galβ1-	4Glcβ1-Cer
NeoLacto (nLc)	Galβ1-	4GlcNAcβ1-	3Galβ1-	4Glcβ1-Cer
Globo (Gb)	GalNAcβ1-	3Galα1-	4Galβ1-	4Glcβ1-Cer
Isoglobo (iGb)	GalNAcβ1-	3Galα1-	3Galβ1-	4Glcβ1-Cer

Modified from reference 8.

1.3 Definition of gangliosides

Gangliosides are a subclass of the larger group of sphingholipids, as gangliosides have a similar structure to other members of this group[15] (Fig. 1.2). Gangliosides contain a sphingosine base with a free -OH group on carbon 3 with a fatty acid linked by an amide to the 2-amino group. A carbohydrate is bound to a glycoside to the -OH group on carbon 1 of ceramide.[16] Sialic acid, is the representative characteristic of gangliosides, which is bound to a carbohydrate residue by a ketoside linkage.[16-18]



R1: Glycan chain containing neutral saccharides and at least one sialic acid .

R2: Chaing of sphingoid base containing 14 to 18 carbons and some double bonds

R3: Chain of fatty acids, containing 14 to 28 carbons (saturated,mono- or poly-unsaturated carbon chain)

Figure 1.2 General structure of gangliosides (modified from reference 18).

1.3.1 Components of gangliosides

1.3.1.1 Ceramide

A ceramide (Fig. 1.3) is a sphingoid long chain base (usually sphingosine, sphinganine or phytosphingosine) attached via N-acylation by an amide linkage to a fatty acid with a chain length greater than 14 carbons bound to an oligosaccharide chain.[18-20] Ceramides change in length, hydroxylation and saturation affecting both sphingoid base and fatty acid portions; this alters the lipid structure and attachment to the glycan portion on the plasma membrane.[22] Ceramides are bound to glycans. Usually, monosaccharides in higher animals such as a galactose or glucose are β-linked to ceramides forming galactosylceramide (GalCer) or glucosylceramide (GlucCer), respectively.[22]

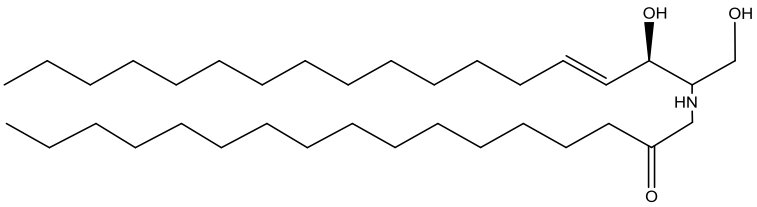


Figure 1.3 Structure of ceramide (d18:1/16:0) (modified from reference 23).

1.3.1.2 Sphingoid bases

All sphingolipids contain one molecule of sphingoid bases.[24] The sphingoid base backbone is composed of long-chain alkanes or long-chain alkenes (or with more unsaturation) 14-20 carbons in length, with an amino group at position 2 and hydroxyl-substituents at positions 1 and 3.[25] Three chemical structures of sphingoid bases are found in nature: C18-sphingosine (trans-4-sphingenine) and, in lesser amounts, C18-sphinganine (dihydrosphingosine), and 4-hydroxysphinganine (phytosphingosine).

Sphingosine or sphingenine (sphing-4-enine, 2-aminooctadec-4-ene-1,3-diol, and trans-D-erytho-2-amino-octadec-4-ene-1,3-diol) is the most common in mammal tissues, including those of humans.[24,25] In humans the sphingosine is 18 carbons in length. Gangliosides with 20-carbon sphingosines appear as humans age.[25] The 18- and 20-carbon atom structures contain a *trans* double bond at position 4-5 (2-amino-1,3-dihydroxyoctadec-4ene and 2-amino-1,3-dihydroxyeicos-4-ene). Of the four possible chemical configurations only 2S, 3R (or 3D(+) erythro) has been found to exist in nature. [25]

Sphinganine is a long-chain amino alcohol not containing a double bond at position 4-5 ((2-amino-1,3-dihydroxyoctadecane and 2-amino-1,3-dihydroxyeicosane).[26,27] Ganglioside species containing sphinganine are very minor components of the total cell ganglioside, and have different chemical and physicochemical characteristics from ganglioside species containing sphingosine.[28-30]

Phytosphingosine is rarely found in mammals, some glycosphingolipids containing phystosphingosine are in plasma membrane of cells in small intestine and kidney.[22]

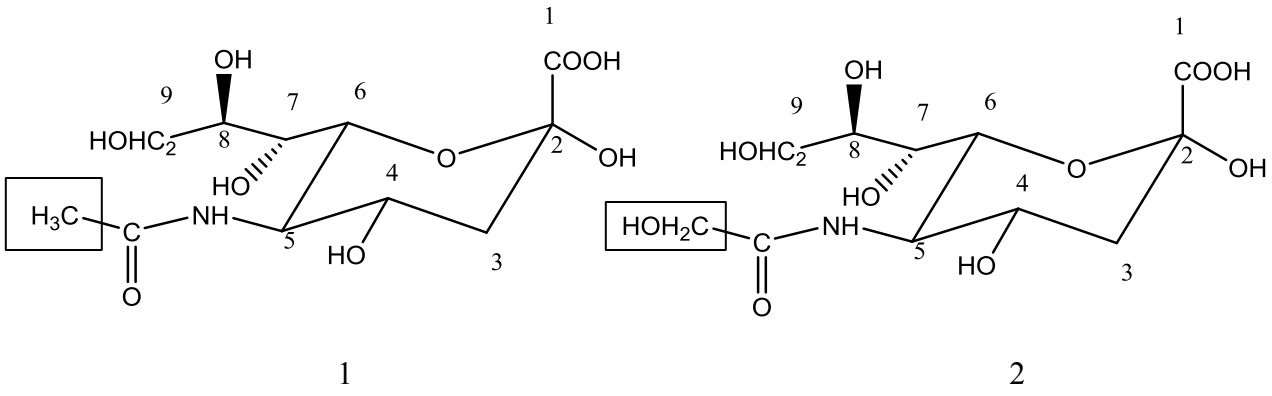
1.3.1.3 Fatty acids

The fatty acid portion varies in chain length (14-32) carbon atoms including odd fatty acids), degree of unsaturation (mostly saturated fatty acid), and presence or absence of a hydroxyl group on the α-carbon atom. Stearic acid is the main fatty acid of mammalian nervous system gangliosides and often forms over 80% of the total ganglioside fatty acid content.[25,31-35] The most abundant fatty acids are C16 and C18 in bovine milk and human milk.[35-37] Monounsaturated fatty acids from 13:1 to 24:1, such as palmitoleic (16:1) and oleic (18:1) acids have been identified. Polyunsaturated fatty acids (18:2, 18:3 and 20:2) are found in gangliosides of human and bovine milk.[37,35]

1.3.1.4 Sialic acids

In the 1960's the nomenclature of sialic acids was established by pioneers in the sialic acid field (Guntar Blix, Ernest Klenk and Alfred Gottshalk).[38] Sialic acid is an electronegative (pka 2.2) monosaccharide occurring in higher animals and some microorganisms.[39,40] Sialic acid is a family of 43 derivatives of a 9-carbon carboxylated monosaccharide.[42] Sialic acid is well-distributed in glycolipids, glycoproteins, gangliosides and mucins.[41] Sialic acid rarely is free in nature.[42]

The most predominant sialic acids in nature and the only two sialic acids present in gangliosides are N-acetylneuraminic acid (NANA, Neu5Ac, SIA) and N-glycolyneuraminic acid (NGNA, Neu5Gc) (Fig. 1.4). Neu5Ac is the most widespread sialic acid and is found in humans.[42] Neu5Gc is common in animal species.[42] Traces of NGNA have been detected in healthy human tissues and in human cancer tissues. [40]



1 2

Figure 1.4 Structure of (1) N-acetylneuraminic and (2) N-glycoloylneuraminic acid (modified from reference 42).

Sialic acid has different substituents at the amino or hydroxyl groups. The amino group of neuraminic acid is acetylated or glycosylated, while in non-glycosidic hydroxyl residues one or various acetyl groups may occur.[39] The distribution of sialic acids depends on the animal and cell species. The distribution of sialic acids is also related to the function of a cell and seems to be strongly regulated on the gene level. The animal with the major diversity of sialic acids known is the cow.[39]

1.3.1.5 Oligosaccharides

Gangliosides in mammals contain uncharged sugars such as glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), and fucose (Fuc) (in other organisms mannose is present).[25] Glucose or galactose is linked to ceramide by β-conformation glycosidic linkage.[35]

1.4 Nomenclature of gangliosides

The nomenclature proposed by Svennerholm is well accepted and popular due to its simplicity and ease of use. The nomenclature is shown in the following steps:

1. The letter G indicates the ganglioside component and is used to refer to all the gangliosides.
2. To indicate the portion of sialic acid, the letter G is followed by the initial Greek term, M (one sialic acid), D (two sialic acids), T (three), Q (four), P (five), H (six) or S (seven). It is then called a monosialoganglioside, a disialoganglioside, a trisialoganglioside, etc.
3. The number of oligossaccharide residues is indicated by a numeral calculated based on 5 minus the number of neutral sugars, so 1: four residues, 2: three residues, 3: two residues and 4: one residue.
4. In addition, to indicate the pathway of a biosynthesized molecule, some additional letters (a, b, c, d) are added, as a subindex.[19]

A more complete and complex nomenclature has been developed by the *Commission of the International Union of Biochemistry*. Using the IUPAC-IUB nomenclature the glycosphingolipids name is formed of (root name) (root size) -osylceramide.[19,43] This nomenclature uses three symbols for the sugar plus Cer for ceramide. GalCer is the abbreviation for galactosylceramide. Complex glycosphingolipids are separated into families using a subscript letter to categorize the sugars in the chain. Roman numeral prefixes indicate the monosaccharide residue counting from the ceramide. The position of the glycosidic linkage is indicated for a superscript Arabic number. II^3-N-acetylneuraminosyl-gangliolactosylceramide is II^3-NeuAc-LacCer (GM3) or II^3-N-acetylneuraminoslyl-digangliolactosylceramide is II_3-$(NeuAc)_2$-LacCer (GD3).[19,43]

1.5 Physical characteristics

Gangliosides are colorless crystallizable substances with a melting point of 189-190°C and in aqueous solutions form micelles with a molecular weight about 200,000-250,000.[16,48] Gangliosides are mainly located on the outer layer of membranes.[29,44] The distribution of gangliosides is assymmetrical.[44] Gangliosides have an amphiphilic behavior due to ganglioside composition of a two tail hydrophobic moiety, ceramide, and a hydrophilic head group, carbohydrate portion with sialic acid.[3] Gangliosides are soluble in solvents such as alcohols, tetrahydrofuran, dimethylformamide, dimethylsulfoxide or chloroform-methanol (with a small part of water).[45] Unlike phospholipids, gangliosides are not flat in structure, and have a curvature. The curvature depends on the number of sugars in the ganglioside.[3,44]

Gangliosides are composed of both hydrophilic and hydrophobic portions and can influence the dynamic environment. Gangliosides are present in domains called lipid rafts in a liquid structure in the presence of cholesterol and form a solid disorder configuration. Cholesterol preferentially interact with sphingolipids that differ from gangliosides. Gangliosides separates from other lipids even in presence of cholesterol, as the liquid arrangement can be formed in mixed systems without cholesterol.[46,47]

1.6 Location and distribution of gangliosides

Gangliosides are distributed in vertebrate tissue, but few have been detected in invertebrates.[50] Ganglioside profile can vary because of different tissue functions and age.[50,51] Gangliosides occur more frequently in neural tissues, especially in the brain.[53]

1.6.1 Brain gangliosides and other neural tissues

Schengrund and Garrigan (1963) extracted brain tissue of 13 different species (cat, dog, rabbit, ox, sheep, pig, rat, chicken, alligator, frog, cod, mink, and electric eel). The brain contains more gangliosides than other tissues, especially in cats, dogs, pigs and sheep.[52] Grey brain matter contains 15 times more gangliosides than the liver, lungs and spleen, and brain grey matter contains 500 times more gangliosides than intestinal mucosa.[42]

In humans, the ganglioside concentration is higher in the grey matter than in the white matter, 2.54 and 0.75 μmol/g of brain tissue, respectively.[50] Gangliosides are the major components of neural membranes, constituting 10-12% of the total lipid content.[49] Four gangliosides, GM1, GD1a, GD1b and GT1b, constitute 96-97% of total gangliosides, in the normal human brain. The same gangliosides are present in mammals and birds.[50,54,55,56] The ceramide portion of brain gangliosides is composed of 18-20 carbon sphingosine linked to a saturated fatty acid amide, e.g. C18:0.[55] Gangliosides are highly abundant in the hippocampus region of the brain, which performs memory and cognitive functions.[57] The concentration and distribution of gangliosides changes during brain development.[58] Ganglioside content increases three fold from 10 weeks of gestation to 5 years of age. Increase in ganglioside concentration level occurs during dendrite arborization, outgrowth of axons and synaptogenesis.[56,59] GM3 and GD3 are also expressed by neuronal and glial precursor cells from 8 to 25 weeks of gestation.[60] During the synaptogenesis period, ganglioside synthesis increases ganglioside biosynthesis via the a-pathway resulting in GD1a and GM1, and gangliosides such as GD1b and GT1b, derived via the b- pathway.[61,62] Ganglioside content increases in the hippocampus from birth to a peak at the age of 40-50 years. Ganglioside

composition changes with aging; however, this change is more remarkable after the age of 80 due to decrease in activity of sialyltransferases.[63]

GD1b and GD3 are the most abundant gangliosides in human cervical and lumbar spinal cord. Different ganglioside content and different ganglioside composition have been found in both tissues, which suggests that gangliosides have different functions in these different regions of human spinal cord.[64]

1.6.2 Gangliosides in gastrointestinal tract

Gangliosides are less abundant in the digestive system than in neural tissues.[65] Ganglioside composition has been characterized in small and large intestinal mucosa in adult monkeys. GM3, GM2 and GD1a were the most abundant in both tissues. The main fatty acids of ceramide ganglioside composition were linoleic acid and oleic acids in the small intestine, while in the large intestine, oleic and palmitic acid were the principal fatty acids.[66] Differences in ceramide composition of gangliosides between different regions of small intestine suggest that gangliosides perform different functions.[66] GM3 is the major ganglioside of rat intestinal epithelium and is distributed more abundantly in differentiated microvillus cells than in undifferentiated crypt cells due to CMP-sialic acid: lactosylceramide sialyltransferase, an enzyme that sialylates lactosylceramide to synthesize GM3, being higher in microvillus cells.[67] In rat, ganglioside variations exist along the small intestinal mucosa. GM3 concentration is highest in the distal segment, intermediate in the middle and lowest in the proximal segment in the small intestine of rats with variations in composition of fatty acids.[68]

Gangliosides have been located in the enterocyte membrane.[69] GM3 is located in the apical membrane. The role of GM3 is related to interception and inactivation of

microorganisms and bacterial toxins.[58] By contrast, GD3 is located in the enterocyte basolateral membrane, and is not directly exposed to the lumen. As a result, the role of GD3 is related to immune cell functions.[58]

Roth J. (1985) studied the distribution of GM1 in enterocytes to be visualized with Cholera toxin and observed distribution of GM1 to be restricted to the apical plasma membrane domain (brush border).[70] The basolateral plasma membrane was free of GM1-Cholera toxin binding.[70]

1.6.3 Gangliosides in liver and other extraneural tissues

Gangliosides have been also identified in plasma membrane of human liver. GM3 is the dominant ganglioside representing around 90% of total gangliosides.[71,72] Nine other gangliosides have been identified by thin layer chromatography (TLC).[72] Monosialogangliosides like GM3, GM2 and GM1, constitute 93.5% of total gangliosides.[71,72] Disialogangliosides, GD3, GD1a, and GD1b, constitute 5.1% of total gangliosides; while polysialogangliosides, GT1a, GT1b, and GQ1b, represent 1.4% of total gangliosides in healthy human liver.[72] The ganglioside fatty acid composition in liver contains fatty acids 16:0 to 25:1 carbon chain length.[72]

Gangliosides have been isolated from other human extraneural tissues and fluids such as skeletal and smooth muscle,[73-75] heart,[76] lung,[77,78] kidney,[79,80] fat tissue,[81] placenta,[82,83,84] amniotic fluid,[85] pancreas,[86,87] spleen,[88,89,90] thymus,[90] lymphocytes,[91] and serum.[92] The ganglioside profile of each extraneural tissue/fluid is described (Table 1.3).

Table 1.3 Distribution of ganglioside in different human extraneural tissues/fluids

Human Tissue/Fluid	Ganglioside (% of total gangliosides)	Fatty acid pattern	Reference
Skeletal muscle	GM3 (37%), GM2 (27%), GM1, GD3, GD1a, GD1b, GT1b	18:0, 24:1, 22:0	74,73
Smooth muscle	GM3 (60%), GM1, GD3, GD1a, GT1b		75
Heart	GM3 (23%), GD3 (22%), GM1 (16%), GM2 (13%), GD1a (11%), GD1b (5%), GT?(10%)		76
Lung	GM3, GM1, GM2, GD1a, GT3 (40%)	24:1, 23: 0, 22:0	77,78
Kidney	GM3, GD1b, GT1, GD1a	16:0, 22:0, 24:0	79,65
Pancreas	GM3 (67%), GD3 (23%), GM1 (2%), GM2 (2%), GD1a (6%), GM2	16:1 to 24:1	86,87
Fat tissue	GM3, GD3, GD1a, GM1, GT1		81
Placenta	GM3, GD3 and other undefined gangliosides		83,84
Amniotic fluid	GM3		85
Lymphocytes	GM3, GM1		90,91,93
Spleen	GM3 (84.5%), GD3 (1.9%), GM1, GD1a		65,90,94
Thymus	GM3 (64%), GD3 (30.6%)		90
Serum	GM3, GD3, GD1, GM2, GT1b, GD1b and GQ1b		92

1.7 Metabolism of gangliosides

1.7.1 *de Novo* ganglioside biosynthesis

Biosynthesis of ganglioside starts with formation of ceramide on membranes of the endoplasmic reticulum by serine palmitoyl transferase.[15,19,45,95] Ceramide is derived from palmityl-CoA, L-Serine and Acyl-CoA.[19] Ganglioside biosynthesis results from the

accumulation of carbohydrate units.[95] After ceramide biosynthesis, ceramide is transported to the cytosolic surface of the Golgi apparatus, where glucosylceramide synthase (UDPglucose:ceramide glucosyltransferase; encoded by GlcT) transfers a glucose residue to ceramide to produce glucosylceramide.[15] Glucosylceramide is transferred to the luminal leaflet of the Golgi where lactosylceramide (precursor of almost all gangliosides) is formed through galactosylation of GlcCer.[15,96,97]

In ganglioside biosynthesis, three pathways have been assessed: series a-, b- and c-. GM3 is the precursor of series a- and is transformed into GD3 and GT3, which are precursors of series b- and series c-, respectively[98-100] (Fig. 1.5). LacCer is the precursor of the 0- or asialo-gangliosides. The 0- series, meaning asialo, indicates absence of sialic acid moieties. The number (0, 1, 2, 3) of sialic acid residues at the "inner galactose" determines which series (0-, a-, b-, or c-, respectively) a ganglioside of the ganglio-family belongs.[96] Glycosyltransferases are enzymes that catalyze addition of monosaccharide units.[97,101,102] Sialyltransferases (SAT I, SAT II, and SAT III) catalyze formation of the three precursor gangliosides, GM3, GD3, and GT3, respectively. Sialyltransferases are highly specific toward lipid substrate, while other sialyltransferases can sialylate different lipid substrates.[96,103] SAT I, IV and V catalyze formation of α 2→3 sialosyl linkage to galactose. SAT II and III catalize α-2→8 sialosyl linkage to sialic acid.[49] When gangliosides have been synthesized; gangliosides are transported from the site of synthesis to the plasma membrane within 20 min.[104,105]

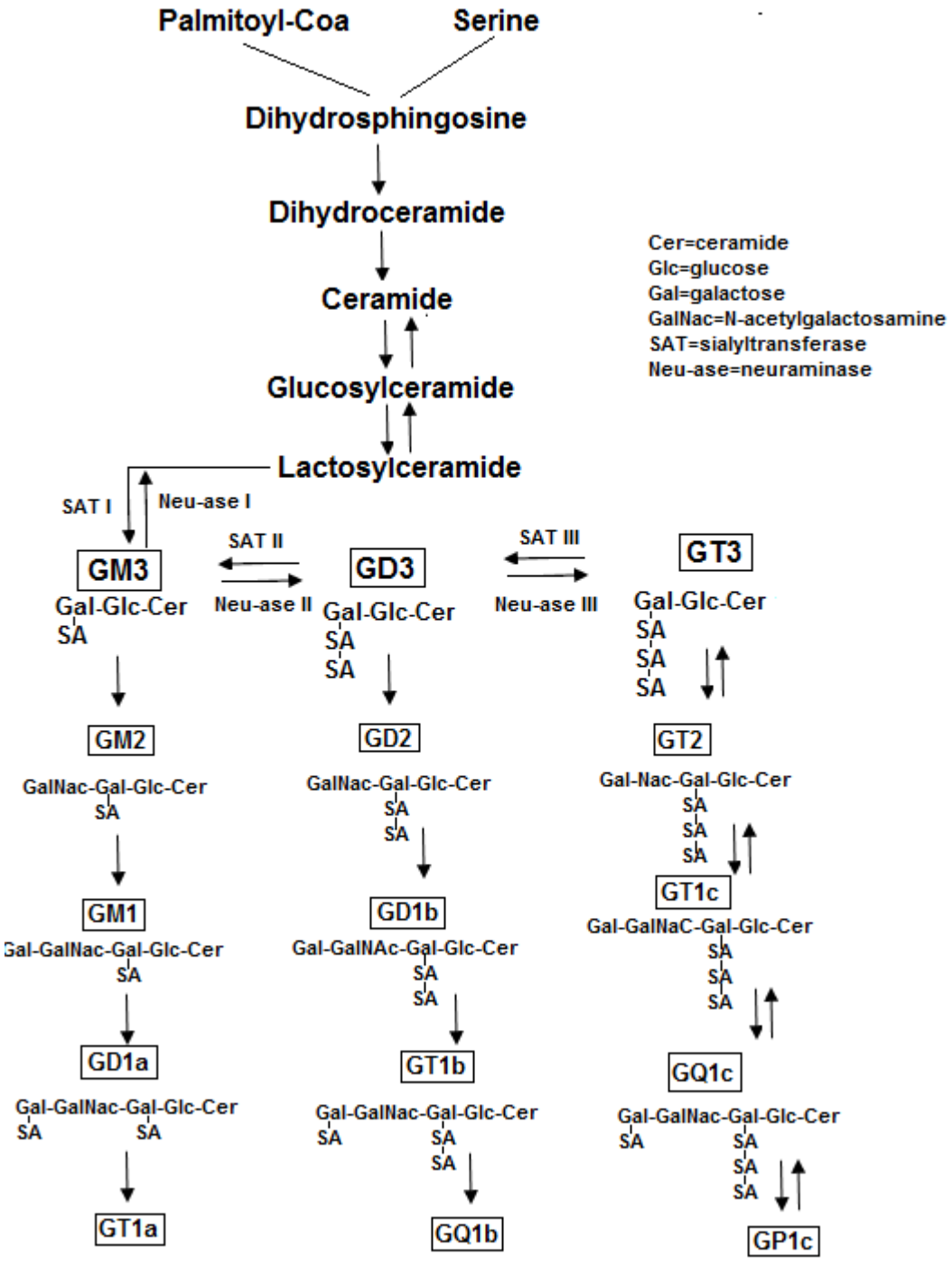


Figure 1.5 Biosynthetic pathway for gangliosides (modified from reference 98).

1.7.2 Catabolism of gangliosides

Ganglioside catabolism takes place in the lysosome[98] by sequential removal of sugar moieties. Exohydrolases (or neuraminases) remove carbohydrates from the hydrophilic end of the ceramide core.[49,98] This process requires an acidic pH inside the organelle.[106] The first step is degradation of the sialic acid terminal residue in the polysialogangliosides.[98] Transformation of polysialogangliosides by corresponding sialidase occurs in order to form monosialogangliosides. From GM1, galactose is removed to generate GM2, and from GM2, the N-acetyl-galactosamine is divided to form GM3, producing lactosylceramide. Lactosylceramide is later converted to ceramide by β-galactosidase and β-glucosidase.[106] The final products of degradation are individual monosaccharides, long chain bases and fatty acids.[49]

1.7.3 Digestion and absorption of gangliosides

Digestion and absorption of gangliosides has been studied using human milk *in vivo*. As GM3 and GD3 gangliosides reach the stomach, the sialic acid does not undergo change in the acidic conditions.[107] In an infant stomach, over 80% of the sialic acids contained in GM3 and GD3 structure remain intact until reaching the intestinal tract.[108] Exogenous gangliosides are absorbed in the small intestine and transported to different cells.[69] The small intestine absorbs dietary gangliosides through passive diffusion. One mechanism proposed for ganglioside absorption in the intestine explains that micellar gangliosides are taken up by endocytosis or dissociation of gangliosides from micelles, crossing the water layer and being inserted into the brush border membrane. Gangliosides may stay in the plasma membrane or may be transported by endosomes or proteins.[109] Gangliosides may also be modified in the Golgi apparatus, or may cross through the basolateral membrane into the blood to be

incorporated into different tissues.[110] Dietary gangliosides are modified in the enterocyte and provoke changes in membrane content of gangliosides.[69] An increase in concentration of gangliosides in the apical membrane affects permeability of the intestine and also modifies enterocyte function.[111]

1.8 Dietary gangliosides

Gangliosides have been found in eggs, chicken livers and dairy products. The GM4, GM3 and GD3 ganglioside content isolated from egg yolk are 2.5, 8.5 and 1.5 mg/yolk, respectively.[58] In chicken liver, the amount of ganglioside, GM4 and GM3, is 330 nmol/g.[58] Gangliosides were extracted from dairy products[112] and illustrated (Table 1.4).

Table 1.4 Ganglioside concentration from dairy products

Dairy Product	**Ganglioside concentration (μg/g dry wt.)**
Sweet Buttermilk	42 ± 10
Cultured Buttermilk	26 ± 4
Yogurt	36 ± 10
Whole milk	~ 4
Skim milk	~1

$MW_{average}$ 2000g/mol of gangliosides(modified from reference 112).

Recently, one Canadian study determined ganglioside intake in a healthy human population that consumed egg, tuna, beef, yogurt, milk and cheese. Minimal amounts of ganglioside intake, around <200 mg/day, were detected in a healthy diet of 2000 kcal/day.[113] There is no literature to suggest an optimal intake of gangliosides.

1.9 Milk gangliosides

1.9.1 Milk fat globule membrane

Bovine milk contains 3.5 to 4.7% fat, which forms an unstable emulsion. The emulsion is composed of very small spherical droplets, approximately 0.2 to 15 μm in size.[114]

Milk fat is made up of triglycerides surrounded by a complex membrane called the milk fat globule membrane (MFGM). The thickness of the membrane is about 4-20 nm. MFGM functions are to emulsify and protect the fat from enzymatic and microbiological attack, flocculation and coalescence.[115,116] The MFGM may play a role in transport and delivery of lipid soluble nutrients in the gastrointestinal tract.[117] This membrane represents 2-6% of the total fat in milk, and is also composed of proteins, lipids and carbohydrates in a ratio 4:3:1.[118] The most common MFGM proteins are Mucin 1, Mucin 15, CD36, Butyrophilin, Lactadherin, Xanthine Oxidoreductase, Adipophilin, and FABP, the last three of which are unglycosylated.[119] The composition of MFGM in bovine milk is shown (Table 1.5).

Table 1.5 Composition of milk fat globule membrane (MFGM)

Constituent	% of total lipids	Reference
Triglycerides	43-68,	120,121,122,123,124,125,126,127
Diglycerides	2-9	128, 123, 124, 125, 126, 127
Monoglycerides	0.4-8 ± 0.5	128, 123, 124, 125
Free fatty acids	0.6-7	128, 130, 123, 124, 125, 126, 127
Polar lipids (phospholipids and glycolipids)	15-54	121, 131, 132, 133, 123, 125, 127
Neutral lipids	56-80	130, 123
Esters	0.1-0-8	128, 130
Cholesterol	0.2-6	128, 121, 128

1.9.2 Advances on different models of MFGM

Models for the structure of milk fat globule membrane have been proposed. These MFGM models are explained in chronological order.

Ascherson (1840) and Babcock (1885), cited by Brunner in his report *Physical Equilibria* in milk in 1974, were the first to describe a membrane surrounding milk fat globules. Ascherson reported that this membrane stabilizes emulsions, and he called it the "haptogenic membrane". Babcock also claimed to observe a membrane surrounding the fat globule.[134]

Since the 1950s, the MGFM has been recognized as a membrane derived from cellular membranes.[135] In 1969, Copius Peereboom, in his research regarding milk phosphate reactivation, elaborated one of the first illustrated models representing the physical structure of the milk fat globule membrane. In this model, the fat membrane was represented as being surrounded by three layers: the inner layer, made up of lipid-proteins, the intermediate layer, and the outer layer, constituted by lipoproteins and some minerals.[136]

In 1972, Heinz Bauer observed the fat membrane using electron microscopy. Bauer explained how fat globules are enveloped in a monomolecular layer of phospholipids associated with other monomolecular layers of triglycerides[137] and proposed a new vision of the composition and structure of the MFGM.

The component arrangement is still not well known; however, several structural models have been proposed to describe the way that the different proteins and lipids are ordered in this membrane. Kanno (1980) introduced enzymes to his MFGM model.[138] In the 1980s, McPherson and Kitchen (1983) incorporated the models proposed by Copius Peereboom in 1969; Bauer in 1972 and Kanno in 1980.[131,136-138] This MFGM representation

incorporated knowledge of different components, including polypeptides, triglycerides, cholesterol and phospholipids.

Danthine (2000) showed two simple models taken from King's studies and Morton's studies.[123] The most important components of these two different models are proteins, lipoproteins, phospholipids, and vitamins.[123] Danthine also proposed a new model which incorporated many components including, polypeptides, glycolipids and different classes of phospholipids.

Singer and Nicholson (1972) proposed the fluid mosaic model in order to understand biological membrane behavior and function. A fluid mosaic model is composed of proteins, lipids, and oligosaccharides. In this model, the highly polar moiety of proteins is oriented to the membrane into the exterior of the membrane, while the non-polar groups are oriented to the hydrophobic interior of the membrane.[139] The Singer and Nicholson model was proposed to explain the structure of MFGM structure, which has been analyzed as a phospholipid discontinuous bilayer serving as a backbone in the fluid membrane phase. Proteins are located through the phospholipid bilayer.[120,140,141]

Keenan and Mather (2002) described the structure of the MGFM as consisting of a trilaminar bilayer consisting of droplets, a dense proteinaceous coat, and finally a true bilayer membrane originating from the secretory cell apical plasma membrane.[142] The phospholipid bilayer, which is in a fluid state, is the backbone of the membrane. Membrane proteins are inserted in the phospholipid bilayer. Glycolipids and glycoproteins are located toward the exterior. Cholesterol is associated with polar lipids.[116]

Recently, the MFGM has been described as a trilaminar membrane. The fat core is surrounded by a surface-active layer from intracellular fat dropet. This is the first layer. The

second layer is an active layer composed of proteins surrounding intracellular neutral lipids. The external part or third layer is a bilayer membrane secreted by the apical plasma membrane. Glycolipids and proteins are inserted into the phospholipid bilayer, forming a glycolalyx.[119,143]

Phospholipids are located in the outer membrane of the fat globule. Phospholipids are organized in two phases, liquid-disordered phase coexisting with a liquid-ordered phase (called as lipid rafts), the latter rich in sphingomyelin and cholesterol. Glycoproteins and glycolipids are heterogeneously distributed in the fat globule membrane and not located in the lipid rafts.[144,145,119]

The importance of gangliosides in the MFGM structure was described for the first time by Keenan et al. (1975).[146] Gangliosides are inserted in the MFGM, and are not hydrolyzed by neuraminidase.[146] After components are extracted from MFGM by trypsin or EDTA-2 mercaptoethanol, the membrane gangliosides are accessible, and degraded by neuraminidase. This extraction demonstrates that the carbohydrate portion of ganglioside is headed to the external environment.[128]

1.9.3 Modification of MFGM by mechanical treatments

The MGFM undergoes structural changes during transformation and preservation of milk. Physical treatments such as cooling, drying, separation, shearing, agitation, heating and homogenization can modify this membrane.[147]

The fatty acid composition and content were studied for milk sub-products. Progressive heat treatment altered the content of total saturated and unsaturated fatty acids.[148].

Greenbank et al. (1961) found that in bovine milk, losses of phospholipids of the MFGM occurred when heated at different temperatures and times.[149]

Homogenization is a physical process that decreases in size of fat globules and increases in the surface area.[150] McPherson et al. (1984) evaluated effects of homogenization on loss of phospholipids in the MFGM. Phospholipid decreased after homogenization compared to unhomogenized milk.[151]

Mechanical operations (automatic milking systems, storage, handling and processing including pumping) introduce air and turbulence into the milk, damaging the MFGM. Aeration and agitation may increase the absorbance of lipase from the milk serum to the MFGM. This absorbance may cause loss of milk fat globule membrane and globule core due to lipolysis and the hydrolytic rancidity of glycerides.[128,114] Oxidation is caused by introduction of air.[128] When a fat globule and a air bubble collapse, the MFGM is fractured because of physical stress, producing coalescence.[152] As a result of the internal and external contact, material of the globule is spread along the oil/water interface. The casein coats are free, and the lipase linked to the casein can induce lipolysis.[153] Lipolysis can be avoided by inactivation of lipase by heat treatment. [154] Measurement of free fatty acid (FFA) and determination of free triacylglycerols evaluates the impact of mechanical treatments on the MFGM.[152,155]

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1.9.4 Milk lipids in MFGM

Milk lipids in the MFGM are divided into three groups: neutral lipids (tri-, di-, and mono, acylglycerols), polar lipids (phospholipids and glycolipids), and miscellaneous lipids (sterols, carotenoids, and vitamins).[156] There are two main kinds of polar lipids in the milk fat globule

membrane, phospholipids and sphingolipids. Phospholipids, also called glycerophospholipids, consist of a glycerol backbone with two esterified fatty acids, containing a phosphate part with different hydroxy compounds (choline, serine, ethanolamine, inositol, serine) that may be united on the third hydroxyl group. Phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin are present in high amounts, while the anionic forms, phosphatidyl inositol and phosphatidyl serine, are less abundant[119] (Table 1.6). Overall, the fatty acid chain on the sn-1 position is more saturated than the sn-2 position of the glycerol backbone.[119] The MFGM phospholipids are important emulsifiers. Phospholipid destruction damages the milk fat globule membrane. The phosphatidyl choline and phosphatidyl ethanolamine contain unsaturated fatty acids (40-60%), and polyunsaturated fatty acids susceptible to oxidation and flavor defects in dairy products.[118]

Table 1.6 Phospholipid concentrations in bovine whole milk (4% fat)

Phospholipid	Concentration (μmol/L)
Phosphatidyl choline	70-150
Phosphatidyl ethanolamine	101
Phosphatidyl inositol	17
Phosphatidyl serine	9
Sphingomyelin	70-125

Modified from reference 157.

1.9.5 Gangliosides in different milk sources

In human and animal milk, gangliosides are associated with the outer membrane of MFGM.[98,158,159] Milk gangliosides differ in content depending on the type of milk (human, bovine and other types).

The two most important and abundant human milk gangliosides are GD3 and GM3. Other gangliosides such as monosialoganglioside GM1.[160] GD1a, GD1b, GT1b, GQ1b[19] GM2 and GT3[161] are present in low amounts. Takamizawa et al. (1986) calculated that GD3 and GM3 together form 70-90% of the total gangliosides in human milk,[162] but Pan and Izumi (1999) later found that GD3 and GM3 ganglioside constitute 50-60% of the total.[163] Some authors have studied secretion of gangliosides in milk during different chronological postpartum periods.[163,159,164,19,168] The largest change is due to stage of lactation affecting ganglioside concentration. GD3 is the major component in colostrum in the first 1-8 days, while GM3 is the major ganglioside at the end of the lactation.[19,164] Pan and Izumi (1999) assessed the total lipid-bound sialic acid content (T-LBSA) in colostrum (2-6 days) and in later milk (7-46 days). The variation is 9.51$\pm$1.61μg/mL and 9.07$\pm$1.15μg/mL, respectively.[164] Pan and Izumi (1999) reported that GD3 is the main ganglioside in human colostrum (GD3, 42-56%; GM3, 2-6.5%), while GM3 decreases at eight days postpartum (GM3, 32%, 28%) and increases after eight days (GM3/GD3, 0.8-2.6%).[164] A more recent study of mothers after preterm delivery demonstrated that GD3 is also the main ganglioside in colostrum; there was difference between preterm and full-term human milk until 8 days postpartum. In preterm human milk, GM3 concentration was very low, compared to full-term human milk even at 10 days postpartum.[165] This chronological alteration suggests that ganglioside species have different roles at different stages of neonatal development.[19] The influence of different ethnic populations, dietary habits and way of life were evaluated in human milk from Spanish and Panamanian mothers. No differences in ganglioside concentrations were found.[166] Other studies have shown that total lipid and ganglioside content are positively correlated. In human milk during milk lactation, total lipid content increases, while higher ganglioside

concentration is only found after the third week of lactation.[167,170] Undefined polysialogangliosides with branched oligosaccharide chains have also been found in human milk suggesting structurally complex gangliosides may play an important role in developing neonatal tissues.[167]

Bovine milk ganglioside originates in the apical plasma membrane in the mammary gland secretory cells.[171] The most abundant gangliosides in bovine milk are GM3 and GD3. GD3 is most abundant in mature bovine milk.[37,19,172,163,112] The lipid backbones of milk gangliosides are composed of sphingosine (d18:1t4, with smaller amounts of sphinganine and other chain length homologs) and 16:0, 22:0, 23:0 and 24:0 as the major fatty acids.[173-175]

The amounts of gangliosides detected in milk vary from different studies. GD3 ganglioside are reported >60% of total ganglioside concentration and GM3 ganglioside > 12% of total ganglioside concentration.[35,161,163] The total concentration of GD3,GM3 and GT3 is 60-92%.[172,19,35] These variations also depend on the stage of lactation.[35,19,172]

Bushway and Keenan (1978) reported other ganglioside species, GM1, GM2, GD1a and GD1b, present in bovine milk.[176] Laegreid et al. (1986) also discovered GM1 in bovine milk. The concentration of ganglioside is 10 times higher in human milk than in bovine milk.[177] Iwamori et al. (2008) assessed other ganglioside concentration, GM2, IV^{3}NeuAcα, V^{3}NeuAcα, GT3 and GM1, as approximately 3.2%, 2.1%, 0.9%, 5.2% and 0.07% of total gangliosides, respectively.[161] Jensen (1995) reported that milk contains ~11 mg/L of gangliosides and the levels of different ganglioside species are ~9–13 mg of GD3, 1.2 mg of GD1b, 0.7 mg of GM2, 0.3 mg of GM3 and 0.001 mg of GM1.[178]

Buttermilk has a higher content of gangliosides. The ganglioside concentration is 1-2% of the total lipid mass in buttermilk.[179] Two different gangliosides in buttermilk, GD3a and

GD3b have the same oligosaccharide structure.[169] The difference is the ceramide moiety and fatty acid content, representing 85% of the total lipid bound sialic acid. Takamizawa et al. (1986) found 0.92 μm of T-LBSA/g dry weight of buttermilk, and GM3, GD3 and GT3 are the major gangliosides in buttermilk.[162]

Martin et al. (2001) reported changes in fatty acid content of bovine milk depending on stage of lactation. 18:0 was the major fatty acid in the colostrum stage and decreased in late lactation. 16:0 followed a different trend, increasing in the late lactation milk, while a smaller amount was present in colostrum. The long chain bases also undergo changes (3-ethoxy-15:0 sphinganine the major fatty acid). These fatty acid changes suggest differences in fluidity of the milk fat globule membrane.[35]

GM3, GD3 and GT3 are predominant gangliosides in goat milk (66-92%). During lactation, GM3 concentration decreases, while GD3 content increases. The highest ganglioside concentration is in colostrum (974 μg T-LBSA/kg), and at the end of the lactation period decreases to 175 μg T-LBSA/kg.[180] Other gangliosides detected in goat milk are GD2, GT1b, GM1, GQ1b and GD1b.[161] Sheep milk contains a smaller amount of gangliosides than goat milk, and only about 6-7% of the ganglioside content of cows milk. Sheep milk gangliosides follow the same trend as cow and goat milk; ganglioside content is higher at the beginning of lactation and ganglioside content is lower at the end of lactation.[181]

Milk of different classes of buffalo contains 40-100% more gangliosides than Swiss cow milk. In Pakistan, buffalo colostrum was higher in ganglioside content (24 nmol/g) than mature buffalo milk (8-11 nmol/g). Buffalo milk has more polysialogangloside content than cows milk.[53]

1.9.6 Factors affecting milk ganglioside profile

Stage of lactation, dietary manipulation, season, species and MFGM size are factors that may modify ganglioside content or ganglioside composition in milk. In cows, goats and ewes milk, ganglioside content change depending on the season; ganglioside content in fall is higher than in other seasons.[182] The amount of gangliosides also changes with animal species. The average in cow and goat milk is very similar, 1.02±0.05 μmol/100 mL of milk (2.04 mg±0.1/100mL) and 1.05±0.05 μmol/100 mL of milk (2.1±0.1 mg/100 mL), respectively; whereas the difference is greater in human milk 1.79±0.28 μmol/100 mL of milk (3.58±0.5 mg/100 mL).[161]

Fat globule size is related to fat content and associated with amount of milk fat. Reducing the milk fat globule size would cause a reduction in MFGM and in the MFGM constituents.[183]

The stage of lactation also seems to be an important factor in variation of gangliosides composition in milk. Lopez et al. (2008) determined the lipid composition of the MFGM after modifying the cow´s feeding with a diet high in polyunsaturated fatty acids; they concluded that this diet modifies the fatty acid composition of phospholipids, triacylglycerols and sphingolipids in the MFGM.[184]

1.10 Biological and physiological functions of gangliosides

Gangliosides perform different functions such as calcium homeostasis, neural repair, and involvement in neurological diseases, immune diseases and infectious diseases in mammalian cells. Other cellular functions of gangliosides are immunological factors, calcium

homeostasis, apoptotic functions, cell recognition, cell signaling, cell differentiation and proliferation, gangliosidoses, cell adhesion.[185]

1.10.1 Immunity functions

Gangliosides regulate cells of the immune system, involved in immunosuppressive activities. Gangliosides are involved in T-cell activation. Gangliosides modulate the response of T cells and modify the immunological environment by promoting type-2 T cell responses.[186] Activation of T cells requires different ganglioside species. In cells from GM2/GD2 synthase-null mice (only synthesizing GM3 and GD3), CD4+ T-cell activation was normal; however, CD8+ T-cell activation was deficient. Supplementation with missing gangliosides restores normal activation.[187] In the intestines, gangliosides are involved in immunologic mechanisms. Weanling mice fed with a gangliosides-enriched diet demonstrated early development and significantly higher numbers of Th1 and Th2 cytokine-secreting cells in lamina propria and Peyer's patch lymphocytes than weanling mice fed with a diet without gangliosides.[188] In weanling mice, dietary ganglioside increased Ig-A secreting cell content as well as increases in the amount of IgA in the lumen.[189,190] In infants, different ganglioside species and content promote intestinal immunity by stimulating or inhibiting proliferative or inhibitory responses in intestinal lymphocytes.[191] Previous studies in our lab show dietary gangliosides reduce pro-inflammatory mediators, platelet activating factor and diglycerides in microdomains of the intestinal mucosa of weanling animals.[192]

1.10.2 Calcium homeostasis

Gangliosides are involved and induce changes in calcium regulatory mechanisms by modulating ion channels, transporting/exchanging proteins and utilizing Ca^{+} enzymes.[193] Gangliosides are abundant in neuronal cells which secrete certain ganglioside profiles, and are involved in Ca+ regulatory mechanisms.[193] Studies have shown that exogenous or endogenous gangliosides modulate calcium mechanisms, enhancing neuritogenic and neuroprotective effects. For example: endogenous gangliosides influence neuritogenesis through influence on Ca+ transport.[194] In one study, treatment with GM1 in the nuclear membrane altered Ca2+ regulatory mechanism in the nucleus, increasing calcium release and stimulating neuritogenesis.[195] In N18 neuroblastoma cells, GM1 modulates L-type calcium channels, suggesting that it plays an important role in development and electrical excitability of neurons.[196] Cerebellar neurons from GM2/GD2 synthase-lacking mice are deficient in a calcium regulatory mechanism that is regulated by the deleted gangliosides. This indicates that calcium homeostasis is dependent on complex gangliosides, and the lack of ganglioside has consequences for neuronal development.[197]

The nuclear envelope of neurons contains GM1 associated with Na^{+}/Ca^{2+}; GM1 potentiates the transfer of Ca2+ from the nucleoplasm to the nuclear envelope. In one study, a neuroprotective effect was observed in Na^{+}/Ca^{2+} - GM1 by culturing cells from GM1 lacking mice that were vulnerable to Ca^{2+} induced apoptosis.[198]

In cardiac myocytes, gangliosides are important to maintain transmembrane Ca gradients. Gangliosides containing negatively charged sialic acid maintain Ca+ permeability of cardiac cells, removing sialic acids on ganglioside increases Ca+ intake and produces a Ca+

leak in the ion channels.[199] In rabbit skeletal muscle, GM3 modulates the calcium level, which regulates muscle contraction.[200]

1.10.3 Apoptotic functions

GM3, GD1 and GD3 are apoptotic inducers.[201] Ceramide is converted into GD3. GD3 and induces apoptosis by disrupting the mitochondrial membrane potential and stimulating cyt c release and caspase activation.[202] Mitochondrial permeabilization is inhibited by antioxidants and cyclosporin A, indicating a role for ROS (reactive oxygen species) production and Ca^{2+} in GD3-mediated mitochondrial effects.[203] The secretion of Fas or TNF-α (Tumor Necrosis Factor--α) produce increase in the cellular concentration of GD3.[204,202] Down regulation of GD3 synthase prevents Fas or β-amyloid-induced cell death.[204-206]

GD3 is present in the plasma membrane as well as in the endosomal/Golgi apparatus. After TNF-α exposure, GD3 is redistributed from plasma membrane to the endosome where GD3 colocalizes with Rab5-positive early endosomes and Rab7-positive late endosomes. This redistribution mechanism contributes to TNF-α mediated hepatocellular cell death.[207] Increasing expression of GD3 synthase in U-1242 MG human glioma cells caused apoptosis determined by cytological appearance, increased annexin V binding, and DNA fragmentation. In glioma cells, GD3 secretion may regulate the mechanism of GD3 induced apotosis.[208]

1.10.4 Cell recognition

Glycosphingolipids are located in the outer leaflet of plasma membrane and cluster to form binding sites. Three different mechanisms explain the effect of glycosphingolipids controlling cell recognition: recognizing lectins; regulating adhesion receptors as integrins and

fibronectins; clusters of specific glycosphingolipids binding clusters from opposite glycosphingolipids.[209] Examples of each of these three mechanisms may be explained as follows.

Glycans on one cell bind to lectins (glycan binding proteins) on a different cell to mediate cell-cell interactions. The effect of lectin-glycan linkage is from cell-cell adhesion to regulate intracellular signaling pathways. Gangliosides are receptors in glycan-driven cell-cell recognition systems.[210] Lectins have greater ability to recognize gangliosides than oligosaccharides alone.[211]

Highly sialylated gangliosides regulate the interaction of epithelial cells and fibronectin through carbohydrate-carbohydrate interactions between GT1b and the α5 subunit of α5β1 integrin.[212] Carbohydrate-carbohydrate interaction has been distinguished by high specificity, weak affinity, and high flexibility. Single carbohydrate-carbohydrate interaction is a weak binding, but many such interactions produce a strong binding affinity.[213] GM3 links to different types of glycosphingolipids, which contains a GalNac ending moiety, and a LacCer. GM3 interacts with different glycolipids such as gangliotriaosylceramide Gg3Cer and globotetraosyl ceramide Gb4Cer. GM3-Gg3 interactions are observed between mouse melanoma B16 (which expresses GM3) and mouse lymphoma L5178 (which expresses Gd3Cer).[214] GM3-Gg3Cer or GM3-LacCer interactions play a physiological role in adhesion of melanoma cells to mouse endothelial SPE-1cells, suggesting that melanoma metastasis is initiated by interaction of GM3 with Gd3Cer or LacCer.[214]

1.10.5 Cell signaling

Some glycosphingolipids are located close to proteins, which are important for cell signaling, so glycosphingolipids can regulate information transduction mechanism through cell membranes.[215,216] While glycosphingolipids in cell-recognition function based on the carbohydrate moiety, cell-signaling depends on glycosphingolipid structure (lipid and carbohydrate moiety). Signaling pathways are activated by cell surface receptors and manage to activation of transducer molecules. There are three different types of receptor processes obtained from signaling molecules: ion-channel linked; G-protein linked and enzyme linked (tyrosine kynase dependent).[209] Examples of each process are as follows.

Gangliosides are related to membrane proteins, which function as ion channels; the former change the properties and functions of the cell environment.[217-219] For example, gangliosides regulate activity of Na^+/K^{+-}ATPase by altering membrane lipid environment around this enzyme.[220] Exogenous GM1 injected into Mongolian gerbils with global ischemia minimized the losses of Na^+/K^+-ATPase, and Mg^{2+-}ATPase suggesting that gangliosides protect the structure of the plasma membrane against ischemic injury.[221] Exogenous gangliosides affect either muscle fibres or nerve terminals by resisting hypoxia and the absence of K^+.[222] Gangliosides affect head groups of channel molecules, altering membrane fluidity in order to preserve membrane viscosity around ion channels independently of environmental temperature.[219] Gangliosides also regulate voltage-dependent sodium channels. GD1a, one of the main gangliosides in brain tissue, generates a hyperpolarizing shift in voltage to activate sodium channels in brain to modulate excitability.[223]

G-protein coupled receptors are signaling molecules in charge of signal transduction across plasma membranes responding to neurotransmitters and hormones. A B2 adrenergic

receptor is a type of G-protein couple receptor that modulates catecholamine, epinephrine and norepinephrine effects. A B2 adrenergic receptor varies between active and inactive states. This conformational changing structure is regulated for lipid microdomains. A stable hydrophobic interaction with cholesterol and B2 adregenic receptor in lipid membranes containing GM1 has been observed.[224] Translocation to lipid rafts of Gα°, heterotrimetric of G protein, depends on gangliosides, which function as platforms on neuronal membranes for appropriate interaction of Gα°.[225]

Insulin receptors (IR) are tyrosine kinase receptors on the surface of cells. IR signaling is modulated by gangliosides.[210] The conversion of an insulin-resistant state induced in adipocytes by TNF-α may depend on a high level of secretion of GM3 after up-regulated gene expression of GM3 synthase, indicating that GM3 may suppress insulin signaling during chronic exposure to TNF-α.[226,227]

An epidermal growth factor receptor (EGF) is a glycoprotein that regulates cell growth in normal and cancer cells. The linkage of EFG autophosphorilate tyrosine residues on the receptor stimulates the activation of Ras, Raf and mitogen-activated protein kinase in cell proliferation. Gangliosides modulate the EFG proliferation pathway in many types of cells.[15,228,229]

1.10.6 Cell differentiation and proliferation

Cell differentiation and proliferation are processes most frequently studied in neuronal cells to understand ganglioside neurobiological functions such as neurodifferentiation, neuritogenesis, and synaptogenesis. Ganglioside analysis has noted the impact of composition and structure of ganglioside on different types of cells. Gangliosides concentration changes

among different brain sections. For example, GM1 and GM4 are more abundant in brain white matter than in the cerebral cortex because these gangliosides are enriched in myelin. GT1b is more abundant in the cerebellum than in the cerebrum.[230]

Ganglioside profile on neuronal cells changes during different developmental stages.[231,232] Levels of GM3 and GD3 are highly abundant in the early embryonic vertebrate brain stage and slowly decrease content during aging. In later developmental stages, more complex gangliosides, GM1, GD1a, GD1b and GT1b, increase in concentration.[233,234,231] Mono-, di-, tri- and tetra- sialogangliosides (except for GM4) have the effect of inducing neuritogenesis, while acidic glycosphingolipids, sulfatide and asialogangliosides have no effect on neuritogenesis.[235] GM1 promotes neuritogenesis in a Ca2+ process.[236] GM2 is associated with ectopic dendrite growth and plays a role in modulation of dendritogenesis in neurons.[237] GD3 has been categorized as a marker for cell division and migration. GQ1b is characteristic of nerve cell sprouting and arborisation. GD1a is a marker for synaptogenesis; and GM1 and GM4 are indicative of myelination, in brain cortex.[238] The secretion of ganglioside species suggest the roles that gangliosides play during different developmental stages.[239] Qualitative changes of gangliosides during development are illustrated (Fig. 1.6).

Genetically modified mice lacking gangliosides or glycosyltranferases expression, contain neurological disorders such as axonal degeneration, sensory and motor and behaviour deficits.[239,240] These observations suggest gangliosides are involved in neuritogenesis, axonogenesis and synaptogenesis.[239]

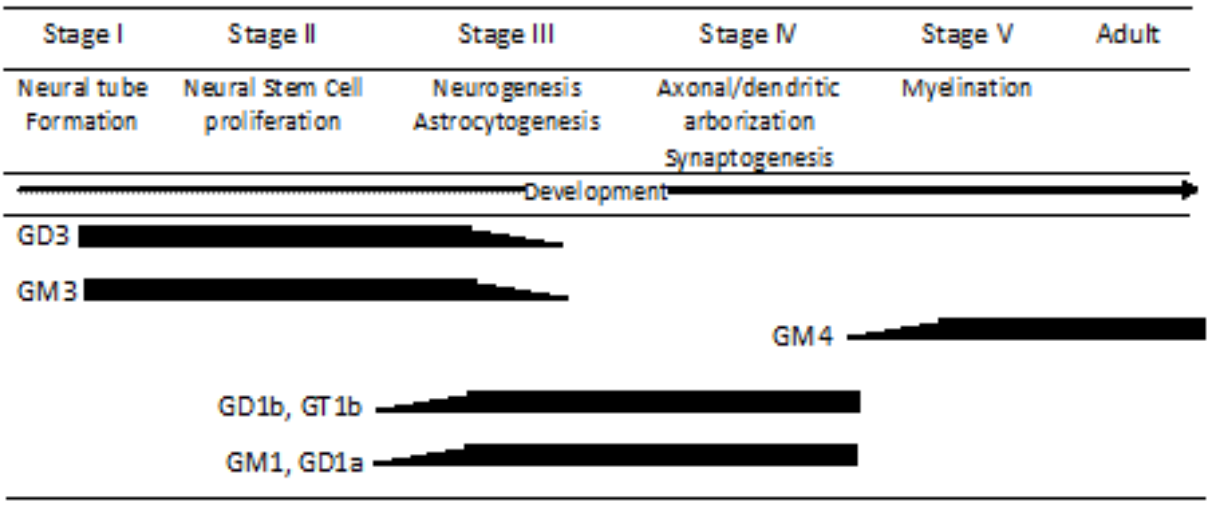

Figure 1.6 Neurodevelopmental milestones and changes in gangliosides (modified from reference 240).

1.10.7 Gangliosidoses

Glycosphingolipids are implicated in the pathology of neuronal diseases. Metabolic disorders implicating glycosphingolid pathway enzymes cause glycosphingolipidoses. This is a deficiency in an enzyme that avoids degradation of specific glycosphingolipids, which then accumulate in lysosomes neurons.[241,201,242] GM1 gangliosidosis and GM2 gangliosidosis (Tay-Sachs disease and Sandhoff disease) are caused by defects in lysosomal glycosidases or co-activators, and thus gangliosides accumulate.[243] GM1 gangliosidosis or β-galactosidase deficiency is a neurological disease, progressively attacking motor skills and contributing to mental retardation. Patients succumb in the first years of life.[241,244] GM2 gangliosidases or β-hexosaminidase deficiency is characterized by accumulation of GM2 in the central nervous system. This abnormality appears in late childhood or adulthood, causing tetraparesis, dementia, blindness. Only ß-hexosaminidase degrades GM2 ganglioside; if it is not present the substrate accumulates.[241,201] Other ganglioside deficiencies are shown on Table 1.7.

Table 1.7 Gangliosidoses and clinical manifestations

Disease		Major clinical manifestations
Glycosphingolipidoses	**Enzyme Deficiencies**	
Fabry disease	α-galactosidase ceramide	Painful numbness in the extremities
Gaucher disease	ß-glucocerbrosidase	enlargement of liver/spleen
Tay-Sachs disease	ß-hexosaminidase A	cherry red retinal spot, seizures
Sandhoff disease	ß-hexosaminidases A & B	mental retardation, seizures
Peripheral neuropathies	**Anti-ganglioside antibodies**	
Guillain-Barre syndrome	anti-GM1, GM2, GQ1b	progressive weakness of limbs, absence of reflexes.
Miller-Fisher syndrome	Anti-GQ1b	ophthalmoplegia, absence of reflexes, lack of voluntary coordination of muscle movements.
Chronic idiopathic ataxic neuropathy	anti-GM3, GD1b, GD3, GQ1b	loss of kinesthesia, lack of voluntary coordination of muscle movements.
Multifocal motor neuropathy	anti-GM1	motor weakness of nerve trunk territories with slight muscular wasting.

Modified from reference 201

1.10.8 Cell adhesion

Microbial attachment to the host is the initial phase of colonization or infection.[245] Bacterial adhesion requires a receptor on the surface of host epithelial cells. The receptors are carbohydrate moieties of glycolipids or glycoproteins on the cell plasma membrane of the host.[108] Various pathogens and toxins recognize sialic acid as targets for binding the host surface. Pathogen-binding proteins, called extrinsic receptors, are produced for pathogens to bind sialic acid on the host cell membrane.[246] Gangliosides are well known receptors due to sialic acid terminals, binding pathogenic and non pathogenic viruses, bacteria and protozoa.[108]

Different pathogenic bacteria that bind sialic acids on human cells are:

Human *Influenza A* binds Siaα2-6Gal(NeuAc) structure. [246]

Avian *Influenza A binds* Siaα2-3Galβ1-.[246]

Vibrio Cholerae toxins bind Galβ1-3GalNAcβ1-4(Siaα2-3)LacCer structure. [246]

Clostridium botulinium binds polysialogangliosides. [246]

Helicobacter pylori binds Siaα2-3Gal structure on gangliosides. [246]

During the initial event of infection, myxoviruses such as influenza and Sendai viruses link to receptors on the host membrane.[247] The attachment of influenza A virus to human erythrocytes and mucins of the respiratory system is controlled by lectins or hemagglutinins. This binding is reversible, but it releases neuramidases (sialidases). Viral neuramidases help to spread viruses in tissues by preventing adhesion to cells and to mucus layers protecting epithelia of the respiratory tract. Inhibitors of neuramidases were synthesized to be used for treatment of influenza.[248,249,39] Markwell et al. (1981) demonstrated that gangliosides with similar structure, GD1a, GT1b, and GQ1b serve as natural receptors for Sendai viruses.[280] Epand et al. (1995) also demonstrated that GD1a functions as a receptor for Sendai virus binding to liposomes of certain lipid compositions.[251] Rotaviruses, which cause gastroenteritis in children, attach to gangliosides as GD1a. It has been suggested that rotavirus can adhere to cell membranes, but gangliosides are required for cell entry.[252,253]

Bacteria develop sialic acid-binding adhesins, lectins or agglutinins which are located in fimbriae or pili, over 100 examples of those have been detected.[39,254] Other pathogenic bacteria express sialic acids on the surface.[246] When present, sialic acid is found in capsular polysaccharides or lipopolysaccharides. Terminal sialic acids are present in

lipooligosaccharides. These terminal sialic acids mimic mammalian structures and function as a protective barrier to avoid attack by the host's immune system.[246,255]

Bacteria and bacteria toxins such as cholera, tetanus and botulinium toxins adhere to sialic acids, situated on gangliosides.[39] Exogenous substances containing gangliosides might inhibit bacteria adhesion to intestinal cells; many studies have been perfomed using breast milk due to high ganglioside content, suggesting that human milk could confer a protective effect against pathogens.[108,42]

Some examples between bacteria toxins and ganglioside interactions are shown as follows. Enterotoxins are produced by *Vibrio cholerae*, which produces cholera toxin (CT), and some strains of *E. coli,* which produce heat labile toxin (LT), both causing acute diarrhea.[256] Both toxins are similar in structure and in action. These toxins consists of an A subunit, which is responsible for enzymatic activities, and a pentameric B subunit, this last is responsible for attachment to the intestinal lumen.[257,258] GM1 ganglioside species has been recognized as a receptor for CT and LT toxins.[259,260,256] Holmgren (1973) reported that CT has more affinity to GM1 than to GM3, GM2, GD1a, GD1b and GTx. Enterotoxins of some *E. coli* strains bind to GM1, but the affinity is much less than cholera toxin.[256] Teneberg et al. (1994) also reported that LT shows a high affinity for GM1 ganglioside species and much less affinity to GM2, GD2 and GD1b species.[261]

To cause toxicity, CT must bind GM1 at the plasma membrane, access the cell by endocytosis and then traffic retrograde into the endoplasmic reticulum. Before causing toxicity, the time between CT binding to GM1 at the cell membrane surface is called the lag phase.[258] An extended lag phase is dependent on temperature, cell membrane viscosity and low levels of GM1. A prolonged lag phase is reduced by adding exogenous gangliosides.[262] It

was suggested that the ceramide tail of GM1 is too short to interact with the submembrane cytoskeleton. GM1 receptor could help activate a membrane protein, involved in translocation of receptor-bound toxin.[263,264] Otnaess et al. (1983) reported that *E. coli* LT and CT toxin activity was inhibited by GM1 human milk ganglioside species and also reported that ganglioside human milk inhibited cholera toxin binding to the rabbit intestine.[265]

Angstrom et al. (1994) reported that tetanus and botulinum toxins bind selectively GT1b, GD1b and GQ1b; and cholera toxin binds specifically GM1.[266] Other bacteria toxins such as Bordetella pertussis, produces pertussis and causes the disease whooping cough in humans, binds GD1a.[260,267]

Idota and Kawakami (1995) reported that GM1 and/or GM3 inhibit adhesion of enteropathogenic (EPEC) and enterotoxigenic (ETEC) *E.coli* to Caco2 cells.[268] Sanchez-Juanes et al. (2009) reported that GD3 and GM3 bind to enterotoxigenic *E. coli* strains.[269] They studied inhibition of the bacterium-mediated agglutination of erythrocytes, and found that bacterial hemagglutination was inhibit by MFGM and glycosphingolipids.[269] Ono et al. (1989) reported the affinity of *E.coli* strains possessing K99 fimbriae binding structures gangliosides containing NeuGc.[270] Microbe-free mice were fed with a solution contained GM3, after 3 days mice were infected with EPEC *E. coli*. The number of adherent bacteria was studied in duodenum, jejunum and colon; mice that were fed with GM3 had a lower number of EPEC *E. coli*. GM3 inhibits adhesion of EPEC *E. coli* to the intestinal mucosa.[108]

GM3, GD3, GM1 ganglioside species and free sialic acid Neu5Ac interfere with adhesion of diarrhea-causing bacteria pathogens such as ETEC *E. coli* ETEC, EPEC *E.coli*, *Listeria monocytogenes*, *Salmonella entericaserovartyphi*, *Shigella sonnei*, *Campylobacter jejuni*, and also *Helicobacter pylori.*[271] The latter bacterium is found in human stomach and

causes gastric inflammation and possibly peptic ulcer disease or gastric cancer. The characteristics and main features of *H. pylori* are shown in the next sections of Chapter 1.

1.11 Epidemiology of *Helicobacter pylori*

Helicobacter pylori was isolated in 1982 by Marshal and Warren[272,273] They found, in 58 patients with chronic gastritis or gastric ulcer, a gram negative, flagellate and microaerophilic bacteria which was related to the genus *Campylobacter.*[272] In 1989, *Campylobacter pylori* was renamed to *Helicobacter pylori*. DNA studies, cellular fatty acids profile, ultrastructural tests, thermoplasmoquinosas profile, growth conditions and enzyme content confirmed the discovery of a new bacterium. Genus *Helicobacter* was established. *Helicobacter* means in Latin "spiral rod of the lower part of the stomach."[274-276] Approximately half of the world's population is infected with *H. pylori.*[277,278] Epidemiology changes depending on the country. In developing countries, between 70-90% of the population is infected, while in developed countries the infection varies from 25 to 50%.[279,280] Only 15% of *H. pylori* infected individuals will develop peptic ulcer disease and <1% develop gastric cancer.[277]

1.11.1 Prevalence

Prevalence of *H. pylori* infection is defined by the proportion of a population that has a particular disease for a given period of time and is calculated as the ratio of those who acquired to those who have recovered from the disease.[281,282] Prevalence of *H. pylori* infection is different from country to country.[283] The World Gastroenterology Organisation reported a global prevalence of *H. pylori* infection (Table 1.8).[284]

Prevalence of infection increases with age (Table 1.8). In the Eurogast study, the detected prevalence was 35% in people 25-34 years of age and 62% in people aged 55-64 years old.[285] Prevalence of *H. pylori* infection in developing countries is more than 50% by the age of 5 years, and increases to about 90% by the age of 20 years.[286] In developing countries such as India, Saudi Arabia or Vietnam, the infection is acquired at an early age; 80% of the population infected by the age of 20.[287] Prevalence of *H. pylori* varies between developing countries, in the same geographical area. For example, in Brazilian adults the prevalence was 82% compared with 65% in Guatemalan adults.[288] Prevalence of infection is decreasing in some Asiatic countries, presumably because of a better socioeconomic status. In China, the prevalence decreased from 63% in 1993 to 47% in 2003. In South Korea, the prevalence of infection decreased from 67% in 1998 to 60% in 2005.[289] In Artic Canada, Aboriginal Communities present high prevalence rates of *H. pylori* infection compared to the rest of Canada.[531]

1.11.2 Incidence

Incidence is defined as the rate of development of a disease in a population and is determined by the number of diagnosed new infections in the population and the proportion of new infections that typically remain undiagnosed.[281] Incidence data are not as abundant as prevalence data. Banni-Hani et al. (2006) suggested that incidence of *H. pylori* in developed countries is about 1% per year until the age of 50 or 60 years, when it stabilizes.[290] In developing countries, the incidence is higher than 1% or 2%. In a study from Bangladesh tracking new infections in children from birth to 2 years, the incidence was 49% by 2 years of

age.[291] In Bolivian children over a period of 2 years, the incidence was 18% per year.[292] The reason for the dramatic difference between developing and developed countries is unknown.

Table 1.8 Prevalence of *H. pylori* globally

Country	Age groups (years)	Prevalence (%)	Country	Age groups (years)	Prevalence (%)
Africa			India	Adults	88
Ethiopia	2-4	48	India, south	30-79	80
Ethiopia	6	80	Japan, 3 areas	20-70+	55.4
Ethiopia	Adultos	>95	Japan, western	Adults	70.1
Nigeria	5-9	82	Siberia	5	30
Nigeria	Adults	91	Siberia	15-20	63
Central America			Siberia	Adults	85
Guatemala	5-10	51	South Korea	16	56
Guatemala	Adults	65	South Korea	>16	40.5
Mexico	5-9	43	Sri Lanka	6-19	67
	Adults	70-90	Sri Lanka	Adults	72
North America			Taiwan	9-12	11
Canada	5-18	7.1	Taiwan	13-15	12.3
Canada	50-80	23.1	Taiwan	>25	45.1
Canada	Adults	95		Adults	
(Manitoba aboriginal communities)	Kids	56			
Aboriginal communities	Adults	51	**Middle East**		
USA / Canada	Adults	30	Egypt	3	50
* Other Canadian aboriginal communities			Egypt	Adults	90
Aklavik		61	Autralasia		
Old Crow		69	Australia	1-59	15.4
Tuktoyaktuk		58		Adults	20
South America			**Europe**		
Bolivia	5	54	(Eastern)	Adults	70
Brazil	6-8	30	(Western)	Adults	30-50
Brazil	10-19	78	Albania	16-64	70.7
Brazil	Adults	82	Bulgaria	1-17	61.7
Chile	3-9	36	Czech Republic	5-100	42.1
Chile	Adults	72	Estonia	25-50	69
Asia			Germany	50-74	48.8
Bangladesh	0-2	50-60	Iceland	25-50	36
Bangladesh	0-4	58	Netherlands	2-4	1.2
Bangladesh	8-9	82	Serbia	7-18	36.4
Bangladesh	Adults	>90	Sweden	25-50	11
Hong Kong	6-19	13.1	Switzerland	18-85	26.6
India	0-4	22			
India	10-19	87			

Abstracted and modified from reference 284, 528, 529, 530,531.

1.12 Factors of transmission

Epidemiological studies have suggested that the transmission of *H. pylori* is associated with three risk factors: socioeconomic status; childhood acquisition; and hygienic conditions.[273] Other factors such as ethnicity and gender have also been studied.

1.12.1 Socioeconomic status

H. pylori prevalence is associated with low socioeconomic status including variables such as low income, high density of children in the household, low education and low hot water supply.[293,280,283] Low-income families have high numbers of children or adults living in a home, high number of people in a room, having to share a bed with parents or other siblings, or living in overcrowded spaces are also associated with *H. pylori* infection.[294] These factors could explain differences in *H. pylori* prevalence rates between Mexico and the United States. O'Rourke et al. (2003) studied prevalence of *H. pylori* infection in children living on both sides of the Rio Grande River.[295] The proportion of Mexican cases infected with *H. pylori* was higher than that of the US cases because of the higher number of low income-families in Mexico.[295] Malaty et al. (1996) studied socioeconomic factors such as income, living conditions and education levels influencing the acquisition and prevalence of *H. pylori* infection in Russia. Income was not relevant due to similar salaries among families.[296] However, *H. pylori* prevalence in children whose mothers completed only 8 to 10 years of school was higher than that of children whose mother completed college.[296] Bastos et al. (2013) also detected the importance of living in a poor environment. Individuals living in a more deprived neighbourhood were associated with higher risk of infection.[297]

1.12.2 Childhood acquisition

Acquisition of the *H. pylori* likely takes place in childhood. The infected childhood population varies from 10 to 80%, depending on the region. By the age of 10 years more than 50% of children are infected.[277] Rothenbacher et al. (2000) suggested that the acquisition of *H. pylori* occurs in the first or second year of life.[298] Jafar et al. (2013) reported that in Iran, acquisition of the *H. pylori* infection occurs before the fourth month of age.[300] In Turkish children living in Germany, prevalence of the infection was 9% among one-year-old children and 37% among two-year-old children.[298] Thomas et al. (1999) reported that in Gambian children prevalence of *H. pylori* infection increased from 19% at 3 months of age to 84% by 30 months of age.[301] In Mexican school-age children, *H. pylori* infection is dynamic. *H. pylori* infection has different intermittent phases: persistent acquisition, spontaneous clearance, and transient events of reinfection and clearance. The loss of the *H. pylori* infection is due to antimicrobial drugs used for other infections, but incidence of *H. pylori* infection is due to the crowded environments and the number of siblings where children are reared.[303] In Russia, 30% of 5-year olds and 49% of 19- year olds and 29-year olds are infected.[296] Mitchell et al. (1992) suggested that acquisition of *H. pylori* occurs during early childhood and also during adult life. They mentioned two important points to consider: how infection in childhood affects the prevalence in adults in different regions or countries, and the importance of determining the factors of acquisition.[303]

1.12.3 Hygienic conditions

Effects of sanitation and hygienic conditions on prevalence of *H. pylori* infection have been evaluated. Ahmed et al. (2007) examined the importance of hygienic conditions, such as

accessing clean water, taking showers and storing and reusing water safely, to reduce transmission of *H. pylori* infection. Acquisition of *H. pylori* will likely decrease with improved household hygienic practices such as establishing waste disposal as well as boiling water for drinking.[278] By contrast, poor hygienic practices that can be implicated in *H. pylori* transmission are not using soap when washing hands, not washing hands after going to the washroom, sharing chopsticks or kitchen utensils and living in a small area with limited sanitary facilities.[294]

1.12.4 Gender

H. pylori infects both males and females at the same rate.[279] Zaterka et al. (2007) detected in a Brazilian study, prevalence of *H. pylori* infection in both genders; in men it was 67% and in women 63%. The rate of infection increases with age but independent of gender.[304]

1.12.5 Ethnicity

Ethnicity is likely an important factor in *H. pylori* infection. Staat et al. (1996) studied American children and adolescents from different ethnic groups. Seroprevalence of *H. pylori* was 42% for Mexican-Americans, 40% for Non-Hispanic African-Americans and 17% for Non-Hispanic Caucasians.[305] Staat et al. (1996) attributed to genetic susceptibility the fact that social and cultural factors differ in the prevalence among ethnicities.[305] *H. pylori* infection was higher in African-American children than in Caucasian children.[306]

1.13 Mechanisms of transmission

H. pylori is an opportunistic bacterium that seeks any possible access to the human stomach.[287] Different studies have suggested that the transmission of *H. pylori* occurs in multiple modes such as zoonotic, iatrogenic, person-to-person (either oral-to-oral or fecal-to-oral), foodborne or waterborne.[273,280,294,307]

1.13.1 Person-to-person transmission

Person-to-person transmission within the same family is called vertical transmission; or when the infection is transmitted outside of the family is called horizontal transmission. Horizontal transmission is typical in developed countries.[308,309]

1.13.1.1 Intrafamilial transmission

Intrafamilial transmission has been evaluated as a major contributor to *H. pylori* transmission, due to the close person-to-person association within families.[310] Goodman and Correa (2000) evaluated whether sibling-to-sibling transmission was the source of *H. pylori* infection.[311] Age of rural Colombian children (being younger than 10 year old), birth order and birth spacing influence prevalence of *H. pylori* infection independently of the number of children in the home. *H. pylori* infection is more frequently transmitted between siblings close in age, and from older to younger siblings.[311] In Taiwan, number of children in the family (below 17 years), birth order and current number of children in the home had a positive association with the risk of *H. pylori* acquisition.[312] A Brazilian study reported how mothers infect their offspring, and how younger siblings are the infected source for older siblings.[313] In

Japanese communities, grandmother-child transmission is an important mechanism to spread *H. pylori* infection in a three generation household.[314]

Morris (2000) analyzed intraspousal transmission of *H. pylori*. The risk depends on the amount of time the spouse has lived with the partner. The same *H. pylori* strain was found in a small percentage of spouses and siblings because family members had close interpersonal contact, shared a genetic predisposition to *H. pylori* infection or were exposed to the same source of infection.[294]

Twins studies are often conducted to determine environmental risk and genetic factors involved in acquiring *H. pylori* infection. Malaty et al. (1994) performed a twin study and found that genetic conditions influence acquisition of *H. pylori* infection, as suggested by the greater similarities within Swedish monozygotic twin pairs. Environmental factors are also important to the susceptibility to *H. pylori* infection, since twins who were reared together were more susceptible to *H. pylori* infection than twins reared apart.[315]

Person-to-person transmission can occur as oral-to-oral or fecal-oral transmission.[310] Oral-to-oral mode has been hypothesized as a transmission route. *H. pylori* is an intermittent microorganism of the mouth. Routes of transmission are saliva, dental plaque and vomitus.[294] *H. pylori* has been identified in saliva of African women who fed premasticated food to their children. Deepika et al. (2010) found a relation between *H. pylori* in dental plaque and dyspepsia. Dental plaque can act as a reservoir for *H. pylori.*[316] *H. pylori* has been cultured from vomitus.[317] Perry et al. (2006) found that being in contact with the vomit of *H. pylori*–infected person increased the risk of a new infection.[318]

Fecal-oral is another route of transmission because *H. pylori* has been isolated from human feces. Namavar et al. (1995) used PCR to detect feces with *H. pylori* in 10% of

patients evaluated with *H. pylori* which can suggest that diagnosis of *H. pylori* by fecal analysis is not an efficient tool to detect the infection.[319] Mapstone et al. (1993) using PCR found that *H. pylori* was present in 90% of patients with dyspepsia.[320] The challenge is to investigate whether *H. pylori* in stool can represent a pathway of transmission.[293] Montaz et al. (2012) compared different genotypes of *H. pylori* extracted from saliva, dental plaques, gastric biopsies and stools. Montaz et al. (2012) found that *H. pylori* strains isolated from saliva, gastric biopsy and stool had the same genotype.[321] Moreno and Ferrus (2012) studied viable and cultivable *H. pylori* strains isolated from wastewaters to understand the mechanism of transmission and positive *H. pylori* cultures were detected by molecular techniques. Fecal transmission could be a route of transmission for *H. pylori* infection.[322,324]

1.13.2 Zoonotic transmission

Houseflies are a vector for transmission of enteric bacteria.[323] If *H. pylori* is transmitted by flies it has been examined since culturing *H. pylori* from body flies in contact with colonies of *H. pylori* under lab conditions.[324] Domestic cats, primates or sheep can be zoonotic reservoirs for *H. pylori*. *H. pylori* has been isolated from saliva and gastric juice of cats, and from milk and gastric juices of sheep. No evidence that these animals may be routes of transmission for humans has been shown.[282,325] Grubel and Cabel (1998) suggested that fly control is essential in eradicating *H. pylori* in developing countries.[326]

1.13.3 Waterborne transmission

Water has been studied as a source for *H. pylori* transmission, probably by way of fecal contamination.[277] Hulten et al. (1996) reported that *H. pylori* might be present in

drinking water in Peruvian communities.[327] Then, *H. pylori* may be considered a waterborne pathogen. This microorganism has been found for a long period of time in water, present as coccoid form and in nonculturable condition.[328]

1.13.4 Foodborne transmission

Food might be a source of infection by *H. pylori*. *H. pylori* can survive in fresh fruits, vegetables, fresh poultry, meat, fish and some dairy products in which the level of acid is low and the level of moisture is high under low temperature conditions. *H. pylori* found in foods has been isolated in a viable but nonculturable state.[329,273] Food could be a vehicle for fecal contamination to spread the infection, but the precise process has still not been established with certainty.[273]

1.13.5 Iatrogenic transmission

The least common route of infection is iatrogenic, since endoscopic equipment comes in contact with gastric mucosa.[279] Good practices of disinfection on fiberoptic endoscopes kill *H. pylori.*[330]

1.14 Diagnosis

Since the discovery of *H. pylori* in 1982, many tests have been developed to identify *H.pylori* infection. Diagnosis involves either invasive tests requiring endoscopy and biopsy, such as culture, histology, rapid urease test and molecular techniques, or non-invasive tests such as urea breath test, serology and stool testing.

1.14.1 Invasive tests

1.14.1.1 Endoscopy

Gastrointestinal endoscopy is an expensive dyspeptic technique that is not always readily available. Endoscopy is recommended in patients over 45-50 years with alarm symptoms (vomiting, dysphagia, bleeding, anemia, weight loss).[331] Endoscopy may be necessary to examine the stomach mucosa to determine presence, size and location of ulcers.[332]

Endoscopy is needed to obtain multiple mucosal biopsies for culture, PCR, histology and rapid urease test.[331] At least two biopsy samples must be taken from 5 cm proximal to pylorus.[333] If the patient has been on recent acid inhibitory therapy, additional two biopsies are taken from the gastric body due to the presence of hypoclorhydria, *H. pylori* migrates proximatly.

1.14.1.2 Culture

H. pylori cannot be reliably cultured from stools.[332] Culture is a tedious, challenging and time consuming method, but it is the most reliable method to diagnose *H. pylori* from biopsies.[331,333,334] In experienced laboratories, the sensitivity of culture is greater than 95%, but the sensitivity falls dramatically when it is performed in laboratories which are inexperienced with methods used for *H. pylori* culture or not enough biopsy samples have been taken.[279,331,335]

Histologically, *H. pylori* can be identified as translucent colonies with positive catalase, oxidase and urease reactions.[331,336] Positive cultures grow after 3 or 5 days. Use of multiple media with antibiotics and albumin could increase the sensitivity. Salts of tetrazolium

help to identify *H. pylori* colonies.[279,331] Two biopsies from the antrum or the corpus of the stomach as samples are recommended. Taking samples of two sites of the stomach is the optimum condition to obtain specific results.[333]

1.14.1.3 Histology

Histology is the standard method to diagnose *H. pylori* infection.[336,337] Histology detects the presence of *H. pylori* as well as the presence of gastric inflammation (acute or chronic inflammation), lymphoid aggregates, intestinal metaplasia, glandular atrophy, dysplasia or cancer.[331,336] Histology has a great sensitivity (from 66 to 100%), but it depends on site, size and numbers of the biopsy, as well as expertise of the pathologist.[335]

1.14.1.4 Rapid urease test

H. pylori produces large amounts of urease to the special niche where the concentration of urease that comes from blood to the gastric mucosa is low, *H. pylori* produces large amounts of urease. Rapid urease test (RUT) is based on activity of urease produced by *H. pylori*. RUT consists of a gel or membrane containing urea and phenol red. In presence of urease produced by *H. pylori*, urea is hydrolyzed into carbon dioxide and ammonia. Ammonium ions increase pH and change the colour of the indicator from yellow to red or violet.[279,338] PyloriTek® and CLO® tests are the most popular kits which are commercially available. PyloriTek® has a sensitivity of 99% after 2 h in incubation of analysis and CLO® test of 90-95% after 24 h of analysis.[334,335,339] The sensitivity of the test depends on the load of bacteria: to have valid results (based on histology detection), it is necessary to have a bacteria load of 10^5.[338]

1.14.1.5 Molecular methods

Polymerase Chain Reaction (PCR) technique is a highly sensitive and specific technique to identify *H. pylori* infection. PCR detects and quantifies some *H. pylori*-associated genes (*cagA, vacA*). Factors that affect the accuracy of the PCR are the primers, DNA target, bacterial charge and procedure.[279] The sensitivity of the PCR for *H. pylori* is between 96 and 100%. When using gastric mucosal biopsies, the sensitivity of PCR for *H. pylori* falls when testing saliva or environmental sources.[279,332,335] PCR with SYBR green targeting in VacA gene is specific, faster and less expensive than the normal PCR to evaluate the presence of *H. pylori* in wasted water.[340]

PCR can also be used as non-invasive test for *H. pylori* detection in feces to detect antibiotic resistance to clarithromycin.[331]

FISH (Fluorescent in situ hybridization) is a sensitive molecular technique that has the potential to analyze frozen biopsies for *H. pylori*.[332]

1.14.2 Non-invasive techniques

Non-invasive techniques are the simplest and most popular techniques used in epidemiological studies for screening *H. pylori*.[341] Application of diagnostic tests depends on the analysis of the circumstances such as evaluation of cost-benefit and use of tests on pre and post-treatment situations.[331,342]

1.14.2.1 Serology

Serology technique for diagnosis of *H. pylori* is based on the immunological response which the gastric mucosa shows after *H. pylori* infection.[338] Antibodies produced by the

gastric mucosa can be analyzed by three different techniques: ELISA, Latex agglutination and Western Blot. These serological tests detect a specific antibody that is called anti-*H. pylori* Ig antibody. Some tests also can detect IgA in the saliva and IgG in the urine. The sensitivity of the serology tests depends on the test antigen used. Serological tests are easy to perform; however, serological testes cannot distinguish between an active versus a past infection. Furthermore, when serology is negative, there is a high likehood that patients do not have a current *H. pylori* infection. Zuniga-Noriega et al. (2006) compared the serological against the invasive test such as culture, RUT and histology. Serology obtained the better performance for patients with peptic ulcer disease. The combination of invasive RUT and serology could be one of the best options to detect *H. pylori* infection because the high specificity, but it is an invasive approach.[343]

1.14.2.2 Stool tests

Stool tests for *H. pylori* detection have the advantage of detecting bacterial DNA in the stool sample. *H. pylori* is in competition with other bacteria, and is susceptible to biliary salts, and could be in a non-culturable state in the stool sample.[344] Stool tests can detect monoclonal and polyclonal antibodies, using ELISA stool antigen.[344] Stool test antigens are highly sensitive and specific tests and are being recommended for detecting *H. pylori* infection in children with recurrent abdominal pain.[345] Also, stool monoclonal tests have been recognized as very reliable for diagnosis *H. pylori* infection in infants and toddlers.[346]

PCR also can be performed on stools to detect the presence of *H. pylori*. Since *H. pylori* is not the only bacterium living in stools, PCR analysis is a challenge. Detection of *H. pylori* DNA using PCR in children after treatment of their infection showed a sensitivity of

only 69%.[337] PCR allows to detect the pathogenicity of *H. pylori,* and to compare genotypes in stool and biopsy samples.[338] In stools, PCR can be performed to determine antibiotic resistance to clarithromycin, macrolides, and quinolones.[334]

1.14.2.3 Urea breath test

Urea breath test (UBT) is based on the capability of *H. pylori* to transform orally taken ^{13}C-urea or ^{14}C-urea to ammonia.[336] A solution of C^{14} or C^{13} labelled urea is ingested, and it is hydrolyzed to C^{14} or $C^{13}O_2$ by *H. pylori* urease. If this bacterium is present in the stomach, ammonium and $^{13}CO_2$ or $^{14}CO_2$ are produced and gone through the blood stream where are exhaled in the breath. If the patient is not infected with *H. pylori*, labelled CO_2 is not produced.[338] UBT is the most common test used today to detect *H. pylori* infection. This technique is also used to evaluate eradication of infection in epidemiological and pharmacological studies.[347]

Proton Pump inhibitors can affect sensitivity of UBT, causing false negative test results.[347] Since urease activity is greater in acidic pH. Citric acid may be taken to improve results. Dominguez-Muñoz et al. (1997) observed that ^{13}C-UBT performed with a solution of a citric acid improves $^{13}CO_2$ recovery values and intraduodenal infusion that inhibits the antral motility and relaxes the fundus in the stomach.[348] A ^{13}C-urea breath test is a highly accurate test in young and elderly patients, compared to invasive and other non-invasive tests. In elderly patients, diagnosis of *H. pylori* infection is difficult since prevalence of chronic atrophic gastritis is higher than in adult patients, and *H. pylori* may be located in the fundus or body and not in the antral gastric mucosa. ^{13}C urea breath has shown to be a reliable test and

to be unaffected by co-treatments representing an option for patients whom endoscopy is not recommended before or after the eradication treatment.[347,349,350]

1.15 Morphology of *H. pylori*

H. pylori is a non-spore forming gram negative bacterium, 2.0 - 4.0 μm long and 0.5-1.0 μm wide. The cellular morphology can be spiral or rod-shaped.[294,351,352] The morphology is shown in Fig. 1.7. *H. pylori* can be transformed into coccoid forms after a long time under *in vitro* conditions, milk or water, antibiotic treatment, nutrient starvation, osmotic stress, oxygen tension, an increase in nitric oxide. Coccoid forms are a viable form, but not culturable.[353,308,354]

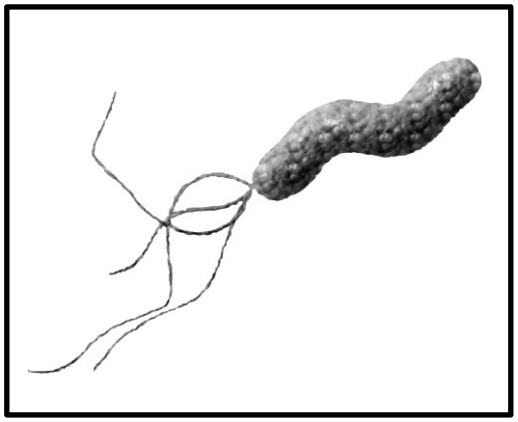

Figure 1.7 Representation of *Helicobacter pylori* (modified from reference 285).

H. pylori has between one and five sheated flagella, with terminal bulbs located at one pole.[355-357] Flagella measure 3-5 μm in length and 30-35 nm in diameter.[358]

1.15.1 Growth characteristics

H. pylori is a microaerophilic bacterium growing at O_2 levels of 2-5%, in CO_2 enriched environment of 5-10% CO_2, and a high humidity (96-100%)[276,351] Optimal conditions in the laboratory to culture *H. pylori* are 85% N_2, 10% CO_2 and 5% O_2[351] on a standard

Campylobacter medium.[359] *H. pylori* can survive at pH <3 by changing its helical form to coccoid form, but it grows in the range of 5.5 to 8.0, and optimal pH is 7.0.[360,351]

The optimal temperature for the growth of *H. pylori* is 37°C. *H. pylori* does not need glucose to grow, but does depend on amino acids, such as arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine. Some strains need alanine, serine, proline and trypthophan.[361,362]

To be cultured, *H. pylori* requires agars supplemented with blood, serum, corn starch, or egg yolk emulsion and appropriate antibiotics.[363] Davood et al. (2009) demonstrated that the optimum growth of *H. pylori* occurs in horse blood agar with trimethoprim, vancomycin, amphotericin, L-cysteine and ferric pyrophosphate. Glucose, some lipids and high amounts of antibiotics are factors that encourage *H. pylori* to develop in coccoid forms.[364]

H. pylori has been cultured in different media including: Skirrows medium,[359] Mueller-Hinton agar, Brain-Heart infusion agar,[325] Wilkings-Chalgren agar,[325,353] Columbia agar enriched with 5% horse blood and Dent-Supplement as a selective medium, BBL liquid medium supplemented with 5% horse serum, Johnson-Murano agar, *H. pylori* special peptone agar (HPSPA),[273] Brucella agar supplemented with either (lysed) horse or sheep blood newborn or fetal calf serum,[351] Brugamann agar, *Pylori* agar, BD *Helicobacter* agar and *Helicobacter* agar.[365]

Positive results can be detected 3-5 days after incubation at 37°C. *H. pylori* colonies are grown from 0.5 to 2 mm in size as translucent or yellowish, on horse blood agar; or greyish in appearance when cultured on blood agar. *H. pylori* colonies look like motile curved rods. *H. pylori* is positive to oxidase, catalase and urease tests.[366]

1.15.2 Lewis antigens in *H. pylori* LPS

Cell envelope of *H. pylori* is composed of an inner membrane, a periplasm with peptidoglycan and an outer membrane. The outer membrane is composed of phospholipids and lipopolysaccharides (LPS). The phospholipid moiety contains cholesterol glucosides that is very rare in bacteria. The LPS moiety consists of lipid A, core oligosaccharide and O-side chain. The lipid A moiety of *H. pylori* LPS has low biological activity compared to lipid A from other bacteria.[351] The O-side chain of *H. pylori* can be fucosylated and mimics Lewis blood group antigens (Le^x and Le^y). The O antigen can also mimic other blood group antigens.[367] O-polysaccharide side chains of *H. pylori* LPS that have been involved in colonization and pathogenesis of *H. pylori*-related diseases.[368] Around 80-90% of *H. pylori* strains express Type 2 chains such as Le^x and Le^y, while the expression of Type 1 chain such as Le^a and Le^b is common in Oriental or South American isolates. In *H. pylori* isolates from Europe, Le^x and Le^y is the predominant expression. Some strains that lack of Lewis antigens are disclosed in asymptomatic hosts. One study showed that infected with *H. pylori* expressing Lewis antigen had more severe diseases that those people infected with *H. pylori* that did not express Lewis antigen.[369] *H. pylori* strains with high Le^x expression mediated through anti-Le^x antibodies to adhere on the host's Le^x gastric epithelium.[370]

1.16 Colonization

Mucosal surface of the human stomach is the main habitat for *H. pylori.*[359] *H. pylori* is also rarely found into the proximal duodenum or distal esophagus.[371]

After *H. pylori* is introduced into the human stomach, the process of colonization of the gastric mucosa begins.[372] Some factors limit the colonization of the human gastric mucosa

to other microorganisms; these include acidity, peristalsis, nutrient availability, immunity and competing microorganisms.[371] Unlike other microorganisms, *H. pylori* has different characteristics such as urease, flagella, a particular shape and adhesins to colonize and to infect the human gastric cells. [372]

1.16.1 Flagella

Motility and chemotaxis allow the bacteria to detect nutrients and to reach niches to colonize.[373] The chemotactic motion using flagella is essential to *H. pylori* colonization.[374] In a study with gnotobiotic piglets, the most motile strain of *Campylobacter pylori* was the most virulent with a 100% infection rate, and the least motile strain was the least virulent, with an infection rate of only17%.[375]

H. pylori has a right-handed helical morphology with unipolar flagella. The flagellum is composed of a basal part (which is embedded into the cell membrane and is made up of the proteins required for rotation and chemotaxis) which contains the flagellar motor and hook structure, the central filament, and a membranous sheath that envelops each filament.[376]

The filament is composed mainly of two proteins of similar molecular weight (around 53 kDa), and the amino acid composition of the two flagellins is 58% similar. The minor flagellin, FlaB, is located proximal to the hook whereas FlaA forms the bulk and the rest of the filament.[356,378] The importance of these two flagellins has been studied. Gnotobiotic piglets, inoculated with *H. pylori* strains lacking FlaA and FlaB do not colonize; demonstrating that flagellins are important to full colonization of the stomach by *H. pylori*. Colonization over 4 days is possible in the absence of one flagellin, but not both.[378]

The flagellar hook is a flexible structure that binds the filament to the motor structure in the basal body. The flagellar hook protein, FlaE, is larger than in other bacteria such as *Salmonella* or *E. coli*.[379]

The flagellum contains a sheath that protects the flagellar filaments against mechanical and enzymatic digestion. The flagellar sheath is composed of proteins, phospholipids and lipopolysaccharides (LPS). This flagellar sheath composition is also similar to that of the outer membranes of bacteria, although some differences in protein and fatty acid composition were noted.[380] The flagellar motor is powered by proton force and *H. pylori* moves quickly at low pH. *H. pylori* can evade the acidic periphery of the mucous layer into the gastric cells by chemoattraction of substances. While urease neutralizes the gastric acid, it may also facilitate the rotation of the flagellar motor.[374]

H. pylori shows a chemotactic movement toward various compounds such as glutamine, histidine, lysine, alanine, mucin, urea, sodium bicarbonate and sodium chloride. Flagellar motility is regulated by different signal transduction proteins, referred to as the "chemotaxis pathway". The function of this pathway is to drive the bacteria into a safe environment, away from harmful conditions. This migration involves a smooth and straight swimming response, with some tumbles. Smooth swimming occurs when the flagella rotate counterclockwise while the tumbles occur when the flagella rotate clockwise.[381]

Transduction of the signal from the receptors to the flagellar motor is achieved by four different regulatory proteins: CheA, CheY, CheW, and CheZ. Chemotaxis pathway is composed by a chemoreceptor bound to the protein CheW; in turn CheW bound to the CheA histidine kinase sensor in the cytoplasm. CheA phosphorylates the regulator CheY. Phosphorylated CheY (CheY-P) interacts with a protein called flagellar switch [methyl-

accepting chemotaxis proteins (MCP)], switching the direction of movement the flagellum from counterclockwise to clockwise. Chemotaxis helps *H. pylori* to arrive up at the mucosa and to locate *H. pylori* in the antrum.[381,382] Under alkaline conditions in the antrum (such as when the infected person is taking a proton pump inhibitor), the flagellum drive the *H. pylori* upwards to colonize the mucus of the gastric body.[376] One recent study reported that FaaA (flagella-associated autotransporter A) protein is localized to a sheath that overlies *H. pylori* flagella. The absence of FaaA decreases motility and stability of the *H. pylori* flagella.[383]

1.16.2 Cag Pathogenicity and Vac A

Bacterial determinants also contribute to the disease appearance. *H. pylori* strain factors may influence the pathogenicity of different *H. pylori* isolates. *H. pylori* strains can be divided into types I and II. Type I express *cagA* and *vacA*. Type II strains do not express. Virulent strains induce morphological changes, vacuolizations and successive degeneration.[466]

H. pylori gene *cagA* is a component of the Cag pathogenicity island (*cag*PAI). Several genes inside this island encode other proteins such as adhesions and neutrophil activing proteins.[463] The cagA (cytotoxin-associated gene A) gene is the most studied of the *H. pylori* genes.[277] CagA leads to the development adenocarcinoma thought the derangement of cellular architecture and signalling.[467] The CagA protein is a highly immunogenic protein encoded by the *cagA* gene.[468] This gene is present in 50 to 70% of *H. pylori* strains.[451] Patients infected with CagA positive strains have a higher inflammatory response and at more risk of developing peptic ulcer or gastric cancer in Western populations[439,469] demonstrated the association between *H.pylori* CagA positive strains and the risk for gastric cancer. CagA positive *H. pylori* strains are more virulent and associated with higher grades of inflammation

compared to CagA negative *H. pylori* strains.[469] The *cagA* gene product, CagA, is translocated into gastric epithelial cells and localizes to the inner surface of the plasma membrane, in which it undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif.[470] Phosphorylated CagA interacts with host signaling compounds which results in morphological changes in the epithelial cells.[471,472] *Cag*PAI alters cell signaling, cell-cell junctions, host cell polarity, and also stimulates gastric epithelial cells to secrete pro-inflammatory cytokines, for example IL-8. It has been demonstrated that 15 genes on the *cag*PAI (*cagd, cagc, virB11, cagY, cagX, cagW, cagV, cagU, cagT, cagM, cagL, cagI, cagH, cagE, cagC*) are crucial for *H. pylori* to induce IL-8 production; in some *H. pylori* strains, cagA is also necessary.[473-476,478]

H. pylori has a single copy of the vacA (vacuolating cytotoxin A) gene encoding the VacA proteins. VacA induce apoptosis in epithelial cells. TVacA protein has been proposed to be a potent immunomodulatory toxin that targets the adopted immune system in order to suppress the local immune response and prolong the outcome of infection.[351,463] Around 50% of all *H. pylori* strains secrete VacA and it is mostly associated with populations of northern Europe, and Southeast Asia.[477] VacA and CagA might function together to acquire iron from the host and establish a replicative environment.[476]

1.16.3 Urease

Urease (EC 3.5.1.5; urea amidohydrolase) production is essential for *H. pylori* to survive in the acidic environment of the stomach.[384,385] *H. pylori* produces large amounts of urease; approximately 10-15% of *H. pylori* total proteins by weight, to neutralize its environment.[386] Urease has a low Michaelis constant (Km 0.17 to 0.48 mM) and a high

maximum velocity, which indicate that it is an efficient catalyst for converting millimolar concentrations of urea.[279,357]

Urease hydrolyzes small amounts (~3 mM) of urea in the stomach to produce ammonia and carbon dioxide, thereby neutralizing pH close to the gastric membrane for a short period, allowing *H. pylori* to penetrate the gastric mucosa.[387,385,376,388]

The enzymatic procedure is described as urease hydrolyses urea to produce ammonia and carbamate. The latter products spontaneously decompose to yield another molecule of ammonia and carbonic acid:

$H_2N\text{-}CO\text{-}NH_2 + H_2O \rightarrow NH_3 + H_2N - C(O)OH$

$H_2N\text{-}C(O)OH + 2H_2O \rightarrow NH_3 + H_2CO_3$

In aqueous solutions, the carbonic acid and ammonia (two molecules) are in equilibrium with deprotonated and protonated forms. The effect is an increase in pH.[377]

$H_2CO_3 \leftrightarrow H + HCO_3^-$

$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^-$

The neutralization of gastric acid by ammonia is produced by urea hydrolysis and helps *H. pylori* to retain 50-60% of its metabolic activity at pH of 2.5. In the absence of the urease, *H. pylori* only performs metabolic functions at pH 4.0-8.5. *H. pylori* can survive in acidic pH only in presence of urea.[389]

Urease production and motility of *H. pylori* are likely associated. Chemotactic activity of the bacteria increases in response to urea and sodium bicarbonate as attractants. Urea is synthesized in the liver and circulates in the blood stream, and through gastric capillaries into the gastric lumen. Bicarbonates are secreted by the chloride-bicarbonate (Cl^--HCO_3^-) exchanger located in parietal cells and sodium ions are secreted by the sodium-proton (Na^+-

H^+) exchanger in the mucous cells. Urease and sodium bicarbonate play a chemotactic role in a high-viscosity environment, since the cytoplasmic urease is more important for the chemotactic motility of *H. pylori* under viscous conditions, and treatment with fluorodamide (urease inhibitor) decreases the chemotactic motility to sodium bicarbonate under high-viscosity conditions.[389,390]

Urease has a molecular mass of 540 Da, and is a nickel-containing hexameric enzyme containing 2 main subunits, UreA (30 kDa) and UreB (62 kDa).[351,391,279] *H. pylori* urease gene cluster encodes nine genes. Only seven of these genes are essential for urease expression and functional activity: *ureA, ureB, ureI, ureE, ureF, ureG* and *ureH. ureA* and *ureB* encode the urease structure. *ureE, ureF, ureG* and *ureH* encode accessory proteins for joining and inserting Ni^{2+} to activate urease.[391,308,351,392,393]

ureI increases permeability of the bacterial membrane to urea. Urea helps to maintain the appropriate intracellular ammonia (NH_3) concentration and to export any excess of intracellular ammonium (NH_4^+)[391,308,351,392] *H. pylori* urease is located in the cytoplasm and cell surface.[394] Urease in the outer membrane has the function of neutralizing the gastric acid, whereas cytoplasmic urease hydrolyzes the urea produced from arginine. Ammonia produced by cytoplasmic urease is used to produce the amino acid glutamine. Cytoplasmic urease is also used to establish the proton gradient. Proton motion and redox potential from respiration are a motor for flagella movement.[374] Cytoplasmic urease activity is important to maintain neutral conditions in which the organism can survive in spite of a harsh (low pH, high H^+ concentration) intragastric environment.[388]

Each active site of the urease contains two-nickel ions. *H. pylori* has six active sites, so it contains 12 nickel ions. Nickel is present in very low concentrations in human serum. To

overcome this nickel limitation, *H. pylori* has developed at least two mechanisms for obtaining nickel ions. The first mechanism is NixA, a protein located in its cytoplasmic membrane which transports nickel ions. *H. pylori* by NixA transports Ni^{2+} from the outside the cell across the membrane into the cytoplasm.[395] Mutation of the nixA gene results in a low activity of urease, and also reduces colonization efficiency in a mouse model *H. pylori* infection. The second mechanism is a putative nickel transport system which may be encoded by the abcCD locus.[396,351]

Urea produces ammonium ions which are not toxic to mucosal cells, however hydroxide ions generated by the equilibrium between NH_4, NH_3 and water.[357,385] Urease activity may also damage the gastric epithelium through its interaction with immune system interaction.[395] *H. pylori* can stimulate an oxidative burst in human neutrophils. When neutrophils, *H. pylori*, and urea were incubated with rabbit fetal gastric mucosal cells; cytotoxicity was observed due to the presence of shrunken gastric cells.[395,397]

1.16.4 Other enzymes

Besides urease, *H. pylori* secretes other enzyme, such as catalase, superoxide dismutase (SOD) and protease to assist in the colonization of the gastric mucosa.[376] Catalase protects *H. pylori* from neutrophilic attack. Catalase enzyme protects bacteria against the damaging effects of hydrogen peroxide.[526] SOD mediate the attack by oxygen-dependent mechanisms of phagocytes since this enzyme catalyzes the dismutation of superoxide to H_2O_2 ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) which is subsequently remove for catalase and peroxidase.[348,399] The role of protease produced by *H. pylori* is to degrade gastric mucus.[376] *H. pylori* infection

weakens the gastric mucosal defense by causing proteolytic digestion of mucin component of the protective mucus layer.[400]

1.17 Adhesins

To colonize gastric cells, *H. pylori* has moved across the gastric mucus must adhere to membrane of gastric cells. This adhesin is a protein-carbohydrate interaction mediated by proteins on the outer membrane of *H. pylori* binding to carbohydrate structures on the host gastric cell surface.[401]

H. pylori genome (1.65 million bp) codes about 1500 proteins including approximately 4% of outer membrane proteins (OMP). Adhesins are OMP virulent factors that deliver toxins into the gastric epithelium, help to colonize and maintain *H. pylori* infection, delivering toxins into the gastric epithelium.[285,402,384] Several adhesins have been identified, including blood antigen binding adhesion (BabA), sialic acid binding adhesion (SabA), adherence associated lipoprotein (AlpA and AlpB), outer membrane inflammatory protein (OipA), HopaA and HopZ.

1.17.1 BabA

Ilver et al. (1998) showed that 66% of 95 *H. pylori* isolates produced a surface-associated protein. This protein is a 78 kDa adhesin on *H. pylori* that recognizes Lewis b (Le^b). This adhesion is designated as "blood group antigen binding adhesion" (BabA).[403] The function of BabA is to bind Le^b antigen (the dominant antigen in the gastric mucosa), and to fucosylate ABO blood group antigens such as O-Le^b, A-Le^b, or B-Le^b.[404,405] Subsequent analyses demonstrate two clones which encode two similar proteins, BabA and BabB, but

only BabA has Le^b binding ability.[405] There are two different BabA alleles, *babA1* and *babA2*, but only *babA2* is functionally active in binding Le^b antigen. The difference between *babA1* and *babA2* is that *bbA1* lacks a 10 bp deletion in the signal peptide sequence that makes *babA1* be inactive.[404]

A mucous gel layer covers the gastric mucosa; mostly of mucins.[404] Mucins are high molecular glycosylated compounds that are synthesized by epithelial cells. Mucins act as a physical barrier, and protect cells in the respiratory and gastrointestinal tracts.[407] Human mucins include 21 different types (MUC1 to MUC 21). These mucins are constituted by a repeated sequence of amino acids (Proline-Threonine-Serine) as well as by a glucosylated structure (GalNAc-O-linkages).[407] The healthy human mucosa is constituted by MUC1, MUC5AC and MUC6. MUC5AC is composed of Le^a and Le^b blood group antigens; MUC6 is associated with Le^x and Le^y antigens.[406] *H. pylori* may be colonized with MUC5AC gastric mucin. BabA mediates the binding of *H. pylori* binding to MUC5AC, even in "non-secretors", i.e. those without Le^b.[405]

Bacterial adhesive factors are recognized by the immune system. BabA-positive strains have higher levels of IL-8 in the mucosa of infected patients. BabA also promotes expression of sialyl-Lewis x/a.[408] In western countries, the prevalence of *babA2*-genopositivity of *H. pylori* is relatively lower (from 38 to 43%). In contrast, in Japan, the prevalence of *H. pylori* BabA strains is over 96%.[409] Yamaoka (2008) analyzed geographically diverse patients by immunoblot analysis.[404] A total of 150, 100, 150 and 120 strains from Colombia, the US, Korea and Japan, respectively. All of the strains from Asia expressed BabA protein. Twenty-four (9.8%) of the Western strains were BabA-negative and were associated with milder

gastric damage and lower *H. pylori* infection rate.[404] More studies are needed since BabA increases the risk for cancer gastric precursor lesions and gastric adenocarcinoma.[410]

1.17.2 SabA

In 2002, Mahdavi et al. reported another adhesin that adheres to sialated glycoproteins, specifically to sialyl-Lewis-X/A (sLe^x and sLe^a).[412] This protein is called SabA (sialic-acid binding adhesion).[385] The concentration of sialylated glyconjugates is very low in healthy human mucosa. Severe gastric mucosa inflammation due to *H. pylori* infection produces Lewis antigens (sLe^X and sLe^a). The minimal structure required for SabA binding is NeuAcα2-3Gal.[402] *SabA* is not a universal gene among *H. pylori* isolates.[413] SabA is encoded by *sabA* gene and is regulated by an on off switch,[412] suggesting that SabA expression can respond to different conditions in the stomach or different regions in the stomach.[413]

SabA likely increases the inflammatory response to *H. pylori* and facilitates the utilization of nutrients from damaged host cells.[413] The expression of SabA may be switched off when the inflammatory response is too strong.[412]

Mahdavi et al. (2002) proposed that when there is chronic gastritis, *H. pylori* regulates the inflammation process associated with sLe^x/sLe^a antigens in the host tissue. SabA binds sLe^x/sLe^a glycosphingolipids. In some sites with inflammatory response including neutrophils carrying sialytated carbohydrates on their surface, *H. pylori* might lose s-Le^x binding because of "on/off" switching of SabA.[412]

SabA, unlike BabA, may mediate binding of *H. pylori* to sialylated carbohydrates on neutrophils and erythrocytes to induce an oxidative burst in these cells causing oxidative damage to the gastric epithelium.[414] SabA is involved in the nonopsonic activation of human

neutrophils, and is a virulent factor in the pathogenesis of *H. pylori* infection.[413] SabA expression correlates inversely with the acidic environment of the stomach; *H. pylori* grown under high acid conditions (pH 5.0) shows a dramatic decrease in SabA expression compared to *H. pylori* grown at pH 7.0.[413]

SabA-positive infection and high s-Le^x cause a higher *H. pylori* load in the host stomach. This effect is more remarkable in *H. pylori* with weak or lacking Le^b expression. SabA may induce serious gastric inflammation and expression of sLe^x.[405]

SabA production is also positively associated with gastric cancer, intestinal metaplasia and corpus atrophy. SabA positive status is associated with decreased neutrophil infiltration. SabA production is negatively associated with development of duodenal ulcer in patients from the United States and Colombia.[413]

1.18 Binding sites for *H. pylori*

Bacterial adherence to gastric cells of the target tissue is an important virulence factor of *H. pylori*. Most microbial attachment sites on host cells are glycoconjugates. A number of different techniques, such as hemoagglutination or hemoagglutination-inhibition, binding to glycosphingolipids separated on thin-layer plates or in-situ analysis of the binding of *H. pylori* to gastric epithelial cells, have been performed for elucidating carbohydrate receptors for *H. pylori*.[415]

Hemoagglutination (in vitro assay) has been performed to investigate the interaction of *H. pylori* with red blood cells (RBC). In vivo, *H. pylori* is unlikely to interact with RBC.[416] *H. pylori* has at least two kinds of hemoagglutinins, of which one binds an N-acetylneuraminic part of receptors. The interaction is mediated by different sialic acid linkages to carbohydrate

moieties of host glycoconjugates (α-2,3 or α-2,6). The second hemoagglutinin has a molecular weight of 59 kDa and this interaction is sialic acid-independent, but the receptor is unknown.[416,417]

A TLC-immunostaining procedure with rabbit anti-*H. pylori* antiserum has been used to characterize glycolipid receptors isolates from human gastric mucosa. Solid-phase overlay has been able to demonstrate that gangliosides and sulfatides are receptors for *H. pylori*.[419]

In-situ analysis, alternative animal models such as ferrets and non-human primates which are naturally infected with closely related *Helicobacter* spp. as well as gnobiotic piglets and dogs models which are experimentally infected with *H. pylori* have been used to study *H. pylori* infection.[419] Sections of human gastric mucosa are also used to study carbohydrate-protein adhesion of *H. pylori* infection.[420] Single carbohydrates have been identified as potential *H. pylori* carbohydrate receptors.

1.18.1 Sulfated carbohydrates.

Structure of sulfatides from human gastric mucosa was confirmed as SO3-3Galβ1Cer with α-hydroxy fatty acids. Sulfatides are present in all the regions of the stomach and duodenum; the highest content of sulfatides is found in the antral mucosa. Saitoh et al. (1992) demonstrated *H. pylori* binds sulfatides.[421]

1.18.2 Lactosylceramide

A large number of bacteria express lactosylceramide specificity including *H. pylori*. Lactose saccharide (Galβ4Glcβ) is recognized by the bacterial adhesin and the extension

(ceramide) is tolerated for steric reasons, while in other molecules, the extensions make the lactose inaccessible to bind.[422] Angstrom et al. (1998) found that a high percentage of cholesterol (~40%) is required for maximal binding of *H. pylori* to lactosylceramide having phytosphingosine and hydroxyl fatty acids, coupled to the low amount of incorporated glycosphingolipid.[423]

1.18.3 Lactotetraosylceramide

Lactotetrasylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) has been identified in human meconium, normal human gastric mucosa and human gastric cancer tissue. An immunohistochemical study using polyclonal antibodies binding to the Galβ3GlcNac sequence showed the presence of lactotetraosylceramide in the brush border of human jejunum and ileum of blood group O-Le of non secretor individuals.[424] Teneberg et al. (2002) analyzed 74 *H. pylori* isolates and *65 H. pylori* strains (88%) expressed the lactotetraosylceramide binding specificity. *H. pylori* binding to lactotetraosylceramide was inhibited by incubating *H. pylori* with free univalent lactotetraose but no with lactose.[425]

1.18.4 Galactosylceramide and glucosylceramide

Abul-Milh et al. (2001) reported that *H. pylori* binds galactosylceramide (GalβCer) with sphingosine and both hydroxyl and non-hydroxy fatty acids. *H. pylori* bound glucosylceramide and sphingosine and hydroxy fatty acids. Binding of *H. pylori* strains to GlcβCer and LacCer glycoconjugates was very similar, which can indicate *H. pylori* recognizes the internal glucose moiety of the Galβ4Glcβ1Cer. The galactose linked at the 4 position of glucose is likely accepted in the binding but not the binding epitope.[426]

1.18.5 NeoLacto carbohydrate chains

H. pylori also binds the neolacto (GalβaGlcNAcβ) core structure, this bacterium prefers binding carbohydrate chains with repetitive neolacto elements. Degradation of sialylneolactohexasylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) showed that the preferred binding epitope in this context is the GlcNacβ3Galβ4GlcNAcβ sequence. The terminal β3-linked GlcNAc can be exchanged for GalNAcβ3, GalNAcα3 or Galα3 without loss of *H. pylori* binding.[427]

1.18.6 Sialic acid-dependent binding

H. pylori sialic acid binding was initially discovered by hemoagglutinating activity in 1988.[428] Hirmo et al., 1996 demonstrated that some *H. pylori* strains express sialic acid binding adhesions. Some strains recognize oligosaccharides chains with terminal α-2,3- linked sialic acids. Only one of fourteen *H. pylori* strains, that was studied, bind α-2,6-linked besides α-2,3-linked sialic acids, although with 10-fold lower affinity.[429] Johansson et al. (1999) investigated sialic acid dependent binding by *H. pylori* to polyglycosylceramides (glycosphingolipids with branched poly N-acetyllactosamine cores and terminal structures which are composed of unsubstituted N-acetyllactosamine, blood group determinants or sialic acid) of human erythrocytes. They found that the binding is dependent on Neu5Acα3 rather than Neu5Acα6.[429] Polyglycosylceramides (PGC) can constitute different epitopes to bind *H. pylori*, although PGC are present in lower amounts in cells.[431]

1.18.7 Host Lewis antigens

Human Lewis antigens are polymorphic fucosylated glycoconjugates and are biochemically associated with the ABO blood group.[279] Human Lewis antigens are present in many epithelial cells including those in gastric mucosa.[279] The structure of human Lewis antigens is composed of two different backbones. The type 1 chain contains galactose (Gal) and N-acetylglucosamine (GlcNAc) composed of Gal-β(1,3)-GlcNAc, which is also termed N-acetyllactosamine (LacNAc); Lewis antigens give rise to Lewis a (Lea), sialyl-Le^a and Le^b.[405] The type 2 chain is composed of Gal-β(1,4)-GlcNAc, also termed N-acetyl-lactosamine (LacNac); Lewis antigens give rise to Lewis x (Le^x), sialyl-Le^x and Lewis y (Le^y).[432]

The terms secretor and non-secretor indicate the capacity of an individual to secrete such substances. Secretors produce ABH, Le^b and Le^y and non-secretors produce Le^a and Le^x in their saliva.[433] In the human stomach, Le^a and Le^b are distributed on the surface of the gastric epithelium. Le^x and Le^y are located deeper in the glands, such as mucous, chief and parietal cells of the gastric glands. In non-secretors the surface and foveolar epithelia express Le^a, whereas in secretors Le^b and Le^y are expressed.[432]

1.18.8 Gangliosides

Effects of gangliosides on the hemoagglutinating activity of *H. pylori* have been studied. GM3, GD1a, GD1b and GM1, inhibited the erythrocytes agglutination by *H. pylori*. Inhibition of hemagglutination by GM3 was 4-fold greater than that by GD1a ganglioside and 8-fold greater than that by GD1b and GM1 gangliosides.[434] Hemoagglutination inhibitory

effect tests are present in Table 1.9 and the inhibitory effect of gangliosides was abolished by the removal of sialic acid is also shown.

Table 1.9 Gangliosides and asialogangliosides inhibit *H. pylori* adhesion to erythrocytes

Type of ganglioside	Hemagglutination inhibition ratio
GM3	1:512
GM1	1:64
Asialo -GM3 and -GM1	1:8
GD1a	1:128
GD1b	1:64
Asialo -GD1a and -GD1b	1:4

Modified from reference 434.

Saitoh et al. (1992) found that GM3 is a receptor for *H. pylori*. Glycoconjugates are abundant in the gastric mucosa and sialic-acid containing chains can be used as receptors for the attachments of *H. pylori* to the gastric epithelium.[421]

Hata et al. (2004) examined the inhibitory effect of milk gangliosides on *H. pylori* adhesion on MKN-45 cells. GD3 inhibited *H. pylori* adhesion more significantly than GM3 and sulfatides.[435]

Bennett and Roberts (2005) identified a *H. pylori* protein that can be an outer membrane protein to bind gangliosides. Recombinant HP0721 protein was expressed in *E. coli* and binds different gangliosides. Binding was determined by TLC This protein shows strong binding to GD1a and GM3 moderate binding to GM1 and weak bindint to asialo GM1.[436]

Data on the distribution of gangliosides in human gastric mucosa show that the gangliosides in the antral mucosa are higher than those in the fundus mucosa. The antral amount of GM3 was found to be around 72% higher than that of the fundus (Table 1.10). The

difference in ganglioside content could explain why *H. pylori* prefers antral mucosa more than fundus mucosa.[437]

Table 1.10 Ganglioside content of human gastric mucosa (in μg/g of dry mucosa)

Ganglioside Species	Gastric Mucosa	
	Fundus	Antrum
GM3	140	240
GD3	400	560
GM1	200	280

$MW_{average}$ 2000 g/mol of gangliosides (modified from reference 437).

1.19 *H. pylori*-associated diseases

H. pylori has been recognized as the main cause of chronic gastritis and is associated with the development of gastric carcinoma, peptic ulcer and mucosa-associated lymphoid tissue (MALT lymphoma). Although the role of *H. pylori* infection is a causative factor for many gastrointestinal diseases, questions remain for dyspepsia and GERD.

1.19.1 Gastritis

Inflammation of the gastric mucosa is called gastritis which can be grouped as acute or chronic gastritis.[387,438,351] Acute gastritis refers to the transient inflammation of the gastric mucosa.[439] Clinical symptoms of acute gastritis include heartburn or sour stomach, transient gastric distress, vomiting, fullness, nausea and in some severe situations bleeding.[387]

Acute *H. pylori* gastritis can cause transient hypoclorhydria and can last for months.[440,351] Hypoclorhydria was also noted in asymptomatic subjects with *H. pylori* infection and gastritis.[441]

Chronic gastritis will develop in almost all individuals who are colonized with *H. pylori*, 80% to 90% will never have symptoms or clinical disease.[384] Chronic gastritis is identified by the absence of visible erosions and by the presence of chronic inflammatory changes as atrophy of the glandular epithelium of the stomach.[439] *H. pylori* infection increases levels of gastrin (hormone that regulates acid secretion). Effects of *H. pylori* on gastric acid are likely mediated through somatostatin. Somatostatin (hormone that suppresses the release of gastrin hormone) seems that is up or down regulated due to the severity and distribution of *H. pylori*-associated inflammation.[442] Whether *H. pylori* is eradicated, gastrin levels return to normal.[440]

Atrophic gastritis is the endpoint of a chronic gastritis that is associated with *H. pylori* infection.[443] Individuals with atrophic gastritis have a high risk for gastric cancer. About 10% of the patients with moderate-severe atrophic gastritis will develop gastric malignancies into 7.8 years.[443-445]

Gastric mucosal atrophy is defined as the loss of appropriate glands. When glands are damaged by inflammatory response; these glands are replaced either by connective tissue or by glandular structures in appropriate for location.[443,446]

Effects of *H. pylori* infection on gastric physiology are complex. *H. pylori* induces a chronic, active inflammation in the mucosa releasing chemokines and cytokines such as interleukin-8, tumour necrosis factor-α and interleukin-1β have a suppressive effect on parietal cell function. This immune response cannot eradicate the organism and leads to persistent gastric mucosal damage, secreting neutrophils, lymphocytes and plasma cells lead to perpetuate the inflammatory state.[438,384]

1.19.2 Peptic Ulcer

Gastric or duodenal ulcers (peptic ulcers as common name) are mucosal defects with a minimum diameter of 0.5 cm, which penetrates through the muscularis mucosa.[351] Gastric ulcers mostly occur along the curvature of the stomach between the corpus and the antrum mucosa.[447] While duodenal ulcers occur in the duodenal bulb, which it is the site most exposed to gastric acid.[351]

H. pylori infection is present in all the subjects with duodenal ulcer and between 70% and 85% of those with gastric ulcer.[387,351] Duodenal ulcers occur five times more frequently than gastric ulcers. Duodenal ulcers affect people between 20 and 50 years of age; gastric ulcers mainly occurs in individuals over 40 years old.[351]

Peptic ulcer can damage one or all layers of the stomach or duodenum. In some cases, peptic ulcers can penetrate the outer wall of the stomach or duodenum.[387]. Ulcer occurs where mucosal inflammation is more severe.[447] In people with low acid secretion levels, this occurs in the gastric transitional site between corpus and antrum, rising to gastric ulcer disease. If acid production is normal to high, the most severe inflammation is located in the distal stomach and proximal duodenum, rising to juxtapyloric and duodenal ulcer disease.[448,351]

Clinical symptoms of peptic ulcer are discomfort and pain. Pain is manifested as burning, gnawing or cramp-like and frequently occurs when the stomach is empty. Pain is located in a site proximate to the pain middle of the epigastrium near the xiphoid and may radiate below the costal margins, into the back.[449] Another feature of peptic ulcer pain is that the pain tends to recur for periods of weeks or months. During exacerbation, it occurs daily for a period of several weeks and then resolves until the next recurrence. Pain is relieved by food or antacids.[387]

Complications of peptic ulcers include haemorrhage, obstruction and perforation.[387,381] Haemorrhage is caused by bleeding from granulation tissue or from erosion of an ulcer into an artery or vein. It occurs to 10–20% of persons with peptic ulcer.[450] Bleeding may be severe or be insidious, producing only occult blood in stool.[387] Obstruction of blood flow is caused by oedema, spasm or contraction of tissue and interference with the passage of gastric contents through the pylorus or adjacent areas. A feeling of epigastric fullness and heaviness is produced after meals; with severe obstruction and there is vomiting of undigested food. Perforation of peptic ulcer develops in around 5% of subjects with peptic ulcers from the anterior wall of the stomach and duodenum.[387] Peptic ulcer perforation requires surgical therapy.[450]

H. pylori is not the only cause of peptic ulcer disease; the other most common cause of peptic ulcer is non-steroidal anti-inflammatory drug (NSAID) and aspirin.[449,351,451] The prevalence of gastric ulcers is between 10-20% and 2-5% prevalence of duodenal ulcers among NSAID users. Aspirin is the most ulcerogenic of NSAIDS. Ulcers that are caused by NSAID are dose-dependent, the risk dose is >325 mg/day.[449] Pathogenesis of NSAID-induced ulcers involves a mucosal injury and inhibition of prostaglandin synthesis. In some cases, NSAID-induced gastric injury does not produce clinical manifestations.[387]

1.19.3 Dyspepsia

Dyspepsia is defined as epigastric or discomfort in the upper abdomen. Patients who are analyzed by endoscopy for upper gastrointestinal symptoms with no abnormalities detected are diagnosed with functional dyspepsia.[451] Clinical manifestations include anaemia, vomiting, weight loss, dysphagia and previous history of peptic ulcer.[451] Those symptoms are

present by 20 to 40% of the adult population at the western world. Some patients, from 30% to 60%, with functional dyspepsia have *H. pylori* infection.[452]

The effect of *H. pylori* eradication on patients with dyspeptic symptoms is unclear. A randomized controlled trial reported that after one year, the proportion of patients suffering from dyspepsia is more that 80% after acid suppression therapy or *H. pylori* eradication.[453]

In contrast, Patchett et al. (1991) evaluated patients with dysfunctional dyspepsia at *H. pylori* eradication after a follow-up of 5 years and demonstrated that patients were asymptomatic.[454]

1.19.4 Gastric cancer

Gastric cancer is the second frequent cancer worldwide approximately one million new diagnoses appear each year.[455] Gastric cancer is more common in Japan, Europe, the Scandinavian countries, South and Central America, China and Korea.[387,455] Incidence of gastric cancer is the most important risk factor in all geographic areas. *H. pylori* has been classified as a class I human carcinogen by the International Agency for Research on Cancer of the World Health Organization.

Gastric cancer begins with the acquisition of *H. pylori* and subsequent chronic active gastritis, developing multifocal gastritis and intestinal metaplasia. *H. pylori* begins with a sequential paradigm of gastric carcinogenesis that it has become known as Correa's cascade[456,457]

Chronic active nonatrophic gastritis is characterized by infiltration of the gastric mucosa by lymphocytes, plasma cells and macrophages. Eosinophils and mast cells are characteristics in this type of gastritis. Gastritis is called "active" when neutrophils are present.

Neutrophils may form small aggregates and are prominent in the glandular necks; sometimes neutrophiles form intraglandular microabscesses. This phase does not show loss of glands (athrophy), so it is called nonatrophic gastritis. Active non-atrophic gastritis is located in the corpus mucosa.[456]

Multifocal atrophic gastritis is the focal loss of glands (atrophy) and takes place in the antrum-corpus junction. The mechanism of cell loss is related to effect of bacterial products and the cytokine environment in the gastric mucosa. Fibrous tissue replaces the lost glands. Loss of cell-cell cross-talk and fibrous stromal tissue controls the influx of blood borne stem cells responsible for subsequent tissue changes.[456]

In Intestinal metaplasia, the original glands and foveolar epithelium are replaced by cells with intestinal phenotype. Metaplastic intestinal cells in the initial phases seem the small intestinal mucosa: lined by eosinophilic absorptive enterocytes with a brush border.[456] This type of metaplasia has been named as small intestinal type due to its morphologic structure; or type 1, based on mucin histochemistry.[458] It reflects the fact that secrets the normal set of digestive enzymes such as sucrose, trehalase and alkaline phosphatase.[459] Later, the metaplastic cells lose their small intestinal phenotype in order to acquire morphologic characteristic of the large intestine and are lined only by goblet cells of different sizes and shapes. This type of metaplasia is called incomplete or colonic metaplasia and includes type II and type III. The mucin histochemistry reveals that MUC2 (absent in normal gastric mucosa) is positive in goblet cells in complete and incomplete intestinal metaplasia. MUC5AC is absent in complete intestinal metaplasia and present in the incomplete type. MUC6 is absent in both types of intestinal metaplasia.[460]

Dysplasia is characterized by atypical changes in nuclear morphology and tissue structure. The nuclei of the epithelium in dysplasia phase, are enlarged, hyperchromatic, irregular in shape and devoid of polarity. The structure is irregular, frequently forming adenomas (tubular structures) with irregular lumens.[456]

Invasive cancer can be described as an abnormal organ composed of multiple diverse cell types in several stages of differentiation and with different proliferative capacities.[456] Recently, a population of cancer cells within tumours has been identified that serves to provide of the cancer cells of the tumour, named "cancer stem cell."[461,462] All tumours are adenocarcinomas present from mucus-secreting cells in the base of the gastric crypts. Between 50 and 60% of gastric cancers occur in the pyloric region or adjacent to the antrum.[449]

An important factor in the pathogenesis of *H. pylori* is the ability to produce an inflammatory response. Proinflammatory cytokine IL-1β is regulated during *H. pylori* infection.[463] El-Omar (2001) found that IL-1β polymorphisms significantly increase the risk of precancerous conditions.[464] IL-8 is another interleukin related with high risk of cancer. IL-8 functions as a key regulator for proliferation, angiogenesis and metastasis. Studies focus on IL-8 represent a scenario for succeessing in the treatment of gastric cancer.[465]

Gastric cancers are asymptomatic until the late phase. Clinical symptoms, when they occur, are indigestion, anorexia, weight loss, vague epigastric pain, vomiting and an abdominal mass.[387]

1.19.5 MALT lymphoma

In healthy humans, MALT (mucosa-associated lymphoid tissue) is found in the intestine and is more common in the terminal ileum in the form of Peyer's patches. The

function of MALT is to protect the permeable membrane of the gastrointestinal tract. A Peyer's patch is an encapsulated nodular site of lymphoid tissue. Lymphoid follicle is the most important component of a Peyer's patch, which is composed of small B lymphoid cells.[479] *H. pylori* infection is associated with the accumulation of organized lymphoid tissue since lymphoid follicles appear in the stomach after *H. pylori* infection. This accumulation of lymphoid tissue in the stomach forms the soil form which low grade gastric MALToma emerges. *H. pylori* negative subjects rarely have lymphoid tissue in their gastric mucosa.[480,481] Gastric MALT lymphoma is a B-cell lymphoma but results from chronic T-cell antigenic stimulation.[438] Gastric MALT lymphoma is less common than gastric carcinoma. MALT lymphoma represents only 3% of all gastric tumours.[482]

MALT lymphoma may be associated with *H. pylori* infection. *H. pylori* is present in the gastric mucosa of more that 90% of patients with gastric MALT lymphoma.[481] Wotherspoon et al. (1991) observed that 92% patients with gastric MALT lymphoma were infected with *H. pylori.*[479]

Incidence of MALT lymphoma associated with *H. pylori* infection affects only some geographical regions. Feltre, Italy is a region with a high incidence of gastric lymphoma and a high prevalence of gastritis related to *H. pylori* infection. In contrast, in some regions from UK, *H. pylori* infection is very low and MALT lymphomas are uncommon.[481]

H. pylori eradication can lead to a complete recovery in around 80% of cases of low grade MALT lymphoma. High-grade lymphoma rarely returns after *H. pylori* treatment.[483] MALT lymphomas progress very slowly. Clinical manifestations are very different rom dyspepsia to vomiting and gastrointestinal bleeding. [484]

1.20 Treatment for *H. pylori* eradication

The purpose of *H. pylori* treatments is the eradication of the organism.[384] A successful *H. pylori* elimination must have cure rates >80% without severe side effects and with minimal bacterial resistance.[384,485,285] The most popular regimen is a triple therapy that consists of two different antibiotics and a proton pump inhibitor (PPI) or ranitidine bismuth citrate. PPI, such as omeprazole or ezomeprazole, increases the effectiveness of some antibiotics because luminal acidity affect it.[384,486,485] This treatment requires from 7 to 14 days for a successful *H. pylori* eradication.[384] The combination of two or more antibiotics increases rates of cure and reduces the risk of *H. pylori* resistance.[384] The main antimicrobial agents used in these therapies are amoxicillin, clarithromycin, metronidazole, tetracycline and bismuth.

1.20.1 First-line therapy

The Maastricht Consensus Conference recommended that first-line therapy be a legacy therapy that consists of PPI-clarithromycin-amoxicillin taken twice a day for 7 to 14 days. Metronidazole has been recommended to substitute clarithromycin, if the resistance to clarithromycin exceeds 15-20%.[486] Treatment with clarithromycin and metronidazole may contribute to development of antibiotic resistance of *H. pylori* and other important bacterial pathogens.[487,488] *H. pylori* resistance to amoxicillin has not been described.[485]

First-line therapy fails in approximately 20% of patients. Failures in first therapy have been attributed to inadequate patient compliance, poor choice of regimen, inadequate duration of treatment and the development of antibiotic resistance. A more effective second-therapy is obviously needed.[285,489,486]

1.20.2 Second-line therapy

The failure of first line therapy is related to secondary antibiotic resistance. It is recommended a quadruple therapy that includes bismuth in addition to the two antibiotics and a PPI. An alternative is PPI, bismuth, tetracycline and metronidazole have has reported an efficacy of 76% in patients who failed first-line therapy.[490] This therapy has to be given for 10 days and taken four times daily or a 14 day therapy taken twice daily. The exact action of bismuth is not clear, but *H. pylori* is susceptible to these compounds in vivo and in vitro.[491]

1.20.3 Third-line therapy

Patients who fail both first-line and second-line therapy for *H. pylori* represent an interesting challenge. The challenge is to treat the patient with two underused antimicrobials such as ribafutin and furizoladone.[492,486] Ribafutin is an antituberculous agent with low minimum inhibitory concentration (MIC) for *H. pylori.*[492,486] For *H. pylori* elimination, it must be prescribed PPI, ribafutin (150 mg), amoxicillin (1g), twice at day for 14 days. Ribafutin might increase resistance tuberculosis infection.[492] Furazolidone is a monoamine with broad antibacterial activity that interferes with bacterial enzymes. *H. pylori* resistance to furazolidone is rare.[493] Furazolidone-based therapy with amoxicillin and PPI eradicated 60% of *H. pylori* infection.[494]

1.20.4 Sequential therapy

Sequential therapy has been suggested as an alternative treatment to first-line therapy. This treatment reduces the bacterial load, which makes the presence of an existing small population less resistant to antibiotics. A sequential treatment regimen uses PPI plus

amoxicillin for 5 days subsequently clarithromycin (500 mg) and tinidazole (500 mg) for an additional 5 days. During the first part of the therapy, amoxicillin weakens the bacteria cell wall to prevent the formation of the channels that block clarithromycin from binding to the bacterium and hence causes resistance to the antibiotic. *H. pylori* eradication levels have succeeded in approximately 90% of patients. Sequential therapy has not been approved in the United States.[495]

1.20.5 Adverse effects

Adverse effects associated with *H. pylori* eradication treatment occur in up to 50% of patients. At least 10% of patients stop treatment because of side effects.[496] Nausea is the most common side effect and is related to most antibiotics. Metallic taste can occur with metronidazole or clarithromycin use. Diarrhea is associated with all antibiotics, but it is more common with amoxicillin. Tetracycline can induce a photosensitivity reaction. Bismuth salts produce dark stools.[486]

1.20.6 *H. pylori* resistance to antimicrobial agents

Antimicrobial resistance is the main cause of a *H. pylori* eradication failure.[497] The most commonly used antibiotics for eradication of *H. pylori* infection that present a well studied mechanism of resistance are, clarithromycin, amoxicillin, metronidazole and tetracycline.

Prevalence of clarithromycin resistance is between 20 and 25%.[338] After a first-line therapy failure with a treatment including clarithromycin; the prevalence of clarithromycin resistance is very high, in the range of 60%.[498] In southern Europe, the prevalence of

clarithromycin-resistant *H. pylori* strains in children is higher than in adults because in this region the use of macrolides for other infections is common.[499] Clarithromycin resistance is caused by mutations of *H. pylori* 23S ribosomal RNA inside 50s ribosomal subunits.[500] These mutations decrease the linkage of clarithromycin to *H. pylori* ribosomes which reduces or prevents inhibition of protein expression.[501] In Japan, the origin of clarithromycin resistance starts in the pediatric, respiratory and otorhinolaryngology fields.[502] Clarithromycin resistance cannot be overcome by increasing the dose or duration of therapy.[501]

Prevalence of *H. pylori* resistance to amoxicillin and to tetracycline is very low (<1%).[503] Except in Japan and South Korea where the resistance to tetracycline is around 5%.[504] Amoxicillin inhibits the synthesis of the bacterial cell wall.[486] The site of action of tetracycline is the bacterial ribosome which interrupts protein biosynthesis of the bacteria.[486]

The prevalence rate of metronidazole resistance among *H. pylori* strains is high; in developed countries; the prevalence of resistance ranges from 11 to 70% and in developing countries is greater than 95%.[279] The high resistance to metronidazole is associated with the frequent use of metronidazole to treat parasitic infections such as giardiasis and amebiasis.[323] Metronidazole penetrates into the bacteria and the nitro group of its imidazole ring is reduced to form derivative. The reduced product causes damage to the DNA and cell death.[487] Alteration in the bacterial enzymes that is required to transform the toxic derivative results in metronidazole resistance.[505] Metronidazole resistance can be partially overcome by increasing the dose of metronidazole and the duration of treatment.[501]

Ierardi et al. (2013) reported no ideal treatment to eradicate *H. pylori* infection since antibiotic resistance is different among geographic regions. Ierardi et al. (2013) suggested

evaluating minimal and maximal values of resistance to different antibiotics for different countries to identify the best therapy.[506]

1.20.7 *H. pylori* and probiotics

Probiotics are living microorganisms that help maintain normal gut flora and prevent colonization by pathogens.[495] Probiotics prevent infection from pathogens activating the host's immune system and competing with the probiotic bacteria with the pathogenic bacteria.[351] Probiotics could represent an adjunctive therapy to prevent or to decrease *H. pylori* colonization.[507,495]

Various mechanisms have been identified by which probiotics can inhibit *H. pylori:*[507] Non-immunological mechanisms. The intake of probiotics strengthens the gastric mucosal barrier since probiotics produce antimicrobial agents that compete with *H. pylori* for adhesion receptors. Probiotics also stimulate mucin production and stabilize the gut mucosal barrier.[495] Antimicrobial substances. Probiotics secrete antibacterial substances, such as lactate and bactericins that can inhibit *H. pylori* growth. *Lactobacilli* are known to produce large amounts of lactate (sometimes more than 10 mM). Lactate has been implicated as inhibitory factor in vitro studies. *Lactobacilli* strains implicate in this bactericidal mechanism are *Lactobacillus acidophilus* CRL 639, *L. casei* dairy starter*; L. johnsonii* L1a; *L. salivarius* WB 1004.[508]

In the gastric mucosa, *H. pylori* adheres to epithelial cells through secretory components.[509] Some in vitro studies, *L. johnsonii* La1, *L. salivarius*, *L. acidophilis* and *W. confusa* inhibit attachment of *H. pylori* to intestinal HT-29 cells.[510-513] or to MKN gastric cell lines.[512,513] *L. reuteri* can inhibit the attachment of *H. pylori* to asialo-GM1 and sulfatide.[514]

Animal studies have demonstrated that previous colonization by probiotics prevented *H.pylori* infection in germ-free mice.

Mucosal barrier reduces mucus secretion and is frequently found in patients with *H. pylori*-associated gastritis. *H. pylori* suppresses MUCI and MUC5A gene expression in a human gastric cell line.[527] In vitro, *L. plantarum* and *L. rhamnosus* increased the expression of MUC3 genes and the subsequent extracellular secretion of mucin by colon cell cultures.[518]
The inflammatory response to gastric *H. pylori* infection is characterized by the production of various inflammatory mediators such as chemokines and cytokines. The immunologic response by the host could be modified by probiotics. Probiotics could interact with the epithelial cells and could modulate the secretion of anti-inflammatory cytokines that results in a reduction of gastric inflammation.[516] In vitro, *L. salivarius* inhibited IL-8 release in *H. pylori* infected gastric epithelial cells.[513]

1.20.8 Probiotics as an adjunctive therapy

Probiotics could represent an alternative treatment as adjunctive therapy for *H. pylori* elimination. Probiotics improve gastrointestinal adverse effects such as nausea, taste disturbance, diarrhea and epigastric discomfort.[495] Therapy with probiotics co-administrated with antibiotic-PPI treatment would improve *H. pylori* eradication rates. Lactic acid or other antimicrobial substances secreted by probiotic bacteria could increase the potential of antibiotic therapy.[507]

One hundred and twenty *H. pylori* positive patients randomly assigned to a 7 day antibiotic-PPI therapy supplemented with a lyophilized culture of *L. acidophilus* increased significantly the eradication of *H. pylori* from 72 to 87%.[517] However, results of 7 studies

evaluating the effect of probiotics added to conventional antibiotic therapy are inconsistent (Table 14). Four studies reported better eradication rates with probiotics, whereas the other 3 studies observed no effect. Limitations probably to account for the discrepant results include the lack of placebo controls and the short duration of probiotics treatment. The frequency of side effects was decreased by the administration of probiotics in 3 studies and 1 study found no effect (Table 1.11). Administration of probiotics can decrease the frequency of diarrhea, a frequent side effect of the triple therapy *H. pylori* elimination.

1.20.9 Other adjuvants

Lactoferrin is a natural antibacterial glycoprotein that is found in mammalian milk. Lactoferrin possesses activity for gram negative bacteria, including *H. pylori.*[486] In a 1 week triple-antibiotic regimen that supplemented with bovine lactoferrin was more efficient than did the same regimen without lactoferrin. This suggests that lactoferrin administration with a conventional therapy might represent a possible *H. pylori* eradication treatment.[524]

N-acetylcysteine (NAC) is a mucolytic agent and a thiol-containing antioxidant. NAC inhibited the growth of *H. pylori* in agar and broth susceptibility tests. NAC may alter the establishment of *H. pylori* infection in the gastric mucus layer and mucosa.[486]

Other components have been studied for anti-*H. pylori* activity such as garlic has a synergistic in vitro effect with omeprazole; honey and propolis since its phenolic constituents were shown to inhibit *H. pylori*. Cranberry juice due to the sialic acid and sialyllactose that has a specific adhesion of *H. pylori* to human gastric mucus is inhibited by a high molecular constituent of this juice.[525]

Table 1.11 Clinical trials using probiotics in association with antibiotics in the treatment of *H. pylori* eradication

Subjects, n	Eradication therapy	Probiotics	Results E.R.	Results A.E.	Reference
Asymptomatic, 60	Clarithromycin, tinidazole, rabeprazole for 1 week	*L. casei* for 2 weeks	No effect	↓	518
Dyspeptic adults, 120	Rabeprazole, clarithromycin, amoxicillin for 1 week	*L. acidophilus* LB for 10 days	↑	No effect	517
Dyspeptic adults, 160	Lansoprazole, clarithromycin, amoxicillin for 1 week	*L. acidophilus* + *B. lactis* for 4 weeks	↑	-	519
Asymptomatic adults, 85	Rabeprazole, clarithromycin, Tinidazole for 1 week	*L. rhamnosus, S. boulardii Lactobacillus* LA5 + *B. lactis* , for 2 weeks	No effect	↓	520
Duodenal ulcer, Dyspepsia, 84	Amoxicilin, Omeprazole, for 2 weeks	*L. acidophilus*	↑	-	521
Symptomatic children, 86	Clarythromycin, amoxicillin, omeprazole for 1 week	*L. casei* for 10 days	↑	↓	522
Asymptomatic adults, 52	Clarythromycin for 2 weeks	*L. johnsonii* for 3 weeks	No effect	-	523

E.R.= Eradication Rate A.E.= Adverse effects

1.21 References

1. Sourkes, T.L. (1993). How Thudichum came to study the brain. *Journal of the History of Neurosciences*, 2(2):107-119.

2. Yamakawa, T. (1996). A reflection on the early history of glycosphingolipids. *Glycoconjugate Journal*, 13(2):123-126.

3. Sonnino, S.; Mauri, L.; Chigorno, V.; Prinetti, A. (2006). Gangliosides as components of lipid domains. *Glycobiology*, 17(1): 1R-13R

4. Sourkes, T.L. (2007). Thudichum's successors. *Neurochemical Research*, 32(10): 1808-1812.

5. Glyco-Forum section (2001). In Memoriam: Lars Svennerholm 1925-2001. *Glycobiology*, 11(5): 21G-22G.

6. Futerman, A.H.; Hannun, Y.A. (2004). The complex life of simple sphingolipids. *EMBO Reports*, 5(8): 777-782.

7. Haynes, C.A.; Allegood, J.C.; Park, H.; Sullards, M.C. (2009).Sphingolipidomics: methods for the comprehensive analysis of sphingolipids. *Journal of Chromatography B*, 877(26): 2696-2708.

8. Merrill, A.H. (2008). Chapter 13 Sphingolipids. In: Vance D.E and Vance J.E. Biochemistry of Lipids, Lipoproteins and Membranes 5th ed. pp364- 397.

9. Merrill, A.H.; Schmelz, E.M.; Dillehay, D.L.; Spiegel, S.; Shayman, J.A.; Schroeder, J.J.; Riley, R.T.; Voss, K.A.; Wang, E. (1997). Sphingolipids--the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicology Applied Pharmacology*, 142(1): 208-225.

10. Wehrmuller, K. (2007). Occurrence and Biological Properties of Sphingolipids - A review. *Current Nutrition and Food Science*, 3:161-173.

11. Rao, R.P.; Acharya, J.K. (2008). Sphingolipids and membrane biology as determined from genetic models. *Prostaglandins and Other Lipid Mediators*, 85(1-2): 1-16.

12. Fuller, M. (2010) Sphingolipids: the nexus between Gaucher disease and insulin resistance. *Lipids in Health Disease*, 9:113-125

13. Makita, A.; Taniguchi, N. (1985). Chapter 1 Glycosphingolipids. In: Wiegandt, H. Glycolipids, 10: 1-99.

14. Varki, A. Chapter 9. Glycosphingolipids. In: Varki, A.; Cummings, R.; Esko, J.D.; Freeze, H.; Hart, G.W.; Marth, J. (Eds.). Essentials of Glycobiology, Cold Spring Harbor Press, Cold Spring Harbor, NY (1999), pp. 1–653

15. Allende, M.L.; Proia, R.L. (2002). Lubricating cell signaling pathways with gangliosides. *Current Opinion in Structural Biology*, 12(5): 587-592.

16. Wiegandt, H. (1968). The structure and the function of gangliosides. *Angewandte Chemie International*, 7(2): 87-96.

17. Kolter, T.; Proia, R.L.; Sandhoff, K. (2002). Combinatorial ganglioside biosynthesis. *The Journal of Biological Chemistry*, 277(29): 25859-25862

18. Lacomba, R.; Salcedo, J.; Alegría, A.; Jesus Lagarda, M.; Reyes Barberá; Matencio, E. (2010). Determination of sialic acid and gangliosides in biological samples and dairy products: A review. *Journal of Pharmaceutical and Biomedical Analysis*, 51(2): 346-357.

19. Rueda, R.; Maldonado, J.; Narbona, E.; Gil, A. (1998). Neonatal dietary gangliosides. *Early Human Development,* 53 Supplement: S135-S147.

20. Wiegandt, H. (1994). Principles of glycosphingolipid-oligosaccharide constitution. *Progress in Brain Research*, 101: 63–73.

21. Buccoliero, R.; Futerman, A.H. (2003). The roles of ceramide and complex sphingolipids in neuronal cell function. *Pharmacological Research,* 47(5): 409-419.

22. Schnaar, R.L.; Suzuki, A.; Stanley, P. (2009). Part II. Structure and Biosynthesis 10. Glycosphingolipids. In: Varki, A.; Cummings, R.D.; Esko, J.D.; Freeze, H.H.; Stanley, P.; Bertozzi, C.R.; Hart, G.W.; Etzler, M.E. editors. *Essentials of Glycobiology*. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

23. Zheng, W.; Kollmeyer, J.; Symolon, H.; Momin, A.; Munter, E.; Wang, E.; Kelly, S.; Allegood, J.C.; Liu, Y.; Peng, Q.; Ramaraju, H.; Sullards, M.C.; Cabot, M.; Merrill, A.H. Jr. (2006). Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy. *Biochimica et Biophysica Acta*, 1758(12): 1864-1884.

24. Kobayashi, T.; Mitsuo, K.; Goto, I. (1988). Free sphingoid bases in normal murine tissues. *European Journal of Biochemy*, 172(3): 747-752.

25. Merrill, A.H. Jr.; Sullards, M.C.; Allegood, J.C.; Kelly, S.; Wang, E. (2005). Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods*, 36(2): 207-224.

26. Sonnino, S.; Chigorno, V. (2000). Ganglioside molecular species containing C18- and C20-sphingosine in mammalian nervous tissues and neuronal cell cultures. *Biochimica et Biophysica Acta*, 1469(2): 63-77.

27. Carter, H.E.; Fujino, Y. Biochemistry of the spingolipides. IX. Configuration of cerebrosides.*The Journal Biological Chemistry*. 1956, 221(2):879-884.

28. Sonnino S, Acquotti D, Riboni L, Giuliani A, Kirschner G, Tettamanti G. New chemical trends in ganglioside research. *Chemistry Physics Lipids*. 1986, 42(1-3):3-26.

29. Cantu, L.; Corti, M.; Sonnino, S.; Tettamanti, G. (1986). Light scattering measurements on gangliosides: dependence of micellar properties on molecular structure and temperature. *Chemistry and Physics Lipids*, 41(3-4): 315-328.

30. Samuelsson, B.; Samuelsson, K.(1969). Gas-liquid chromatography-mass spectrometry of synthetic ceramides. *Journal of Lipid Research*. 10(1):41-46.

31. Vanier, M.T.; Holm, M.; Månsson, J.E.; Svennerholm, L. (1973). The distribution of lipids in the human nervous system-V. Gangliosides and allied neutral glycolipids of infant brain. *Journal of Neurochemistry*, 21(6): 1375-1384.

32. Mansson, J.E.; Vanier, M.T.; Svennerholm, L. (1978). Changes in the fatty acid and sphingosine composition of the major gangliosides of human brain with age. *Journal of Neurochemistry*, 30(1): 273-275.

33. Martín, M.J.; Martín-Sosa, S.; Hueso, P. (2001). Bovine milk gangliosides: changes in ceramide moiety with stage of lactation. *Lipids*, 36(3): 291-298.

34. Handa, S.; Burton, R.M. Biosynthesis of glycolipids: incorporation of N-acetylgalactosamine by a rat brain particulate preparation. *Lipids*, 1969, 4(6): 589-598.

35. Thompson, T.E.; Tillack, T.W. (1985) Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. *Annual Review of Biophysics and Biophysical Chemistry*, 14:361-86.

36. Ando, S.; Yu, RK. Fatty acid and long-chain base composition of gangliosides isolated from adult human brain. *Journal of Neuroscience Research*, 1984, 12(2-3): 205-211.

37. Bode, L.; Beermann, C.; Mank, M.; Kohn, G.; Boehm, G. (2004). Human and bovine milk gangliosides differ in their fatty acid composition. *The Journal of Nutrition*, 134(11): 3016-3020.

38. Lamari, F.N.; Karamanos, N.K. (2002). Separation methods for sialic acids and critical evaluation of their biologic relevance. *Journal of Chromatography B Analytical Technologies in he Biomedical of Life Sciences*, 781(1-2): 3-19.

39. Schauer, R. (2004). Sialic acids: fascinating sugars in higher animals and man. *Zoology*, 107(1): 49-64.

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

41. Shaw, C.J.; Chao, H.; Xiao, B. (2001). Determination of sialic acids by liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 913(1-2):365-370.

42. Wang, B; Brand-Miller, J. (2003). The role and potential of sialic acid in human nutrition. *European Journal of Clinical Nutrition*, 57(11):1351-1369.

43. Chester, M.A. (1997). Nomenclature of Glycolipids (IUPAC Recommendations. *Pure and Applied Chemistry*, 69(12): 2475–2488.

44. Cantu, L.; Corti, M.; Brocca, P.; Del Favero, E. (2009). Structural aspects of ganglioside-containing membranes. *Biochimica et Biophys Acta*, 1788(1): 202-208.

45. Wiegandt, H. (1985). Chapter 3. Gangliosides New Comprehensive Biochemistry In: Wiegandt, H. *Glycolipids*, 10:199–260.

46. Ferraretto, A.; Pitto, M.; Palestini, P.; Masserini, M. (1997). Lipid domains in the membrane vesicles containing GM1 ganglioside and cholesterol. *Biochemistry,* 36 (30): 9232–9236.

47. Ramstedt, B.; Slotte, J.P. (2006). Sphingolipids and the formation of sterol-enriched ordered membrane domains. *Biochimica et Biophysica Acta*, 1758:1945–1956.

48. Trams, E.G.; Lauter, C.J. (1962). On the isolation and characterization of gangliosides. *Biochimica et Biophysica Acta*, 60:350-358.

49. Tettamanti, G. (2004). Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconjugate Journal*, 20(5):301-17.

50. Ledeen, R.W. (1978). Ganglioside structures and distribution: are they localized at the nerve ending? *Journal of Supramolecular Structure*, 8(1):1-17.

51. Mansson, J.E.; Vanier, M.T.; Svennerholm, L. (1978). Changes in the fatty acid and sphingosine composition of the major gangliosides of human brain with age. *Journal of Neurochemistry*, 30(1):273-275.

52. Schengrund, C. L.; Garrigan, O. (1969). A comparative study of gangliosides from the brains of various species. *Lipids*, 4(6): 488-495.

53. Colarow, L.; Turini, M.; Teneberg, S.; Berger, A. (2003). Characterization and biological activity of gangliosides in buffalo milk. *Biochimica et Biophysica Acta -Molecular and Cell Biology of Lipids*, 1631(1): 94-106.

54. Vajn, K.; Viljetić, B.; Degmečić, I.V.; Schnaar, R.L.; Heffer, M. (2013). Differential distribution of major brain gangliosides in the adult mouse central nervous system. *PLoS One*, 8(9):1-11.

55. Schnaar, R.L. (2010). Brain gangliosides in axon-myelin stability and axon regeneration. *FEBS Letters*, 584(9): 1741-1747.

56. Svennerholm, L.; Boström, K.; Fredman, P.; Månsson, J.E.; Rosengren, B.; Rynmark, B.M. (1989). Human brain gangliosides: developmental changes from early fetal stage to advanced age. *Biochimica et Biophysica Acta*, 1005(2): 109-117.

57. Ryan, J.M.; Rice, G.E.; Mitchell, M.D. (2013). The role of gangliosides in brain development and the potential benefits of perinatal supplementation. *Nutrition Research*, 33(11): 877-887.

58. McJarrow, P.; Schnell, N.; Jumpsen, J.; Clandinin, T. (2009). Influence of dietary gangliosides on neonatal brain development. *Nutrition Reviews*. 67(8): 451-463.

59. Wang, B. (2012). Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Advances in Nutrition,* (3): 465S-472S.

60. Goldman, J.E.; Hirano, M.; Yu, R.K.; Seyfried, T.N. (1984). GD3 ganglioside is a glycolipid characteristic of immature neuroectodermal cells. *Journal of Neuroimmunology*, 7(2-3): 179-192.

61. Skaper, S.D. Leon, A.; Toffano, G. (1989). Ganglioside function in the development and repair of the nervous system. *Molecular Neurobiology*, 173(3): 173-199.

62. Svennerholm, L.; Rynmark, B.M.; Vilbergsson, G.; Fredman, P.; Gottfries, J.; Månsson, J.E.; Percy, A. (1991). Gangliosides in human fetal brain. *Journal of Neurochemistry*, 56(5): 1763-1768.

63. Svennerholm, L.; Boström, K.; Jungbjer, B.; Olsson, L. (1994). Membrane lipids of adult human brain: lipid composition of frontal and temporal lobe in subjects of age 20 to 100 years. *Journal of Neurochemistry*, 63(5): 1802-1811.

64. Vorwerk, C.K. (2001). Ganglioside patterns in human spinal cord. *Spinal Cord*, 39(12): 628-632.

65. Nagai, Y.; Iwamori, M. (1983). Ganglioside Distribution at differet levels of organization and its biological implications. In: Advancese in Experimental Medicicne and Biology Vol. 174. Ganglioside Structure, Function and Biomedical Potential. Plenum Press. USA. Pp135-146.

66. Dahiya, R.; Brown, M.D.; Brasitus, T.A. (1986). Distribution of glycosphingolipids of monkey small and large intestinal mucosa. *Lipids*, 21(2): 107-111.

67. Glickman, R.M.; Bouhours, J.F. (1976). Characterization, distribution and biosynthesis of the major ganglioside of rat intestinal mucosa. *Biochimica et Biophysica Acta*, 424(1):17-25.

68. Dahiya, R.; Brasitus, T.A. (1986). Distribution of glycosphingolipids and ceramide of rat small intestinal mucosa. *Lipids*, 21(2): 112-116.

69. Park, E.J.; Suh, M.; Ramanujam, K.; Steiner, K.; Begg, D.; Clandinin, M.T. (2005). Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *Journal of Pediatric Gastroenterology and Nutrition*, 40(4): 487-495.

70. Roth, J. (1985). Polarized Distribution of GM1-Ganglioside in Human Duodenal Absorptive Enterocytes as Visualized with Cholera Toxin-Gold Complex. *Glycoconjugate Journal*, 2: 315-321.

71. Kwiterovich, P.O. Jr.; Sloan, H.R.; Fredrickson, D.S. (1970). Glycolipids and other lipid constituents of normal human liver. *Journal of Lipid Research*, 11(4): 322-330.

72. Nilsson, O.; Svennerholm, L. (1982). Characterization and quantitative determination of gangliosides and neutral glycosphingolipids in human liver. *Journal of Lipid Research*, 23(2): 327-334.

73. Svennerholm, L.; Bruce, A.; Månsson, J.E.; Rynmark, B.M.; Vanier, M.T. (1972). Sphingolipids of human skeletal muscle. *Biochimica et Biophysica Acta*, 280(4): 626-636.

74. Nakamura, K.; Ariga, T.; Yahagi, T.; Miyatake, T.; Suzuki, A.; Yamakawa, T. (1983). Interspecies comparison of muscle gangliosides by two-dimensional thin-layer chromatography. *Journal of Biochemistry*, 94(5):1359-1365.

75. Mukhin, D.N.; Prokazova, N.V.; Bergelson, L.D.; Orekhov, A.N. (1989). Ganglioside content and composition of cells from normal and atherosclerotic human aorta. *Atherosclerosis*, 78: 39–45.

76. Levis, G.M.; Karli, J.N.; Moulopoulos, S.D. (1979). Isolation and partial characterization of the neutral glycosphingolipids and gangliosides of the human heart. *Lipids*, 14(1): 9-14.

77. Narasimhan, R.; Murray, R.K.(1979). Neutral glycosphingolipids and gangliosides of human lung and lung tumours. *Biochemical Journal*, 179:199-211.

78. Månsson, J.E.; Mo, H.Q.; Egge, H.; Svennerholm, L. (1986). Trisialosyllactosylceramide (GT3) is a ganglioside of human lung. *FEBS Letters*, 196(2): 259-262.

79. Rauvala, H. (1976). Isolation and partial characterization of human kidney gangliosides. *Biochimica et Biophysica Acta*, 424(2):284-295.

80. Rauvala, H. (1976). Gangliosides of human kidney. *The Journal of Biological Chemistry*, 251(23): 7517-7520.

81. Ohashi, M. (1979). A comparison of the ganglioside distributions of fat tissues in various animals by two-dimensional thin layer chromatography. *Lipids*, 14(1): 52-57.

82. Levery, S.B.; Nudelman, E.D.; Salyan, M.E.; Hakomori, S. (1989). Novel tri-and tetrasialosylpoly-N-acetyllactosaminyl gangliosides of human placenta: structure determination of pentadeca- and eicosaglycosylceramides by methylation analysis, fast atom bombardment mass spectrometry, and 1H NMR spectroscopy. *Biochemistry*, 28(19):7772-7781.

83. Svennerholm, L. (1965). Gangliosides and other glycolipids of human placenta. *Acta Chemica Scandinavica*, 19(6):1506-1507.

84. Taki, T.; Matsuo, K.;Yamamoto, K.; Matsubara, T.; Hayashi, A.; Abe, T.; Matsumoto, M. (1988). Human placenta gangliosides. *Lipids*, 23(3): 192-198

85. Rueda, R.; Tabsh, K.; Ladisch, S. (1993). Detection of complex gangliosides in human amniotic fluid. *FEBS Letters*, 328(1-2):13-16.

86. Dotta, F.; Colman, P.G.; Lombardi, D.; Sharp, D.W.; Andreani, D.; Pontieri, G.M.; Di Mario, U.; Lenti, L., Eisenbarth, G.S.; Nayak, R.C. (1989). Ganglioside Expression in Human Pancreatic Islets. *Diabetes,* 38:1478-1483.

87. Dotta, F.; Previti, M.; Lenti, L.; Dionisi, S.; Casetta, B.; D'Erme, M.; Eisenbarth, G.S.; Di Mario, U. (1995). GM2-1 pancreatic islet ganglioside: identification and characterization of a novel islet-specific molecule. *Diabetologia*, 38(9): 1117-1121.

88. Wiegandt, H.; Bücking, H.W. (1970). Carbohydrate components of extraneuronal gangliosides from bovine and human spleen, and bovine kidney. *European Journal of Biochemistry*, 15(2): 287-292.

89. Svennerholm, L. (1963). Isolation of the major ganglioside of human spleen. *Acta Chemica Scandinavica*, 17(3): 860-862.

90. Yuasa, H.; Scheinberg, D.A.; Houghton, A.N. (1990). Gangliosides of T lymphocytes: evidence for a role in T-cell activation. *Tissue Antigens*, 36(2):47-56.

91. Sorice, M.; Parolini, I.; Sansolini, T.; Garofalo, T.; Dolo, V.; Sargiacomo, M.; Tai, T.; Peschle, C.; Torrisi, M.R.; Pavan, A. (1997). Evidence for the existence of ganglioside-enriched plasma membrane domains in human peripheral lymphocytes. *Journal of Lipid Research*, 38(5): 969-980.

92. Senn, H.J.; Orth, M.; Fitzke, E.; Wieland, H., Gerok, W. (1989). Gangliosides in normal human serum. Concentration, pattern and transport by lipoproteins. *The FEBS Journal*, 181(3):657–662.

93. Revesz, T.; Greaves, M. (1975). Ligand-induced redistribution of lymphocyte membrane ganglioside GM1. *Nature*, 257:103-106.

94. Vanier, M.T. (1983). Biochemical Studies in Niemann-Pick Disease I. Major Sphingolipids of Liver and Spleen. *Biochimica et Biophysica Acta*, 750:178-184.

95. Venerando, B.; Fiorilli, A.; Ghidoni, R.; Sonnino, S.; Chigorno, V.; Tettamanti, G. (1984).Ganglioside metabolism: new experimental approaches. *Italian Journal of Biochemistry*, 33(4): 295A-297A.

96. van Echten-Deckert, G.; Guravi, M. (2008). Golgi localization of glycosyltransferases involved in ganglioside biosynthesis. *Current DrugTargets*, 9(4): 282-291.

97. Sandhoff, K.; Kolter, T. (2003). Biosynthesis and degradation of mammalian glycosphingolipids. *Philosophical Transactions of the Royal Society Biological Sciences*, 358(1433):847-861.

98. Rueda, R.; Gil, A. (1998). Chapter 15 Role of Gangliosides in Infant Nutrition. In: Huang Y-S., Sinclair, A.J. Lipids in Infant Nutrition. USA AOCS.pp213-234.

99. Lloyd, K.O.; Furukawa, K.(1998). Biosynthesis and functions of gangliosides: recent advances. *Glycoconjugate Journal*, 15(7): 627-636.

100. van Echten, G.; Sandhoff, K. (1993). Ganglioside metabolism. Enzymology, Topology, and regulation. *Journal of Biological Chemistry*, 268(8): 5341-5344.

101. Fishman, P.H.; Brady, R.O. (1976). Biosynthesis and function of gangliosides. *Science,* 194 (4268): 906-915.

102. Keenan, T.W. (1974). Composition and Synthesis of Gangliosides in Mammary Gland and Milk of the Bovine. *Biochimica et Biophysica Acta*, 337: 255-270.

103. Prokazova, N.V.; Samovilova, N.N.; Gracheva, E.V.; Golovanova, N.K. (2009). Ganglioside GM3 and its biological functions. *Biochemistry* (Moscow), 74(3): 235-249.

104. Valivullah, H.M.; Dylewski, D.P.; Keenan, T.W. (1986). Distribution of terminal transferases of acylglycerol synthesis in cell fractions from lactating mammary gland. *International Journal of Biochemy,* 18(9): 799-806.

105. Miller-Podraza, H.; Fishman, P.H. (1982). Translocation of newly synthesized gangliosides to the cell surface. *Biochemistry*, 21(14):3265-3270.

106. Tettamanti, G.; Bassi, R.; Viani, P.; Riboni, L. (2003). Salvage pathways in glycosphingolipid metabolism. *Biochimie*, 85(3-4): 423-437.

107. Idota, T.; Kawakami, H. (1995). Inhibitory effects of milk gangliosides on the adhesion of *Escherichia coli* to human intestinal carcinoma cells. *Bioscience, Biotechnology, and Biochemistry*, 59(1): 69-72.

108. Nakano, T.; Sugawara, M.; Kawakami, H. (2001). Sialic acid in Human Milk: *Composition and Functions. Acta Pediatrica Taiwanica*, 42(1): 11-17.

109. Schwarzmann, G. (2001). Uptake and metabolism of exogenous glycosphingolipids by cultured cells. *Seminars in Cell and Developmental Biology*, 12:163–171.

110. Schnabl, K.L.; Larcelet, M.; Thomson, A.B.; Clandinin, M.T. (2009). Uptake and fate of ganglioside GD3 in human intestinal Caco-2 cells. *The American Journal of Physiology: Gastrointestinal and Liver Physiology*, 2297(1):G52-G59.

111. Birecki, C.J.; Drozdowski, L.A.; Suh, M.; Park, E.J.; Clandinin, M.T.; Thomson, A.B. (2006). Dietary gangliosides enhance in vitro lipid uptake in weanling rats. *The Journal of Pediatric Gastroenterology and Nutrition*, 42:59 –55.

112 Moore, K. H.; Ettinger, A. C.; Yokoyama, M. T. (2000). Variation in ganglioside content of bovine dairy products. *Journal of Food Composition and Analysis*, 13(5): 783-790.

113. Pham, P.H.; Duffy, T.L.; Dmytrash, A.L.; Lien, V.W.; Thomson, A. B.; Clandinin, M.T. (2011). Estimate of dietary ganglioside intake in a group of healthy Edmontonians based on selected foods. *Journal of Food Composition and Analysis*, 24:1032–1037.

114. Fox, P.F. (2003). Advanced dairy chemistry. Vol. 2, Lipids. Springer, USA.

115. Singh, H. (2006). The milk fat globule membrane-A biophysical system for food application. *Current Opinion in Colloid & Interface Science*, 11:154 – 163.

116. Dewettinck, K.; Rombaut, R.; Thienponta, N.; Le, T.T.; Messens, K.; Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal*, 18(5):436–457.

117. Liu , H. X.; Adachi , I.; Horikoshi , I.; Ueno, M. (1995). Mechanism of promotion of lymphatic drug absorption by milk fat globule membrane. *International Journal of Pharmaceutics,* 118:55-64.

118. Deeth, H.C. (1997). The role of phospholipids in the stability of milk fat globules. *Australian Journal Of Dairy Technology*, 52(1): 44-46.

119. Contarini, G.; Povolo, M. (2013). Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. *International Journal of Molecular Sciences,* 14(2): 2808-2831.

120. Anderson, M.; Cawston, T.E. (1975). Reviews of the progress of dairy science. The milk fat globule membrane. *Journal of Dairy Research*, 42:459-483.

121. Patton, S. (1982). Release of remnant plasma membrane from milk fat globules by Triton X-100. *Biochimica et Biophysica Acta*, 688:727–734.

122. Chen, Z.Y.; Nawar W.W. (1991). Role of milk fat globule membrane in autoxidation of milk fat. *Journal of Food Science,* 56:398–401.

123. Danthine, S.; Blecker, C.; Paquot, M.; Innocente, N.; Deroanne, C. (2000). Progress in milk fat globule membrane research: a review. Évolution des connaissances sur la membrane du globule gras du lait: synthèse bibliographique. *Lait*, 80(2): 209-222.

124. Bracco, U.; Hidalgo, J.; Bohren, H. (1972). Lipid composition of the fat globule membrane of human and bovine milk. *Journal of Dairy Science*, 55(2):165-172.

125. Bertram, Y.; Fong, B.Y.; Norris, C.S.; MacGibbon, A.K.H. (2007). Protein and lipid composition of bovine milk-fat-globule membrane. *International Dairy Journal*, 17(4): 275–288

126. Keenan, T.W.; Dylewski, D.P.(1995) Intracellular origin of milk lipid globules and the nature and structure of the milk fat globule membrane. In: Advanced Dairy Chemistry, Lipids, vol. 2, (ed. P.F. Fox).Chapman and Hall, London, pp. 89–130.

127. Kim, S.K.; Nawar, W.W. (1992). Oxidative interactions of cholesterol in the milk fat globule membrane. *Lipids*, 27(11): 928-932.

128. Keenan, T.W.; Patton, S. (1995). The structure of milk: implications for samplig and storage A. The milk lipid globule membrane. In Jensen. Handbook of milk composition pp.5-50. San Diego Academic Press.

129. Patton, S. And T.W. Keenan (1975). The milk fat Globule Membrane. *Biochimica et Biophysica Acta*, 415: 273-309.

130. Keenan, T.W.; Moon, T.W.; Dylewsky, D.P.(1983). Lipid globules retain membrane material after Homogenization. *Journal of Dairy Science*, 66: 196–203.

131. McPherson, A.V.; Kitchen, B.J. (1983). Reviews of the progress of Dairy Science: The bovine milk fat globule membrane-its formation, composition, structure and behaviour in milk and dairy products. *Journal of Dairy Research*, 50:107-133.

132. Dapper, C.H.; Valivullah, H.M.; Keenan, T.W. (1987). Use of polar aprotic solvents to release membranes from milk lipid globules. *Journal of Dairy Science*,70: 760–765.

133. Houlihan, A.V.; Goddard, P.A.; Nottingham, S.M.; Kitchen, B.J.; Masters C.J. (1992). Interactions between the BMFGM and skim milk components on heating whole milk. *Journal of Dairy Research*, 59:187–195.

134. Brunner, J.R. (1974). Physical equilibria in milk: The lipid phase. In: Fundamentals of Dairy Chemistry, 2nd ed. BH Webb. A.H. Johnson, and J.A. Alford (Editors). AVI Publishing Co., Westport, Conn, 474-602.

135. Keenan, T.W. (2001). Milk lipid globules and their surrounding membrane: a brief history and perspectives for future research. *Journal of Mammary Gland Biology and Neoplasia*, 6(3): 365-371.

136. Copius Peereboom, J.W. (1969). Theory on the renaturation of alkaline milk phosphates from pasteurized cream. *Milchwissenschaft*, 24(5): 266-269.

137. Bauer, H. (1972). Ultrastructural Observations on the Milk Fat Globule Envelope of Cow's Milk. *Journal of Dairy Science,* 55(10):1375-1387.

138. Kanno, C. (1980). Recent studies on milk fat globule membrane with special reference to the constituent proteins. *Japanese Journal of Zootechnical Science,* 51(2): 75-88.

139. Singer ,S.J.; Nicholson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. *Science,* 175(4023): 720-731.

140. Evers, J.M. (2004). The milkfat globule membrane-compositional and structural changes post secretion by the mammary secretory cell. *International Dairy Journal*, 14(8): 661–674.

141. van Boekel, M.A.J.S.; Walstra, P. (1989) Chapter 2. Physical Changes in the Fat Globules in Unhomogenized and homogenized milk. *Bulletin International Dairy Federation*, 238:13-16.

142. Keenan, T.W.; Mather, I.H.(2002). Milk fat globule membrane.In: Encyclopedia of Dairy Sciences (H. Roginski, J.W. Fuquay, P.F. Fox). Academic Press, London, pp. 1568–1576.

143. Evers, J.M.; Haverkamp, R.G.; Holroyd, S.E.; Jameson, G.B.; Mackenzie, D.D.S.; McCarthy, O.J. (2008). Heterogeneity of milk fat globule membrane structure and composition as observed using fluorescence microscopy techniques. *International Dairy Journal*, 18; 1081–1089.

144. Gallier, S.; Gragson, D.; Cabral, C.; Jimenez-Flores, R.; Everett, D.W.(2010). Using confocal laser scanning microscopy to probe the milk fat globule membrane and associated proteins. *Journal of Agricultural and Food Chemistry*, 58:4250–4257.

145. Lopez, C.; Madec, M.N.; Jimenez-Flores, R. (2010). Lipid rafts in the bovine milk fat globule membrane revealed by the lateral segregation of phospholipids and heterogeneous distribution of glycoproteins. *Food Chemistry*, 120: 22–33.

146. Keenan, T.W.; Schmid, E.; Franke, W.W.; Wiegandt, H. (1975). Exogenous Glycosphingolipids Supress Growth Rate of Transformed and Untransformed T3 Mouse Cells. *Experimental Cell Research*, 92: 259-270.

147. Lee, S.J.; Sherbon, J.W. (2002). Chemical changes in bovine milk fat globule membrane caused by heat treatment and homogenization of whole milk. *Journal of Dairy Research*, 69(4): 555-567.

148. Janbandhu, T.J.; Khedkar, C. D.; Singh, A. (2000). Effect of heating and chilling of recombine milk on fatty acid composition of fat globule membrane lipids. *Indian Journal of Dairy Science*, 53(3): 231-234

149. Greenbank, G.R.; Pallansch, M. J. (1961). Migration of Phosphatides in Processing Dairy Products. *Journal of Dairy Science,* 44(9): 1597-1602.

150. Buchheim, W. (1985) Membranes of the milk fat globules-ultrastructural, biochemical and technological aspects. *Kiel Milchwirtschaft. Forschugsber*, 38: 227-246.

151. McPherson, A.V.; Dash, M.C.; Kitchen, B.J. (1984). Isolation and composition of milk fat globule membrane material. I. From pasteurized milks and creams. *Journal of Dairy Research*, 51, 279-28

152. Wiking, L; Bjorck, L; Nielsen, JH. (2003). Influence of feed composition on stability of fat globules during pumping of raw milk. *International Dairy Journal*, 13(10): 797-803.

153. Walstra, P.; Jenness, R. (1984). Dairy chemistry & physics. Dairy chemistry & physics. John Wiley & Sons, New York; USA.

154. Weihrauch, J.L. (1988). Lipids of Milk: Deterioration. In: Wong, N.P.; Jenness, R., Keeney, M.; Marth, E.H. editors. Fundamentals of dairy chemistry. 3rd ed., New York: Van nostrand Reinhold Company, pp. 215-278.

155. Michalski, M.C.; Michel, F.; Sainmont, D.; Briard, V. (2002). Apparent zeta-potential as a tool to assess mechanical damages to the milk fat globule membrane. *Colloids and Surfaces B: Biointerfaces*, 23(1): 23-30.

156. Huppertz, T.; Kelly, A.L.; Fox. P.F. (2009). Milk Lipids – Composition, Originand Properties. In: Dairy fats and related products. Tamime, A. Y.Blackwell Publising Limited UK pp 1-27.

157. Sprong, RC; Hulstein, MFE; Van der Meer, R. (2001).Bactericidal activities of milk lipids. *Antimicrobial Agents and Chemotherapy,* 45(4):1298-1301.

158. Martín, M.J.; Martín-Sosa; S.; García-Pardo, L.A.; Hueso, P. (2001). Distribution of Bovine Milk Sialoglycoconjugates during Lactation. *Journal of Dairy Science*, 84: 995-1000.

159. Martín-Sosa, S.; Martín, M. J.; Castro, M. D.; Cabezas, J. A.; Hueso, P. (2004). Lactational changes in the fatty acid composition of human milk gangliosides. *Lipids*, 39(2):111-116.

160. Laegreid, A.; Kolstø Otnaess, A.B. (1987). Trace amounts of ganglioside GM1 in human milk inhibit enterotoxins from *Vibrio cholerae* and *Escherichia coli*. *Life Sciences*, 40(1): 55-62.

161. Iwamori, M.; Takamizawa, K.; Momoeda, M.; Iwamori, Y.; Taketani, Y. (2008). Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin. *Glycoconjugate Journal,* 25: 675–683.

162. Takamizawa, K.; Iwamori, M.; Mutai, M.; Nagai, Y. (1986). Gangliosides of bovine buttermilk. Isolation and characterization of a novel monosialoganglioside with a new branching structure. *Journal of Biological Chemistry*, 261(12): 5625-5630.

163. Pan, X. L.; Izumi, T. (2000). Variation of the ganglioside compositions of human milk, cow's milk and infant formulas. *Early Human Development*, 57(1):25-31.

164. Pan, X.L.; Izumi, T. (1999). Chronological changes in the ganglioside composition of human milk during lactation. *Early Human Development*, 55(1):1-8.

165. Uchiyama, S.; Sekiguchi, K.; Akaishi, M.; Anan, A.; Maeda, T.; Izumi, T.(2011). Characterization and chronological changes of preterm human milk gangliosides. *Nutrition,* 27: 998–1001.

166. Rueda, R.; Maldonado, J., Gil, A. (1996). Comparison of content and distribution of human milk gangliosides from Spanish and Panamanian mothers. *Annals of Nutrition and Metabolism*; 40(4):194-201.

167. Rueda, R.; Puente, R.; Hueso, P.; Maldonado, J.; Gil, A. (1995). New data on content and distribution of gangliosides in human milk. *Biological Chemistry Hoppe Seyler*. 376(12):723-727.

168. Takamizawa, K.; Iwamori, M.; Mutai, M.; Nagai, Y. (1986). Selective changes in gangliosides of human milk during lactation: a molecular indicator for the period of lactation. *Biochimica et Biophysica Acta*, 879(1): 73-77.

169. Hauttecoeur, B.; Sonnino, S.; Ghidoni, R. (1985). Characterization of two molecular species GD3 ganglioside from bovine buttermilk. *Biochimica et Biophysica Acta*, 833(2):303-307.

170. Jensen, R.G.(1989). The Lipids of Human Milk CRC Press, INC.: Boca Raton, Florida, USA.

171. Keenan, T. (1974b). Membranes of mammary gland. IX. concentration of glycosphingolipid galactosyl and sialyltransferases in golgi apparatus from bovine mammary gland. *Journal of Dairy Science*, 57(2): 187-192.

172. Puente, R.; Garcia-Pardo, L. A.; Hueso, P. (1992). Gangliosides in bovine milk. changes in content and distribution of individual ganglioside levels during lactation. *Biological Chemistry Hoppe-Seyler*, 373(5):283-288.

173. Morrison, W.R. (1969). Polar Lipids In Bovine Milk I. Long-Chain Bases in Sphingomyelin. *Biochimica et Biophysica Acta,* 176:537–546.

174. Schmelz, E.M.; Dillehay, D. L.; Webb, S. K.; Reiter, A.; Adams, J.; Merrill, A. H. Jr. (1996). Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with1,2 dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. *Cancer Research*, 56: 4936–4941.

175. Zeisel, S. H.; Char, D.; Sheard, N. F. (1986). Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. *Journal of Nutrition,* 116: 50–58.

176. Bushway, A.A.; Keenan, T.W. (1978). Composition and synthesis of three higher ganglioside homologs in bovine mammary tissue. *Lipids*, 13(1):59-65.

177. Laegreid, A.; Otnaess, A.B.; Fuglesang, J.(1986). Human and bovine milk: comparison of ganglioside composition and enterotoxin-inhibitory activity. *Pediatric Research*, 20(5): 416-421.

178. Jensen, R. G.(1995) Handbook of Milk Composition. Academic Press,New York, NY.

179. Huang, R.T. (1973). Isolation and characteristics of the gangliosides of buttermilk. *Biochimica et Biophysica Acta*, 306(1):82-84.

180. Puente, R.; Garcia-Pardo, L.A.; Rueda, R.; Gil, A.; Hueso, P. (1994). Changes in ganglioside and sialic acid contents of goat milk during lactation. *Journal of Dairy Science*, 77:39–44.

181. Puente, R.; Garcia-Pardo, L.A.; Rueda, R.; Gil, A.; Hueso, P.(1995b). Ewes' milk: changes in the contents of ganglioside and sialic acid during lactation. *Journal of Dairy Research*, 62:651–654.

182. Puente, R.; Garcia-Pardo, L.; Rueda, R.; Gil, A.; Hueso, P. (1996). Seasonal variations in the concentration of gangliosides and sialic acids in milk from different mammalian species. *International Dairy Journal*, 6(3):315-322.

183. Couvrer, S.; Hurtaud, C. (2007). Le globule gras du lait : sécrétion, composition, fonctionset facteurs de variation. *INRA Productions Animales*, 20 (5):369-382.

184. Lopez, C.; Briard-Bion, V.; Menard, O.; Rousseau, F.; Pradel, P.; Besle, J.M. (2008). Phospholipid, sphingolipid, and fatty acid compositions of the milk fat globule membrane are modified by diet. *Journal of Agricultural Food Chemistry*, 56(13): 5226-5236.

185. Ryan, J.M.; Rice, G.E.; Mitchel, M.D. (2013). The role of gangliosides in brain development and the potential benefits of perinatal supplementation. *Nutrition research*, 33: 877-887.

186. Crespo, F.A.; Sun, X.; Cripps, J.G.; Fernandez-Botran, R. (2006). The immunoregulatory effects of gangliosides involve immune deviation favoring type-2 T cell responses. *Journal of Leukocyte Biology*, 79:586-595.

187. Nagafuku M, Okuyama K, Onimaru Y, Suzuki A, Odagiri Y, Yamashita T, Iwasaki K, Fujiwara M, Takayanagi M, Ohno I, Inokuchi J. (2012). CD4 and CD8 T cells require different membrane gangliosides for activation. *The Proceedings of the National Academy of Sciences of the United States of America*, 109(6): E336-E342.

188. Vázquez, E.; Gil, A.; Rueda, R. (2001). Dietary gangliosides positively modulate the percentages of Th1 and Th2 lymphocyte subsets in small intestine of mice at weanling. *Biofactors*, 15(1):1-9.

189. Vazquez, E.; Gil, A.; Rueda, R. (2000). Dietary gangliosides increase the number of intestinal IgA-secreting cells and the luminal content of secretory IgA in wealing mice. Conference: World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition Location: Boston, Massachusetts, USA. *Journal of Pediatric Gastroenterology and Nutrition*, 31(2): S133-S134.

190. Vazquez, E.; Gil, A.; Garcia-Olivares, E.; et al. (1999). Dietary gangliosides increase the number of intestinal IgA-secreting cells in weanling mice. Conference: 10th International Congress of Mucosal Imunology Location: Amsterdam, Netherlands. *Immunology Letters*, 69(1):168.

191. Rueda, R. (2007). The role of dietary gangliosides on immunity and the prevention of infection. *British Journal of Nutrition*, 98(1): S68-S73.

192. Park, E.J.; Suh, M.; Thomson, B.; Thomson, A.B.; Ramanujam, K.S.; Clandinin, M.T. (2005). Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. *Glycobiology*, 15(10):935-942.

193. Ledeen, R.W.; Wu, G. (2002). Ganglioside function in calcium homeostasis and signaling. *Neurochemistry Research*, 27(7-8): 637-647.

194. Wu G.; Vaswani, K.K.; Lu, Z.H.; Ledeen, R.W. (1990). Gangliosides stimulate calcium flux in Neuro-2a cells and require exogenous calcium for neuritogenesis. *Journal of Neurochemistry*, 55:484-491.

195. Wu, G.; Lu, Z.H.; Ledeen, R.W.(1995). GM1 ganglioside in the nuclear membrane modulates nuclear calcium homeostasis during neurite outgrowth. *Journal of Neurochemistry*, 65(3):1419-1422.

196. Carlson, R.O.; Masco, D.; Brooker, G.; Spiegel, S. (1994). Endogenous ganglioside GM1 modulates L-type calcium channel activity in N18 neuroblastoma cells. *Journal of Neuroscience*, 14(4): 2272-2281.

197. Wu, G.; Xie, X.; Lu, Z.H.; Ledeen; R.W. (2001). Cerebellar neurons lacking complex gangliosides degenerate in the presence of depolarizing levels of potassium. *The Proceedings of the National Academy of Sciences of the United States of America*, 98(1): 307-312.

198. Ledeen, R.; Wu, G. (2007). GM1 in the nuclear envelope regulates nuclear calcium through association with a nuclear sodium-calcium exchanger. *Journal of Neurochemistry*, 103(1):126-134.

199. Marengo, F.D.; Wang, S.Y.; Wang, B.; Langer,G.A. (1998). Dependence of cardiac cell Ca2+ permeability on sialic acid-containing sarcolemmal gangliosides. *Journal of molecular and cellular cardiology*, 30(1): 127-137.

200. Müthing, J.; Maurer, U.; Weber-Schürholz, S. (1998). Glycosphingolipids of skeletal muscle: II. Modulation of Ca2(+)-flux in triad membranes by gangliosides. *Carbohydrate Research*, 307(1-2):147-157.

201. Zhang, X.; Kiechle, F.L. (2004). Review: Glycosphingolipids in health and disease. *Annals of clinical and laboratory science*, 34(1):3-13.

202. García-Ruiz, C.; Colell, A.; París, R.; Fernández-Checa, J.C. (2000). Direct interaction of GD3 ganglioside with mitochondria generates reactive oxygen species followed by mitochondrial permeability transition, cytochrome c release, and caspase activation. *The FASEB Journal*, 14(7): 847-858.

203. Young, M.M.; Kester, M.; Wang, H.G. (2013). Sphingolipids: regulators of crosstalk between apoptosis and autophagy. *Journal of Lipid Research*, 54(1): 5-19.

204. De Maria, R.; Lenti, L.; Malisan, F.; d'Agostino, F.; Tomassini, B.; Zeuner, A.; Rippo, M.R.; Testi, R. (1997). Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. *Science*, 277(5332):1652-1655.

205. Rippo, M.R.; Malisan, F.; Ravagnan, L.; Tomassini, B.; Condo, I.; Costantini, P.; Susin, S.A.; Rufini, A.; Todaro, M.; Kroemer, G.; Testi, R. (2000). GD3 ganglioside directly targets mitochondria in a bcl-2-controlled fashion. *The FASEB Journal*, 14(13):2047-2054.

206. Copani, A.; Melchiorri, D.; Caricasole, A.; Martini, F.; Sale, P.; Carnevale, R.; Gradini, R.; Sortino, M.A.; Lenti, L.; De Maria, R.; Nicoletti, F. (2002). Beta-amyloid-induced synthesis of the ganglioside GD3 is a requisite for cell cycle reactivation and apoptosis in neurons. *Journal of Neuroscience*, 22(10): 3963-3968.

207. García-Ruiz, C.; Colell, A.; Morales, A.; Calvo, M.; Enrich, C.; Fernández-Checa, J.C.(2002). Trafficking of ganglioside GD3 to mitochondria by tumor necrosis factor-alpha. *Journal of Biological Chemistry*, 277(39):36443-36448.

208. Saqr, H.E.; Omran, O.; Dasgupta, S.; Yu, R.K.; Oblinger, J.L.; Yates, A.J. (2006). Endogenous GD3 ganglioside induces apoptosis in U-1242 MG glioma cells. *Journal of Neurochemistry*, 96(5):1301-1314.

209. Hakomori, S.; Igarashi, Y. (1995). Functional role of glycosphingolipids in cell recognition and signaling. *Journal of Biochemistry*, 118(6):1091-1103.

210. Lopez, P.H.; Schnaar, R.L. (2009). Gangliosides in cell recognition and membrane protein regulation. *Current Opinion of Structural Biology*, 19(5):549-557.

211. Tiemeyer, M.; Yasuda, Y.; Schnaar, R.L. (1989). Ganglioside-specific binding protein on rat brain membranes. *Journal of Biological Chemistry*, 264(3): 1671-1681.

212. Wang, X.; Sun, P.; Al-Qamari, A.; Tai, T.; Kawashima, I.; Paller, A.S. (2001). Carbohydrate-carbohydrate binding of ganglioside to integrin alpha(5) modulates alpha(5)beta(1) function. *Journal of Biological Chemistry*, 276(11):8436-8444.

213. Hakomori, S. (1991). Carbohydrate-carbohydrate interaction as an initial step in cell recognition. *Pure and Applied Chemistry*, 63(4):473-482.

214. Kojima, N.; Hakomori, S. (1989). Specific interaction between gangliotriaosylceramide (Gg3) and sialosyllactosylceramide (GM3) as a basis for specific cellular recognition between lymphoma and melanoma cells. *Journal of Biological Chemistry*, 264(34):20159-20162.

214. Kojima, N.; Hakomori, S. (1991). Cell adhesion, spreading, and motility of GM3-expressing cells based on glycolipid-glycolipid interaction. *Journal of Biological Chemistry*, 266(26):17552-17558.

215. Kabayama, K.; Sato, T.; Saito, K.; Loberto, N. Prinetti, A.; Sonnino, S.; Kinjo, M.; Igarashi, Y.; Inokuchi, J. (2007) Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*,104:13678–13683.

216. Sonnino, S.; Mauri, L.; Ciampa, M.G.; Prinetti, A. (2013). Gangliosides as regulators of cell signaling: ganglioside-protein interactions or ganglioside-driven membrane organization? *Journal of Neurochemistry*, 124(4): 432-435.

217. Tettamanti, G.; Riboni, L. (1994). Gangliosides turnover and neural cells function: a new perspective. *Progress in Brain Research*, 101:77-100.

218. Nagai, Y. (1995). Functional roles of gangliosides in biosignaling. *Behavioural Brain Research*, 66(1-2):99-104.

219. Kappel, T.; Anken, R.H.; Hanke, W.; Rahmann, H. (2000). Gangliosides affect membrane-channel activities dependent on ambient temperature. *Cellular and Molecular Neurobiology*, 20(5): 579-590.

220. Leon, A.; Facci, L.; Toffano, G.; Sonnino, S.; Tettamanti, G. (1981). Activation of (Na^+/K^+)-ATPase by nanomolar concentrations of GM1 ganglioside. *Journal of Neurochemistry*, 37(2):350-357.

221. Mahadik, S.P.; Hawver, D.B.; Hungund, B.L.; Li, Y.S.; Karpiak, S.E.(1989). GM1 ganglioside treatment after global ischemia protects changes in membrane fatty acids and properties of Na^+/K^+-ATPase and Mg^{2+}-ATPase. *Journal of Neuroscience Research*, 24(3):402-412.

222. Vyskocil, F.; Di Gregorio, F.; Gorio, A. (1985). The facilitating effect of gangliosides on the electrogenic (Na+/K+) pump and on the resistance of the membrane potential to hypoxia in neuromuscular preparation. *Pflugers Archives*, 403(1):1-6.

223. Salazar, B.C.; Castaño, S.; Sánchez, J.C.; Romero, M.; Recio-Pinto, E. (2004). Ganglioside GD1a increases the excitability of voltage-dependent sodium channels. *Brain Research*, 1021(2):151-158.

224. Mahmood, I.; Liu, X.; Neya, S.; Hoshino, T. (2013). Influence of lipid composition on the structural stability of g-protein coupled receptor. *Chemical and Pharmaceutical Bulletin*, 61(4):426-437.

225. Yuyama, K.; Sekino-Suzuki, N.; Sanai, Y.; Kasahara, K. (2007). Translocation of activated heterotrimeric G protein Galpha(o) to ganglioside-enriched detergent-resistant

membrane rafts in developing cerebellum. *Journal of Biological Chemistry*, 282(36):26392-26400.

226. Nojiri, H.; Stroud, M.; Hakomori, S. (1991). A specific type of ganglioside as a modulator of insulin-dependent cell growth and insulin receptor tyrosine kinase activity. Possible association of ganglioside-induced inhibition of insulin receptor function and monocytic differentiation induction in HL-60 cells. *Journal of Biological Chemistry*, 266(7):4531-4537.

227. Tagami, S.;, Inokuchi, J.J.; Kabayama, K.; Yoshimura, H.; Kitamura, F.; Uemura, S.; Ogawa, C.; Ishii, A.; Saito, M.; Ohtsuka, Y.; Sakaue, S.; Igarashi,Y. (2002). Ganglioside GM3 participates in the pathological conditions of insulin resistance. *Journal of Biological Chemistry*, 277(5):3085-3092.

228. Bremer, E.G.; Schlessinger, J.; Hakomori, S. (1986). Ganglioside-mediated modulation of cell growth. Specific effects of GM3 on tyrosine phosphorylation of the epidermal growth factor receptor. *Journal of Biological and Chemistry*, 261(5):2434-2440.

229. Rebbaa, A.; Hurh, J.; Yamamoto, H.; Kersey, D.S.; Bremer, E.G. (1996). Ganglioside GM3 inhibition of EGF receptor mediated signal transduction. *Glycobiology*, 6(4): 399-406.

230. Yates, A.J. (1986). Gangliosides in the nervous system during development and regeneration. *Neurochemical Pathology,* 5(3):309-329.

231. Ledeen, R.W.; Wu, G.; Lu, Z.H.; Kozireski-Chuback, D.; Fang, Y. (1998). The role of GM1 and other gangliosides in neuronal differentiation. Overview and new finding. *Annals of the New York Academy of Sciences*, 845:161-175.

232. Vanier, M.T.; Holm, M.; Ohman, R.; Svennerholm, L. (1971). Developmental profiles of gangliosides in human and rat brain. *Journal of Neurochemistry*, 18(4):581-592.

233. Ngamukote, S.; Yanagisawa, M.; Ariga, T.; Ando, S.; Yu, R. K. (2007). Developmental changes of glycosphingolipids and expression of glycogenes in mouse brains. *Journal of Neurochemistry*, 103:2327–2341.

234. Yu, R.K.; Macala, L.J.; Taki, T.; Weinfield, H.M.; Yu, F.S. (1988). Developmental changes in ganglioside composition and synthesis in embryonic rat brain. *Journal of Neurochemistry*, 50(6):1825-1829.

235. Byrne, M.C.; Ledeen, R.W.; Roisen, F.J.; Yorke, G.; Sclafani, J.R. (1983). Ganglioside-induced neuritogenesis: verification that gangliosides are the active agents, and comparison of molecular species. *Journal of Neurochemistry*, 41(5):1214-1222.

236. Fang, Y.; Wu, G.; Xie, X.; Lu, Z.H.; Ledeen, R.W. (2000). Endogenous GM1 ganglioside of the plasma membrane promotes neuritogenesis by two mechanisms. *Neurochemical Research*, 25(7): 931-940.

237. Walkley, S. U.; Siegel, D.A.; Dobrenis, K. (1995). GM2 Ganglioside and Pyramidal Neuron Dendritogenesis. *Neurochemical Research*, 20(11): 1287-1299.

238. Hilbig, R.; Lauke, G.; Rahmann, H. (1983-1984). Brain gangliosides during the life span (embryogenesis to senescence) of the rat. *Developmental Neuroscience*, 6(4-5):260-270.

239. Yu, R.K.; Tsai, Y.T.; Ariga, T. (2012). Functional roles of gangliosides in neurodevelopment: an overview of recent advances. *Neurochemical Research*, 37(6):1230-1244.

240. Yu, R.K.; Nakatani, Y.; Yanagisawa, M. (2009). The role of glycosphingolipid metabolism in the developing brain. *Journal of lipid research*, 50 Suppl:S440-S445.

241. Jeyakumar, M.; Butters, T.D.; Dwek, R.A.; Platt, F.M. (2002). Glycosphingolipid lysosomal storage diseases. *Neuropathology and Applied Neurobiology*, 28:343–357.

242. Konrad Sandhoff, K.; Harzer, K. (2013). Gangliosides and Gangliosidoses: Principles of Molecular and Metabolic Pathogenesis. *The Journal of Neuroscience*, 33(25):10195–10208.

243. Yu, R.K.; Tsai, Y.T.; Ariga, T.; Yanagisawa, M. (2011). Structures, biosynthesis, and functions of gangliosides-an overview. *Journal of Oleo Science*, 60(10):537-544.

244. Suzuki Y, Sakuraba H, Oshima M. GM1 gangliosidosis and Morqui B disease.(1995). In The Metabolic and Molecular Bases of Inherited Diseases, 7th edn. Eds Scriver CR, Beaudet AL, Sly WS, Valle D. New York: McGraw-Hill; 2785– 2824.

245. Karlsson, K.A. (1989). Animal Glycosphingolipids as Membrane Attachment Sites for Bacteria. *Annual Review of Biochemistry*, 58:309-350.

246. Varki, A. (2008). Sialic acids in human health and disease. *Trends in Molecular Medicine*, 14(8): 351-360.

247. Muthing, J. (1996). High-resolution thin-layer chromatography of gangliosides. *Journal of Chromatography A*, 720: 3-25.

248. Hirst, G.K. (1942). Adsorption of influenza virus hemagglutinins and virus by red blood cells. *Journal of Experimental Medicine*,76:195-209.

249. von Itzstein, M.; Wu, W.Y.; Jin, B. (1994). The synthesis of 2,3-didehydro-2,4-dideoxy-4-guanidinyl-N-acetylneuraminic acid: a potent influenza virus sialidase inhibitor. *Carbohydrate Research*, 259: 301-305.

250. Markwell, M.A.K.; Svennerholm, L.; Paulson, J.C. (1981). Specific gangliosides function as host cell receptors for Sendai virus. *Proceedings of the National Academy of Sciences of the United States of America*, 78(9):5406-5410.

251. Epand, R.M.; Nir, S.; Parolin, M.; Flanagan, T.D. (1995). The Role of the Gangliosides GD1a as a Receptor for Sendai Virus. *Biochemistry*, 34:1084-1089.

252. Taube, S.; Jiang, M.; Wobus, C.E. (2010). Glycosphingolipids as Receptors for Non-Enveloped Viruses. *Viruses*, 2:1011-1049.

253. Martínez, M.A.; López, S.; Arias, C.F.; Isa, P. (2013). Gangliosides Have a Functional Role during Rotavirus Cell Entry. *Journal of Virology*, 87(2): 1115-1122.

254. Lewis, A.L. Lewis, W.G. (2012). Host sialoglycans and bacterial sialidases: a mucosal perspective. *Cellular Microbiology*, (14)8: 1174-1182.

255. Angata, T.; Varki, A.(2002). Chemical Diversity in the Sialic Acids and Related α-Keto Acids: An Evolutionary Perspective. *Chemical Reviews*, 102:439-469.

256. Holmgren, J. (1973). Comparison of the Tissue Receptors for Vibrio cholerae and *Escherichia coli* Enterotoxins by Means of Gangliosides and Natural Cholera Toxoid. *Infection and Immunity*, 8(6):851-859.

257. Fukuta, S.; Magnani, J.L.; Twiddy, E.M.; Holmes, R.K.; Ginsburg, V. (1988). Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. *Infection and Immunity*, 56(7):1748-1753.

258. Wolf, A.A.; Fujinaga, Y.; Lencer, W.I. (2002). Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *Journal of Biological Chemistry*, 277(18):16249-16256.

259. Moss, J.; Fishman, P.H.; Manganiello, V.C.; Vaughan, M.; Brady, R.O. (1976). Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. *The Proceedings of the National Academy of Sciences of the United States of America*,73(4):1034-1037.

260. Karlsson, K.A. (1995). Microbial recognition of target-cell glycoconjugates. *Current Opinion in Structural Biology*, 5(5):622-635.

261. Teneberg, S.; Hirst, T.R.; Angström, J.; Karlsson, K.A. (1994). Comparison of the glycolipid-binding specificities of cholera toxin and porcine *Escherichia coli* heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine. *Glycoconjugate Journal*, 11(6):533-540.

262. Middlebrook, J.L.; Dorland, R.B. Bacterial toxins: cellular mechanisms of action. *Microbiology Reviews*, 48(3):199-221.

263. Craig, S. W.; Cuatrecasas, P.(1975) Mobility of cholera toxin receptors on rat lymphocyte membranes. *The Proceedings of the National Academy of Sciences of the United States of America*, 72:3844-3848.

264. Hagmann, J., Fishman, P.H. (1981). Inhibitors of protein synthesis block action of cholera toxin. *Biochemical and Biophysical Research Communications*, 98:677-684.

265. Otnaess, A.B.; Laegreid, A.; Ertresvåg, K. (1983). Inhibition of enterotoxin from Escherichia coli and Vibrio cholerae by gangliosides from human milk. *Infection and Immunology*, 40(2): 563-569.

266. Angström, J.; Teneberg, S.; Karlsson, K.A. (1994). Delineation and comparison of ganglioside-binding epitopes for the toxins of *Vibrio cholerae*, *Escherichia coli*, and *Clostridium tetani*: evidence for overlapping epitopes. *The Proceedings of the National Academy of Sciences of the United States of America*, 91(25):11859-11863.

267. Singh, A.K.; Harrison, S.H.; Schoeniger, J.S. (2000). Gangliosides as receptors for biological toxins: development of sensitive fluoroimmunoassays using ganglioside-bearing liposomes. *Analytical Chemistry*, 72(24):6019-6024.

268. Idota, T.; Kawakami, H. (1995). Inhibitory effects of milk gangliosides on the adhesion of *Escherichia coli* to human intestinal carcinoma cells. *Bioscience, Biotechnology, and Biochemistry*, 59(1):69-72.

269. Sánchez-Juanes, F.; Alonso, J.M.; Zancada, L.; Hueso, P. (2009). Glycosphingolipids from bovine milk and milk fat globule membranes: a comparative study. Adhesion to enterotoxigenic *Escherichia coli* strains. *Biological Chemistry*, 390(1):31-40.

270. Ono, E.; Abe, K.; Nakazawa, M.; Naiki, M. (1989). Ganglioside epitope recognized by K99 fimbriae from enterotoxigenic *Escherichia coli*. *Infection and Immunology*, 57(3):907-911.

271. Salcedo, J.; Barbera, R.; Matencio, E.; Alegría, A.; Lagarda, M.J. (2013). Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: an in vitro study. *Food Chemistry*, 136(2):726-734.

272. Marshall, B.J.; Warren, J.R. (1984). Undentified cured bacilli in the stomach of patients with gastritis and peptic ulceration. *The Lancet, i*:1311-1315.

273. Gomes, B.C.; De Martinis, E.C.P. (2004). The significance of *Helicobacter pylori* in water, food and environmental samples. *Food Control,* 15: 397-403

274. Goodwin, C.S.; Armstrong, J. A.; Chilvers, T.; Peters, M.; Collins, D.V.; Sly, L.; Mc Connell, W and Harper W.E.S. (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. Nov. and *Helicobacter mustelae* comb. Nov., Respectively. *International Journal of Systematic Bacteriology* 39(4): 397-405

275. Goodwin, C.S. (1994). How *Helicobacter pylori* acquired its name and how it overcomes gastric defence mechanisms. *Journal of Gastroenterology and Hepatology*, 9: S1-S3.

276. Velazquez, M.; Feirtag J.M. (1999) *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. *International Journal of Food Microbiology,* 53: 95–104.

277. Go, M.F. (2002). Review article: natural history and epidemiology of *Helicobacter pylori* infection. *Alimentary Pharmacology & Therapeutics,* 16(Suppl. 1): 3-15.

278. Ahmed, K.S.; Khan, A.A.; Ahmed, I.; Tiwari, S.K.; Habeeb, A.; Ahi, J.D.; Abid, Z.; Ahmed, N.; Habibullah, C.M. (2007). Impact of household hygiene and water source on the prevalence and transmission of *Helicobacter pylori*: a South Indian perspective. *Singapore Medical Journal*, 48(6): 543-549.

279. Dunn, B.E.; Cohen, H.; Blaser M.J. (1997). *Helicobacter pylori*. *Clinical Microbiology Reviews*, 10(4):720-741.

280. Perez-Perez G.I., Rothenbacher D. and Brenner H. (2004). Epidemiology of *Helicobacter pylori* Infection. *Helicobacter*, 9 (Suppl. 1):1–6.

281. Parsonnet, J.(1995). The incidence of *Helicobacter pylori* infection. *Alimentary Pharmacology & Therapeutics,* 9(suppl2):45-51.

282. Everhart J. E. (2000). Recent Development in the epidemiology of *Helicobacter pylori*. *Gastroenterology Clinics of North America*, 29(3): 559-578.

283. Strebel, K.; Rolle-Kampczy, U.; Richter, M.; Kindler, A.; Richter, T.; Schlink, U. (2010). Arigorous small area modelling-study for the *Helicobacer pylori* epidemiology. *Science of the Total Epidemiology*, 408: 3931-3942.

284. World Gastroenterology Organisation Global Guidelines (2010). *Helicobacter pylori* in developing countries. Available in: http://www.worldgastroenterology.org/assets/downloads/en/pdf/guidelines/11_helicobacter_pylori_developing_countries_en.pdf. Consulted: November 2011.

285. Reyes, V.E.; Suárez, G.; Sierra, J.C.; Beswick, E. J. (2009). Chapter 50 *Helicobacter pylori*. from Barrett, A.DT. and Stanberry, L.R. *Vaccines for Biodefense and Emerging and Neglected Diseases.* Elsevier, 983-1012.

286. Bardhan, P.K.(1997). Epidemiological features of *Helicobacter pylori* infection in developing countries. *Clinical Infectious Diseases*, 25 (5):973 – 978.

287. Malaty, H.M. (2007). Epidemiology of *Helicobacter pylori* infection. *Best Practice & Research Clinical Gastroenterology*, 21(2): 205-214.

288. Dowsett, S.A.; Archila, L.; Segreto, V.A.; Gonzalez, C.R.; Silva, A.; Vastola, K.A.; Bartizek, R.D.; Kowolik, M.J. (1999). *Helicobacter pylori* infection in indigenous families of Central America: serostatus and oral and fingernail carriage. *Journal of Clinical Microbiology*, 37(8): 2456 – 2460.

289. Fock KM and Ang TL. (2010). Epidemiology of *Helicobacter pylori* infection and gastric cancer in Asia. *Journal of Gastroenterology and Hepatology*, 25: 479–486.

290. Bani-Hani, K.E.; Nawaf, J.S.; Qaderi, S.E.; Khader, Y.S.; Bani-Hani, B.K. (2006). Prevalence and risk factors of *Helicobacter pylori* infection in healthy schoolchildren. Chinese *Journal of Digestive Diseases*,7:55–60.

291. Bhuiyan, T.R.; Qadri, F.; Saha, A.; Svennerholm, A.M. (2009) Infection by *Helicobacter pylori* in Bangladeshi children from birth to two years, relation to blood group, nutritional status, and seasonality. *Pediatric Infectious Disease Journal*, 28:79–85.

292. Glynn, M.K.; Friedman, C.R.; Gold, B.D.; Gold, B.; Khanna, L.; Hutwagner, N.; Iihoshi, C.; Revollo, R. (2002). Seroincidence of *Helicobacter pylori* infection in a cohort of rural Bolivian children: acquisition and analysis of possible risk factors. *Clinical Infectious Diseases*. 35:1059–1065.

293. Axon, A. (2006). *Helicobacter pylori* What Do We Still Need to Know? Alimentary tract: Clinical review. *Journal of Clinical Gastroenterology*, 40(1): 1-5.

294. Morris-Brown, L. (2000). *Helicobacter pylori*: Epidemiology and Routes of Transmission. *Epidemiology Reviews*, 22(2): 283-297.

295. O'Rourke, K.; Goodman, K.J.; Grazioplene, M.; Redlinger, T.; Day, R.S. (2003). Determinants of Geographic Variation in *Helicobacter pylori* Infection among Children on the US-Mexico Border. *American Journal of Epidemiology*, 158: 816–824.

296. Malaty, H.M.; Paykov, V.; Bykova, O.; Ross, A.; Graham, P.; Anneger, J.F.; Graham, D.Y.(1996). *Helicobacter pylori* and Socieconomic Factors in Russia. *Helicobacter*. 1(2): 82-87.

297. Bastos, J.; Peleteiro, B.; Barros, R.; Alves, L.; Severo, M.; de Fátima Pina M.; Pinto, H.; Carvalho, S.; Marinho, A.; Guimarães, J.T.; Azevedo, A.; La Vecchia, C.; Barros, H.; Lunet, N. (2013). Sociodemographic determinants of prevalence and incidence of *Helicobacter pylori* infection in Portuguese adults. *Helicobacter*. 18(6): 413-422.

298. Rothenbacher, D.; Bode, G.; Berg, G.; Gommel, R.; Gonser, T.; Adlerb, G.; Brenner H. (1998). Prevalence and determinants of *Helicobacter pylori* infection in preschool children: a population-based study from Germany. *International Journal of Epidemiology*, 27:135-141.

299. Pan, K.; Tanaka, C.; Inagaki, M.; Ryuichi Higuchi, R.; Miyamoto, T. (2012). Isolation and Structure Elucidation of GM4-Type Gangliosides from the Okinawan Starfish Protoreaster nodosus. *Marine Drugs*, 10: 2467-2480.

300. Jafar, S.; Jalil, A.; Soheila, N.; Sirous, S. (2013). Prevalence of *Helicobacter pylori* infection in children, a population-based cross-sectional study in west Iran. *Iran Journal of Pediatrics*, 23(1):13-18.

301. Thomas, J.E.; Dale, A.; Harding, M.; Coward, W.A.; Cole, T.J.; Weaver, L.T. (1999). *Helicobacter pylori* colonization in early life. *Pediatric Research*, 45:218-223.

302. Duque, X.; Vilchis, J.; Mera, R.; Trejo-Valdivia, B.; Goodman, K.J.; Mendoza, M.E.; Navarro, F.; Roque, V.; Moran, S.; Torres, J.; Correa, P. (2012). Natural history of *Helicobacter pylori* infection in Mexican schoolchildren: incidence and spontaneous clearance. *Journal of Pediatric Gastroenterology and Nutrition*, 55(2):209-216.

303. Mitchell, H.M.; Li, Y.Y.; Hu, P.J.; Liu, Q.; Chen, Du G.G.; Wang, Z.J.; Lee, A.; Hazell, S.L.(1992). Epidemiology of *Helicobacter pylori* in Southern China: Identification of Eary Childhood as the Critical Period for Acquisition. *The Journal of Infectious Diseases*, 166: 149-153.

304. Zaterka, S.; Eisig, J.N.; Chinzon, D.; Rothstein, W. (2007). Factors related to *Helicobacter pylori* prevalence in an adult population in Brazil. *Helicobacter*.12: 82–88.

305. Staat, M.A.; Kruszon-Moran, D.; McQuillan, G.M.; Kaslow, R.A. (1996). A Population-Based Serologic Survey of *Helicobacter pylor*i Infection in Children and Adolescents in the United States . *The Journal of Infectious Diseases*, 174: 1120-1123

306. Fiedorek, S.C.; Malaty, H.M.; Evans, D.L.; Pumphrey, C.L.; Casteel, H.B.; Evans D.J. Jr; Graham, D.Y. (1991). Factors Influencing the Epidemiology of *Helicobacter pylori*. *Infection in Children Pediatrics,* 88: 578-582.

307. Ford, A.C.; Axon, A.T.R.(2010). Epidemiology of *Helicobacter pylori* infection and Public Health Implications. *Helicobacter,* 15 (Suppl. 1):1–6.

308. Vale, F.F.; Vítor, J.M.B. (2010). Transmission pathway of *Helicobacter pylori*: Does food play a role in rural and urban areas? *International Journal of Food Microbiology*, 138: 1–12.

309. Schwarz, S.; Morelli, G.; Kusecek, B.; Manica, A.; Balloux, F.; Owen, R.J.; Graham, D.Y.; van der Merwe, S.; Achtman, M.; Suerbaum, S. (2008). Horizontal versus Familial Transmission of *Helicobacter pylori*. *PLoS Pathogens,* 4(10):1-10.

310. Azevedo, N.F.; Huntington, J.; Goodman, K.J. (2009). The Epidemiology of *Helicobacter pylori* and Public Health Implications. *Helicobacter,* 14 (Suppl. 1): 1–7.

311. Goodman, KJ; Correa, P. (2000). Transmission of *Helicobacter pylori* among siblings. *The Lancet*, 355:358–362.

312. The, B.H.; Lin, J.T.; Pan, W.H. (1994) Seroprevalence and associated risk factors of *Helicobacter pylori* infection in Taiwan. *Anticancer Research,* 14: 1389–1392.

313. Fialho, A.M.N.; Braga, A.B.C.; Neto, M.B.B.; Carneiro, J.G.; Rocha, A.M.C.; Rodrigues, M.N.; Queiroz, D.M.M; Braga, L.L.B.C. (2010). Younger Siblings Play a Major Role in *Helicobacter pylori* Transmission Among Children From a Low-Income Community in the Northeast of Brazil. *Helicobacter,* 15: 491–496.

314. Urita, Y.; Watanabe, T.; Kawagoe, N.; Takemoto, I.; Tanaka, H.; Kijima, S.; Kido, H.; Maeda, T.; Sugasawa, Y.; Miyazaki, T.; Honda, Y.; Nakanishi, K.; Shimada, N.; Nakajima, H.; Sugimoto, M.; Urita, C. (2013). Role of infected grandmothers in transmission of *Helicobacter pylori* to children in a Japanese rural town. *Journal of Paediatrics and Child Health*, 49(5):394-398.

315. Malaty, H.; Engstrand, L.L.; Pedersen, N.; Graham Y.D. (1994). *Helicobacter pylori* Infection: Genetic and Environmental Influences. *American College of Physicians*, 120:982-986.

316. Deepika, B.; Rosamma, J.; Bali, A. (2010). The Association Of Dental Plaque And *Helicobacter Pylori* Infection In Dyspeptic Patients Undergoing Endoscopy. *Journal of Clinical and Diagnostic Research,* (4): 3614-3621.

317. Lehours, P.; Yilmaz, O. (2007). Epidemiology of *Helicobacter pylori* infection. *Helicobacter*, 12(suppl. 1):1-3.

318. Perry, S.; Sanchez, M.L.; Yang, S.; Haggerty, T.D.; Hurst, P.; Perez-Perez, G.; Parsonnet, J. (2006).Gastroenteritis and Transmission of *Helicobacter pylori* Infection in Households. *Emerging Infectious Diseases*, 12(11):1701-1708.

319. Namavar, E.; Roosendaal, R.E.I.; Kuipers, E.J.; Groot, P.; van der Bij, I.M.W., Pefia, A.S.; Graaff, J. (1995). Presence of *Helicobacter pylori* in the Oral Cavity, Oesophagus, Stomach and Faeces of Patients with Gastritis. *European Journal of Clinical Microbiology & Infectious Diseases*, 14:234- 237.

320. Mapstone N.P., Lynch, Lewis F.A., Axon A.T.R., Tomkins D.S., Dixon M.F. and Quirke P. (1993). PCR identification of *Helicobacter pylori* in faeces from gastritis patients. *The Lancet,* 341:447.

321. Momtaz, H.; Souod, N.; Dabiri, H.; Sarshar, M. (2012). Study of *Helicobacter pylori* genotype status in saliva, dental plaques, stool and gastric biopsy samples. *World Journal Gastroenteroly*, 18(17): 2105-2111.

322. Moreno, Y.; Ferrús, M.A. (2012). Specific detection of cultivable *Helicobacter pylori* cells from wastewater treatment plants. *Helicobacter*, 17(5):327-332.

323. Frenck, R.Jr.; Clemens, J. (2003). *Helicobacter* in the developing world. Editions scientifiques et médicales. *Microbes and Infection*, 5:705–713.

324. Grubel, P.; Hoffman, J.S.; Chong, F.K.; Burstein, N.A.; Mepani, C.A.; Cave, D.R. (1997). Vector potential of houseflies (Musca domestica) for *Helicobacter pylori*. *Journal of Clinical Microbiology*, 35: 1300–1303.

325. Ramirez-Ramos, A.; Sanchez-Sanchez. (2009). *Helicobacter pylori* 25 años despues (1983-2008): Epidemiologia, Microbiologia, Patogenia y Diagnostico y Tratamiento. *Reviews Gastroenterology*, 29(2): 158-170.

326. Grubel, P.; Cave, D.R. (1998). Sanitation and houseflies (Musca domestica): factors of the transmission of *Helicobacter pylori*. *Bulletin de l'Institute Pasteur*, 96: 83-91.

327. Hulten, K.; Han, S.W.; Enroth, H.; Klein, P.D.; Opekun, A.R.; Gilman, R.H.; Evans, G.; Engstrand, L.; Graham, D.Y.; El-Zaatari, F.A.K. (1996). *Helicobacter pylori* in the drinking water in Peru. *Gastroenterology*, 110: 1031-1035

328. Bruce, M.G.; Maaroos, H.I. (2008). Epidemiology of *Helicobacter pylori* Infections No claim to original works. *Helicobacter,* 13 (Suppl. 1): 1–6.

329. van Duynhoven Y., T.H.P.; de Jonge, R. (2001). Transmission of *Helicobacter pylori*: a role for food? *Bulletin of the World Health Organization*, 79(5): 455-460.

330. Akamatsu, T.; Tabata, K.; Hironga, M.; Kawakami, H.; Yyeda, M. (1996) Transmission of *Helicobacter pylori* infection via flexible fiberoptic endoscopy. *American Journal of Infection Control*, 24 (5):396-401.

331. Ricci, C. (2007). Diagnosis of *Helicobacter pylori*: Invasive and non-invasive tests. *Best Practice & Research Clinical Gastroenterology*, 21(2): 299-313.

332. Granstrom, M.; Lehours, P.; Bengtsson, C.; Mégraud, F. (2008). Diagnosis of *Helicobacter pylori*. *Helicobacter,* 13 (Suppl. 1):7–12.

333. Ndip, R.N.; MacKay, W.G.; Farthing, M.J.G.; Weaver, L.T. (2003). Culturing *Helicobacter pylori* from Clinical Specimens: Review of Microbiologic Methods. *Journal of Pediatric Gastroenterology and Nutrition*, 36:616–622.

334. Guarner, J.; Kalach, N.; Elitsur, Y.; Koletzko, S. (2010). *Helicobacter pylori* diagnostic tests in children: review of the literature from 1999 to 2009. *European Journal of Pediatrics*, 169:15–25.

335. Burucoa, C. (2010). Diagnostic de l'infection à *Helicobacter pylori* chez l'enfant. *Archives de Pédiatrie*; 17:814-815.

336. Hirschl, A.M.; Makristathis, A. (2007). Methods to Detect *Helicobacter pylori*: From Culture to Molecular Biology. *Helicobacter,* 12 (Suppl. 2): 6–11.

337. Makristathis, A.; Barousch, W.; Pasching, E. (2000). Two enzyme immunoassays and PCR for detection of *Helicobacter pylori* in stool specimens from pediatric patients before and after eradication therapy. *Journal of Clinical of Microbiology*, 38:3710–3714.

338. Megraud, F.; Lehours, P. (2007). *Helicobacter pylori* Detection and Antimicrobial Susceptibility Testing. *Clinical Microbiology Reviews,* 20(2):280–322.

339. Calvet, X.; Lehours, P.; Lario, S.; Megraud, F. (2010). Diagnosis of *Helicobacter pylori* Infection. *Helicobacter*, 15(Suppl. 1): 7–13.

340. Nayak, A.K.; Rose, J.B. (2007). Detection of *Helicobacter pylori* in sewage and water using a new quantitative PCR method with SYBR green. *Journal of Applied Microbiology*, 103:1931–1941.

341. Mahmood, S. Hamid, A. (2010). Comparision between invasive and no invasive test in diagnosis of *Helicobacter pylori* infection. *Pakistan Journal of Biological Sciences*, 13 (10): 509-512.

342. Jiang, J.H.; De-Zhong, X.; Yan, Y.P.; Men, K.; Shao, Z.J. (2007). Diagnosis of *Helicobacter pylori* infection: A meta-analysis. *Journal of Medical College*, 22(4): 246-249.

343. Zuñiga-Noriega, J.R.; Bosques-Padilla, F.J.; Perez-Perez, G.I.; Tijerina-Menchaca, R.; Flores-Gutierrez, J.P.; Maldonado-Garza, H.J.; Garza-Gonzalez, E. (2006). Diagnostic Utility of Invasive Tests and Serology for the Diagnosis of *Helicobacter pylori* Infection in Different Clinical Presentations. *Archives of Medical Research,* 37:123–128.

344. Razaghi, M.; Boutorabi, S.M.; Mirjalili, A.; Norolahi, S.; Hashemj, M.; Jalalian, M. (2010). Diagnosis of *Helicobacter pylori* infection by ELISA stool antigen and comparison with the other diagnostic methods. *HealthMED*, 4 (3): 545-551.

345. Iranikhah, A.; Ghadir, M.R.; Sarkeshikian, S.; Saneian, H.; Heiari, A.; Mahvari, M. (2013). Stool antigen tests for the detection of *Helicobacter pylori* in children. *Iranian Journal of Pediatrics*, 23(2):138-142.

346. Queiroz, D.M.; Saito, M.; Rocha, G.A.; Rocha, A.M.; Melo, F.F.; Checkley, W.; Braga, L.L.; Silva, I.S.; Gilman, R.H.; Crabtree, J.E. (2013). *Helicobacter pylori* infection in

infants and toddlers in South America: concordance between [13C] urea breath test and monoclonal *H. pylori* stool antigen test. *Journal of Clinical Microbiology*, 51(11): 3735-3740.

347. Braden B. (2009). Methods and functions: Breath tests. *Best Practice & Research Clinical Gastroenterology*, 23:337-352.

348. Dominguez-Munoz, J.E.; Leodolter, A.; Sauerbruch, T.; Malfertheiner, P. (1997). A citric acid solution is an optimal test drink in the13C-urea breath test for the diagnosis of *Helicobacter pylori* infection. *Gut*, 40: 459-462.

349. Gomollon, F.; Ducons, J.A.; Santolaria, S.; Lera Omiste, I.; Guirao, F.; Ferrero, M. Montoro, M. (2003). Breath test is very reliable for diagnosis of *Helicobacter pylori* infection in real clinical practice. *Digestive and Liver Disease*, 35: 612-618.

350. Di Rienzo, T.A.; D'Angelo, G.; Ojetti, V.; Campanale, M.C.; Tortora, A.; Cesario, V.; Zuccalà, G.; Franceschi, F. (2013). 13C-Urea breath test for the diagnosis of *Helicobacter pylori* infection. *European Review for Medical and Pharmacological Sciences*, 17 Suppl 2:51-58.

351. Kusters, J.G.; van Vliet, A.H.M.; Kuipers, E.J. (2006). Pathogenesis of *Helicobacter pylori* Infection. *Clinical Microbiology Reviews*, 19(3): 449-490.

352. Solnick, J.V.; Vandamme, P. (2001). From Mobley Harry LT *Helicobacter pylori*. ASM Press. Washington. Pp 39-51.

353. Bode, G.; Mauch, F.; Malfertheiner, P. (1993). The coccoid forms of *Helicobacter pylori*. Criteria for their viability. *Epidemiology Infection,* 111: 483-490.

354. Couturier, M.R.; Stein, M. (2008). *Helicobacter pylori* produces unique filaments upon host contact in vitro. *Canadian Journal of Microbiology,* 54: 537-548.

355. Ottemann K.M. and Lowenthal A.C. (2002). *Helicobacter pylori* uses motility for initial colonisation and to attain robust infection. *Infection and Immunity,* 70(4): 1984-1990.

356. Kostrzynska, M.; Betts, J.D.; Austin, J.W.; Trust, T.J. (1991). Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. *Journal of Bacteriology*, 173(3): 937-946.

357. Blaser, M.J. (1993). *Helicobacter pylori*: microbiology of a slow bacterial infection. *Trends in Microbiology,* 1(7): 255-270.

358. Geis, G.; Leying, H.; Suerbaum, S.; Mai, U.; Opferkuch, W.(1989) Ultrastructure and chemical analysis of *Campylobacter pylori* flagella. *Journal of Clinical Microbiology,* 27(3): 436-441.

359. Hill, M. (1997). The microbiology of *Helicobacter pylori*. *Biomedicine & Pharmacotherapy*, 51:151-153.

360. Meyer-Rosberg, K.; Scott, D.R.; Rex, D.; Melchers, K.; Sachs, G. (1996). The Effect of Environment pH on the Proton Motive Force of *Helicobacter pylori*. *Gastroenterology,* 111: 886-900.

361. Albertson, N.; Wenngren, I.; Sjostrom, J.E. (1998). Growth and survival of *Helicobacter pylori* in difined medium and susceptibility to Brij 78. *Journal of Clinical Microbiology*, 36(5): 1232-1235.

362. Reynolds, D.J.; Penn, C.W. (1994). Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirement. *Microbiology*, 140:2649-2656.

363. Hachem, C.Y.; Clarridge, J.E.; Evans, D.G.; Graham, D.Y. (1995). Comparison of agar based media form primary isolation of *Helicobacter pylori*. *Journal of Clinical Pathology*, 48: 714-716.

364. Davood, E.; Mobarez, A.M; Ali Hatef, S.; Hosseini, A. Z. (2009). Optimization of *Helicobacter pylori* culture in order to prepare favourable antigens. *Journal of Bacteriology Research,* 1(9): 101-104.

365. Miendje, Deyia, V.Y.; Van den Borrea, C.; Fontaine, V. (2010). Comparative evaluation of 3 selective media for primary isolation of *Helicobacter pylori* from gastric biopsies under routine conditions. *Diagnostic Microbiology and Infectious Disease* 68: 474–476.

366. Andersen and Wadstrom (2001). Basic Bacteriology and culture. From Mobley Harry LT *Helicobacter pylori*. ASM Press. Washington. 27-38.

367. Sherburne, R., Taylor, D.E. (1995). *Helicobacter pylori* expresses a complex surface carbohydrate, Lewis X. *Infection and Immunity*, 63, 4564–4568.

368. Eaton, KA.; Logan, SM.; Baker, P.E.; Peterson, R.A.; Monteiro, M.A.; Altman, E. (2004). *Helicobacter pylori* with a Truncated Lipopolysaccharide O Chain Fails To Induce Gastritis in SCID Mice Injected with Splenocytes from Wild-Type C57BL/6J Mice. *Infection and Immunity,* 22(7): 3925-3931.

369. Monteiro, M. A.; Michael, F.St.; Rasko, D. A.; Taylor, D.E; Conlan, J.W. K. H. Chan, K.H.; Logan, S.M.; Appelmelk, B.J.; Perry, M.B. (2001). *Helicobacter pylori* from asymptomatic hosts expressing heptoglycan but lacking Lewis O-chains: Lewis blood-group O-chains may play a role in *Helicobacter pylori* induced pathology. *Biochemistry and Cell Biology*, 79:449–459.

370. Sheu, S.M.; Sheu, B.S.; Yang, H.B.; Lei, H.Y. (2007). Anti-Lewis WUJJ. X antibody promotes *Helicobacter pylori* adhesion to gastric epithelial cells. *Infection and Immunology*, 75: 2661–2667.

371. Cover, L.T.; Blaser, J.M. (2009). *Helicobacter pylori* in Health and Disease. *Gastroenterology,* 136: 1863–1873.

372. Andersen, L.P. (2007) Colonization and Infection by *Helicobacter pylori* in Humans. *Helicobacter*, 12 (2): 12–15.

373. Josenhans, C.; Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *International Journal Medical Microbiology*. 291, 605-614.

374. Yoshiyama, H.; Nakazawa, T. (2000). Unique mechanism of *Helicobacter pylori* for colonizing the gastric mucus. *Microbes and Infection* 2: 55−60.

375. Eaton, K.A.; Morgan, D.R.; Krakowka, S. (1989). *Campylobacter pylori* virulence factors in gnotobiotic piglets. *Infection and Immunology*, 57: 1119–1125.

376. Clyne, M.; Drumm, B. (1996). Envelope characteristics of *Helicobacter pylori*: their role in adherence to mucosal surfaces and virulence. *FEMS Immunology and Medical Microbiology* 16: 141-155.

377. Spohn, G.; Scarlato, V. (2001). Chapter 21 Motility, Chemotaxis and Flagella from Mobley H.LT., Mendz G.L. and Hazell S.L. *Helicobacter pylori*: physiology and Genetics ASM Press.USA, pp 239-257.

378. Eaton, K.A.; Suerbaum, S.; Josenhans, C.; Krakowka, S. (1996). Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes. *Infection and Immunology*, 64: 2445–2448.

379. O'Toole, P.W.; Lane, M.C; Porwollik, S. (2000). *Helicobacter pylori* motility. *Microbes and Infection* 2:1207-1214.

380. Geis G., et al.(1993). Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. *Journal of Medical Microbiology*. 38:371–377.

381. Lowenthal, A.C.; Hill, M.; Sycuro, L.K,; Mehmood, K.; Salama, N.R.; Ottemann, K.M. (2009). Functional Analysis of the *Helicobacter pylori* Flagellar Switch Proteins. *Journal of Bacteriology*, 191(23): 7147–7156.

382. Terry, K.; Williams, S.M.; Connolly, L.; Ottemann, K.M. (2005). Chemotaxis Plays Multiple Roles during *Helicobacter pylori* Animal Infection. *Infection and Immunity*, 73 (2): 803–811.

383. Radin, J.N.; Gaddy, J.A.; González-Rivera, C.; Loh, J.T.; Algood, H.M.; Cover, T.L. (2013). Flagellar localization of a *Helicobacter pylori* autotransporter protein. *MBio*. 4(2):1-10.

384. Suerbaum, S.; Michetti, P. (2002). *Helicobacter pylori* infection. *The New England Journal of Medicine,* 347(15): 1175-1186.

385. Amieva, R.M.; El–Omar, M.E. (2008). Host-Bacterial Interactions in *Helicobacter pylori* Infection. *Gastroenterology,* 134:306–323.

386. Kobayashi, M.; Heesob, L.; Nakayama, J.; Fukuda, M. Roles of gastric mucin-type O-glycans in the pathogenesis of *Helicobacter pylori* infection. *Glycobiology,* 19(5): 453-461.

387. Tanih, F.N.; Ndip, M.L.; Clarke, M.A.; Ndip, N.R. (2010). An overview of pathogenesis and epidemiology of *Helicobacter pylori* infection. *Journal of Microbiology Research*, 4(6): 426-436.

388. Stingl, K.; Altendorf, K.; Bakker; P.E. (2002). Acid survival of *Helicobacter pylori*: how does urease activity trigger cytoplasmic pH homeostasis. *Trends in Microbiology,* 10(2): 70-74.

389. Follmer, C. (2010). Ureases as a target for the treatment of gastric and urinary infections. *Journal of Clinical Pathology*, 63: 424-430.

390. Mizote, T.; Yoshiyama, H.; Nakazawa, T. (1997). Urease-Independent Chemotactic Responses of *Helicobacter pylori* to Urea, Urease Inhibitors, and Sodium Bicarbonate. *Infection and Immunity*, 65(4): 1519-1521.

391. Israel, A.D.; Peek, M.R.(2001). Review article: pathogenesis of *Helicobacter pylori*-induced gastric inflammation. *Alimentary Pharmacology & Therapeutics,* 15: 1271-1290.

392. Skouloubris S., Thiberge J.M., Labigne A., DeReuse H. (1998). The *Helicobacter pylori* UreI protein is not involved in urease activity but is essential for bacterial survival in vivo. *Infection and Immunology*, 66:4517–4521.

393. Dunn, B.E.; Phadnis, S.H.; Structure, function and localization of *Helicobacter pylori* urease. *Yale Journal of Biology and Medicine*, 71(2): 63-73.

394. Scott, R. D.; Marcus, A. E.; Weeks, L.D.; Sachs, G. (2002). Mechanisms of Acid Resistance Due to the Urease System of *Helicobacter pylori*. *Gastroenterology*, 123: 187–195.

395. Mobley, H.L.; Garner, R.M.; Bauerfeind, P. (1995). *Helicobacter pylori* nickel-transport gene nixA: synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. *Molecular Microbiology*, 16(1): 97-109.

396. Mobley, H.L.; Island, M.D.; Hausinger, R.P. (1995). Molecular biology of microbial ureases. *Microbiology Reviews*, 59:451–480.

397. Suzuki, M.; Miura, S.; Sofematsu, M.; Fukumura, D.; Kurose, I.; Suzuki, H.; Kai, A.; Kudoh, Y.; Ohashi, M.; Tanchiya, M. (1992). *Helicobacter pylori* associated ammonia production enhances neutrophil-dependent gastric mucosal cell injury. *American Journal of Physiology*, 263:G719–G725.

398. Spiegelhalder, C.; Gerstenecker, B.; Kersten, A.; Schiltz, E.; Kist, M. (1993) Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. *Infection and Immunology*, 61: 5315-5325.

399. Wang, G.; Alamuri, P.; Maier, R.J. (2006). The diverse antioxidant systems of *Helicobacter pylori*. *Molecular Microbiology,* 61(4): 847-860.

400. Slomiany, B.L.; Bilski, J.; Sarosiek, J.; Murty, V.L.; Dworkin, B.; Van Horn, K.; Zielenski, J.; Slomiany, A. (1987). *Campylobacter pyloridi*s degrades mucin and undermines gastric mucosal integrity. *Biochemical and Biophysical Research Communications*, 144:307-314.

401. Pieters, R.J. (2007). Intervention with Bacterial Adhesion by Multivalent Carbohydrates. *Medicinal Research Reviews*, 27 (6): 796-816.

402. Magalhães, A.; Reis, C.A. (2010). *Helicobacter pylori* adhesion to gastric epithelial cells is mediated by glycan receptors. *Brazilian Journal of Medical and Biological Research*, 43: 611-618.

403. Ilver, D.; Arnqvist, A.; Ogren, J.; Frick, I.M.; Kersulyte, D.; Incecik, E.T.; Berg, D. E.; Covacci, A.; Engstrand, L.; Boren, T. (1998). *Helicobacter pylori* Adhesin Binding Fucosylated Histo-Blood Group Antigens Revealed by Retagging. *Science*, 279: 373-377.

404. Yamaoka, Y. (2008). Roles of *Helicobacter pylori* BabA in gastroduodenal pathogenesis. *World Journal of Gastroenterology*, 14(27): 4265-4272.

405. Sheu, B.S.; Yang, H.B.; Yeh, Y.C.; Wu, J.J. (2010). *Helicobacter pylori* colonization of the human gastric epithelium: A bug's first step is a novel target for us. *Journal of Gastroenterology and Hepatology*, 25: 26–32.

406. Kufe, D.W. (2009) Mucins in cancer: function, prognosis and therapy. *Nature Reviews*, 9: 874-885.

407. De Bolos, C.; Garrido, M.; Real, F.X. (1995). MUC6 Apomucin Shows a Distinct Normal Tissue Distribution That Correlates With Lewis Antigen Expression in the Human Stomach. *Gastroenterology*, 109: 723-734.

408. Rad, R.; Gerhard, M.; Lang, R.; Schöniger, M.; Rösch, T.; Schepp, W.; Becker, I.; Wagner, H.; Prinz, C. (2002). The *Helicobacter pylori* Blond group antigen-binding adhesion facilities bacterial colonization and augments a nonspecific immune response. *The Journal Immunology*, 168: 3033-3041.

409. Con, S.A.; Takeuchi, H.; Nishioka, M.; Morimoto, N.; Sugiura, T.; Yasuda, N.; Con-Wong, R. (2010). Clinical relevance of *Helicobacter pylori* babA2 and babA2/B in Costa Rica and Japan. *World Journal Gastroenterology*, 16(4): 474-478.

410. Israel, D.A.; Peek, R.M.Jr. (2006). The role of persistence in *Helicobacter pylori* pathogenesis. *Gastroenterology*, 22: 3–7.

411. Mahdavi, J.; Sondén, B.; Hurtig, M.; Olfat, F.O.; Forsberg, L.; Roche, N.; Ångström, J.; Larsson, T.; Teneberg, S.; Karlsson, K.A.; Altraja, S.; Wadström, T.; Kersulyte, D.; Berg, D.E.; Dubois, A.; Petersson, C.; Magnusson, K.E.; Norberg, T.; Lindh, F.; Lundskog, B.B.; Arnqvist, A.; Hammarström, L.; Borén, T. (2002). *Helicobacter pylori* SabA Adhesin in Persistent Infection and Chronic Inflammation. *Science*, 297(5581): 573–578.

412. Yamaoka, Y. (2008). Increasing evidence of the role of *Helicobacter pylori* SabA in the pathogenesis of gastroduodenal disease. *Journal of Infection in Developing Countries*, 2(3): 174–181.

413. Wen, S.; Moss, S.F. (2009). *Helicobacter pylori* virulence factors in gastric carcinogenesis. *Cancer Letters*, 282: 1–8.

414. Unemo, M.; Aspholm-Hurtig, M.; Ilver, D.; Bergstrom, J.; Boren, T.; Danielsson, D.; Teneberg, S. (2005). The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. *The journal of biological chemistry*, 280(15): 15390–15397.

415. Teneberg, S. (2009). The Multiple Carbohydrate binding specificities of *Helicobacter pylori*. *Topics in Current Chemistry*, 288: 121–138.

416. Testerman, TL.; McGee, DJ and Moble y H.LT. (2001). Chapter 34 Adhere and Colonization from Mobley H.LT., Mendz G.L. and Hazell S.L. *Helicobacter pylori*: physiology and Genetics ASM Press.USA, 381-417.

417. Huang, J.; Keeling, P.W.N.; Smyth, C.J. (1992) Identification of erythrocyte binding antigens in *Helicobacter pylori*. *Journal of General Microbiology*, 138, 1503-1513.

418. Saitoh, T.; Natomi, H.; Zhao, K.; Okozumi, K.; Sugano, K.; Iwamori, M.; Nagai, Y. (1991). Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. *FEBs*, 282(2): 385-387.

419. Ferrero, R.L.; Fox, J.G. (2001). Chapter 45 In vivo modeling of Helicobacter-Associated Gastrointestinal diseases from Mobley H.LT., Mendz G.L. and Hazell S.L. *Helicobacter pylori*: physiology and Genetics ASM Press.USA pp 565-582.

420. Borén, T.; Falk, P.; Roth, K.A.; Larson, G.; Normark, S. (1993). Attachment of *Helicobacter pylori* to Human Gastric Epithelium Mediated by Blood GroupAntigens. *Science New Series*, 262(5141):1892-1895.

421. Saitoh, T.; Sugano, K.; Natomi, H.; Zhao, W.; Okuzumi, K.; Iwamori, M.; Yazaki, Y. (1992). Glycosphingolipid receptors in human gastric mucosa for *Helicobacter pylori*. *European Journal of Gastroenterology & Hepatology,* 4:S49-S53.

422. Karlsson, K. (1989). Glycosphingolipids as membrane attachment sites for bacterial. *Annual Review Biochemistry*, 58:309-350.

423. Angstrom, J.; Teneberg, S.; d, M.; Larsson, T.; Leonardsson, I.; Olsson B.M.; Olwergard, M.; Danielsson, D.; Naslund, I.; Ljungh, A.; Waldstrom, T.; Karlsoon, K.A. (1998). The lactosylceramide binding specificity of *Helicobacter pylori*. *Glycobiology*, 8(4): 297-309.

424. Henry, S. M.; Samuelsson, B.E.; Oriol, R. (1994). Immunochemical and immunohistological expression of Lewis histo-blood group antigens in small intestine including individuals of the Le(a+b+) and Le(a-b-) nonsecretor phenotypes. *Glycoconjugate Journal*, 11: 600–607.

425. Teneberg, S.; Leonardsson, I.; Karlsson, H.; Jovall, P.; Ångstrom, J.; Danielsson, D.; Naslund, I.; Ljungh, Å.; Wadstro, T.; Karlsson, K. (2002). Lactotetraosylceramide, a Novel glycosphingolipid receptor for *Helicobacter pylori*, present in human gastric epithelium. *The journal of biological chemistry*, 277(22): 19709–19719.

426. Abul-Milh, M.; Foster, D.B.; Lingwood, C.A. (2001). In vitro binding of *Helicobacter pylori* to monohexosylceramides. *Glycoconjugate Journal*, 18: 253–260.

427. Miller-Podraza, H.; Lanne, B.; Ångstrom, J.; Teneberg, S.; Milh, M.A.; Jovall, P.; Karlsson, H.; Karlsson, K. (2005). Novel binding epitope for *Helicobacter pylori* found in neolacto carbohydrate chains. *The Journal of Biological chemistry*, 280: (20) 19695–19703.

428. Evans, D.G.; Evans, D.J.Jr.; Moulds, J.J.; Graham, D.Y. (1988). N-Acetylneuraminyllactose-Binding Fibrillar Hemagglutinin of *Campylobacter pylori*: a Putative Colonization Factor Antigen. *Infection and Immunity*, 56(11):2896-2906.

429. Hirmo, S.; Kelm, S.; Schuer, R.; Nilsson, B.; Wadstrom, T. (1996). Adhesion of *Helicobacter pylori* strains to α-2,3-1inked sialic acids. *Glycoconjugate Journal*, 13: 1005-1011.

430. Johansson, L.; Johansson, P.; Miller-Podraza, H. (1999). Neu5Aca3Gal is part of the *Helicobacter pylori* binding epitope in polyglycosylceramides of human erythrocytes. *European Journal of Biochemistry*, 266: 559-565.

431. Miller-Podraza, H.; Bergstrom J.; Teneberg S.; Abul Milh, M.; Longard, M.; Olsson B.M.; Uggla, L.; Karlsson, K.A. (1999). *Helicobacter pylori* and Neutrophils Sialic Acid-Dependent Binding to various isolated glycoconjugates. *Infection and Immunity*, 67(12): 6309-6313.

432. Moran, A.P. (2008). Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen *Helicobacter pylori*. *Carbohydrate Research,* 343:1952–1965.

433. Sakamoto, J.; Yin, B.W.T.; Lloyd, K.O. (1984). Analysis of the expression of H. Lewis, X, Y and precursor blood group determinants in saliva and red cells using a panel of mouse monoclonal antibodies. *Molecular Immunology*, 21(11):1093-1098.

434. Slomiany, B.L.; Piotrowski, J.; Samanta, A.; VanHorn, K.; Murty, V.L.N.; Slomiany, A.(1989). *Campylobacter pylori* colonization factor shows specificity for Lactosylceramide sulfate and GM3 Ganglioside. *Biochemistry International,* 19(4): 929-936.

435. Hata, Y; Murakami, M.; Okabe, S. (2004). Glycoconjugates with NeuAc-NeuAc-Gal-Glc are more effective at preventing adhesion of *Helicobacter pylori* to gastric epithelial cells than glycoconjugates with NeuAc-Gal-Glc. *Journal of physiology and Pharmacology*. (55) 3: 607-625.

436. Bennett, H.; Roberts; I.S. (2005). Identification of a new sialic acid-binding protein in *Helicobacter pylori*. *FEMS Immunology and Medical Microbiology*, 44: 163–169.

437. Slomiany, B.L.; Slomiany, A (1992). Mechanism of *Helicobacter pylori* pathogenesis: focus on mucus. *Journal of Clinical Gastroenterology*, 14(Suppl 1): S114-S121.

438. Makola, D.; Peura, D.A.; Crowe, S.E. *Helicobacter pylori* infection and related gastrointestinal disesases. *Journal of Clinical Gastroenterology,* 2007, 41(6): 548-558.

439. Kuipers, E. J.; Perez-Perez, G.I.; Meuwissen, S.G.; Blaser, M.J. (1995). *Helicobacter pylori* and atrophic gastritis: importance of the cagA status. *Journal of the National Cancer Institute*, 87:1777–1780.

440. Calam, J.; Gibbons, A.; Healey, Z.V. (1997) How does *Helicobacter pylori* cause mucosal damage? Its effect on acid and gastrin physiology. *Gastroenterology*, 113:S43–S49.

441. Barthel, J.S.; Westblom, T.U.; Havey, A.D. (1988). Gastritis and *Campylobacter pylori* in healthy, asymptomatic volunteers. *Archives of International Medicine*, 148:1149-1151.

442. Beales, I.L.; Calam, J. (1998). The histamine H3 receptor agonist N alphamethylhistamine produced by *Helicobacter pylori* does not alter somatostatin release from cultured rabbit fundic D-cells. *Gut*, 43:176–181.

443. Dai, Y.C.; Tang, Z.P.; Zhang, Y.L. (2011). How to assess the severity of atrophic gastritis. *World Journal of Gastroenterology*, 17(13):1690-1693.

444. Kurosawa, M.; Kikuchi, S.; Inaba, Y.; Ishibashi, T.; Kobayashi, F. (2000). *Helicobacter pylori* infection among Japanese children. *Journal of Gastroenterology and Hepatology*, 15:1382–1385.

445. Uemura, N.; Okamoto, S.; Yamamoto. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *The New England Journal Medicine*, 345:784–789.

446. Rugge, M.; Correa, P.; Dixon, M.F.; Fiocca, F.; Hattori, T.; Lechago, J.; Leandro, G.; Price, A.G.; Sipponen, P.; Solcia, E.; Watanabe, H.; Genta, R.M. (2002). Gastric mucosal atrophy: interobserver consistency using new criteria for classification and grading. *Alimentary Pharmacology & Therapeutics,* 16: 1249-1259.

447. Veldhuyzen van Zanten, S.O.J.; Dixon, M.F.; Lee, A. 1999. The gastric transitional zones: neglected links between gastroduodenal pathology and *Helicobacter* ecology. *Gastroenterology*, 116: 1217–1229.

448. Allen, P. (2001). What's the story *H. pylori*? *Lancet*, 357(9257):694.

449. Furuta, T.; Delchier, J.C. (2009). *Helicobacter pylori* and non-malignant diseases. *Helicobacter*, 14(Suppl 1): 29-35.

450. Graham, D.Y.; Hepps, K.S.; Ramirez, F.C.; Lew, G.M.; Saeed, Z.A. (1993). Treatment of *Helicobacter pylori* reduces the rate of rebleeding in peptic ulcer disease. *Scandinavian Journal of Gastroenterology*, 28(11): 939-942.

451. Kandulski, A.; Selgrad, M.; Malfertheiner, P. (2008). *Helicobacter pylori* infection: A clinical overview. *Digestive and liver disease*, 40: 619–626.

452. Talley, NJ; Hunt, R.H.(1997). What role does *Helicobacter pylori* play in dyspepsia and nonulcer dyspepsia? Arguments for and against *H-pylori* being associated with dyspeptic symptoms. *Gastroenterology*, 113(6):S67-S77.

453. Delaney, B.C.; Qume, M.; Moayyedi, P.; Logan, R.F.; Ford, A.C.; Elliott, C.; McNulty, C.; Wilson, S.; Hobbs, F.D. (2008). *Helicobacter pylori* test and treat versus proton pump inhibitor in initial management of dyspepsia in primary care: multicentre randomised controlled trial (MRC-CUBE trial). *British Medical Journal,* 336:651–654.

454. Patchett, S.; Beattie, S.; Leen, E.; Keane, C.; O'Morain, C. (1991). Eradicating *Helicobacter pylori* and symptoms of non-ulcer dyspepsia. *British Medical Journal,* 303: 1238-1240.

455. Kamangar, F.; Dores, G.M.; Anderson, W.F. (2006). Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *Journal of Clinical Oncololgy,* 24: 2137–2150.

456. Correa, P.; Houghton, J. (2007). Carcinogenesis of *Helicobacter pylori*. *Gastroenterology*, 133: 659–672.

457. Malfertheiner, P.; Bornschein, J.; Selgrad, M. (2010). Role of *Helicobacter pylori* infection in gastric cancer pathogenesis: A chance for prevention. *Journal of Digestive Diseases*, 11(1): 2-11.

458. Filipe, M.I.; Potet, F.; Bogomoletz, W.V. (1985). Incomplete sulpho mucin secreting intestinal metaplasia for gastric cancer. Preliminary data from a prospective study from three centres. *Gut,* 26:1319–1326.

459. Matsukura, N.; Suzuki, K.; Kawachi, T. (1980). Distribution of marker enzymes and mucin in intestinal metaplasia in human stomach and relation to complete and incomplete types of intestinal metaplasia to minute gastric carcinomas. *Journal of the National Cancer Institute*, 65: 231–240.

460. Piazuelo, M.B.; Haque, S.; Delgado, A. (2004). Phenotypic differences between esophageal and gastric intestinal metaplasia. *Modern Pathology*, 17:62–74.

461. Sell, S. (2004). Stem cell origin of cancer and differentiation therapy. *Critical Reviews in Oncology and Hematology*, 51:1–28

462. Kim, S.S.; Ruiz, V.E.; Carrol, J.D.; Moss, S.F. (2011). *Helicobacter pylori* in the pathogenesis of gastric cancer and gastric lymphoma. *Cancer Letters*, 305: 228-238.

463. Venkateshwari, A.; Krishnaveni, D.; Venugopal, S.; Shashikumar, P.; Vidyasagar, A.; Jyothy, A. (2011). *Helicobacter pylori* infection in relation to gastric cancer progression. *Indian Journal of Cancer*, 48(1): 94-98.

464. El-Omar, E.M. (2001). The importance of interleukin 1β in *Helicobacter pylori* associated disease. *Gut*, 48: 743–747.

465. Lee, K.E.; Khoi, P.N.; Xia, Y.; Park, J.S.; Joo, Y.E.; Kim, K.K.; Choi, S.Y.; Jung, Y.D. (2013). *Helicobacter pylori* and interleukin-8 in gastric cancer. *World Journal of Gastroenterology*, 19(45): 8192-8202.

466. Leunk, R. D.; Johnson, P.T.; David, B.C.; Kraft, W.G.; Morgan, D.R..(1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *Journal of Medical Microbiology*. 26: 93–99.

467. Tummuru, M.R.; Cover, T.L.; Blaser, M.J. (1994). Mutation of the Cytotoxin-Associated cagA Gene Does Not Affect the Vacuolating Cytotoxin Activity of *Helicobacter pylori*. *Infection and Immunity*, 62(6): 2609-2613.

468. Covacci, A.; Censini, S.; Bugnoli, M.; Petracca, R.; Burroni, D.; Macchia, G.; Massone, A.; Papini, E.; Xiang, Z.; Figura, N.; Rappuoli, R. (1993). Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *The Proceedings of the National Academy of Sciences of the United States of America*, 90: 5791–5795.

469. Huang, J.Q.; Zheng, G.F.; Sumanac, K.; Irvine, E.J.; Hunt, R.H. (2003). Meta-analysis of the relationship between cagA seropositivity and gastric cancer. *Gastroenterology*, 125:1636–1644.

470. Higashi, H.; Yokoyama, K.; Fujii, Y.; Ren, S.; Yuasa, H.; Saadat, I.; Murata-Kamiya, N.; Azuma, T.; Hatakeyama, M. (2005). EPIYA Motif Is a Membrane-targeting Signal of *Helicobacter pylori* Virulence Factor CagA in Mammalian Cells. *The Journal of Biological Chemistry*, 280(24): 23130-23137.

471. Moese, S.; Selbach, M.; Kwok, T.; Brinkmann, V.; Konig, W.; Meyer, T.F.; Backert, S. (2004). *Helicobacter pylori* induces AGS cell motility and elongation via independent signaling pathways. *Infection and Immunology*, 72:3646–3649.

472. Naumann, M.; S. Wessler, C.; Bartsch, B.; Wieland, A.; Covacci, R.; Haas, Meyer, T.F. (1999). Activation of activator protein 1 and stress response kinases in epithelial cells colonized by *Helicobacter pylori* encoding the cag pathogenicity island. *Journal of Biological Chemistry*. 274: 31655–31662.

473. Fischer, W.; Puls, J.; Buhrdorf, R.; Gebert, B.; Odenbreit, S., Hass, R. (2001) Systematic mutagenesis of the *Helicobacter pylori* cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Molecular Microbiology*, 42: 1337–1348.

474. Shaffer, C.L.; Gaddy, J.A.; Loh, J.T.; Johnson, E.M.; Hill, S.; Hennig, E.E.; McClain, M.S.; McDonald, W.H.; Cover, T.L. (2011). *Helicobacter pylori* exploits a unique repertoire of type IV secretion system components for pilus assembly at the bacteria-host cell interface. *PLoS Pathogens*, 7(9): e1002237.

475. Murata-Kamiya, N. (2011). Pathophysiological functions of the CagA oncoprotein during infection by *Helicobacter pylori*. *Microbes and Infection*, 13(10):799-807.

476. Tan, S.; Noto, J. M.; Romero-Gallo, J.; Peek, R. M. Jr.; Amieva, M. R. (2011). *Helicobacter pylori* perturbs iron trafficking in the epithelium to grow on the cell surface. *PLoS Pathogens,* 7(5): e1002050.

477. Barrozo, R.M.; Cooke, C.L.; Hansen, L.M.; Lam, A.M.; Gaddy, J.A.; Johnson, E.M.; Cariaga, T.A.; Suarez, G.; Peek, R.M. Jr.; Cover, T.L.; Solnick, J.V. (2013). Functional plasticity in the type IV secretion system of *Helicobacter pylori*, *PLoS Pathogens*, 9(2): e1003189.

478. Pandey, R.; Misra, V.; Misra, S.P.; Dwivedi, M.; Kumar, A.; Kumar, Tiwari, B. (2010). *Helicobacter pylori* and Gastric Cancer. *Asian Pacific Journal of Cancer Prevention,* 11: 583-588.

479. Wotherspoon, A.C.; Ortiz-Hidalgo, C.; Falzon, M.R. (1991). *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet*, 338:1175–1176.

480. Eidt, S.; Stolte, M. (1993). Prevalence of lymphoid follicles and aggregates in *Helicobacter pylori* gastritis in antral and body mucosa. *Journal of Clinical Pathology,* 46:832–835.

481. Ahmad, A.; Govil, Y.; Franck, B.B. (2003). Gastric Mucosa-Associated Lymphoid Tissue Lymphoma. *The American Journal of Gastroenterology*, 98(5): 975-986.

482. Du, M.Q.; Atherton, J.C. (2006). Molecular subtyping of gastric MALT lymphomas: implications for prognosis and management. *Gut*, 55:886–893.

483. Atherton, J.C. (2006). The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annual Reviews of Pathology*, 1:63-96.

484. Marshall, B.J.; Windsor, H.M. (2005). The relation of *Helicobacter pylori* to gastric adenocarcinoma and lymphoma: pathophysiology, epidemiology, screening, clinical presentation, treatment, and prevention. *Medical Clinics of North America*, 89:313–344.

485. Sachs, G.; Wen, Y.; Scott, D.R. (2009). Gastric Infection by *Helicobacter pylori*. *Current Gastroenterology Reports*, 11: 455-461.

486. Vilaichone, R.K.; Mahachai, V.; Graham, D.Y. (2006). *Helicobacter pylori* Diagnosis and Management. *Gastroenterology Clinics of North America*, 35: 229–247.

487. Megraud, F. (1997). Resistance of *Helicobacter pylori* to antibiotics. *Alimentary Pharmacology and Therapeutics*, 11(Suppl 1):43–53.

488. Penston, J.G.; McColl, K.E.. (1997). Eradication of *Helicobacter pylori*: an objective assessment of current therapies. *British Journal of Clinical Pharmacology*, 43:223–243.

489. Gisbert, J.P.(2008). "Rescue'' regimens after *Helicobacter pylori* treatment failure. *World Journal of Gastroenterology*, 14(35):5386-5402

490. Hojo, M.; Miwa, H.; Nagahara, A.; (2001). Pooled analysis on the efficacy of the second-line treatment regimens for *Helicobacter pylori* infection. *Scandinavian Journal of Gastroenterology*, 36:690–700.

491. Armstrong, J. A.; Wee, S.H.; Goodwin, C.S.; Wilson, D.H. (1987). Response of *Campylobacter pyloridis* to antibiotics, bismuth and an acid reducing agent in vitro—an ultrastructural study. *Journal of Medical Microbiology*, 24: 343–350.

492. O'Connor, A.; Gisbert, J.P.; McNamara, D.; O'Morain, C. (2010). Treatment of Helicobacter pylori Infection. *Helicobacte*r, 15(Suppl. 1): 46–52.

493. Segura, A.M.; Gutierrez, O.; Otero, W. (1997) Furazolidone, amoxycillin, bismuth triple therapy for *Helicobacter pylori* infection. *Alimentary Pharmacology and Therapeuti*cs, 11(3): 529–532.

494. Qasim, A.; Sebastian, S.; Thornton, O.; Dobson, M.; McLoughlin, R.; Buckley, M.; O'Connor, H.; O'Morain, C. (2005). Rifabutin and furazolidone based *Helicobacter pylori* eradication therapies after failure of standard first- and second-line eradication attempts in dyspepsia patients. *Alimentary Pharmacology and Therapeutics*, 21:91–96.

495. Wilhelm, S.; Johnson, J.L.; Kale-Pradhan, P.B. (2011). Treating Bugs with Bugs: The Role of Probiotics as Adjunctive Therapy for *Helicobacter pylori*. *The Annals of Pharmacotherapy*, 45: 960-966.

496. de Boer, W.A.; Tytgat, G.N. (1995).The best therapy for *Helicobacter pylori* infection: should efficacy or side-effect profile determine our choice? *Scandinavian Journal of Gastroenterology*, 30(5):401–407.

497. Graham, D.Y.; Shiotani, A. (2008). Newer concepts regarding resistance in the treatment *Helicobacter pylori* infections. *Nature Clinical Practice Gastroenterology and Hepatology*, 5(6):321-331.

498. Heep, M.; Kist, M.; Strobel, S.; Beck, D.; Lehn, N. (2000). Secondary resistance among 554 isolates of *Helicobacter pylori* after failure of therapy. *European Journal of Clinical Microbiology of Infectious Diseases*, 19: 538–541.

499. Koletzko, S.; Richy, F.; Bontems, F. (2006). Prospective multicenter study on antibiotic resistance of *Helicobacter pylori* strains obtained from children living in Europe. *Gut,* 55:1711–1716.

500. Versalovic, J. (1996). Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy*, 40: 477–480.

501. Rimbara, E.; Fischbach, L.A.; Graham, D.Y. (2011). Optimal therapy for *Helicobacter pylori* infections. *Nature Reviews Gastroenterology & Hepatology*, 8:79-88.

502. Asaka, M.; Kato, M.; Takahashi, S.; Fukuda, Y.; Sugiyama, T.; Ota, H.; Uemura, N.; Murakami, K.; Satoh, K.; Sugano, K. (2010). Guidelines for the Management of *Helicobacter pylori* Infection in Japan: 2009 Revised Edition. *Helicobacter*, 15(1):1-20.

503. Kim, J.J.; Reddy, R.; Lee, M.; Kim, J.G.; El-Zaatari, F.A.K.; Osato, M.S.; Graham, D.Y.; Kwon, D.H. (2001). Analysis of metranodizale, clarithromycin and tetracycline resistance of *Helicobacter pylori* isolates from Korea. *Journal of Antimicrobial Chemotherapy*, 47: 459-461.

504. Kwon, D.H.; Kim, J.J.; Lee, M. (2000). Isolation and characterization of tetracycline-resistant clinical isolates of *Helicobacter pylori*. *Antimicrobial Agents Chemotherapy*, 44(11): 3203–3205.

505. Dore, M.P.; Leandro, G.; Realdi, G. (2000). Effect of pretreatment antibiotic resistance to metronidazole and clarithromycin on outcome of *Helicobacter pylori* therapy: a meta-analytical approach. *Digestive Diseases and Sciences*, 45(1): 68–76.

506. Ierardi, E.; Giorgio, F.; Losurdo, G.; Di Leo, A.; Principi, M. (2013). How antibiotic resistances could change *Helicobacter pylori* treatment: A matter of geography? *World Journal of Gastroenterology*, 19(45):8168-8180.

507. Lesbros-Pantoflickova, D.; Corthésy-Theulaz, I.; Blum, A.L. (2007). *Helicobacter pylori* and probiotics. *Journal of Nutrition*, 137(3 Suppl 2):812S-818S.

508. Hamilton-Miller, J.M.T. (2003). The role of probiotics in the treatment and prevention of *Helicobacter pylori* infection. *International Journal of Antimicrobial Agents*, 22: 360-366.

509. Smoot, D.T.(1997) How does *Helicobacter pylori* cause mucosal damage? Direct mechanisms. *Gastroenterology*, 113: suppl 6:S31–S34.

510. Michetti, P.; Dorta, G.; Wiesel, P.H.; Brassart, D.; Verdu, E.; Herranz, M.; Felley, C.; Porta, N.; Rouvet, M. (1999). Effect of whey-based culture supernatant of Lactobacillus acidophilus (johnsonii) La1 on *Helicobacter pylori* infection in humans. *Digestion*, 60:203–209.

511. Coconnier, M.H.; Lievin, V.; Hemery, E.; Servin, A.L. (1998) Antagonistic activity against *Helicobacter* infection in vitro and in vivo by the human Lactobacillus acidophilus strain LB. *Applied and Environmental Microbiology*, 64: 4573–4580.

512. Nam, H.; Ha, M.; Bae, O.; Lee, Y. (2002). Effect of Weissella confusa strain PL9001 on the adherence and growth of *Helicobacter pylori*. *Applied and Environmental Microbiology*, 68(9):4642-4645.

513. Kabir, A.M.; Aiba, Y.; Takagi, A.; Kamiya, S.; Miwa, T.; Koga, Y. (1997). Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut*, 41: 49–55.

514. Mukai, T.; Asasaka, T.; Sato, E.; Mori, K.; Matsumoto, M.; Ohori, H. (2002) Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. *FEMS Immunology Medical Microbiology*, 32:105–110.

515. Mack, D.R.; Ahrne S, Hyde L, Wei S, Hollingsworth MA. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus strains* to intestinal epithelial cells in vitro. *Gut*, 52:827–833.

516. Gill, H.S. (2003). Probiotics to enhance anti-infective defences in the gastrointestinal tract. *Best Practice & Research Clinical Gastroenterology*, 17(5) :755–773.

517. Canducci, F.; Armuzzi, A.; Cremonini, F.; Cammarota, G.; Bartolozzi, F.; Pola, P.; Gasbarrini, G.; Gasbarrini, A. (2000). A lyophilized and inactivated culture of Lactobacillus acidophilus increases *Helicobacter pylori* eradication rates. *Alimentary Pharmacology and Therapeutics*, 14:1625–1629.

518. Armuzzi, A.; Cremonini, F.; Bartolozzi, F.; Canducci, F.; Candelli, M.; Ojetti, V.; Cammarota, G.; Anti, M.; De Lorenzo, A. (2001). The effect of oral administration of Lactobacillus GG on antibiotic-associated gastrointestinal side-effects during *Helicobacter pylori* eradication therapy. *Alimentary Pharmacology and Therapeutics*,15:163–169.

519. Sheu, B.S.; Wu, J.J.; Lo, C.Y. (2002). Impact of supplement with Lactobacillus- and Bifidobacterium- containing yogurt on triple therapy for *Helicobacter pylori* eradication. *Alimentary Pharmacology and Therapeutics*, 16: 1669–1675.

520. Cremonini, F.; Di Caro, S.; Covino, M. (2002). Effect of different probiotic preparations on anti-*Helicobacter pylori* therapyrelated side effects: a parallel group, triple blind, placebo-controlled study. *American Journal of Gastroenterology*, 97: 2744–2749.

521. De Francesco, V.; Stoppino V, Sgarro C, Faleo D. (2000). *Lactobacillus acidophilus* administration added to omeprazole/amoxycillin-based double therapy in *Helicobacter pylori* eradication. *Digestive and Liver Disease*, 32:746–747.

522. Sykora J, Valeckova K, Amlerova J, Siala K, Dedek P, Watkins S, Varvarovska J, Stozicky F, Pazdiora P, Schwarz J. (2005). Effects of a specially designed fermented milk product containing probiotic *Lactobacillus casei* DN-114 001 and the eradication of *H. pylori* in children: a prospective randomized, double-blind study. *Journal of Clinical Gastroenteroly*, 39: 692–698.

523. Felley, C.P.; Corthesy-Theulaz, I.; Rivero, J.L. (2001). Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man. *European Journal of Gastroenterology & Hepatology*, 13: 25–29.

524. Di Mario, F.; Aragona, G.; Bo, N.D. (2003). Use of lactoferrin for *Helicobacter pylori* eradication. Preliminary results. *Journal of Clinical Gastroenterology*, 36(5):396–398.

525. Kamiji, M.M.; de Oliveira, R.B. (2005). Non-antibiotic therapies for *Helicobacter pylori* infection. *European Journal Gastroenterology & Hepatology*, 17(9):973-981.

526. Hazell, S.L.; Evans, D.J.; Graham, D.Y. (1991) *Helicobacter pylori* catalase. *Journal of General Microbiology*, 137, 57-61.

527. Byrd, J.C.; Yunker, C.K.; Xu, Q.S, Sternberg, L.R.; Bresalier, R.S. (2000) Inhibition of gastric mucin synthesis by *Helicobacter pylori*. *Gastroenterology*, 118:1072–1079.

528. Bernstein, C.N.; McKeown, I.; Embil, J.M.; Blanchard, J.F.; Dawood, M.; Kabani, A.; Kliewer, E.; Smart, G.; Coghlan, G.; MacDonald, S.; et al. (1999). Seroprevalence of *Helicobacter pylori*, incidence of gastric cancer, and peptic ulcer-associated hospitalizations in a Canadian Indian population. *Digestive diseases and sciences*, 44:668-674.

529. Zhu, J.; Davidson, M.; Leinonen, M.; Saikku, P.; Gaydos, C.A.; Canos, D.A.; Gutman, K.A.; Howard, B.V.; Epstein, S.E.; GOCADAN Study Investigators.(2006) Prevalence and per- sistence of antibodies to herpes viruses, Chlamydia pneumoniae and *Helicobacter pylori* in Alaskan Eskimos: the GOCADAN Study. *Clinical Microbiology and Infection*, 12:118-122.

530. McKeown, I.; Orr, P.; Macdonald, S.; Kabani, A.; Brown, R.; Coghlan, G.; Dawood, M.; Embil, J.; Sargent, M.; Smart, G. (1999) *Helicobacter pylori* in the Canadian arctic: seropreva- lence and detection in community water samples. *American Journal of Gastroenterology*, 94:1823-1829.

531. Hastings, E.V.; Yasui, Y.; Hanington, P.; Goodman, K.J.; The CanHelp Working Group. (2014). Community-driven research on environmental sources of H. pylori infection in arctic Canada. *Gut Microbes*, 5(5):606-617.

CHAPTER II. Research Plan

2.1 Rationale

Cell membrane lipids consist of phospholipids, cholesterol and glycolipids such as gangliosides. The content of these components varies with the cell cycle or age and in response to a variety of stimuli or changes in environment and physiological state (for example, with diet and in disease states).[1] A great deal of research has investigated how alterations in diet phospholipid and cholesterol composition can change membrane lipid composition. Much less is known about the ability of alterations in diet ganglioside composition to alter membrane composition and function.

Dietary gangliosides are taken up by cells. For example: Caco2 cells were exposed to GD3 on the apical side or basolateral side and membrane ganglioside increased in content.[2] Gangliosides are located and distributed in different sides of intestinal cells. GM3 is located on the apical membrane domain while GD3 is located in the basolateral membrane domain. Location of gangliosides might determine the role of the gangliosides.[3]

Membrane phospholipids might be affected for the metabolism of gangliosides. Ceramide is a lipid mediator for the metabolism of sphingomyelin, glycosphingolipids and sphingosine-1-phosphate. Sphingosine-1-phosphate can be degradated to phosphoethanolamine and fatty acids.[4] Ganglioside increase in plasma membranes may affect lipid mediators important for the synthesis of phospholipids.

Adhesion is mediated by bacterial surface glycoproteins to receptors of host gastric epithelial cells. The receptors appeared to be carbohydrate residues of glycoconjugates on the gastric cell membrane such as gangliosides.[5] The mucosal surface of the human stomach is the main habitat for *H. pylori*.[6] After *H. pylori* is introduced into the human stomach, it starts the

process of colonization of the gastric mucosa.[7] Gangliosides act as receptors for *H. pylori* due to gangliosides contain sialic acids which are a binding site for this bacterium.[8,9]

H. pylori has different affinity for different ganglioside species. *H. pylori* adhesion to MKN-45 cells (undifferentiated gastric cells) was inhibited by GD3, but not by GM3.[9] In other study, GM3 has been recognized as a strong epitope for *H. pylori.*[10] Abul-Milh (2001) reported that some *H.pylori* strains bind GM3 but no other glycosphingolipids.[18] Tang et al. (2001) reported the importance and requirement of ganglioside structure for *H. pylori* adhesion to bind glycolipids consisting of both carbohydrate portions and ceramides with α-hydroxyl fatty.[19]

In vitro models provide a useful tool to study *H. pylori* adhesion assays, but these studies were performed with undifferentiated gastric cells or colon cells[9,11] which do not imitate the characteristics of the human stomach. A well-differentiated polarized gastric cell line forming tight junctions (zonula-occludens-1), adherens junctions (E-cadherin, forming monolayers, secreting mucus, pepsinogen and gastric lipase such as NCI-N87 cell line that might provide more reliable information about *H. pylori* adhesion assays.[12] There is no information about alterations in ganglioside plasma membranes of gastric human epithelial cells by dietary gangliosides (GD3 and GM3) as a nutritional strategy to prevent *H. pylori* adhesion.

Colostrum, raw whole milk and powder buttermilk from cows have been recognized for the abundance and contribution of gangliosides to the diet. [13,14,15] However, the ganglioside profile and ganglioside composition of milk products have not been fully analyzed by LC/MS method. On the other hand, pasteurization remains the main method to preserve milk products. Previous studies in human and bovine milk show no alteration of ganglioside content, but

concentration was analyzed by TLC and colorimetric methods which are not very accurate.[16,17] Other analytical methods such as LC/MS may provide information about the effects of pasteurization on ganglioside content and composition.

2.2 Hypothesis

Exogenous gangliosides will modify ganglioside content in human gastric epithelial cells, which will alter *H. pylori* adhesion to human gastric cells. It is specifically hypothesized that:

Hypothesis 1. Pasteurization treatment does not affect ganglioside content measured in whole milk.

Hypothesis 2. Ganglioside profile will be different in the three different dairy products analyzed (whole raw milk, colostrum, buttermilk).

Hypothesis 3. Human gastric epithelial cell membrane will be modified by GD3, GM3 and a ganglioside mix enriched powder.

Hypothesis 4. Human gastric epithelial cell membranes enriched with GD3 and GM3 will modify apical membrane domain and not the basolateral membrane domain

Hypothesis 5. Exogenous gangliosides will modify phospholipids (PC and PE) in human gastric epithelial cells.

Hypothesis 6. Human gastric cells treated with a ganglioside mix enriched powder will decrease *H. pylori* adhesion.

Hypothesis 7. Human gastric cells treated with purified GD3 ganglioside will reduce *H. pylori* adhesion more than human gastric epithelial cells treated with GM3.

2.3 Objectives

This research is conducted to determine if dietary gangliosides from milk modify the ganglioside content in the apical membrane domain of human gastric epithelial cells. The main focus of this study is to determine if dietary gangliosides from milk reduces *H. pylori* adhesion to human gastric epithelium.

The particular objectives of this project research are to determine:

1. the composition of gangliosides from bovine whole raw milk, bovine colostrum and powder buttermilk using LC/MS.

2. ganglioside content of the human gastric epithelial cell line (NCI-N87)

3. whether the alteration of GM3:GD3 ratio of the apical and basolateral membrane of human gastric cell line can be modified by exogenous gangliosides.

4. the influence of dietary ganglioside on the composition and content of PC and PE.

5. whether adhesion of *H. pylori* decreases in GD3 and GM3-modified human gastric epithelial cells.

2.4 References

1. Clandinin, M.T.; Field, C.J. Hargreaves, K.; Morson, L.; Zsigmond, E. (1985). Role of diet fat in subcellar structure and function. *Canadian Journa of Physiology and Pharmacology*, 63:94-106.

2. Schnabl, K.L.; Larcelet, M.; Thomson, A.B.; Clandinin, M.T. (2009). Uptake and fate of ganglioside GD3 in human intestinal Caco-2 cells. *The American Journal of Physiology: Gastrointestinal and Liver Physiology*, 2297(1):G52-G59.

3. Park, E.J.; Suh, M.; Ramanujam, K.; Steiner, K.; Begg, D.; Clandinin, M.T. (2005). Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *Journal of Pediatric Gastroenterology and Nutrition*, 40(4): 487-495.

4. Duan, R.D. Physiological functions and clinical implications of sphingolipids in the gut. *Journal of Digestive Diseases*, 12(2):60-70.

5. Idota T & Kawakami H. (1995). Inhibitory effects of milk gangliosides on the adhesion of *Escherichia coli* to human intestinal carcinoma cells. *Bioscience, Biotechnology, and Biochemistry,* 59(1):69-72.

6. Hill, M. (1997). The microbiology of *Helicobacter pylori*. *Biomedicine and Pharmacotherapy*, 51:151-153.

7. Andersen L.P. (2007) Colonization and Infection by *Helicobacter pylori* in Humans. *Helicobacter,* 12(2): 12–15.

8. Saitoh, T.; Natomi, H.; Zhao, K.; Okozumi, K.; Sugano, K.; Iwamori, M.; Nagai, Y. (1991). Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. *FEBs*, 282(2): 385-387.

9. Hata, Y; Murakami, M.; Okabe, S. (2004). Glycoconjugates with NeuAc-NeuAc-Gal-Glc are more effective at preventing adhesion of *Helicobacter pylori* to gastric epithelial cells than glycoconjugates with NeuAc-Gal-Glc. *Journal of physiology and Pharmacology*. (55) 3: 607-625.

10. Saitoh, T.; Sugano, K.; Natomi, H.; Zhao, W.; Okuzumi, K.; Iwamori, M.; Yazaki, Y. (1992). Glycosphingolipid receptors in human gastric mucosa for *Helicobacter pylori*. *European Journal of Gastroenterology & Hepatology,* 4:S49-S53.

11. Salcedo, J.; Barbera, R.; Matencio, E.; Alegria, A.; Lagarda, M.J. (2013). Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: an in vitro study. *Food Chemistry*, 136:726-734.

12. Basque, J.R.; Chénard, M.; Chailler, P.; Ménard, D. (2001). Gastric cancer cell lines as models to study human digestive functions. *Journal of Cellular Biochemistry*, 81(2):241-251.

13. Huang, R.T.C. (1973). Isolation and characterization of the gangliosides buttermilk. *Biochimica et Biophysica Acta*, 306, 82–84.

14. Moore, K.H.; Ettinger, A.C.; Yokoyama, M.T. (2000). Variation in Ganglioside Content of Bovine Dairy Products . *Journal of Food Composition and Analysis*, 13:783-790.

15. Pham, P.H.; Duffy, T.L.; Dmytrash, A.L.; Lien, V.W.; Thomson, A. B.; Clandinin, M.T. (2011). Estimate of dietary ganglioside intake in a group of healthy Edmontonians based on selected foods. *Journal of Food Composition and Analysis*, 24:1032–1037.

16. Ewaschuk, J.B.; Unger, S.; O'Connor, D.L.; Stone, D.; Harvey, S., Clandinin, M.T.; Field, C.J. (2011). Effect of pasteurization on selected immune components of donated human breast milk. *Journal of Perinatology:* 31:593-598.

17. Puente, R.; Garcia-Pardo, L.A.; Rueda, R.; Gil, A., Hueso, P. (1996). Seasonal variations in the concentration of gangliosides and sialic acids in milk from different mammalian species. *International Dairy Journal*, 6:315-322.

18. Abul-Milh, M.; Foster, D.B.; Lingwood, C.A. (2001). In vitro binding of *Helicobacter pylori* to monohexosylceramides. *Glycoconjugate Journal*, 18: 253–260.

19. Tang W, Seino K, Ito M, Konishi T, Senda H, Makuuchi M, Kojima N & Mizuochi T. (2001). Requirement of ceramide for adhesion of *Helicobacter pylori* to glycosphingolipids. *FEBS Letters*, 504(1-2):31-35.

CHAPTER III. Profiling gangliosides from milk dairy products using LC/MS

3.1 Introduction

Gangliosides are complex amphiphilic lipids associated with the plasma membrane of mammalian cells and other biological membranes.[1-4] Gangliosides and all glycosphingolipids are composed of a glycan moiety linked to a ceramide portion.[5] The distinguishing characteristic of gangliosides is the presence of one or more sialic acid residues[6] within the glycan chain. Gangliosides are divided into different classes based on the number of sialic acids present; GM (mono-sialilated), GD (di-sialilated) GT (tri-sialilated).[7,8] Further classification is based on oligosaccharide chain length. The ceramide consists of N-acylsphingosine in which the acyl residue is linked by an amide bond to a long-chain fatty acid (Fig. 3.1).[2,9]

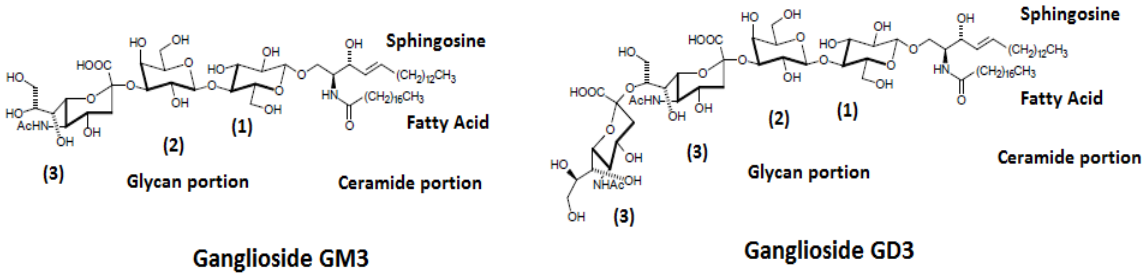

Figure 3.1 Schematic representation of GM3 and GD3. 1= glucose, 2=galactose, 3= sialic acid (modified from 58).

The amphiphatic nature of milk gangliosides determines the biological function; the hydrophobic ceramide determines how gangliosides are embedded in biological membranes while the glycan moiety modulates various recognition and adhesion processes.[10,11] In milk, for example, gangliosides are linked to the milk fat globule membrane (MFGM) where the ceramide portion is anchored into the membrane and the glycan moiety is interacting with the external environment.[12,13] Dietary milk gangliosides may promote intestinal immunity

development in the neonate, growth of beneficial microflora such as *Bifidobacteria* and suppress growth of pathogens such as *E. coli*. [9,11,14] Gangliosides have been recognized as regulators in signaling pathways,[15] immunomodulators[16] and modulators of ion channels.[17]

Thus gangliosides represent a very interesting, although challenging, class of biomolecules to study. Challenges in ganglioside analysis are due to high levels of variability in ceramide composition (carbon number, degree of saturation) and carbohydrate core (number and nature of saccharides) and also due to the relatively low abundance of gangliosides in biological membranes. Conventional analysis involve the use of thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC),[18,19] followed by colorometric methods using-hydrochloric (HCl) acid or orcinol-sulfuric acid reagents which quantify total lipid bound sialic acid (T-LBSA).[9,20] These approaches are very time-consuming and do not provide simultaneous information regarding ceramide or carbohydrate composition.

3.1.1 Methods of extraction

Methods for the extraction and separation of gangliosides from tissues and body fluids are based on the use of solvents. Chloroform:methanol solvent system is the most common first step to extract gangliosides. The second step is the use of a salt such as $CaCl_2$ to enhance phase separation, to increase ganglioside yield and to minimize the loss of phospholipids in the aqueous phase.[33] Folch et al. (1957) has been recognized as the first to extract gangliosides from neural tissues. Some authors such as Suzuki (1964) and Svennerholm and Fredman (1980) have modified the Folch method; however, the Folch method remains as the most important due to simplicity. After Folch extraction, the use of reverse phase chromatography is an option to remove contaminants. Solid phase extraction (SPE) using C18 Sep-Pak

cartridges removes salts, water-contaminants and glucose which possibly remain after Folch extraction.[21]

Folch extraction has been recognized as a satisfactory and acceptable method to analyze milk with different fat content.[22,23] Folch extraction inactivates lipolytic activity and recovers phospholipids effectively.[22] The simplicity of the Folch method is the separation of total lipid extraction in two partition phases and the recovery of individual lipid classes.[24] The upper phase or aqueous phase is composed of sphingolipids including cerebrosides and gangliosides.[23,25] Lactosylceramides and monohexosylceramides are in the organic phase.[25] The Folch extraction method was modified by Timmen and Dimmick to extract lipids in human and bovine milk.[26,27] Other methods such as Svennerholm and Fredman, 1980, also involve modification of the the Folch-extraction procedure.[28,37]

3.1.2 Ganglioside analysis by MS

An emerging tool in ganglioside analysis is mass spectrometry (MS). [18, 29, 30] An MS based approach offers several key advantages, including sensitivity (>1 pg) and selectivity (distinguising different species).[19] MS based methods offer direct information on both the ceramide and carbohydrate composition.[31] The application of gas-phase dissociation experiments, such as collision induced dissociation (CID), can also provide structural information. Using CID the position and anomeric configuration of the glycosidic linkages can be determined.[29]

Although these MS-based approaches represent significant improvement in analysis of gangliosides, some limitations remain. For example, the existing MS methodology is still quite time-consuming, with long liquid chromatography (LC) runs prior to MS detection.

Existing techniques require the use of expensive high resolution mass instruments, such as Orbitrap or Fourier transform ion/cyclotron resonance mass spectrometers.[29,31] I present a method for rapid profiling of gangliosides from various biological membranes using a low resolution triple-quadrupole MS operating in multiple reaction monitoring mode (MRM).

In the MRM mode, the first quadrupole scans multiple masses of gangliosides selected with precursor ion to pass (Fig. 3.2). In this case the precursor ion is one sialic acid (Neu5Ac). In the second quadrupole or collision cell, some fragmented ions are generated. Finally, the third quadrupole is set to allow sialic acid to pass and to collect fragments. [32]

This method provides key advantages in terms of sensitivity, specificity and quantitative analysis. Every aspect of the method was optimized for MS detection; this included optimizing the extraction of gangliosides from dairy products and the LC separation prior to MS analysis. This method was optimized using bovine whole milk, colostrum and powder buttermilk as a source of gangliosides and is applied to evaluate the effect of the pasteurization, decrease sample size, modify ganglioside extraction and detect the ganglioside profile in these milk products.

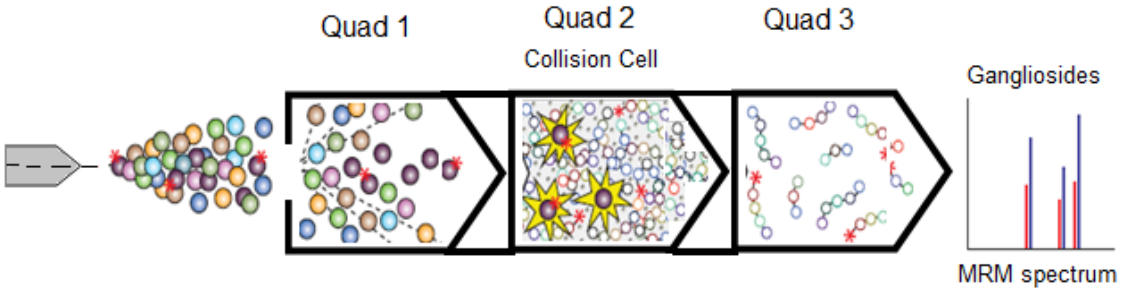

Figure 3.2. Illustration of MS operating in MRM. Quad 1 only target specific ganglioside masses. Quad 2 or collision cell breaks ion 290 (Neu5Ac) apart by inert gas. Quad 3 monitors only characteristic ions from ion *m/z* 290 for detection (modified from reference 32).

3.2 Materials and Methods

3.2.1 Materials. Bovine buttermilk powder (7% of fat) was provided by Parmalat (Toronto, Ontario, Canada). Bovine buttermilk powder was reconstituted with Milli-Q water at 25% (w/v). Fresh unpasteurized pooled whole milk (~3.5% of fat) and colostrum (~5.5% of fat) was obtained from the University of Alberta Dairy Research Technology Centre from Holstein cows. All LC/MS solvents were of LC/MS grade and solvents for ganglioside extraction were ACS grade (Fisher Scientific Company, Ottawa, Ontario, Canada). Whole milk samples were pasteurized in a preheated water bath at 63.7°C for 30 min for the low temperature, long time (LTLT) pasteurization treatment (62°C for 30 min), and at 72.7°C for 15 s for the treatment of high temperature, short time (HTST) pasteurization. Both pasteurization treatments were followed by cooling in a water bath (<5°C).

3.2.2 Ganglioside extraction and purification. Samples were extracted according to a Folch method for extracting animal tissue lipids.[33] This method consisted of a chloroform: methanol (2:1, v/v) extraction with a solvent to aqueous ratio of 20:1. The mixture was homogenized and shaken vigorously (20 min). After shaking, $CaCl_2$ (0.025%, w/v) was added to the mixture followed by another 20 min of shaking. The solution was either left overnight at 4°C or centrifuged to allow the phases to settle. The upper methanolic phase (aqueous phase) was purified by SPE passing through Sep-Pak C18 cartridges. Prior to sample loading, cartridges were prewashed with 5 mL of Milli-Q water, 5 mL of methanol, 5 mL of chloroform-methanol (2:1, v/v), 5 mL of methanol and 5 mL of Milli-Q water. The aqueous phase was passed through a prewashed Sep-Pak C18 cartridge and then washed with 10 mL of Milli-Q water to remove salts. Gangliosides were eluted with 2 mL of methanol and 10 mL of

chloroform-methanol (2:1, v/v),[34] dried under N_2 gas, and re-dissolved with 500 uL of methanol/water (1:1, v/v). To evaluate the optimal sample size for a MS-based approach, the Folch method was applied to various milk aliquots, from 50 uL to 5 mL. The ratio of all organic solvents to sample size was kept constant at 20:1. The necessity of the solid phase cleanup was also evaluated, as direct injection of the aqueous phase phase was analyzed as well as a reconstituted sample of the entire aqueous phase after drying under nitrogen.

3.2.3 Standard ganglioside preparation. Gangliosides were extracted from whole milk as described by Jennemann and Wiegandt (1994).[35] Nonhomogenized whole fresh milk was centrifuged (Beckman Coulter Avant J-26XP centrifuge) at 6238 xg at 4°C. Methanol was then added to the milk fat at a ratio of (2:1, w/v). This mixture was stirred for 15 min at room temperature then centrifuged (Jouan CR422) at 1811 xg at 4°C. The supernatant was collected and kept at 4°C. The pellet was extracted two more times with the same amount of methanol. Gangliosides were extracted from the supernatants using a Folch extraction as described in the previous section.

To separate gangliosides based on the degree of sialylation, TLC using silica gel G plates (20x20 cm, 250 μm) (Analtech Inc., Newark, DE) was used. TLC plates were washed with methanol, dried at room temperatura and washed with hexane. TLC plates were activated by heating at 110°C for 60 min. Aqueous phase from Folch extraction was used to prepare TLC plates. The solvent system used for TLC separation of individual gangliosides was chloroform/methanol/28% ammonia/H_20 (60:35:7:3, by volume). Filter paper was collocated in the walls of the standard separation chambers to balance the solvent. Gangliosides were visualized using 8-anilino-1-naphthalene-sulfonic acid (ANSA) 0.1%.

Bands containing gangliosides were excised and gangliosides were extracted from silica by vortexing and sonicating in methanol. Tubes were centrifuged at 250 xg for 10 min and an aliquot of supernatant was subjected to LC/MS analysis to confirm ganglioside identity. The remaining supernatant was dried under nitrogen and subjected to quantitative analysis using the resorcinol-HCl based assay.[36] The resorcinol-HCl based assay detects the sialic acid bonds and produce a blue-violet color.[36] The resorcinol-HCl based assay was used to quantify the standards that will be use in LC/MS method.

3.2.4 High performance liquid chromatography. Prior to MS analysis, ganglioside extracts were subjected to reverse-phase chromatography with a Poroshell 120 EC-C18 column (3.0 x 50 mm, 2.7 μm particle size) using an Agilent 1260 Infinity LC system (Santa Clara, CA). Two mobile phases were used; phase A was composed of a 50:50 water/isopropanol mixture containing 5 mM ammonium acetate and 0.05% acetic acid, phase B consisted of 100% methanol. A gradient elution consisting of an increase in mobile phase B from 70% to 95% over 6 min was used, with a total LC run time of 12 min at a flow rate of 0.5 μL/min. The retention time of the detected ganglioside species ranged from 3 to 9 min.

3.2.5 Mass spectrometry. All MS measurements were obtained using an Agilent 6430 Triple-Quad LC/MS system (Santa Clara, CA) operating in negative ion mode. Deprotonated gas-phase ions of various ganglioside species were obtained using electrospray ionization (ESI), with the electrospray needle held at -4500 V. The MS was operated in multiple reaction monitoring mode (MRM). A library of theoretical precursor ions was generated for over 600 gangliosides of specific ceramide and carbohydrate composition. 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 CID. Gangliosides readily lose a sialic acid moiety when subjected to CID, thus the second quadrupole mass filter was set to only allow this characteristic fragment ion (*m/z* 290) to reach the detector. The CID and ion source voltages for each ganglioside class (GM, GD, and GT) were optimized using the Agilent Optimizer software. Data acquisition and analysis was carried out using the Agilent Mass Hunter software package.

3.2.6 Ganglioside library screening. For each class of ganglioside (GM, GD, and GT), a theoretical *m/z* library was generated in which the ceramide composition was varied in its' extent of saturation and total carbon chain length. Ganglioside masses were calculated assuming a sphingosine of d18:1 and d18:0 (prefix d indicates dihydroxy bases). The fatty acid (FA) side chain was then varied from 10 to 26, and included the odd carbon FA species 19, 21, 23, and 25. The library also included mono- and polyunsaturated FA. For each ganglioside of a specific carbohydrate composition, there were 52 variations on the ceramide composition included in the library search. The library searched Neu5Ac, and different sugar units (glucose, galactose, N-acetylgalactosamine following Fig. 1.6). Thus in total, the library consisted of 624 gangliosides from 12 classes (GM1, GM2, GM3, GM4, GD1, GD2, GD3, GD4, GT1, GT2, GT3, GT4).

3.2.7 Statistical analysis. Statistical analysis was performed using SPSS software (version 21). The effect of pasteurization of whole milk on ganglioside profile was determined via one-way Analysis of Variance (ANOVA) and Tukey test. The effect of modifying ganglioside

extraction was determined via repeated measures ANOVA with Tukey test. Data presented as mean ± SD and significance was established at $P<0.05$.

3.3 Results

3.3.1 Modifying sample size for a LC/MS based assay. A Folch extraction was performed on a range of whole milk aliquots (5 mL, 250 µL, and 50 µL). After extraction, the dried ganglioside extracts were dissolved in 500 µL of a 50/50 water/methanol solution and analyzed by LC/MS to determine GM3 and GD3 content. In total, 25 GD3 and GM3 species were detected with varying ceramide composition. MRM chromatograms for the 5 mL, 250 µL, and 50 µL milk aliquots were presented for a range of high and low abundant gangliosides (Fig. 3.3). The high abundant gangliosides were detected in a 5 mL aliquot and also detected in a 50 µL aliquot of milk, including the low abundant GM3 (C34,1). Thus the ganglioside profile can be accurately obtained from 50 µL of whole milk. However, the optimum milk sample size might be between 50 and 250 µL probably detecting many ganglioside species with small volumes of sample and well-defined peaks.

3.3.2 Modifying ganglioside extraction for a LC/MS based assay. Whole milk was extracted using three different protocols: a Folch extraction with sample cleanup on a solid phase extraction (SPE) cartridge, a Folch extraction without SPE dried top aqueous phase, and a Folch extraction followed by direct analysis of the top aqueous phase. The ganglioside profile for the most abundant GD3 ganglioside is presented with these additional extraction steps eliminated (Fig. 3.4). The relative amount of each specific ganglioside remains unchanged, indicating that altering the extraction protocol does not alter the measured

ganglioside profile. The highest ganglioside response was consistently obtained for samples that were subjected to Folch without SPE ($P<0.001$).

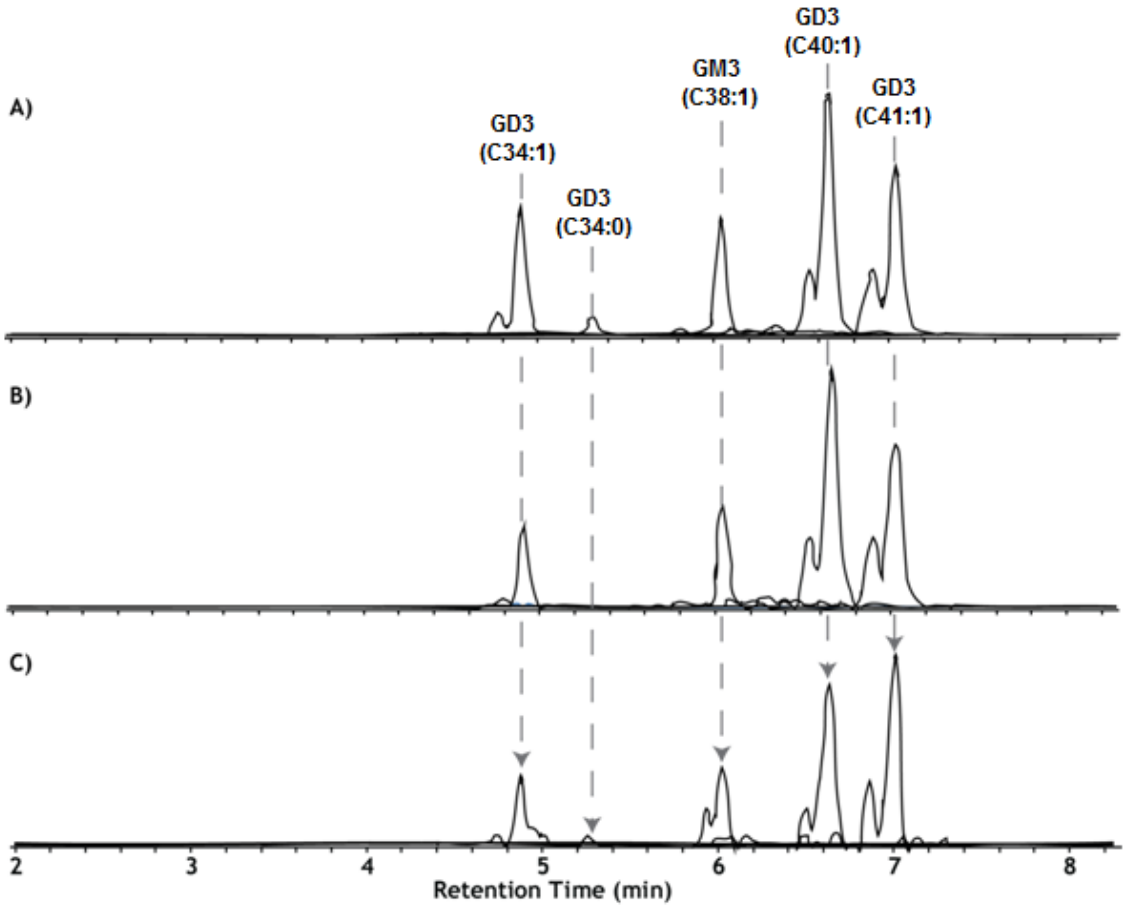

Figure 3.3 Illustrative MRM chromatograms for gangliosides of varying abundance in A) 5 mL, B) 250 µL, and C) 50 µL of bovine whole milk. Highly abundant ganglioside species are still present at in 50 µL of bovine whole milk sample. C, indicates number of carbons and bonds in ceramide portion.

3.3.3 Effect of pasteurization on the gangliosides profile of bovine whole milk. Two common pasteurization methods were investigated; high temperature, short time (HTST) pasteurization in which milk was heated to 72°C for 15-20 s, and low temperature, long time (LTLT) pasteurization in which the milk is heated to 63°C for 30 min. Different gangliosides species were scanned GM1, GM3, GD3, GD1 and GT3 varying the ceramide portion, for

analysis of 37 gangliosides species in total (Fig. 3.5). Both samples subjected to pasteurization showed a high MS response. LTLT pasteurization increased MS response compared to unpasteurized milk ($P<0.05$). MS response was 20% higher in LTLT pasteurized milk samples than in HTST pasteurized milk samples.

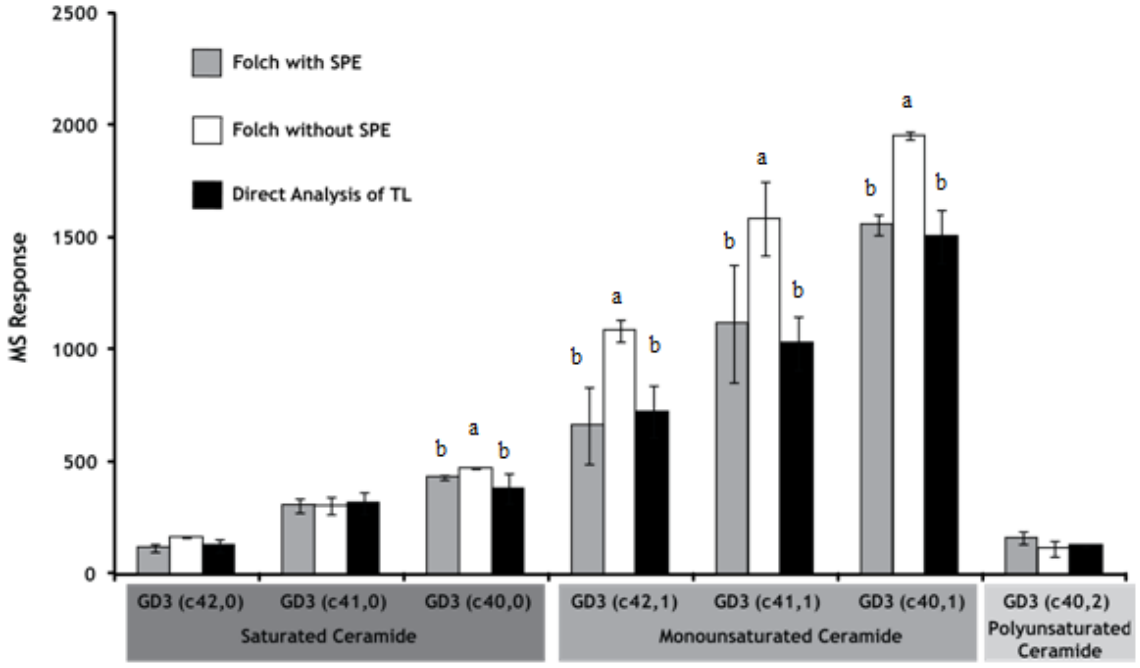

Figure 3.4 MS response for various GD3 gangliosides in bovine whole milk extracted with different extraction protocols; a Folch extraction with sample cleanup on a solid phase extraction (SPE) cartridge (▭), a Folch extraction without SPE (▭), and a Folch extraction followed by direct analysis of the top aqueous phase (▭). Samples extracted without SPE present higher amounts of ganglosides species than samples extracted with SPE ($P<0.001$). Ganglioside species are present in the three different extraction protocols indicating samples have not being altered. Data are expressed as mean±SD from 2 separated day milk collection, and tested in triplicate. Significant difference in ganglioside level was found in the three different extraction protocols. Different letters (a,b) indicate significant difference at $P<0.001$.

3.3.4 Comparing the ganglioside profile in various milk products. The LC/MS based ganglioside assay was applied to measure the ganglioside profile in whole milk, colostrum and buttermilk (Fig. 3.6). Samples were screened against the ganglioside library screening (>600 gangliosides species scanned). The relative amount of each ganglioside with respect to the

total ganglioside response is indicated. This technique was able to detect 4 different classes of gangliosides in whole milk (GM3, GD3, GT2, GT3); in buttermilk, 6 different gangliosides classes (GD1, GD3, GM1, GM3, GT2, GT3); and in colostrum, 7 different ganglioside classes (GD1, GD3, GM1, GM3, GT2, GT3, GT4) (Figure 3.6A). Within each class of ganglioside, several species were identified with varying ceramide composition. Differences in saturation within the ceramide results in ganglioside species with very similar *m/z* values. This study presents a challenge when analyzing gangliosides. To aid in identification of gangliosides with similar *m/z* values, the retention time on the C18 column was used. A linear relationship between carbon number and retention time was observed, and as expected the degree of saturation had a significant effect on the retention time (Appendix A), thus allowing discrimination between gangliosides with similar *m/z*.

3.3.5 Determination of ceramide and fatty acid portion in various milk products. There exists a wide range in ceramide composition within each ganglioside class. The MS results for the three milk products is summarized in Fig. 3.7. In GD3, GM3 and GT3, monounsaturated ceramide portion of gangliosides was the most abundant species in buttermilk, milk and colostrum. Polyunsaturated ceramide portions are less than 10% for GD3 ganglioside. In GM3, polyunsaturated ceramide portions were only found in colostrum. In GT3, small amounts of polyunsaturated portions were also found in the three milk products Assuming a sphingosine composition of d18:0, the fatty acid carbon chain length can be estimated for the three different dairy products (Fig. 3.8). Fatty acid chain containing from 10-26 carbons were detected. Interestingly, GM3 species possessed shorter fatty acid carbon chain (10-12) in the milk products when compared to GD3 and GT3 (14-26). In GM3, GD3

and GT3, long fatty acid carbon chain are present in the three products. C16 fatty acid chain are most abundant in GM3 for the three products. In GT3 and GD3, C22 fatty acid chain are most abundant for the three products.

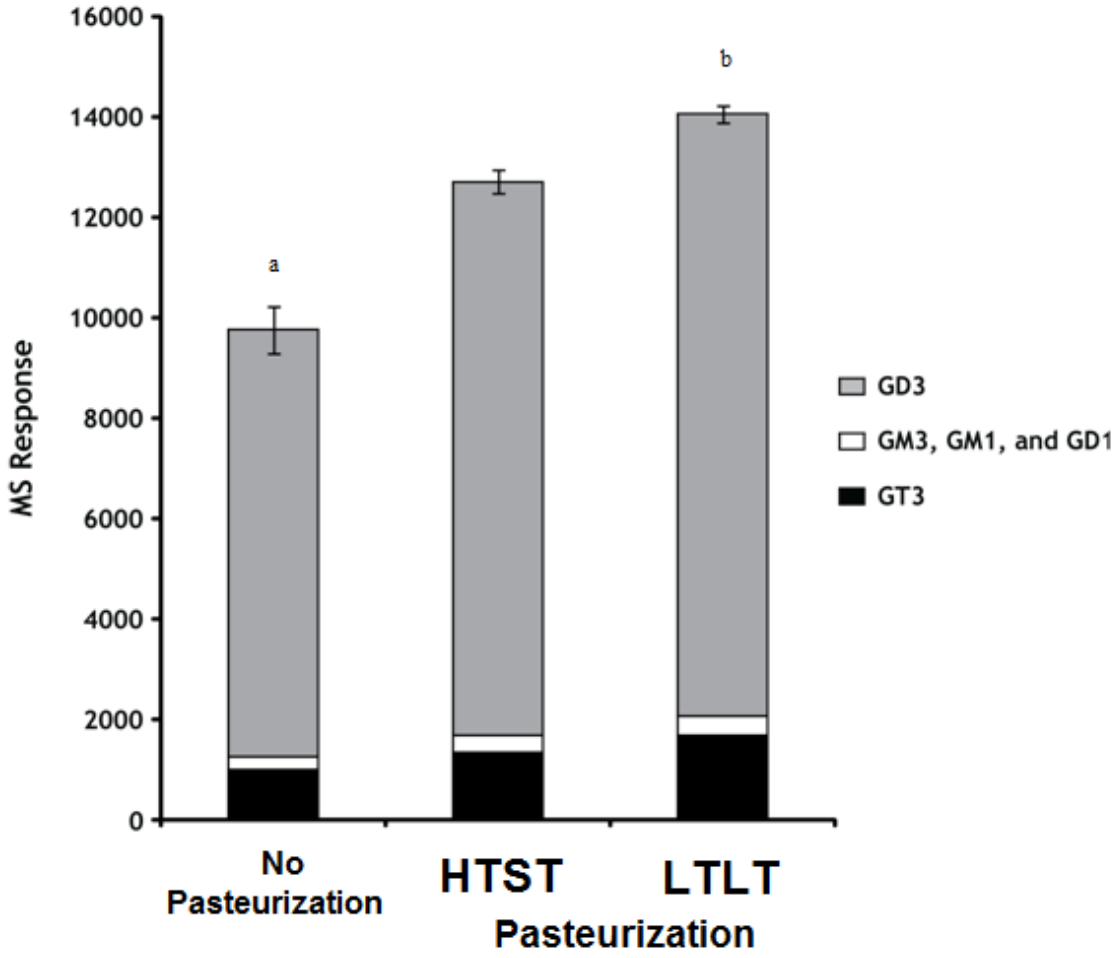

Figure 3.5 Effect of different pasteurization methods on the ganglioside profile in bovine whole milk as measured by LC/MS. Ganglioside profile is not altered by any pasteurization method; however, ganglioside extraction improves with either pasteurization compared to no pasteurized milk. Data are expressed as mean±SD from 2 separated day milk collection and tested in quadruplicate. Significant difference was found between no pasteurization and LTLT pasteurization treatment at $P<0.05$.

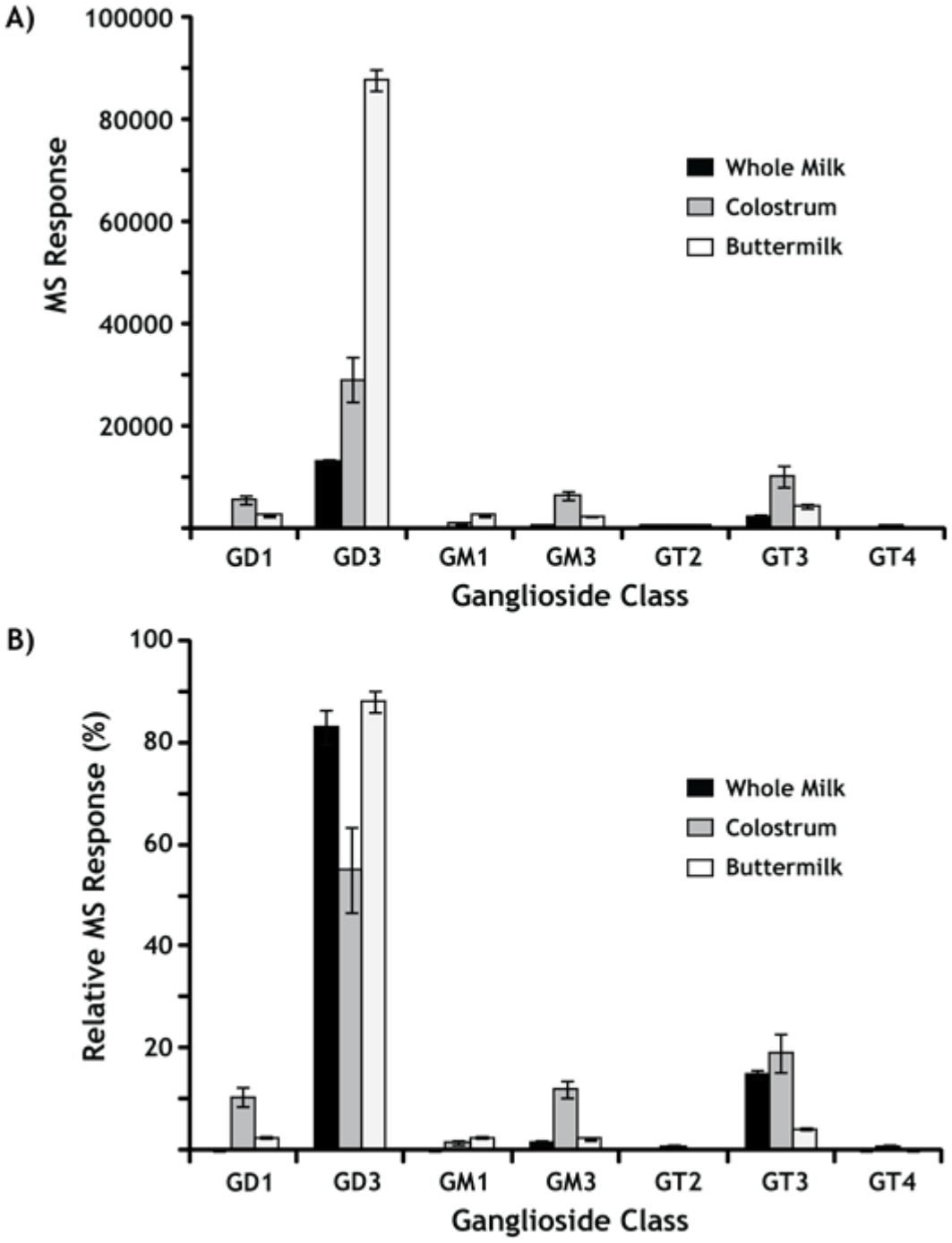

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Figure 3.6 A) Total and B) Relative MS response for each class of ganglioside content in the three bovine milk products (whole milk, buttermilk, and colostrum). Data are expressed as mean±SD from 2 separated days milk products collection and tested in triplicate.

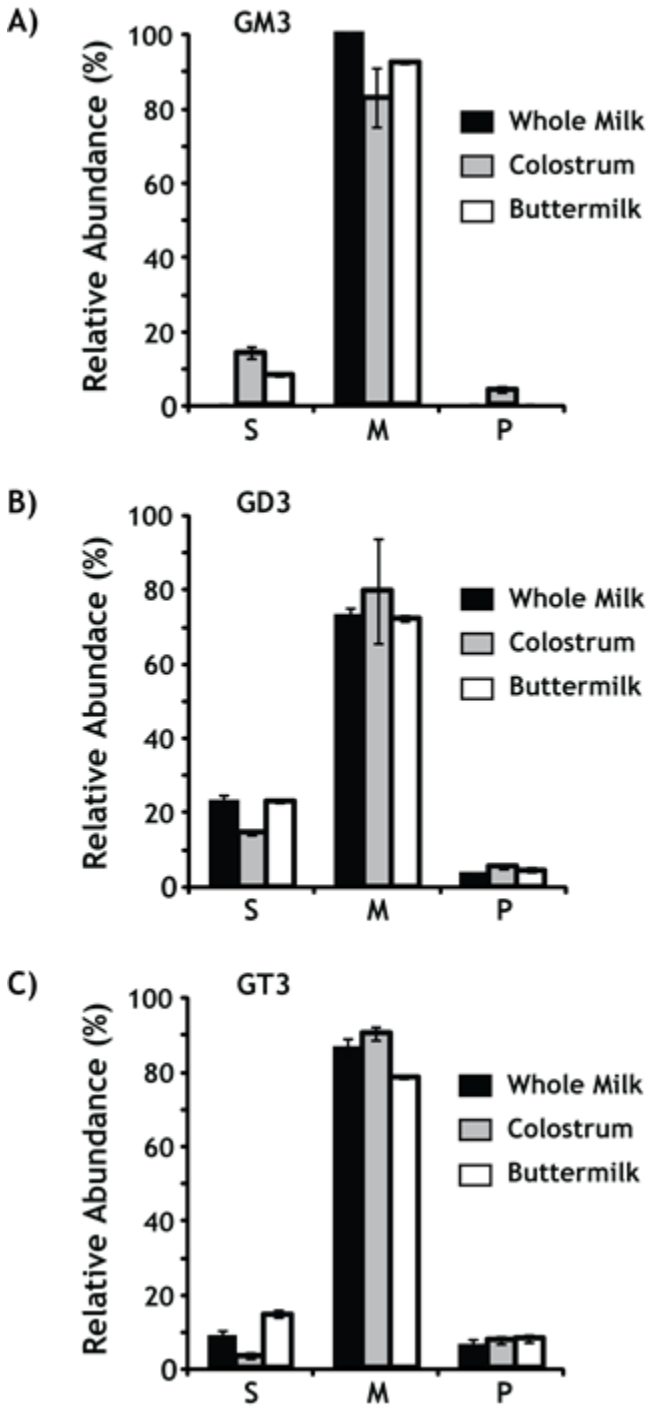


Figure 3.7 Relative MS response of saturated (S), monounsaturated (M) and polyunsaturated (P) ceramides in a) GM3, b) GD3, and c) GT3 gangliosides of bovine whole milk, colostrum and buttermilk. Data are expressed as mean $\pm$ SD from 2 separated day milk collection and tested in quadruplicate.

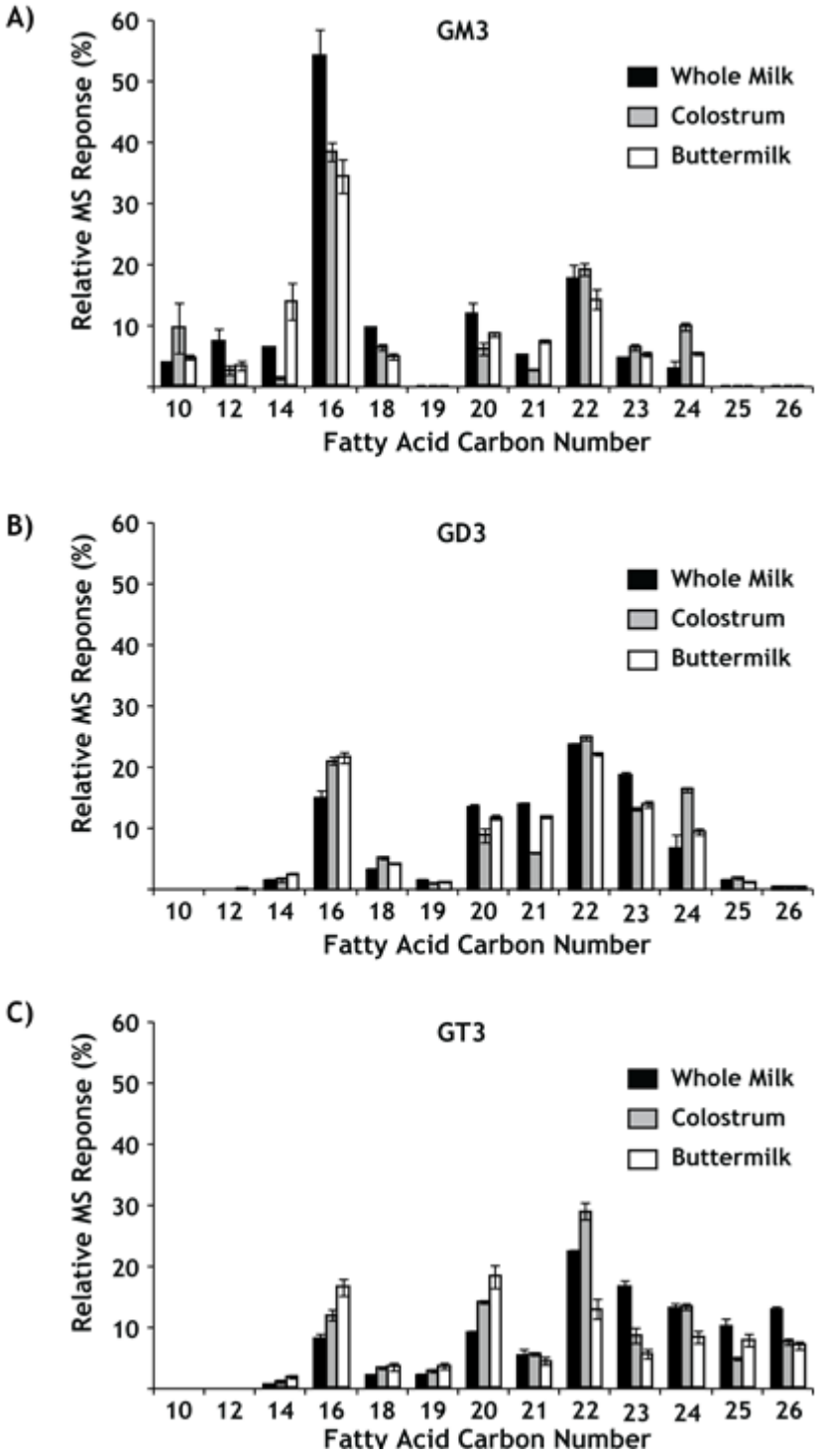


Figure 3.8 Representation of fatty acid carbon number of a) GM3, b) GD3 and c) GT3 ganglioside species in whole milk, buttermilk, colostrum assuming a sphingosine with a composition of d18:0. Data are expressed as mean $\pm$ SD from 2 separated day milk collection and tested in quadruplicate.

3.3.6 Quantitative LC/MS based assay for profiling gangliosides. The total concentration of gangliosides (GM3, GD3, GT3) in each milk product was 3.8 mg/L, 6.6 mg/L, and 30.5 mg/kg for milk, colostrum, and buttermilk, respectively (Table 3.1). GD3 was the most abundant gangliosides in the three different dairy products. GD3 and GT3 are found in aqueous phase (Table 3.2). GM3 is also found in aqueous phase, but large amounts (about 30-50% of GM3) can be found in organic phase (Table 3.2). The organic phase also contains 1-7% of total GD3 and 2.5% of total GT3.

Table 3.1. Comparison of ganglioside content in bovine whole milk, buttermilk and colostrum

Ganglioside species	Colostrum (mg/L)	Whole Milk (mg/L)	Buttermilk (mg/kg)
GD3	4.54±0.64	3.17±0.58	26.8±2
GM3	0.35±0.023	0.01±0.005	0.71±0.124
GT3	1.75±0.217	0.67±0.144	3.3±0.484

Data are expressed as mean ± SD from 2 separated day milk collection and tested in quadruplicate.

Table 3.2 Comparison of ganglioside content from aqueous and organic phase of Folch extraction in bovine whole milk, buttermilk and colostrum

Ganglioside Species	Colostrum (mg/L)		Whole Milk (mg/L)		Buttermilk (mg/kg)	
	A.P.	O.P.	A.P.	O.L.	A.P.	O.P.
GD3	4.229±0.647	0.315 ±080	3.133 ±0.580	0.033±0.011	24.8± 2.5	2.04± 0.03
GM3	0.251±0.034	0.100±0.011	0.009±0.003	0.003±0.001	0.36± 0.05	0.35±0.16
GT3	1.706±0.208	0.048±0.005	0.67±0.144	N.D.	3.3±0.48	N.D.

Data are expressed as mean ± SD from 2 separated day milk collection and tested in quadruplicate. Aqueous phase (A.P.) and Organic phase (O.P.) obtained of Folch Extraction were quantified separately. B.L. constituted were re-Folch two times more. N.D.=Not detected.

3.4 Discussion

Conventional ganglioside analysis by TLC or HPTLC requires large quantities of starting material, resulting in a lengthy extraction that consumes large volumes of organic

solvent. A typical Folch extraction of gangliosides in whole milk requires 5 mL of milk, generates 200 mL of solvent waste and takes over 24 h. A key advantage to an LC/MS based approach is sensitivity. Thus, the ganglioside extraction protocol was optimized to take advantage of this feature by reducing milk simple to 50 μL.

Decreasing the initial sample size yields benefits in terms of speed and decreased solvent waste. Other common and time consuming steps in ganglioside extraction include solid phase extraction and drying samples under nitrogen. Eliminating these steps would yield significant gains in terms of speed of analysis. Eliminating the solid phase extraction step also improves the analysis in terms of time and cost. Drying the entire Folch aqueous phase under nitrogen gave a statistically significant higher response than the other ($P<0.001$). Direct injection of the aqueous phase without drying gave an identical ganglioside profile and was comparable to samples extracted with a solid phase extraction step. This indicates that for a rapid evaluation of ganglioside profile, a direct injection of the Folch aqueous phase is sufficient. Thus the lengthy drying step can be eliminated if sensitivity is not critical. In summary, in comparison to conventional ganglioside extraction protocols, the sample size has been decreased from 5 mL to 50 μL, the solvent waste has been decreased from 200 mL to 1 mL and speed of analysis has been increased from days to hours.

Considering that pasteurization is a ubiquitous processing step in the preparation of commercial milk, the effect of temperature on ganglioside profile was also investigated. Neither pasteurization method altered the ganglioside profile in any pasteurized milk sample compared to unpasteurized milk sample. The MS response for the gangliosides increased in pasteurized milk samples when compared to the untreated milk sample. Thus pasteurizing of milk samples prior to analysis does not alter the ganglioside profile, but appears to improve

the extraction of gangliosides. Puente et al. (1996) studied the effect of heat treatment on bovine milk gangliosides content analyzed by HPTLC analysis and reported the bovine milk ganglioside content was not affected after heating bovine's milk during 65°C for 30 s.[38] Ewaschuk et al. (2011) studied the impact of pasteurization of gangliosides on human milk by HPTLC. Human milk samples pasteurized at 62.5°C for 30 min were not altered in ganglioside content.[39]

The present results agree with previous studies of other milk components.[40,41,42] Evers (2004) suggested the loss of glycosphingolipids on the MFGM since Bandyopadhyay et al. (1975) reported a remarkable decrease of sialic acids content on the MFGM on pasteurized buffalo milk at 63°C for 30 min.[40,41] Fatty acid composition and content was studied on milk sub-products. Progressive heat treatment altered the content of total saturated and unsaturated fatty acids.[42] Heat treatment would affect the stability of MFGM disordering the membrane increasing extraction of the gangliosides by Folch extraction, but not altering ganglioside profile extracted.

Consistent with the literature, GD3 was found to be the most abundant ganglioside class in whole milk, accounting for over 80% of ganglioside measured. The next abundant gangliosides were GT3 (15%) and GM3 (2.5%). GD3, GM3 and GT3 represent 80-90% of the total ganglioside content in whole bovine milk, while GD3 is the major ganglioside representing 60-70%[42] or 85% as reported by Laegreid, Oatness and Fuglesang (1986).[44] GM1, GM2 (2-6%), GD1 and GD2 have also been reported as ganglioside species in whole milk.[12,44,45] Iwamori et al. (2008) reported the presence of GM1, GM2, GM3, GD3, GT3 and others unspecified species.[45] Pan et al. (2000) found only four ganglioside species (GM3, GD3 and two unkown gangliosides) in bovine's milk.[46]

In colostrum samples, which are reported to have a different and quite variable ganglioside profile compared to whole milk,[43] the GM3 and GT3 gangliosides were more abundant, accounting for 12% and 19% of the total ganglioside, respectively. GD3 constitutes 55% of total ganglioside content. GD3 is the major ganglioside in whole milk even though GD3 content decreases from the beginning of lactation to the fifth day of lactation.[43] GM3 increases from the first and fifth day of lactation and GT3 is higher the first day of lactation and decreases.[43]

The ganglioside profile of buttermilk was, similar to whole milk, with GD3 accounting for 90% of total gangliosides, followed by GM3 (3%) and GT3 (4%). In terms of GD3 abundance, buttermilk yielded the highest response followed by colostrum and whole milk samples. Huang (1973) reported that buttermilk is an enriched source of gangliosides due to the content of milk fat globule membrane where gangliosides are mainly found in milk.[47] Takamizaka et al. (1986) found seven undefined ganglioside species in buttermilk.[48] GD3, GM3 and GT3 are detectable by TLC and represent 80-85% of the total gangliosides present.[48-50]

In conventional TLC analysis, TLC bands are extracted and analyzed by an additional method, typically by GC, to obtain information regarding the ceramide moiety. This procedure also requires sample derivatization and thus significantly increases analysis time and potential losses. A unique feature of an MS based approach to ganglioside analysis is the capability to obtain simultaneous information regarding ganglioside class and ceramide composition.

The high abundance of ceramides in bovine whole milk, buttermilk and colostrum containing an odd number of carbon atoms in the fatty acid chain. Although not typically present in humans, odd chain fatty acids are abundant in ruminant fats.[51-53] Huang (1973)

reported that buttermilk contains large amounts of large carbon chains (C22-C24) of fatty acids which indicates that there are no metabolic pathways to convert ceramide portions of milk gangliosides in cow's to other sphingolipids as in neuronal sphingolipids.[47] Hauttecoeur et al. (1985) found two different GD3 species distinguishable by the ceramide portion in buttermilk.[50] One species containing C22-C25 long chain fatty acids and a equimolar proportion of C16:0 and C18:0 sphingosine bases. The second species contained mainly fatty acids C16:0 and C18:0 sphingosine base. Ren et al. (1992) also found even number of fatty acid carbon chains from C14-C20 and odd number of long fatty acid carbon chains from C21-C25 in buttermilk.[49] Beermann et al. (2003) reported different fatty acid chains from C6-C25.[54] Bode et al. (2003) reported C23 fatty acid as the predominant fatty acid in GD3 and GM3 in bovine milk and other long chain fatty acids from C21-C25 are present in larger amounts.[55] Stage of lactation affects the fatty acid composition. Short chain fatty acids have been detected in small amounts in the early stage of lactation.[56] It has been suggested that short chain fatty acids are biosynthesized in the mammary gland while long chain fatty acids are coming from circulating lipoproteins. [56]

Comparing the relative MS signal does not necessarily reflect the true abundance of gangliosides in the sample of interest due to differences in MS response factors within various ganglioside classes. A quantitative LC/MS assay is needed to obtain absolute abundances for gangliosides measured and to obtain a more accurate ganglioside profile. This analysis requires ganglioside standards corresponding to each ganglioside class. Ideally, standards with identical sialic acid content, carbohydrate core, and ceramide composition would be analyzed with the samples at a range of concentrations. Given the heterogeneity in ganglioside composition, this is not feasible due to cost and lack of commercial availability of ganglioside

standards. For gangliosides, difference in number of sialic acids has been shown to be the most important factor affecting the MS response.[30] This difference in MS response is due to the effect of the negatively charged sialic acid residue on ionization efficiency in negative mode ESI. The carbohydrate core is also expected to play a significant role in determining response.

GM3, GD3 and GT3 values are consistent with literature values, but the amount of GM3 compared to GT3 differed. GM3 is consistently reported as the second most abundant ganglioside in milk products, whereas GT3 is the second most abundant species in this study (Table 3.1). A range of ganglioside values is reported in bovine milk, perhaps due to biological variability, extraction procedures and differences in analytical methodology used. Another potential reason for lower GM3 values is the propensity of GM3 to partition in the Folch organic phase. To investigate this partition the organic phase of the Folch extraction was subjected to a second Folch extraction. These values are also provided (Table 3.2). The organic Folch phase contains 30-50% of GM3, while containing only 1-7% of GD3 and 2.5% of GT3. Further Folch extractions of the organic phase yields more GM3, but with less abundance.

This method can be performed to analyze gangliosides in biological systems such as food and cells. This method developed can be used for a routine analysis since the method provides rapid results, saving solvent waste and utilizing a minimum amount of sample

3.5 References

1. Levery, S.B. (2005) Glycosphingolipid structural analysis and glycosphingolipidomics. *Methods of Enzymology*, 405:300-369.

2. Skaper, S.D.; Leon, A.; Toffano, G. (1989). Ganglioside function in the development and repair of the nervous system. From basic science to clinical application. *Molecular Neurobiol*ogy 3:173-199.

3. Sonnino, S.; Mauri, L.; Chigorno, V.; Prinetti, A. (2006). Gangliosides as components of lipid membrane domains. *Glycobiology*, 17:1R-13R.

4. Stoffel, W. (1971). Sphingolipids. *Annual Reviews of Biochemistry*, 40:57-82.

5. Lopez, P.H.; Schnaar, R.L. (2009). Gangliosides in cell recognition and membrane protein regulation. *Current Opinion in Structural Biology,* 19: 549-557.

6. Khatun, U.L.; Gayen, A; Mukhopadhyay, C. (2013). Gangliosides containing different numbers of sialic acids affect the morphology and structural organization of isotropic phospholipid bicelles. *Chemistry Physics of Lipids*,170-171:8-18.

7. Svennerholm, L.; Bruce, A.; Månsson, J.E.; Rynmark, B.M.; Vanier, M.T. (1972). Sphingolipids of human skeletal muscle. *Biochimistry et Biophysica Acta,* 280: 626-636.

8. Rodden, F.A.; Wiegandt, H., Bauer, B.L.(1991). Gangliosides: the relevance of current research to neurosurgery. *Journal of Neurosurgery,* 74:606-619.

9. Rueda, R. (2007). The role of dietary gangliosides on immunity and the prevention of infection. *British Journal of Nutrition,* 98 (Suppl 1): S68-S73.

10. Salcedo, J.; Barbera, R.; Matencio, E.; Alegría, A.; Lagarda, M.J. (2013). Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: an in vitro study. *Food Chemistry*, 136:726-734.

11. Rueda, R.; Maldonado, J.; Narbona, E.; Gil, A. (1998). Neonatal dietary gangliosides. *Early Human. Development, 53: S135-S47.*

12. Keenan, T,W. (1974). Composition and synthesis of gangliosides in mammary gland and milk of the bovine. *Biochimica et Biophysica Acta*, 337: 255-270.

13. van Echten, G.; Sandhoff, K. Ganglioside metabolism. (1993). Enzymology, topology, and regulation. *Journal of Biological Chemistry*, 268:5341-5344.

14. Gil, A.; Rueda, R. (2002). Interaction of early diet and the development of the immune system. *Nutrition Research Reviews,* 15:263-292.

15. Buccoliero, R.; Futerman, A.H. (2003). The roles of ceramide and complex sphingolipids in neuronal cell function. *Pharmacology Research*, 47: 409-419.

16. Potapenko, M.; Shurin, G.V.; de León, J. (2007). Gangliosides as immunomodulators. *Advances in Experimental Medicine and Biology,* 601:195-203.

17. Ledeen, R.W.; Wu, G. (2002). Ganglioside Function in Calcium Homeostasis and Signaling. *Neurochemistry Research*, 27:637-647.

18. Fong, B.; Norris, C.; McJarrow, P. (2011). Liquid chromatography high-resolution electrostatic ion-trap mass spectrometric analysis of GD3 ganglioside in dairy products. *International Dairy Journal,* 21:42-47.

19. Lacomba, R.; Salcedo, J.; Alegría, A.; Lagarda, J.M.; Barberá, R.; Matencio, E. (2010). Determination of sialic acid and gangliosides in biological samples and dairy products: a review. *Journal of Pharmaceutical and Biomedical Analysis,* 51:346-357.

20. Yu, R.K.; Ariga, T. (2000). Ganglioside analysis by high-performance thin-layer chromatography. *Methods of Enzymology*, 312:115-134.

21. Yates, A.J. (1988). Methods to Study the Biochemistry of Gangliosides. *Lipids and Related Compounds*. *Neuromethods*, 7:265-327.

22. Ferris, A.M.; Jensen, R.G. (1984). Lipids in human milk: a review. 1: Sampling, determination, and content. *Journal of Pediatric & Gastroenterology Nutrition*, 3: 108-122.

23. Jensen, R.G.; Clark, R.M.; Ferris, A.M. (1980). Composition of the lipids in human milk: a review. *Lipids*, 15:345-355.

24. Hundrieser, K.E.; Clark, R.M.; Jensen, R.G.; Ferris; A.M. (1984). A comparison of methods for determination of total lipids in human milk. *Nutrition Research,* 4:21-26.

25. Bouhours, J.F.; Bouhours, D. (1979). Galactosylceramide is the major cerebroside of human milk fat globule membrane. *Biochemical and Biophysical Research Commun*ucations, 88:1217-1222.

26. Timmen, H.; Dimick, P.S. (1972). Structure and synthesis of milk fat. X. Characterization of the major hydroxy compounds in milk lipids. *Journal of Dairy Science*, 55:919-925.

27. Jensen, R.G.; Clark, R.M. (1984). Methods of lipid analysis. *Journal of Pediatric Gastroenterology Nutrition*, 3:296-299.

28. Svennerholm, L.; Fredman, P. (1980). A procedure for the quantitative isolation of brain gangliosides. *Biochimica et Biophysica Acta*, 617:97-109.

29. Lee, H.; An, H.J.; Lerno, L.A. Jr.; German, J.B.; Lebrilla; C.B. (2011). Rapid Profiling of Bovine and Human Milk Gangliosides by Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *International of Journal of Mass Spectrometry*, 305:138-150.

30. Sørensen, L.K. (2006). A liquid chromatography/tandem mass spectrometric approach for the determination of gangliosides GD3 and GM3 in bovine milk and infant formulae. *Rapid Communication of Mass Spectrom*etry, 20:3625-3633.

31. Fong, B.; Norris, C.; Lowe, E.; McJarrow, P. (2009). Liquid chromatography-high-resolution mass spectrometry for quantitative analysis of gangliosides. *Lipids*, 44: 867-874.

32. Gillette, M.A.; Carr, S.A. (2013). Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. *Nature Methods*, 10: 28-34.

33. Folch, J.; Lees, M.; Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, 226: 497-501.

34. Williams, M.A.; McCluer, R.H. (1980). The Use of Sep-Pak C18 Cartridges During the Isolation of Gangliosides. *Journal of Neurochemistry*, 35: 266-269.

35. Jennemann, R.; Wiegandt, H. (1994). A Rapid Method for the Preparation of Ganglioside $G_{lac}2$ (GD3). *Lipids,* 29:365-368.

36. Suzuki, K. (1964). A simple and accurate micromethod for quantitative determination of ganglioside patterns. *Life Sciences,* 3:1227-1233.

37. Moore, K.H.; Ettinger, A.C.; Yokoyama, M.T. (2000). Variation in Ganglioside Content of Bovine Dairy Products . *Journal of Food Composition and Analysis*, 13:783-790.

38. Puente, R.; Garcia-Pardo, L.A.; Rueda, R.; Gil, A., Hueso, P. (1996). Seasonal variations in the concentration of gangliosides and sialic acids in milk from different mammalian species. *International Dairy Journal*, 6:315-322.

39. Ewaschuk, J.B.; Unger, S.; O'Connor, D.L.; Stone, D.; Harvey, S., Clandinin, M.T.; Field, C.J. (2011). Effect of pasteurization on selected immune components of donated human breast milk. *Journal of Peritanology,* 31:593-598.

40. Evers, J.M. The milk fat globule membrane—compositional and structural changes post secretion by the mammary secretory cell. *International Dairy Journal,* 14: 661-674.

41. Bandyopadhyay, A.K.; Ganguli, N.C. (1975). Effect of Heating and Chilling Buffalo Milk on the Properties of Fat Globule Membranes Proteins. *Journal of Food Science Technology,* 12:312-315.

42. Janbandhu, T. J.; Khedkar, C. D.; Ajit, S. (2000) Effect of heating and chilling of recombine milk on fatty acid composition of fat globule membrane lipids. *Indian Journal of Dairy Science*, 53:231-234.

43. Puente, R.; García-Pardo, L.A.; Hueso, P. (1992). Gangliosides in bovine milk. Changes in content and distribution of individual ganglioside levels during lactation. *Biological Chemistry Hoppe Seyler*, 373:283-288.

44. Laegreid, A.; Otnaess, A.B.; Fuglesang, J. (1986). Human and bovine milk: comparison of ganglioside composition and enterotoxin-inhibitory activity. *Pediatric Research*, 20:416-421.

45. Iwamori, M.; Takamizawa, K.; Momoeda, M.; Iwamori, Y.; Taketani, Y. (2008). Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin. *Glycoconjugate. Journal,* 25: 675-683.

46. Pan, X. L.; Izumi, T. (2000). Variation of the ganglioside compositions of human milk, cow's milk and infant formulas. *Early Human Development,* 57: 25-31.

47. Huang, R.T.C. (1973). Isolation and characterization of the gangliosides butter milk. *Biochimica et Biophysica Acta*, 306:82–84.

48. Takamizawa, K.; Iwamori, M.; Mutai, M.; Nagai, Y. (1986). Gangliosides of bovine buttermilk. isolation and characterization of a novel monosialoganglioside with a new branching structure. *Journal of Biological Chemistry,* 261:5625-5630.

49. Ren, S.; Scarsdale, J.N.; Ariga, T.; Zhang, Y.; Klein, R.A.; Hartmann, R., Kushi, Y.; Egge, H.; Yu, R.K. (1992). 0-acetylated gangliosides in bovine buttermilk. Characterization of 7-O-acetyl, 9-O-acetyl, and 7,9-di-O-acetyl GD3. *Journal of Biological Chemistry*, 267: 12632-12638.

50. Hauttecoeur, B.; Sonnino, S.; Ghidoni, R. (1985). Characterization of two molecular species GD3 ganglioside from bovine buttermilk. *Biochimica et Biophysica Acta*, 833:303-307.

51. Garton, G.A. (1963). The composition and biosynthesis of milk lipids. *Journal of Lipids Research*, 4:237-254.

52. Jensen, R.G.; Ferris, A.M.; Lammi-Keefe, C.J.; Henderson, R.A. (1990). Lipids in Bovine and Human Milks: A comparison. *Journal of Dairy Science*, 73:223-240.

53. Vlaeminck, B.; Fievez, V.; Cabrita, A.R.J.; Fonseca, A.J.M.; Dewhurst, R.J. (2006). Factors affecting odd- and branched-chain fatty acids in milk: A review. *Animal Feed Science and Technology*, 131:389-417.

54. Beermann, C.; Röhrig, A.K.; Boehm, G. (2003). Chemical and enzymatic transacylation of amide-linked FA of buttermilk gangliosides. *Lipids,* 38:855-864.

55. Bode, L.; Beermann, C.; Mank, M.; Kohn, G.; Boehm, G. (2004). Human and bovine milk gangliosides differ in their fatty acid composition. *Journal Nutrition,* 134:3016-3020.

56. Martín, M.J; Martín-Sosa, S.; Hueso, P. (2001). Bovine Milk Gangliosides: Changes in Ceramide Moiety with Stage of Lactation. *Lipids*, 36:291-298.

57. Suzuki, K. (1964). A simple and accurate micromethod for quantitative determination of ganglioside patterns. *Life Science*, 3, 1227-1233.

58. Farwanah, H.; Kolter, T. (2012). Lipidomics of glycosphingolipids. *Metabolites*, 2(1):134-164.

CHAPTER IV. Exogenous gangliosides alter ganglioside content of apical and basolateral membrane domains in human gastric epithelial cells

4.1 Introduction

Gangliosides are a subclass of glycosphingolipids containing at least one sialic acid moiety.[1] Gangliosides are inserted in the plasma membrane of mammalian cells.[1] Distribution, location and functionality is determined by the number and configuration of sugar units and sialic acid molecules.[2]

Exogenous gangliosides are incorporated from diet into plasma membrane.[3] Incorporation of exogenous gangliosides has been studied by adding gangliosides into culture media of a diversity of cell lines such as pituitary tumor cells,[4] MDCK cells,[5] cerebellar granule cells,[6,7] neuroblastomas and gliomas,[8] astrocytes,[9] hippocampal neurons,[10] chick neurons,[11] fibroblasts,[12,13] blood cells,[14] leukemic and non leukemic lymphocytes,[15] HeLa cells,[16] and intestinal cells.[17] Studies of incorporation of gangliosides into cells demonstrate exogenous ganglioside catabolism and catabolic intermediates,[18] utilization and recycling of catabolic intermediates (lactosylceramide, glucosylceramide, ceramide),[6,18] dietary ganglioside precursors of new gangliosides,[6] factors affecting ganglioside uptake (time, concentration of gangliosides, presence of serum, temperature, pH),[19] different fates of gangliosides,[17] and protein carriers mediating ganglioside uptake.[19-23]

Gangliosides have structural roles since ceramides, present in gangliosides, influence the structure of membrane rafts and caveolae.[24] Gangliosides are also involved in functional roles. GM3 is the main ganglioside of the human body, a component of all non-neuronal cells[25,26] and regulates ganglioside content in cells as GM3 is a precursor for all more complex gangliosides.[27] GM3 is formed by sialyltransferase I or SAT I (GM3 synthase, ST I) . GM3 is

converted to GD3 by GD3 synthase (ST8Sia I or SAT II) for the addition of one sialic acid moiety to GD3.[28] GD3 is a disialoganglioside, precursor of b- and c-ganglioside series.[27-30] Degradation of gangliosides is a sequential removal of sugar moieties. Exohydrolases remove carbohydrates from the hydrophilic end of the ceramide core.[31,32] The first step is degradation of the sialic acid terminal residue in the polysialogangliosides[31] (Fig. 4.1). Transformation of polysialogangliosides by the corresponding sialidase occurs in order to form monosialogangliosides.[32] GD3 ganglioside can be converted to GM3 ganglioside by neuraminidase I (neu-ase I).[33]

GM3 is involved in modulation of cell proliferation, cell adhesion, cell recognition, apoptosis.[27,34] GM3 inhibits growth of tumors by controlling expression of factors and altering the cell cycle.[27,35,36] GM3 regulates activity of certain cell receptors such as fibronectin, integrins and selectins.[37] GM3 is involved in signal transduction processes. GM3 is a main component of detergent insoluble glycosphingolipid enriched microdomains (DIGEM) and GM3 is related to transducer molecules such as protein receptor-associated kinases.[38] GM3 is also involved in cell adhesion functions since GM3 increases interaction among cells and the extracellular matrix.[27]

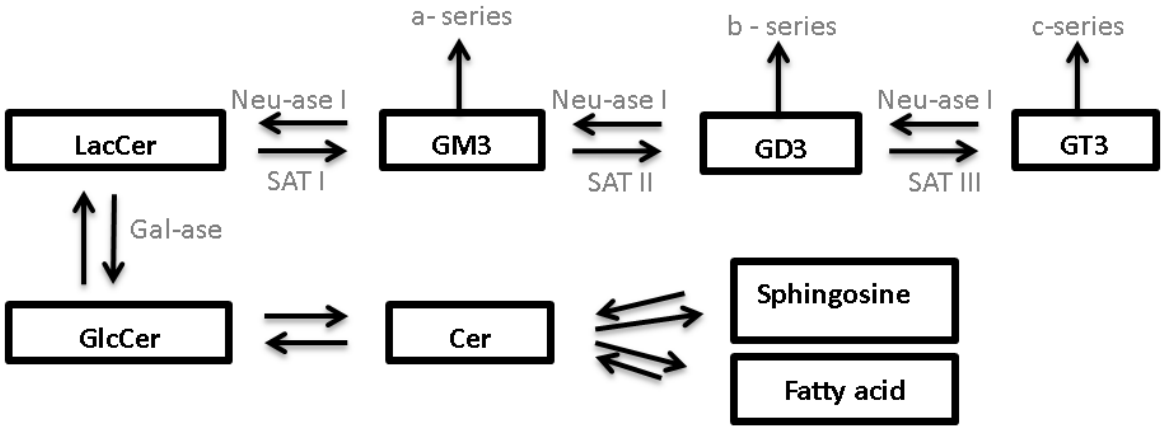


Figure 4.1 Schematic diagram of ganglioside metabolic pathway (modified from reference 4,33).

GD3 regulates cell proliferation, apoptotic mediators, cell adhesion, anti-inflammatory mediators and suppressor of pro-inflammatory signals in intestine.[34,39-42] GD3 is present in high amounts during development, specially in embryonic brains but decreases after birth.[28,43] In some pathological conditions GD3 is overexpressed such as in tumor cells and artherosclerosis.[27] GD3 is present in lower amounts in normal tissues except for placenta and thymus.[28,44,45] GD3 has been related to cell death.[45] Changes in the mitochondria is a main step for cell death. Mitochondria are the target for intracellular GD3 which releases apoptogenic factors such as ROS, cytochrome c, caspase activation and changes in the mitochondrial potential.[28,45-47] GD3 might be an extracellular regulator for anti-inflammatory processes.[46] GD3 also has roles in cell growth and proliferation. Membrane GD3 improves cell permeability to ions and metabolites.[48] GD3 is crucial in cell signalling since GD3 regulates the Src-Family kinase which is important for brain development and function.[49]

In human stomach, GD3 and GM3 are the main gangliosides.[50,51] GM3 is significant higher in antrum mucosa than in fundus mucosa.[51] Natomi et al. (1990) evaluated the ganglioside content in human gastric mucosa in fundic and antral mucosa and found that GM3 and GD3 are the most abundant gangliosides using a TLC method.[51] Keranen (1975) reported the presence of GM3, GM2, GD3, GM1, GD1a, GD1b, GT1 and two unknown ganglioside species were determined by GC-MS. [50]

The ability of gastric cells to take up GM3 and GD3 has not been addressed. NCI-N87 cell line is recognized as an excellent model for gastric studies.[52] NCI-N87 forms tight monolayers secreting E-cadherin and zonula occludens-1 (ZO-1), expressing gastric zymogens, secreting pepsinogen and gastric lipase, expressing mucin-6 glycoprotein and is permeable to gastric drugs.[53,54]

The present research was designed to determine whether exogenous ganglioside increases the content of individual gangliosides and phospholipids [phosphatidylcholine (PC) and phosphatidylethanolamine (PE)] in human gastric epithelial cells when NCI-N87 cell line was modified using different concentrations of ganglioside treatments. The objective of this research was also to determine if dietary gangliosides induce changes in the apical and basolateral membrane domain of NCI-N87 human gastric cell line.

4.2 Materials and Methods

4.2.1 Materials. Ganglioside mix enriched powder was obtained from Fonterra (Cambridge, New Zealand; average molecular weight 1,542 g/mol, GD3 91%, GM3 4.5%, GT3 2.5%, GM1 1%, GD1 0.5%, GT2 0.5%). All cell culture flasks were purchased from Costar (Cambridge, MA). Trypsin-EDTA (0.025%), Fetal Bovine Serum (FBS), AB-human serum, penicillin (10,000 units/mL), trypan blue (0.04%) streptomycin (10,000 μg/mL), RPMI-1640, 1M HEPES, 100 mM sodium pyruvate were ordered from Gibco (Life Technologies, Burlington, ON, Canada). LC/MS solvents were of LC/MS grade and solvents for ganglioside extraction were ACS grade (Fisher Scientific Company, Ottawa, ON, Canada).

4.2.2 Cell culture. NCI-N87 [NCI-N87] (ATCC® CRL-5822™ cell line) were grown under humidified atmosphere, 5% CO_2, and 37°C. Cells were grown for passages 23-32 as adherent monolayers in T-flasks and cultured in RPMI-1640 supplemented with 5% (v/v) FBS heat inactivated, 2% (v/v) AB-human serum, 2.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1 mM sodium pyruvate, 1% penicillin-streptomycin. Medium was changed every 2-3 days. Cell number and cell viability was estimated with a hemocytometer by the trypan blue

exclusion method. Trypan blue exclusion method is based on live cells have intact plasma membranes and do not take up certain dyes such as trypan blue. In dead cells, trypan blue penetrates damaged plasma membranes and appear blue under microscope.

4.2.3 Purification of gangliosides. Total lipids were extracted from a ganglioside mix enriched powder by a Folch extraction.[55] Ganglioside mix enriched powder (35 g) was dissolved in 700 mL chloroform-methanol 2:1 (v/v). This solution was shaken vigorously on a shaker for 30 min. After shaking, 210 mL of 0.025% $CaCl_2$ was added and this solution was shaken vigorously on a shaker for 30 min. The solution was left 48 h at 4°C. The upper aqueous phase was purified by solid phase extraction (SPE) passing through Sep-Pak C18 cartridges (Waters Corporation, Milford, MA). Prior to sample loading, cartridges were prewashed with 5 mL of Milli-Q water, 5 mL of methanol, 5 mL of chloroform-methanol (2:1, v/v), 5 mL of methanol and 5 mL of Milli-Q water. Aliquots of 50 mL of the aqueous phase were passed through a prewashed Sep-Pak C18 cartridge and then washed with 10 mL of Milli-Q water to remove salts. Gangliosides were eluted with 2 mL of methanol and 10 mL of chloroform-methanol (2:1, v/v), dried under nitrogen gas. This extraction was used for ganglioside mix treatment and for GM3 and GD3 separation.

4.2.4 Gangliosides separation. GM3 and GD3 ganglioside species used to treat cells were obtained by TLC. TLC silica gel G plates (20x10 cm, 1000 μm) (Analtech Inc., Newark, DE) were used to separate individual gangliosides based on the degree of sialylation. TLC plates were washed with methanol, dried at room temperature and washed again with hexane. TLC plates were activated by heating at 110°C for 60 min. The solvent system used for TLC

separation of individual gangliosides was chroloform/methanol/28% (w/v) ammonia/H_2O (60:35:7:3, by volume). Standard separation chambers were used. Gangliosides were visualized using 8-anilino-1-naphthalene-sulfonic acid (ANSA) 0.1%. Bands containing gangliosides were removed and gangliosides were extracted from silica by vortexing and sonicating in methanol. Tubes were centrifuged for 10 min at 800 xg and an aliquot of supernatant was subjected to LC/MS analysis to confirm ganglioside identity. Standard gangliosides were prepared using this method and subjected to quantitative analysis using the resorcinol–HCl based assay.[56] The principle of resorcinol-HCl based assay is to detect the sialic acid bonds and produce a blue-violet color.

4.2.5 Optimization of sample size and pH to detect gangliosides in gastric cells by LC/MS. NCI-N87 cell stocks stored in liquid nitrogen were thawed at 37 °C for 2 min. NCI-N87 cells were seeded in T25 flasks and fed with RPMI-1640 media was decreased pH to pH 6.8. NCI-N87 were grown under humidified atmosphere at 37 °C. When NCI-N87 cells were 80-90% confluent, cells were transfered to 12 well plates, 6 well plates, T-25 flasks and T-75 flasks. When reaching confluence, cells were quantified by trypan blue exclusion method. A variety of number of NCI-N87 cells from $1x10^6$ to 35 $x10^6$ were prepared. Ganglioside were extracted with Folch method (see 4.2.7) and were analyzed by LC/MS (see 4.2.8).

4.2.6 Ganglioside uptake by human gastric epithelial cells. NCI-N87 cells were seeded at a cell density of $1x10^5$ cells/cm^2 on a T-25T flask. Monolayers were grown to confluence and 24 h later cells were treated with ganglioside-enriched media that was changed at 24 h and discarded at 48 h. Media contained ganglioside mix enriched powder at different

concentrations (0, 10, 20, 30 μg/mL), GD3 (99% of purity) at different concentrations (0, 10, 30 μg/mL), GM3 (98% of purity) (0, 10 μg/mL). These concentrations were selected base on the content of gangliosides in milk products. Cells were incubated at standard conditions. After 48 h, cells were washed three times with PBS-10% FBS followed by another washing with PBS (three times) and harvested with 0.025% trypsin-EDTA (Gibco, Burlington, ON, Canada). After harvesting, cells were counted by trypan blue exclusion method. Cells were centrifuged with PBS at 200 xg for 5 min. The cell pellet was weighted and kept for ganglioside and phospholipid analysis.

To determine the uptake of gangliosides in media over time, aliquots (100 μL) of media were collected at 8 and 24 h (day 1), media was changed at 8 and 24 h (day 2) and also 100 μL aliquots of media were collected at day 2. These samples were kept at -80°C for ganglioside analysis.

4.2.7 Ganglioside and phospholipid extraction. Cell pellets were extracted according to a modified Folch method for extracting brain lipids.[55] This method consisted of a chloroform: methanol (2:1, v/v) extraction with a solvent to aqueous ratio of 20:1. The mixture was homogenized, sonicated (10 min) and shaken vigorously (20 min). After shaking, $CaCl_2$ (0.025%, w/v) was added to the mixture followed by another 20 min of shaking. The solution was centrifuged to allow the phases to settle. Aqueous phase was dried and reconstituted with 500 μL of methanol:H_2O (1:1) for ganglioside analysis by LC/MS analysis. The 50% of the organiz phase was dried and resuspendend in 1.5 mL of 75% acetonitrile/25% water for phospholipid analysis by LC/MS. The other 50% of the organic phase was used for total phosphorus assay determination. Gangliosides from ganglioside enriched media were

extracted by Folch extraction. Each 100 μL aliquot was mixed with 500 μL of chloroform:metanol (2:1), shaken for 20 min and sonicated (10 min). After shaking, 112 μL of 0.025% (w/v) $CaCl_2$ was added and followed by another 20 min of shaking. The solution was centrifuged and the aqueous phase was dried under N_2 and resuspended in 250 μL of methanol: H_2O (1:1).

4.2.8 Analysis of ganglioside content and composition by LC/MS. Prior to MS analysis, ganglioside extracts were subjected to reverse-phase chromatography with a Poroshell 120 EC-C18 column (3.0 x 50 mm, 2.7 μm particle size) using an Agilent 1260 Infinity LC system (Santa Clara, CA). Two mobile phases were used; phase A was composed of a 50:50 water/isopropanol mixture containing 5 mM ammonium acetate and 0.05% acetic acid, phase B consisted of 100% methanol. A gradient elution consisting of increase in mobile phase B from 70% to 95% over 6 min was used, with a total LC run time of 12 min at a flow rate of 0.5 μL/min.

4.2.9 Mass spectrometry. All MS measurements were obtained using an Agilent 6430 Triple-Quad LC/MS system (Santa Clara, CA) operating in negative ion mode. Deprotonated gas-phase ions of various ganglioside species were obtained using electrospray ionization (ESI), with the electrospray needle held at -4500 V. The MS was operated in multiple reaction monitoring mode (MRM). A library of theoretical precursor ions was generated for over 600 gangliosides of specific ceramide and carbohydrate composition. 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 CID. Gangliosides readily lose

a sialic acid moiety when subjected to CID, thus the second quadrupole mass filter was set to only allow this characteristic fragment ion (*m/z* 290) to reach the detector. The CID and ion source voltages for each ganglioside class (GM, GD, and GT) were optimized using the Agilent Optimizer software. Data acquisition and analysis was carried out using the Agilent Mass Hunter software package.

4.2.10 Analysis of total phosphorus content. Phospholipids were separated from the organic phase of Folch extraction (using 50 % of this phase) by Thin Layer Chromatography (TLC) on silica gel G plates (20x20 cm, 250 μm) (Analtech Inc., Newark, DE) using a chloroform/methanol/water (65:35:6, by vol.) solvent system. Analysis of the total amount of phospholipid was performed by determination of phosphorus assay method of Bartlett (1959).[99] Seventy percent perchloric acid was added to samples and standards and were incubated for 60 min at 180°C. MilliQ water, 2.5% ammonium molybdate and 10% ascorbic acid was added to samples and standards. Samples and standards were incubated at 95°C for 15 min in a water bath. Samples were spun at 200 xg for 5 min. Standards were prepared from sodium phosphate. Absorbance was read at 750 nm in a spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA).

4.2.11 Analysis of individual phospholipids by LC/MS. Prior to MS analysis, phospholipid extracts were subjected to normal phase chromatography with an Agilent Zorbax RX-Sil column (3.0 x 100 mm, 1.8 μm particle size) using an Agilent 1260 Infinity LC system (Santa Clara, CA). 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

minutes at a flow rate of 0.5 μL/min. 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 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 CID. PC species readily undergo head group specific fragmentation, so the second mass filter was set to monitor *m/z* = 184. For PE species, the fragment with the neutral loss of 141 mass units, the second mass filter monitored the precursor *m/z* minus 141. The CID and ion source voltage for each phospholipid class was optimized using the Agilent Optimizer software.

4.2.12 Determination of cell protein. Cell protein was extracted with Radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Scientific, IL, USA). The amount of cell protein was estimated by bicinchoninic assay (BCA) using Pierce™ BCA Protein Assay Kit (Thermo Scientific, IL, USA). Absorbance at 562 nm was measured by a spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA). The principle for the BCA method is that protein reduce Cu^{+2} to Cu^{+1} in an alkaline solution (the biuret reaction) and produces a purple color by bicinchoninic acid.

4.2.13 Optimization of homogenization buffer to membrane fragmentation method. NCI-N87 cells were seeded at a cell density of $1x10^5$ cells/cm^2 on a T-25T flask. Monolayers were grown to confluence, were dissociated using trypsin and counted to be around $20x10^6$ of cells. Before apical and basolateral membrane isolation, two homogenization buffers were tested**.**

One homogenization buffer contained 500 mM sucrose, 90 mM potassium acetate, 2 mM magnesium acetate, 20 mM HEPES-KOH (pH 8.0) and second homogenization buffer contained 5 mM Tris. NCI-N87 cells were suspendend in 400 μL of one of theses homogenization buffers. NCI-N87 were homogenizated by 1) dounce homogenization for 10 min, 2) passing through needle with syringe (22G and 27G) twenty passages with each needle, 3) bead homogenizer for 10 min, 3) dispersor (Ultra Turrax, Wilmingtion, USA) for 10 cycles (each cycle of 1 min), 4) blender using autoclaved tips (OMNI International, Kennesaw, USA) for 15 times (5 s each time) and 5) sonicator (Artek, Farmingdale, NY, USA) (cycle set at 35%) for 2-3 cycles of 10 s each one and allowing 10 s between cycle. NCI-N87 cells were kept in ice during homogenization. After homogenization, the efficacy of sonication was monitored under microscope using trypan blue exclusion method.

4.2.14 Apical and basolateral membrane isolation. To determine whether ganglioside uptake modified the apical or basolateral membrane were used gastric cells treated with ganglioside mix enriched powder at concentrations of 0, 10, 20, 30 μg/mL treated and incubated as in section 4.2.6. Membranes were separated by differential centrifugation and density gradient centrifugation from a modified protocol of Culp and Forte (1981).[57] Cell pellets were lysed with homogenizer buffer containing 500 mM sucrose, 90 mM potassium acetate, 2 mM magnesium acetate, 20 mM HEPES-KOH (pH 8.0) (homogenization buffer modified from Optiprep protocols),[58] complete protease inhibitor cocktail and sonicated (cycle set at 35%) at 4°C for 3 cycles of 10 s each one and allowing 10 s between cycle.[59] The efficacy of sonication was monitored under the microscope. The homogenate was centrifugated at 800 xg (Thermo Scientific Sorvall Legend Micro 17R Centrifuge) for 8 min

to remove unbroken cells, debris and mitochondria. The supernantant was brought to 5 mM EDTA and was centrifuged at 17000 xg (Thermo Scientific Sorvall Legend Micro 17R Centrifuge) for 15 min. The supernantant was used to obtain the apical fraction and the pellet was used to obtain basolateral fraction. The supernantant was resuspended in isolation buffer containing 250 mM of sucrose and 5 mM Tris-HCl (pH 8.0) and was spun at 150,000 xg (Beckman CoulterL-100XP, SW 55 rotor) for 90 min; the pellet was the apical fraction. The basolateral fraction was obtained by density gradient centrifugation. The pellet was suspended with a density gradient buffer composed of 3 parts of 250 mM sucrose, 5mM Tris-HCL (pH 8.0) and 5 mM EDTA and mixed with 1 part of 20% (w/v) of dextran (MW 500,000). This suspension was layered on top of a 20% dextran solution topped with the density gradient buffer and spun at 120,000 xg (Beckman Coulter L-100XP, SW-55Ti rotor) overnight. The three fractions were diluted with isolation buffer and centrifuged at 150,000 xg (Beckman Coulter L-100XP, SW-55Ti rotor) for 90 min. Pellets were reconstituted with PBS and stored at -80°C.

4.2.15 Confirmation of purity of apical and basolateral fractions. Fractions were analyzed for determination of carcinoembryonic antigen (CEA) to confirm separation of apical membrane domain. NCI-N87 cell line expresses CEA antigen. CEA antigen has been found located on apical membranes domains in different cell lines.[60-63] CEA was evaluated by chemiluminiscence immunoassay (CLIA) using CEA (human) CLIA kit (Abnova, Walnut, CA). Samples and standards were incubated with an enzyme conjugate reagent at room temperature. After 60 min, samples and standards were washed. Subsequently, a

chemiluminescence substrate solution was added. Chemiluminiscence was read with a chemiluminescence microwell reader (Perkin Elmer Envision 2104 Multilabel Reactor).

Fractions were analyzed for the determination of Na^+/K^+-ATPase assay to confirm the separation of basolateral membrane domain. Na^+/K^+-ATPase assay was performed as described by Suhail and Rizvi, 1987.[64] The Na^+/K^+-ATPase assay is based on the determination of the concentration of inorganic phosphate produced by the hydrolisis of ATP by Na^+/K^+-ATPase in the presence or abscence of ouabain (Na^+/K^+-ATPase inhibitor).[64]

4.2.16 Statistical analysis. All data are expressed as mean$\pm$SD from two different cell passages. Significant differences between control and experimental groups were determined by a one-way ANOVA and using SPSS statistical software version 22. Significant effects for ganglioside treatments were determined by Games-Howell test (test for non-homogeneity of variance). Significant treatments for GM3 treatment was determined by Student's T-test. Significant differences of the effect of ganglioside treatments in GD3 treatment, apical and basolateral membrane separation, effect of phospholipids were determined by Tukey test. Significant differences for the effect of uptake ganglioside over the time were determined by repeated measures ANOVA. Significant differentences for ganglisoide species at apical and basolateral membrane were determined by 2-way repeated measures ANOVA. Differences between all treatments were considered statistically significant at $P<0.05$

4.3 Results

4.3.1 Optimization of sample size and pH to detect gangliosides in gastric cells by LC/MS. Different number of NCI-N87 cells were evaluated to determine ganglioside content.

Gangliosides extracted from up to $15x10^6$ NCI-N87 cells gave a valid MS response. To resemble the characteristics of the stomach, pH in the cultured media was decreased from pH 7.5 to 7.6 to pH 6.8. Two to four hours after meals, the gastric pH is around pH 5.8-6.7 and goes down to the basal state of 2.0.[54] In the fasted state, the gastric pH is 1.1-1.7, while in the fed state; the pH is around 5.0.[54,66,67] GM3 and GD3 are stable under low pH (pH 3-5). In infant stomach, up to 80% of sialic acid in GM3 and GD3 is absorbed intact in the intestine after passing through the acidic stomach.[68,69]

4.3.2 Ganglioside profile of human gastric epithelial cell line NCI-N87. Twelve different ganglioside species were scanned and eight different ganglioside species were determined in the gastric cell line NCI-N87 (Fig. 4.2) using $3x10^9$ cells. Monosialogangliosides comprised around 65% of total gangliosides. Disialogangliosides are the second most abundant comprising more than 22%. Trisialogangliosides are less abundant ganglioside species (13%).

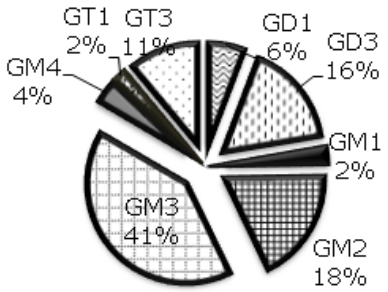


Figure 4.2 Total relative ganglioside content of NCI-N87 cell line. More than 600 ganglioside species were run by LC/MS and 8 different ganglioside species were determined. Data is present as % values of total gangliosides.

4.3.3 Modification of ganglioside content in human gastric cell line NCI-N87. Human gastric epithelial cells NCI-N87 were cultured on the apical membrane with a ganglioside mix enriched powder media at different concentrations 0, 10, 20 and 30 μg/mL and exhibited a significant difference among different concentration treatments ($P<0.001$) (Fig. 4.3). GD3 and GM3 content increased in a concentration dependent manner. At 30 μg/mL of ganglioside mix enriched powder treatment, GD3 increased 70 fold compared to GD3 at 0 μg/mL ($P<0.001$) (Fig. 4.3). At 30 μg/mL of ganglioside mix enriched powder treatment, GM3 increased 13 fold compared to GM3 at 0 μg/mL ($P<0.01$) (Fig. 4.3). Other gangliosides such as GD1 and GT3 were evaluated qualitatively but not quantitavely. Relative MS abundance increased in a concentration dependent manner for GD1 and GT3 (Fig. 4.4). Human gastric epithelial cells were cultured with GD3-enriched media at concentrations of 0, 10 and 30 μg/mL (Fig. 4.5) or GM3-enriched media at concentrations of 0 and 10 μg/mL (Fig. 4.6), on the apical side. In gastric cells treated with GD3 enriched media at 10 μg/mL, GM3 increased 4 fold compared to 0 μg/mL ($P<0.0001$). However, in gastric cells treated with GD3 at 30 μg/mL, GD3 increased 5 fold ($P<0.01$). For GD3 enriched media, GD3 increased 12 fold ($P<0.0001$) at 10 μg/mL and at 30 μg/mL, GD3 increased 15 fold ($P<0.001$) compared to 0 μg/mL. For cells cultured with GM3 enriched media, GD3 increased 8 fold compared to 0 μg/mL ($P<0.0001$), while GM3 increased 4.5 fold ($P<0.00001$) (Fig. 4.5). GD3 and GM3 species found in human gastric cells are illustrated (Table 4.1 and 4.2). Media used to culture human gastric cells contained GD3 and GM3 composed of saturated, monounsaturated and polyunsaturated ceramides varying the carbon chain length (28-43 carbons). Few ganglioside species were incorporated into the cells. In the GD3 treatments (10 μg/mL and 30 μg/mL), gangliosides incorporated into cells were mainly gangliosides present in higher amounts

contained in the media (Table 4.1). For the GM3 treatment, three different GD3 species containing C37:2, C37:1 and C38:0 were found in cells (Table 4.2). GM3 C34:1 was the main ganglioside species found in cells after the three ganglioside treatments (GD3 (10 and 30 μg/mL) and GM3 (10 μg/mL). GM3 C34:1 was the exogenous ganglioside species incorporated into the media present in higher amounts. GM3 C34:2 was the only polyunsaturated ceramide found in gastric epithelial cells.

4.3.4 Uptake of ganglioside by human gastric epithelial cells over time. On the apical side, human gastric epithelial cells were cultured with GD3 or GM3 enriched media for 48 h. After 24 h, media was discarded and changed. Media samples were taken at 8, 24, 32 and 48 h to monitor whether ganglioside uptake is time dependent for exogenous GM3 and GD3 (Table 4.3). After 8h, 50% of exogenous GD3 and GM3 (concentration of 10 μg/mL) was incorporated into the cell and 60% of exogenous GD3 at higher concentration was incorporated into the cell ($P<0.02$). After 24 h, 70% of the gangliosides (GM3 or GD3) were incorporated into the cell. Gangliosides were incorporated 2.3 $\mu g/10^6$ gastric cells/24 h or 7 $\mu g/10^6$ gastric cells/24 h, for 10 μg/mL or 30 μg/mL, respectively. After media was changed on day 2; at 8 h <50% of GD3 and GM3 was incorporated into the cells ($P<0.02$). At 24 h on day 2, 90% of gangliosides were incorporated into the cell. Gangliosides were incorporated 2.7 $\mu g/10^6$ gastric cells/24 h or 9 $\mu g/10^6$ gastric cells/24 h, for 10 μg/mL or 30 μg/mL, respectively (Table 4.3). Repeated measures ANOVA revealed an overall interaction between time and different concentrations ($P<0.0001$).

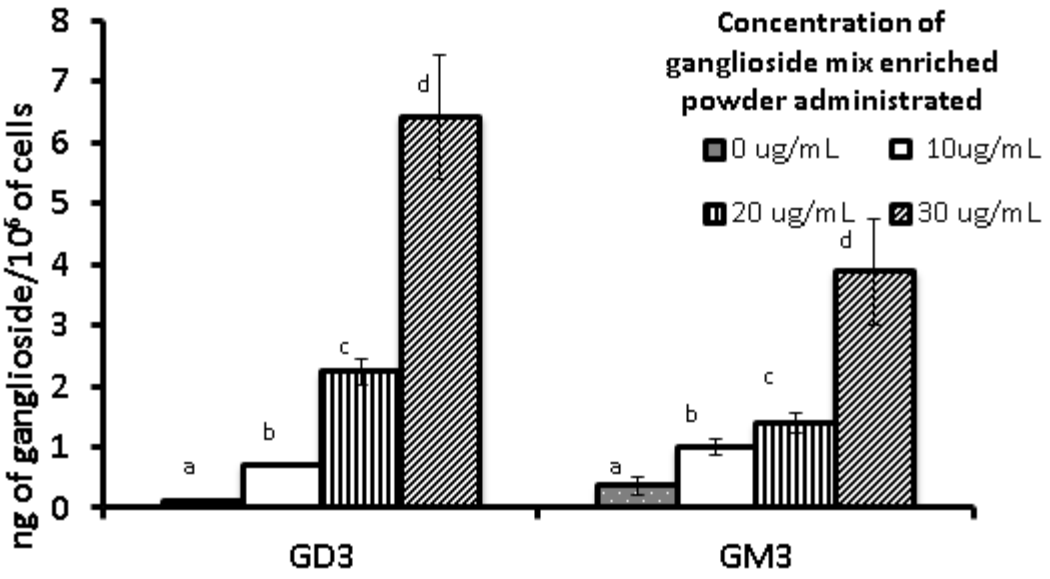


Figure 4.3 Amount of GD3 and GM3 taken up by human gastric cells (NCI-N87) after 48h exposure to different concentrations (0-30 μg/mL) of ganglioside mix enriched powder media (91% GD3, 4.5% GM3). Data represents mean±SD from 3 experiments in triplicate. Different letters (a,b,c,d) indicate significance for GD3 at $P<0.001$ and for GM3 at $P<0.05$.

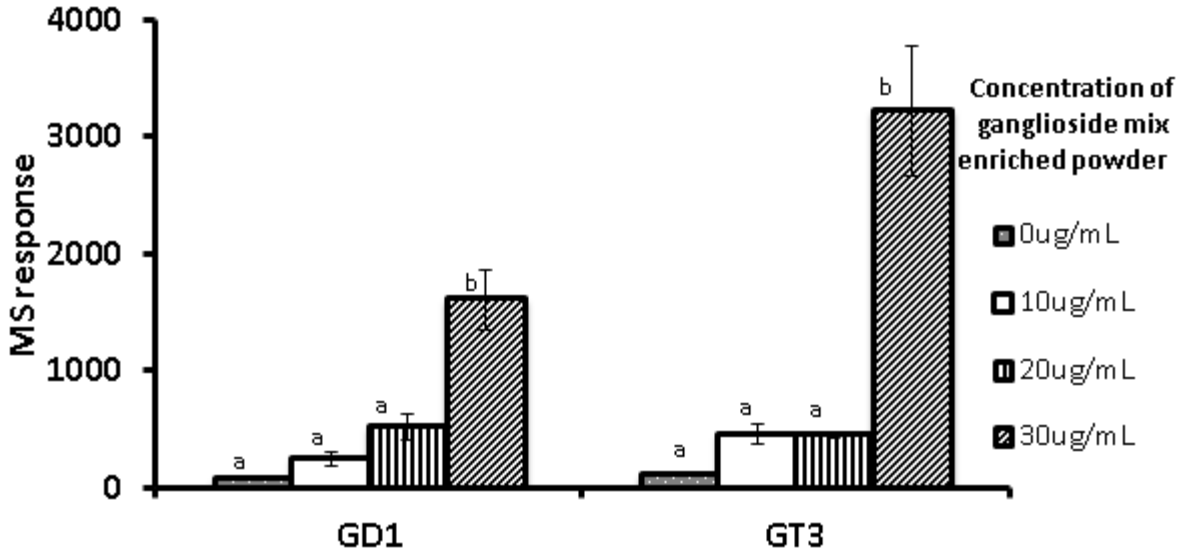


Figure 4.4 Relative MS response of GD1 and GT3 taking up by human gastric cells (NCI-N87) after 48 h exposure to different concentration 0-30 μg/mL of ganglioside mix enriched powder media (91% GD3, 4.5% GM3). Data represents mean±SD from 3 experiments in triplicate. Different letters (a,b,c,d) indicate significance for GD1 at $P<0.01$ and for GT3 at $P<0.01$.

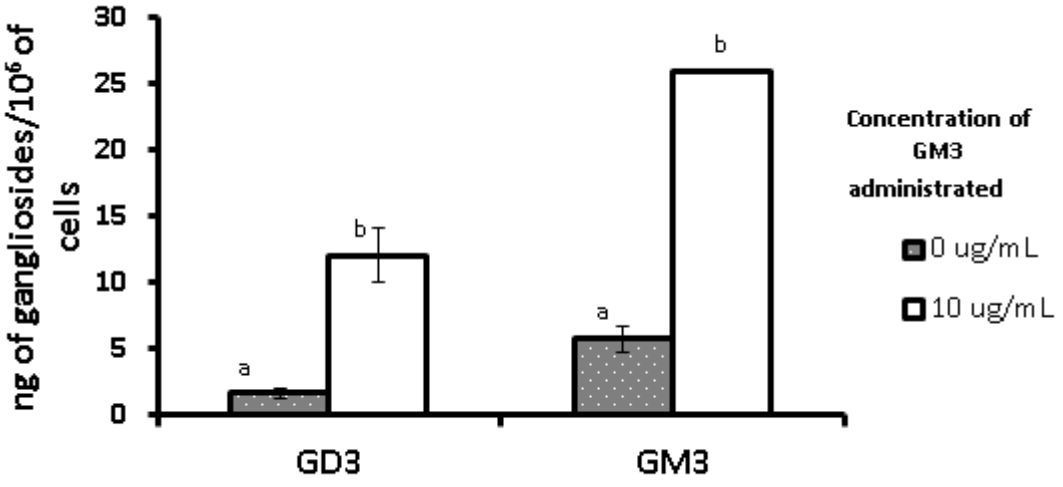


Figure 4.5 Amount of GD3 and GM3 taken up by human gastric cells (NCI-N87) after 48 h exposure to two different concentrations (0, 10 μg/mL) of GM3 enriched media. Ganglioside uptake increases in a dose dependent-manner. Data represents mean±SD from 2 experiments in triplicate. Different letters (a,b) indicate significance for GD3 at $P<0.0001$ and for GM3 at $P<0.00001$.

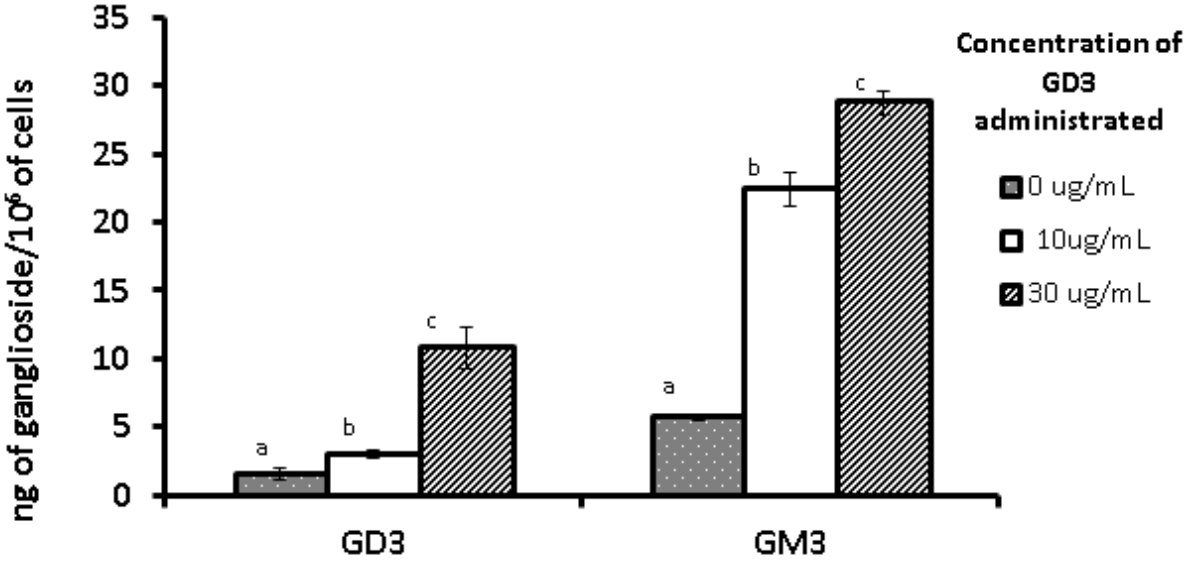


Figure 4.6 Amount of GM3 and GD3 taken up by human Gastric Cells (NCI-N87) after 48h exposure to two different concentrations (0, 10, 30 μg/mL) of GD3 enriched media. Data represents mean ± SD from 2 experiments in triplicate. Different letters (a,b,c) indicate significance for GD3 at $P<0.001$ and for GM3 at $P<0.01$.

4.3.5 Optimization of homogenization buffer to membrane fragmentation method. Two different homogenization buffers were used, method developed for Culp and Forte (1981) and a method using a commercial product, were used to separate the apical and the basolateral membrane domains. Effectiveness of separation of fractions depends on homogenization of the cells. Different methods of homogenization were used such as Dounce homogenizer, needle with syringe (different gauges), bead homogenizer, blenders and sonication. Sonication was the only homogenization method that enabled of breakage of the cells. The homogenization buffer is critical. Methods developed for Culp and Forte (1981) used a buffer containing Tris-Sucrose. Combination of Tris-Sucrose buffer and sonication method completely lysed the cells. The method developed for Culp and Forte (1981) and the buffer, showed in the commercial product protocol, was found to be the optimum to separate membrane fractions.

4.3.6 Separation of membrane domains. Cell membrane separation into apical and basolateral membrane was performed by differential centrifugation and density gradient centrifugation. Confirmation of the purity of each fraction was performed to detect CEA antigen in the apical membrane and Na^+/K^+-ATPAse in the basolateral membrane. Basolateral membrane fraction contained $80\pm3\%$ of the total Na^+/K^+-ATPAse. Apical membrane fraction contained $82\pm4\%$ of the total CEA antigen. This confirms the identity of the basolateral and the apical membrane domains; even though some contamination occurred in other fractions.

4.3.7 Apical and basolateral distribution of gangliosides. GM3 was the most abundant ganglioside in the apical membrane domain. GD3 increased ganglioside content after

ganglioside treatment in a concentration dependent manner; at 10 μg/mL, GD3 increased 14 fold ($P<0.0001$); at 20 μg/mL GD3 increased 32 fold $P<0.0001$), and at 30 μg/mL GD3 increased 50 fold ($P <0.001$). GM3 also exhibited a significant increase at concentrations of 20 and 30 μg/mL ($P<0.01$). In the basolateral membrane domain, gangliosides are present in very small amounts. In human gastric cells, GD3 is 2 fold more abundant in apical membrane than GD3 in the basolateral membrane, while GM3 is 17.8 fold more abundant in apical than in basolateral membrane.

GD3 and GM3 increased in small amounts compared to gangliosides in the apical membrane. Composition of all ganglioside species administrated and incorporated into human gastric cells is shown (Table 4.1 and 4.2). GD3 C34,1 was the most abundant ganglioside present in the apical membrane domain (29%) and in the basolateral membrane domain (40%). GM3 C34,1 (61%) and GM3 C34,0 (39%) were the only two ganglioside present in the basolateral membrane domain in untreated cells. After treatments at different concentrations GM3 C34,1 was the most abundant ganglioside present in basolateral membrane

4.3.8 Phospholipid Analysis. Quantitative and qualitative analysis of PC and PE in the different ganglioside treatments was performed. Gastric epithelial cells modified with gangliosides did not show statistically significant change in amount of PC and PE (Fig. 4.8). Composition of different individual phospholipids did not show statistically significant changes (Table 4.5). PC and PE in the apical and basolateral membrane after modification with gangliosides also did not show statistically significant changes. This indicates that ganglioside treatments used do not have any influence on membrane of phospholipid composition.

Table 4.1 Individual GD3 species incorporated in media and GD3 species found after ganglioside treatments

	% of species relative to total GD3 in media	Composition of plasma membrane before and after treatments			
		GD3 (0 μg/mL) %	GD3 (10 μg/mL) %	GM3 (10 μg/mL) %	GD3 (30 μg/mL) %
GD3 (C32:2)	NA	0	0	0	0.62 ± 0.42
GD3 (C32:1)	NA	0	10.98±0.9	0	16.85±0.91
GD3 (C32:0)	NA	0	6.11±1.3	0	9.41±0.8
GD3 (C34:2)	0.25	0	0	0	1.5±0.02
GD3 (C34:0)	2.35	0	12.78±3.2	0	7.76±0.5
GD3 (C34:1)	11.06	0	52.6±2.4	0	53.42±2.5
GD3 (C36:1)	0.90	0	0	0	1.29+0.1
GD3 (C37:2)	Tr	57 ± 5^{a}	8.58 ± 0.5^{b}	47.54 ± 4^{c}	0
GD3 (C37:0)	0.43	0	0	0	0.81±0.2
GD3 (C37:1)	14.22	43 ± 5^{a}	4.86 ± 1.5^{b}	33.8 ± 6^{ac}	1.31±0.8
GD3 (C38:0)	5.07	0^{a}	4.09 ± 0.7^{a}	18.66 ± 8^{b}	1.15±0.1
GD3 (C38:1)	4.77	0	0	0	1.72±0.5
GD3 (C39:1)	11.58	0	0	0	1.42±1.2
GD3 (C40:1)	12.88	0	0	0	1.7±0.3
GD3 (C41:1)	12.46	0	0	0	1.04±0.3

Representation of GD3 individual species found after treated cells with GD3 at 10 and 30 μg/mL and representation of GD3 individual species found when cells treated with GM3 enriched media to be compared with relative percentage of GD3 individual species present in media. No all ganglioside species are incorporated into plasma membrane. After GM3 treatment, GD3 ganglioside species are also found in plasma membrane. GD3 treatment at higher concentration (30 μg/mL) incorporated more ganglioside from other GD3 species. Data are expressed as mean±SD from 2 experiments in triplicate. Significant difference among 0 μg/mL and GD3 and GM3 treatments at 10 μg/mL were found. Different letters (a,b,c) indicate significance at $P<0.001$. NA = not analysed. Tr = <0.1% of total abundance.

Other species found in media were (and no in plasma membranes) GD3 (C36,0) (0.56%); GD3(C38,2) (0.16%); GD3 (C39:0) (5.52%); GD3 (C39:2) (0.53%); GD3 (C40:0) (4.63%); GD3(C41:0) (1.26%); GD3 (C41:2) (1.57%); GD3 (C42:0) (0.88%); GD3 (C42:1) (6.39%); GD3 (C42:2) (1.64%); GD3 (C43:1) (0.68%).

Table 4.2 Individual GM3 species incorporated in media and GM3 species found after ganglioside treatments

	% of species relative to total GM3 in media	Composition of plasma membrane before and after treatments			
		GD3 (0 μg/mL) %	GD3 (10 μg/mL) %	GM3 (10 μg/mL) %	GD3 (30 μg/mL) %
GM3(C32:1)	NA	0	0	2.56±0.8	1.37±0.85
GM3(C32:0)	NA	0	0	0	2.7±3.7
GM3(C34:2)	0.25	0^{a}	$1.69±0.37^{b}$	0^{a}	1.68±1.2
GM3(C34:0)	3.65	0	21.27±0.2	18.38±5.6	19.5±8.7
GM3(C34:1)	20.72	27.8±13	33.16±4	35.27±3.3	28.73±9
GM3(C37:0)	0.36	$22.1±17^{a}$	$2.39±0.4^{b}$	$2.07±1^{b}$	1.53±0.4
GM3(C40:0)	5.10	5.5±1.5	3.93±0.36	5±2	4.39±0.88
GM3(C40:1)	13.61	4.7±2	3.38±0.5	3.34±1.4	3.49±1
GM3(C41:0)	4.53	$3.8±0.1^{a}$	$5.9±0.3^{b}$	$5.16±1.2^{ab}$	6±4.1
GM3(C42:0)	0.48	6.8±7.6	2.9±1.4	3.47±0.6	6.82±8
GM3(C42:1)	6.00	6.1±5.1	3.77±1.3	3.58±1.4	2.97±0.7
GM3(C43:0)	0.49	15.5±12.3	14.8±3.7	14.64±3.3	14.45±4.4
GM3(C43:1)	0.89	7.6±1.8	6.76±2	7.07±1.7	6.28±0.6

Representation of GM3 individual species found after treated cells with GD3 at 10 and 30 μg/mL and representation of GM3 individual species found when cells treated with GM3 enriched media to be compared with relative percentage of GM3 individual species present in media. No all GM3 species are incorporated into plasma membrane. After GD3 treatment, GM3 ganglioside species are also found in plasma membrane. GD3 treatment at higher concentration (30 μg/mL) incorporated more ganglioside from other treatments. Data are expressed as mean±SD from 2 experiments in triplicate. Significant difference among 0 μg/mL and GD3 and GM3 treatments at 10 μg/mL were found. Different letters (a,b,c) indicate significance at $P<0.01$. NA = not analysed.

Other species found in media (and no in plasma membranes) were GM3 (C36,0) (0.63%); GM3(C36,1) (1.12%); GM3 (C36:2) (0.32%); GM3 (C37:1) (0.34%); GM3 (C38,0) (3.47%); GM3(C38:1) (6.35%); GM3 (C39:0) (3.86%); GM3 (C39,1) (8.38%); GM3 (C39,2) (0.98%); GM3 (C40:2) (1.75%); GM3 (C41:1) (11.36%); GM3 (C41:2) (3.60%); GM3 (C42:2) (1.26%); GM3 (C43:2) (0.73%).

Table 4.3 Uptake of gangliosides by human gastric epithelial cells over time

Type of treatment	Day 1 (%)		Day 2 (%)	
	8h	**24 h**	**8 h**	**24 h**
GD3 10 μg/mL	$49.4\pm5.4^{a,*}$	$70.8\pm3.3^{b,*}$	$47.8\pm5.5^{a,*}$	94 ± 0.9^{c}
GD3 30 μg/mL	$62.6\pm12.3^{a,**}$	$76.4\pm7.7^{b,**}$	$42.9\pm18.7^{c,*,}$	94 ± 0.7^{d}
GM3 10 μg/mL	$52.1\pm9.4^{a,***,*}$	$75.4\pm 7.0^{b,*,**}$	$32\pm11^{c,**}$	93 ± 5.3^{d}

Values indicate the percentage (%) of gangliosides that were taken up from the media. Human gastric epithelial cells were exposed to GD3 or GM3. On day 1, samples of media were taken at 8 and 24 h. After 24 h (day 2), media was changed and samples were taken at 8 and 24 h. At 8 h, gastric cells treated with GD3 30μg/mL take up more gangliosides. At 24 h (day 2) take similar amounts of gangliosides at any concentration for GM3 and GD3 gangliosides. Data means ± SD from 2 experiments in triplicate. Letters (a,b,c,d) represent a significant difference between each type of treatment and time at $P<0.001$. Symbols (*,**,***) represent a significant difference between types of treatment and time at $P<0.02$.

Table 4.4 Relative amounts of main ganglioside species in apical and basolateral membrane

Ganglioside Species	Concentration of ganglioside mix enriched powder media							
	Apical Membrane(%)				Basolateral Membrane (%)			
	0 μg/mL	10 μg/mL	20 μg/mL	30 μg/mL	0 μg/mL	10 μg/mL	20 μg/mL	30 μg/mL
GD3 (C32:0)	8±3a*	13±0.4	13±0.1	14+0.07^{b}	0^{a}*	18±2^{b}	14+0.6^{b}	11±2^{b}
GD3 (C32:1)	11±4^{a}	24±1^{b}	25±0.6^{b}	25±0.1^{b}	15±5	31±7	21±1	24±1
GD3 (C34:0)	7±2*	8±0.8	90±0.2	9±0.3	0a*	10±3^{b}	9±1^{b}	8±1^{b}
GD3 (C34:1)	29±2^{a}	46±1^{b}*	47±0.5^{b}	46±0.4b	40±6^{a}	22±4b*	50±1^{a}	48±1^{a}
GD3 (C39:1)	10±0.2^{a}*	1±0.3^{b}	0.6±0.3^{b}	0.5±0.03^{b}	0*	0	0	0.7+0.5
GD3 (C40:1)	19±0.3^{a}	2±0.5^{b}*	0.6±0.4^{b}	0.6±0.09^{b}	19±3^{a}	12±5^{a}*	1±1^{b}	1±0.5b
GD3 (C41:1)	16±0.3^{a}*	2±0.7^{b}	0.6±0.7^{b}	0.6±0.06^{b}	25±7^{a*}	7±1^{b}	0.9±0.9^{b}	1±0.8^{b}
GM3 (C32:0)	0^{a}	2±0.4^{b}*	±0.3^{c}	6±1^{d}	0^{a}	0^{a}*	0^{a}	5±1.5^{b}
GM3 (C32:1)	0^{a}	4±0.2^{b}*	8±0.5^{c}*	9±0.2^{d}	0^{a}	0^{a}*	0^{a}*	8±3^{b}
GM3 (C34:0)	15±2.5^{a}	16±3^{b}*	18±0.5^{c}*	14+1^{d}	39±2^{a}	13±5^{a}*	25±6a*	16±2^{b}
GM3 (C34:1)	46±1^{a}*	43±1^{a}*	43±0.6^{a}	40±0.7^{b}	61±2*	58±6*	51±6	52±3
GM3 (C40:1)	6±1	6±1*	3±0.3	4±1	0^{a}	14±5^{b}*	3±1^{ac}	7±0.8
GM3 (C43:0)	10±1*	8±1	7±0.3	8±0.8	0^{a}*	14±1^{b}	14±5^{c}	11±1^{d}
GM3 (C43:1)	2±0.5*	2±0.1*	2±0.2*	1±0.2*	0*	0*	0*	0*

After ganglioside mix enriched powder media treatment at different concentrations (0-30 μg/mL), human gastric epithelial cells were fractionated in apical and basolateral membrane. Data means±SD from 2 experiments in triplicate. Letters (a,b,c,d) represent a significant difference between apical or basolateral membrane and each concentration at $P<0.01$. Symbol (*) represents a significant difference between apical and basolateral at different concentrations at $P<0.05$.

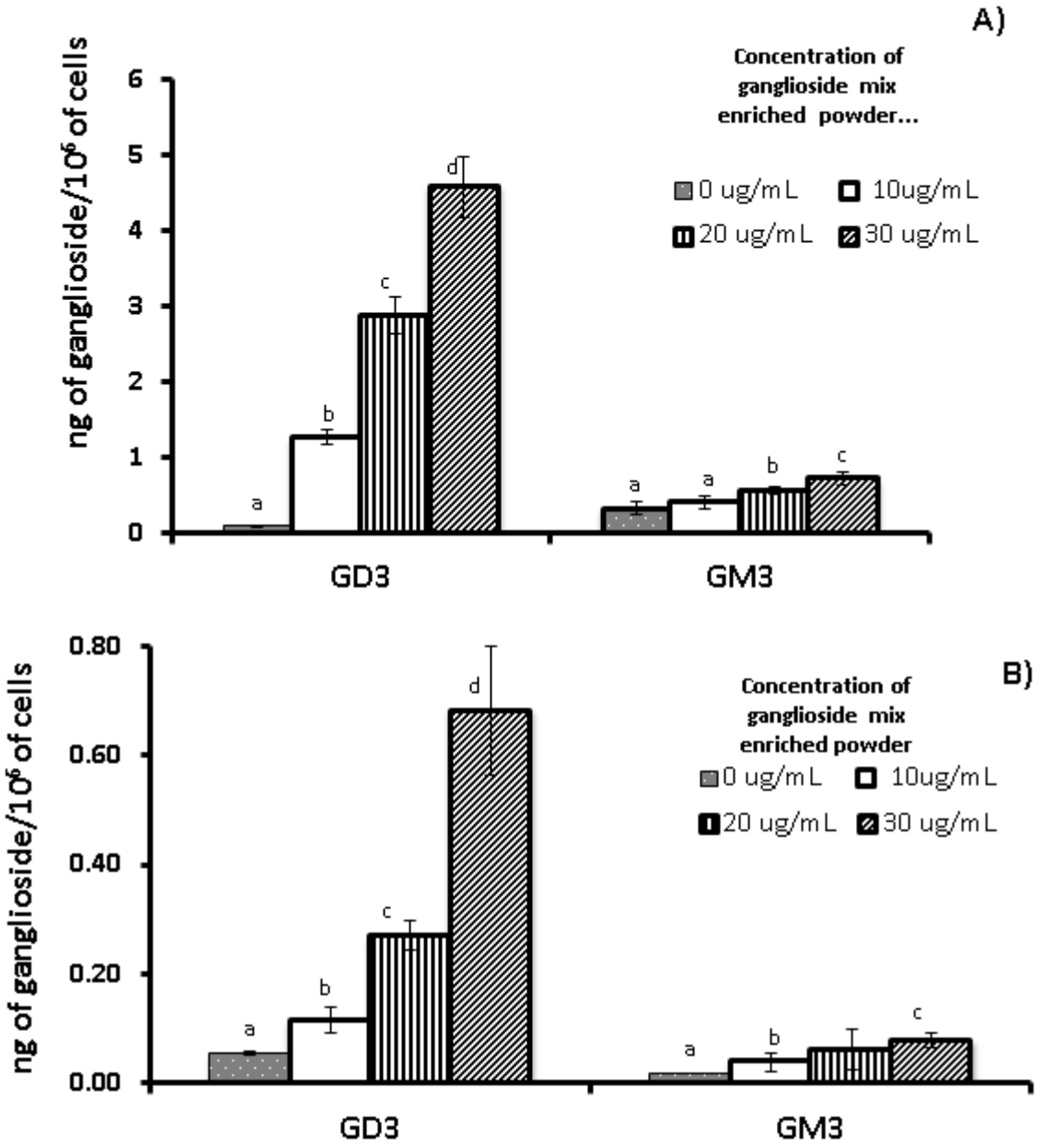


Figure 4.7 A) Distribution of gangliosides in the apical membrane fraction. B) Distribution of gangliosides in the basolateral membrane fraction. Data are expressed as mean $\pm$ SD from from 3 experiments in quintuplicate or sixtuplicate. Letters (a,b,c,d) indicate significant difference at $P<0.01$ except for GD3 apical membrane where the significance level is $P<0.001$.

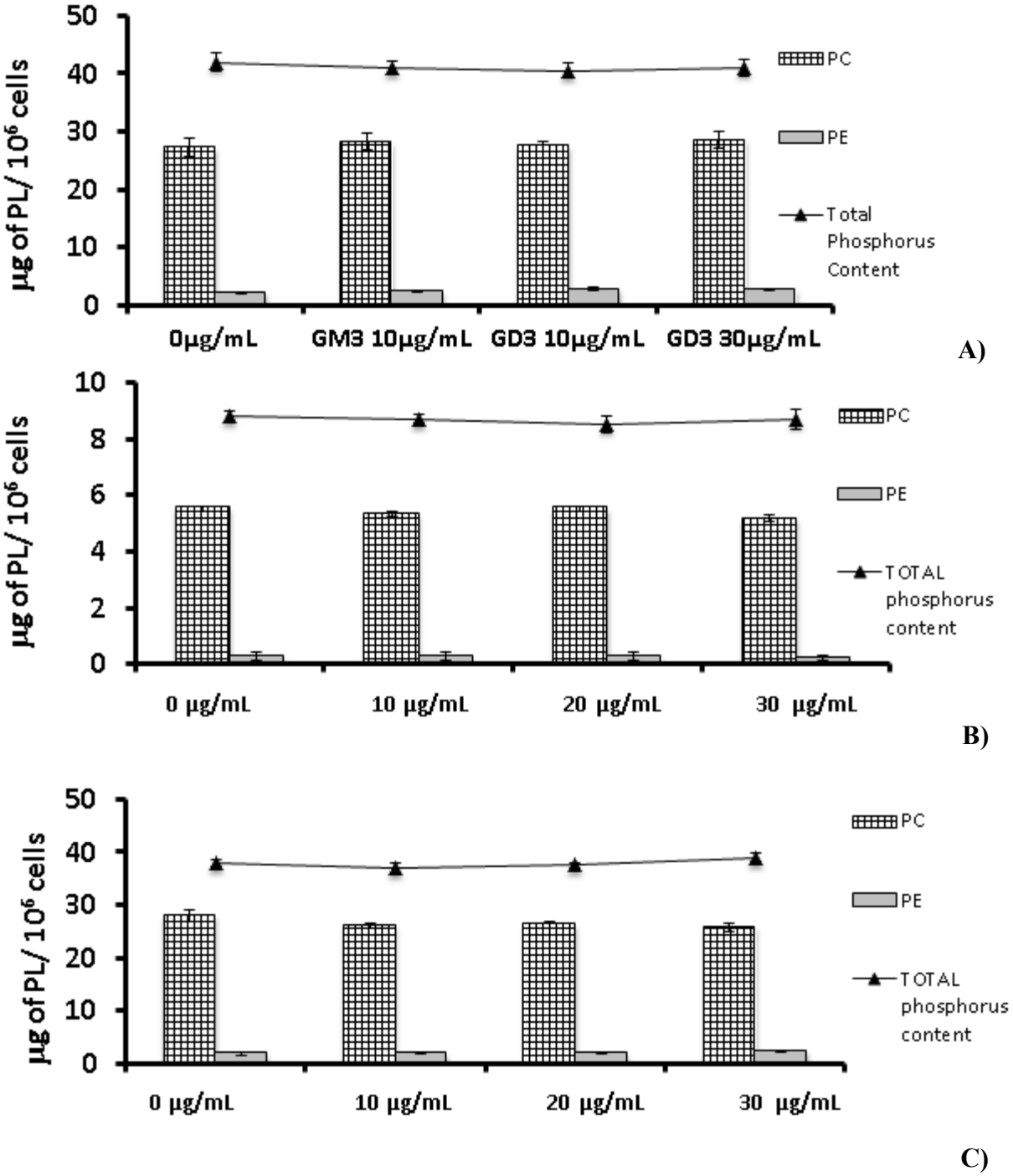


Figure 4.8 The content of total phospholipids, PC and PE in gastric epithelial cells when incubated at different concentrations of GM3 or GD3 or ganglioside mix enriched powder treatment. A) PC and PE present in total cells. B) PC and PE present in apical membrane after ganglioside mix enriched powder treatment. C) PC and PE present in basolateral membrane domain after ganglioside mix enriched powder treatment. Data are expressed as mean±SD from from 2-3 experiments in triplicate. Total Phosphorus content, PC and PE content did not show any statistically significant change after different ganglioside treatments.

Table 4.5 Composition of PC and PE in human gastric epithelial cells

Phospholipids	Total Cells (%)	Apical Membrane (%)	Basolateral Membrane (%)
PC (C32:1)	16.3±0.67	15.39±0.34	15.39±0.79
PC (C34:1)	52.3±2.17	56.09±0.77	55.46±0.71
PC (C36:0)	0.2±0.07	0.17 ±0.83	0.12±0.03
PC (C36:2)	27.4±2.94	25.23±0.60	25.66±0.94
PC (C36:4)	0.8±0.22	0.89±0.19	0.96±0.15
PC (C38:1)	0.3±0.21	0.16±0.04	0.23±0.05
PC (C38:2)	0.9±0.06	0.61±0.07	0.54±0.08
PC (C38:5)	0.7±0.10	0.88±0.34	0.87±0.14
PC (C40:7)	0.6±0.13	0.46±0.11	0.48±0.06
PC (C40:8)	0.2±0.04	0.04±0.03	0.18±0.04
PC (C40:9)	0.2±0.04	0.07±0.02	0.11±0.02
PE (C32:1)	6.1±0.21	5.16±0.24	5.07±0.41
PE (C34:1)	31.8±0.56	27.81±1.44	30.57±0.78
PE (C36:2)	42.8±4.24	51.45±3.35	44.95±2.20
PE (C36:4)	3.0±0.85	3.27±0.23	3.18±0.16
PE (C38:0)	0.7±0.06	0.80±0.16	0.60±0.08
PE (C38:2)	0.4±0.05	0.36±0.10	0.39±0.06
PE (C38:3)	1.7±0.34	1.47±0.32	1.83±0.12
PE (C38:4)	11.9±2.60	8.45±1.71	12.06±1.12
PE (C40:6)	1.5±0.22	1.25±0.11	1.35±0.10

Since no significant difference among treatments data is presented as mean±SD from 2-3 experiments in triplicate of all samples at all concentrations.

4.4 Discussion

The digestive system is distinguished by the diversity of gangliosides, even though gangliosides are present in smaller amounts than in the nervous system. The findings of the present study agree with previous studies where GD3 and GM3 are the major gangliosides in extraneural cells.[50,51] In this cell line, GM2 is present in high levels. In normal gastric mucosa, GM2 is present in gastric carcinomas but not detectable in normal gastric mucosa because upregulation of enzymes that catalizes GM3 to GM2.[65] In the digestive system, there is not enough evidence to explain the presence and functions of different ganglioside species.

Exogenous GM1 incorporated into cell culture has been extensively analyzed in different types of cells to demonstrate cells are taking up gangliosides.[6,12-14,18,19,70,71] Some others gangliosides such as GD3 and GM3 have not been extensively analyzed. The present study observed incorporation of ganglioside in human gastric cells NCI-N87 without using radiolabeled exogenous gangliosides. *In vitro* absorption of GD3 and GM3 has not been previously evaluated in human gastric epithelial cells.

Incorporation of gangliosides (GM3 and GD3) into human gastric epithelial cells is a time-dependent process presenting a rapid increase at 24 h (Table 4.3). After 32 h (day 2), when media was changed the incorporation of ganglioside decreased; however, after 48 h (day 2), the incorporation of gangliosides reached at 90%. The incorporation of gangliosides also depends on the concentration of the ganglioside. At 8 h, gastric cells treated with GD3 at 30 μg/mL incorporated more gangliosides than GD3 and GM3 at 10 μg/mL. In other types of cells, ganglioside uptake is a time dependent process and reached a plateau. Caco2 cells incorporated within 6 h and reached at plateau at 24 h in the apical side and basolateral side.[17] In cerebellar granule cells, GM1 demonstrated a time-dependent incorporation.[6] In non-neuronal cells, after 14 h >50% of GM1 and GM2 were incorporated and 42% of gangliosides were converted to ceramide while GM3 was only 7% incorporated into the cells.[18]

All gangliosides might not be inserted permanently into the plasma membrane. Schwarzmann et al. (1983) suggested that exogenous gangliosides are inserted into the lipid bilayer plasma membrane, not only adsorbed into the surface or trapped in membrane vesicles, similar to endogenous gangliosides.[72] Gangliosides are amphiphilic compounds because of the hydrophobic ceramide tail and the charged hydrophilic glycan portion.[73] This characteristic suggests that gangliosides composed of long oligosaccharide chains form micelles in different

sizes and in disk or rod like aggregates (gangliosides are so large that these molecules form spherical micelles), but GM3 forms unilamellar vesicles.[73,74] A portion of ganglioside micelles can be removed by extensive washes by media containing serum or ganglioside micelles can bind proteases such as trypsin.[75] Trypsinization was the technique used to detach cells in this project, before and after treatment cells were extensively washed with PBS containing FBS and PBS washing that indicate that exogenous gangliosides were trypsin resistant and inserted into the cell membrane.[75]

Gastric epithelial cells are able to incorporate GM3 and GD3 in a concentration dependent-manner. Gastric cells treated with ganglioside mix enriched powder (with higher concentration in GD3 and GM3, 20:1), increased GD3 content, but also GM3 (60% respect to GD3). Other ganglioside species are also present in this ganglioside mix enriched powder that might contribute to increasing GM3. Human gastric epithelial cells incubated with GM3 enriched media also shown an increase in GD3; this might presumably demonstrate that GD3 synthesis from GM3 ganglioside. However, human gastric epithelial cells incubated with GD3 enriched media increased GM3 which might indicate ganglioside catabolism from GD3 to form GM3. GM3 could represent an essential ganglioside to this type of cell.

GM3 is taken up into the cell plasma membrane in Caco2 cells in a concentration dependent manner. Caco2 cells treated with GM3 in the basolateral side increased content of GM3 when concentration was doubled.[17] It has been considered that gangliosides are absorbed in the enterocyte crossing the brush border to the basolateral membrane. After rats were fed with GD3; GD3 increased content in intestinal mucosa lipid rafts and plasma.[42]

Pagano's vesicle sorting theory (1990) suggested different pathways or fates for exogenous lipids absorbed in cells; transportation to plasma membrane cells after endocytosis,

endocytosis to the Golgi apparatus for glycosylation to form more complex ganglioside species, transportation by the endosome to the lysosome for degradation into bioactive mediators and transcytosis.[19] Another evidence of the fate of the gangliosides describes that ganglioside are glycosylated or deglycosylated in the plasma membrane for enzymes presents in this cellular site.[32] These findings do not demonstrate the fate of GM3 and GD3 ganglioside species in gastric cells or demonstrate the site of catabolism and synthesis of other gangliosides.

Media administrated to the human gastric cells contained different GD3 or GM3 species with different saturation/unsaturation in the ceramide. In GD3 treatment, GD3 containing monounsaturated ceramides with more carbons (C38-C41) was incorporated when cells were treated at higher concentrations (30 μg/mL). When cells were treated with GM3 enriched media, only three GD3 species with medium number of carbons in the ceramide concentrations were synthesized.

In epithelial cells, plasma membranes are differentiated in apical and basolateral plasma membrane domains.[77] Different distribution of lipids between apical and basolateral membrane domains have been demonstrated in this study. Apical membrane domains are rich in glycosphingolipids and sphingomyelin while glycerolipid and phosphatidylcholine are more abundant in basolateral membrane domains.[36,77,78] Glycosphingolipids including gangliosides and other glycosphingolipids present in the apical membrane domain are organized in membrane microdomains (lipid rafts or calveolae). Membrane microdomains are liquid-ordered phased and dynamic membranes presenting high amounts of cholesterol and specific proteins for each type of cell and function.[79-82] Glycolipids, present in the apical membrane, might make a protective barrier against hydrolases and pH changes of the luminal

medium.[80,83] In secretory cells, secretion is regulated in the apical plasma membrane domain and may be influenced by membrane lipid composition.[83] For example in gastric apical cell membranes, K^+/H^+-ATPase is associated with the presence of glycosphingolipids.[84] Intragastric GM1 administration prevented ethanol damage to the gastric mucosa.[85] Basolateral membrane domain might resemble non-polar cells and contains proteins for nutrient uptake, cell-substrate attachment and cell growth.[80] Basolateral membrane domain is composed of desmosomes, gap junctions and adherent junctions including tight junctions which are responsible for regulation, cell-cell contact and communication and also control nutrient transference between apical and basolateral membrane domains.[80,81]

In human gastric cells, apical plasma membranes exposed to ganglioside increased ganglioside content. GD3 was present in smaller amounts that GM3. After GD3 exposition using ganglioside mix enriched powder media, increased in dose dependent manner. Ganglioside content in basolateral membrane domain also increased ganglioside content. Intestinal cells incorporate gangliosides *in vitro* and *in vivo*. In Caco2 cells, media supplemented with exogenous GD3 modified GD3 content in a time- and dose-dependent manner. The fate of GD3 depends on the route (apical or basolateral) of the delivery. Gangliosides play different roles depending on the location of the plasma membrane cells indicating that gangliosides perform different biological and physiological roles. In the enterocyte, GM3 is localized in the apical membrane domain while GD3 is located in the basolateral membrane.[42] Thus, GM3 is exposed to the lumen interacting with bacteria and toxins. GD3 might play immunological roles.[86] Jennemann et al. (2012) suggested that glycosphingolipids play an important role in the enterocyte function, but are not crucial for brush border formation.[87]

Spiegel et al. (1985) added exogenous GM1 to the apical membrane of epithelial cells (A6 and MDCK) in a 4 h treatment and concluded ganglioside did not migrate to the basolateral membrane by passing tight junctions.[5] Van Meer and Simons (1988) suggested that lipids might migrate through the cytosol by two different routes by protein carriers or vesicles.[88]

The observations of this study suggest gangliosides are endocytosed and migrated to the basolateral membrane domain in small amounts via a trancystotic pathway or any other migration pathway to transport gangliosides from apical to basolateral membrane to preserve polarity and/or a ganglioside protein carrier is present in this type of human gastric epithelial cells.[76,81] Transcytotic pathways have not been clearly studied in lipids. In the apical and basolateral membrane domains, some GD3 and GM3 species are present. The most abundant are GD3 C34,1 and GM3 C34,1 in the apical side, while in the basolateral side, GD3 C34,1 and GM3 C34,1 (Table 4.3) are most abundant. After cells treated with ganglioside mix enriched powder, modifications in membrane domains were found. The most radical alteration is the presence of new GD3 (GD3 C32,0; GD3 C34,0; GD3 C39,1) and GM3 (GM3 32,0; GM3 32,1; GM3 40,1; GM3 43,0) ganglioside species in the basolateral side (Table 4.3). However, GM3 C43,0 was found in the apical membrane domain as well as in the basolateral membrane domain due to probably transcytosis since GD3 ganglioside species with this number of carbons are not present ($P<0.01$).

In cell adhesion, Blanco and DiRita (2006) reported that GM1 binds *Vibrio cholerae*, GM1 functions in the transcytotic pathway of this bacterium.[89] Some studies report that gangliosides had to be located in the inner plasma membrane to diffuse across the tight junctions to the basolateral membrane and/or diffusing through the cytoplasmic leaflet. Lipids

present in the outer membrane do not move.[5,88,90] Simons and van Meer, 1988 hypothesized that sphingolipids are budding into a membrane vesicle from the apical membrane and are introduced to the exoplasmic leaflet. Sphingolipid microdomains are generated in the trans-Golgi network and bud to the basolateral membrane.[88]

Saslowsky et al. (2013) reported that the ability to cross tight junctions or epithelial barriers depends on ceramide composition. In colon cells (T84 cells), GM1 species were endocytosed on apical membrane and GM1 containing long, saturated C18:0 fatty acids were degraded. In contrast, GM1 containing short or *cis*-unsaturated ceramide were transposed from apical to basolateral membrane domain by transcytosis, after retrogradation or recycling pathways.[91,92] Chinnapen et al. (2012) reported that GM1 containing unsaturated ceramides sorting depends on cholesterol and membrane proteins such as flotilin-1 and actin.[93]

In intestinal cells, phosphatidylcholine represents 2-4 fold reductions in apical membrane than in basolateral membrane. Phosphatidylethanolamine as percentage of total is equal in two domains.[88] In gastric cells, PC represents a 5-fold reduction in apical than in basolateral membrane. Total amount of PE is higher in basolateral membrane than in apical membrane in this study.

Ganglioside uptake in gastric epithelial cells had no effect on the amount and composition of phospholipids in total cells as well as phospholipids in the apical or basolateral membrane domains. Sphingosine-1-phosphate is a lipid mediator related to the synthesis and degradation of ceramide, and also the synthesis of other of phospholipids such as sphingomyelin and PE.[94,95] The addition of gangliosides did not affect the content of PE. Phospholipids are the main components in plasma membranes. Different amounts and composition of phospholipids may possibly change the membrane fluidity.[96] Phospholipids

and metabolites play a structural role enhancing the barrier properties of the gastrointestinal (GI) mucosa and protect the GI mucosa from external drugs like NSAIDS which can damage membranes and natural factors such as HCl, pepsin.[97,98] The mucus-bicarbonate-phospholipid barrier composed of these elements protect from the penetration of pepsin and other proteolytic enzymes into the surface epithelium.[98] Prostaglandins also (PGE2 and PGI2) maintain the stomach mucosal defensive mechanisms against ulcerogenic agents.[98] The addition of gangliosides did not affect the integrity of the phospholipid composition and cellular functions.

In summary, the uptake of GD3 and GM3 in human gastric epithelial cells increases ganglioside content in apical and basolateral membrane domains by metabolism of gangliosides or trancystotic pathways to the basolateral side of human gastric cells. Modifications of gastric apical membrane domain might represent a means for anti-adhesion therapy by altering the binding of gastric pathogens such as *H. pylori.*

4.5 References

1. Sonnino, S.; Mauri, L.; Chigorno, V.; Prinetti, A. (2006). Gangliosides as components of lipid domains. *Glycobiology*, 17(1): 1R-13R

2. Miklavcic, J. J.; Schnabl, K.L.; Mazurak, V.C.; Thomson, A.B.R.; Clandinin, M.T. (2012). Dietary Ganglioside Reduces Proinflammatory Signaling in the Intestine. *Journal of Nutrition and Metabolism*, 1-8.

3. Park, J.; Suh, M.; Clandinin, M.T. (2005) Dietary ganglioside and long-chain polyunsaturated fatty acids increase ganglioside GD3 content and alter the phospholipid profile in neonatal rat retina. *Investigative Ophthalmology and Visual Science*, 46(7): 2571–2575.

4. Whitmore, C.D.; Hindsgaul, O.; Palcic, M.M.; Schnaar, R.L.; Dovichi, N.J. (2007). Metabolic cytometry. Glycosphingolipid metabolism in single cells. *Analytical Chemistry*,79(14):5139-5142.

5. Spiegel, S.; Blumenthal, R.; Fishman, P.H.; Handler, J.S. (1985). Gangliosides do not move from apical to basolateral plasma membrane in cultured epithelial cells. *Biochimica et Biophysica Acta*, 821(2):310-318.

6. Ghidoni, R.; Riboni, L.; Tettamanti, G. (1989). Metabolism of exogenous gangliosides in cerebellar granule cells, differentiated in culture. *Journal of Neurochemistry*, 53(5):1567-1574.

7. Riboni, L.; Prinetti, A.; Pitt, M.; Tettamanti, G. (1990). Patterns of Endogenous Gangliosides and Metabolic Processing of Exogenous Gangliosides in Cerebellar Granule Cells During Differentiation in Culture. *Neurochemical Research*, 15(12): 1175-1183.

8. Nishio, M.; Furukawa, K. (2004). Incorporation, remodeling and re-expression of exogenous gangliosides in human cancer cell lines in vitro and in vivo. *Nagoya Journal of Medical Science*., 67(1-2):35-44.

9. Masco, D.; Flott, B.; Seifert, W. (1989). Astrocytes in cell culture incorporate GM1 ganglioside. *Glia*, 2(4):231-240.

10. Lim, S.T.; Esfahani, K.; Avdoshina, V.; Mocchetti, I. (2011). Exogenous gangliosides increase the release of brain-derived neurotrophic factor. *Neuropharmacology*, 60(7-8):1160-1167.

11. Leray, C.; Ferret, B.; Freysz, L.; Dreyfus, H.; Massarelli, R. (1988). Effect of exogenous gangliosides on the lipid composition of chick neurons in culture. *Biochimica et Biophysica Acta*, 944(1):79-84.

12. Fishman, P.H.; Moss, J.; Manganiello, V.C. (1977). Synthesis and uptake of gangliosides by choleragen-responsive human fibroblasts. *Biochemistry*, 16(9): 1871-1875.

13. Giglioni, A.; Chigorno, V.; Pitto, M.; Valsecchi, M.; Palestini, P.; Ghidoni, R. (1990). Effect of the different supramolecular organization on the uptake and metabolization of exogenous GM1 ganglioside by human fibroblasts. *Chemistry and Physics Lipids*, 55(2):207-213.

14. Ackerman, G.A.; Wolken, K.W.; Gelder, F.B. (1980). Surface distribution of monosialoganglioside GM1 on human blood cells and the effect of exogenous GM1 and neuraminidase on cholera toxin surface labeling. A quantitative immunocytochemical study. *Journal of Histochemistry and Cytochemistry*, 28(10):1100-1112.

15. Krishnaraj R, Saet YA, Kemp RG. (1980). Binding of monosialogan- glioside by murine thymus cells in vitro. *Cancer Research*, 40:2808–2813.

16. Masserini, M.; Palestini, P.; Pitto, M.; Chigorno, V.; Tomasi, M.; Tettamanti, G. (1990). Cyclic AMP accumulation in HeLa cells induced by cholera toxin. Involvement of the ceramide moiety of GM1 ganglioside. *Biochemistry Journal*, 271: 107–111.

17. Schnabl, K.L.; Larcelet, M.; Thomson, A.B.; Clandinin, M.T. (2009). Uptake and fate of ganglioside GD3 in human intestinal Caco-2 cells. *The American Journal of Physiology: Gastrointestinal and Liver Physiology*, 2297(1):G52-G59.

18. Essaka, D.C.; Prendergast, J.; Keithley, R.B.; Hindsgaul, O.; Palcic, M.M.; Schnaar, R.L.; Dovichi, N.J. (2012). Single cell ganglioside catabolism in primary cerebellar neurons and glia. *Neurochemistry Research*, 37(6):1308-1314.

19. Saqr, H.E.; Pearl, D.K.; Yates, A.J. (1993) A review and predictive models of ganglioside uptake by biological membranes. *Journal of Neurochemistry*, 61:395–411.

20. Young, H.P.; Christian, Z.F.; Cabeza, R.; Irwin, L.N. (1998) Uptake of exogenous gangliosides by rat brain synaptosomes. *Neurochemistry Research*, 23(12):1515-1520.

21. Lauc, G.; Heffer-Lauc, M. (2005). Shedding and uptake of gangliosides and glycosylphosphatidylinositol-anchored proteins. *Biochimica et Biophysica Acta*, 1760(4):584-602.

22. O'Brien, J.S.; Kishimoto, Y. (1991). Saposin proteins: structure, function, and role in human lysosomal storage disorders. *FASEB Journal*, 5:301–308.

23. Brown, R.E.; Mattjus, P. (2007). Glycolipid transfer proteins. *Biochimica et Biophysica Acta*, 1771(6):746-760.

24. Zheng, W.; Kollmeyer, J.; Symolon, H.; Momin, A.; Munter, E.; Wang, E.; Kelly, S.; Allegood, J.C.; Liu, Y.; Peng, Q.; Ramaraju, H.; Sullards, M.C.; Cabot, M.; Merrill, A.H. Jr. (2006). Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy. *Biochimica et Biophysica Acta*, 1758(12):1864-1884.

25. Cantú, L.; Del Favero, E.; Sonnino, S.; Prinetti. A. (2011). Gangliosides and the multiscale modulation of membrane structure. *Chemistry and Physics Lipids*, 164(8):796-810.

26. Inokuchi, J. (2011). Physiopathological function of hematoside (GM3 ganglioside). *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, 87(4):179-198.

27. Prokazova, N.V.; Samovilova, N.N.; Gracheva, E.V.; Golovanova, N.K. (2009). Ganglioside GM3 and its biological functions. *Biochemistry (Moscow),* 74(3): 235-249.

28. Malisan, F.; Testi, R. (2005). The ganglioside GD3 as the Greek goddess Hecate: several faces turned towards as many directions. *IUBMB Life*, 57(7):477-482.

29. Diociaiuti, M.; Ruspantini, I.; Giordani, C.; Bordi, F.; Chistolini, P. (2004). Distribution of GD3 in DPPC monolayers: a thermodynamic and atomic force microscopy

combined study. *Biophysica Journal*, 86(1):321-328.

30. van Echten-Deckert, G.; Guravi, M. (2008). Golgi localization of glycosyltransferases involved in ganglioside biosynthesis. *Current Drug Targets*, 9(4): 282-291.

31. Rueda, R.; Gil, A. (1998). Chapter 15 Role of Gangliosides in Infant Nutrition. In: Huang Y-S., Sinclair, A.J. Lipids in Infant Nutrition. USA AOCS.pp213-234.

32. Tettamanti G. (2004) Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconjugate Journal*, 20(5):301-317.

33. d'Azzo A1, Tessitore A, Sano R.(2006). Gangliosides as apoptotic signals in ER stress response. *Cell Death Differentiation*, 13(3):404-414.

34. Posse de Chaves E.; Sipione, S. (2009). Sphingolipids and gangliosides of the nervous system in membrane function and dysfunction. *FEBS Letters*, 584(9):1748-1759.

35. Hakomori, S. (1990). Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *Journal of Biological Chemistry*, 265(31):18713-18716.

36. Hakomori, S. (2001). Membrane Microdomains Defining Cell Adhesion and Signaling. *Trends in Glycoscience and Glycotechnology*, 13(71): 219-230.

37. Yates, A.J.; Rampersaud, A. (1998). Sphingolipids as receptor modulators. An overview. *Annals of the New York Academy of Sciences*, 845:57-71.

38. Hakomori, S.; Yamamura, S.; Handa, A.K. (1998). Signal transduction through glyco(sphingo)lipids. Introduction and recent studies on glyco(sphingo)lipid-enriched microdomains. *Annals of the New York Academy of Sciences*, 845:1-10.

39. Skaper, S.D. Leon, A.; Toffano, G. (1989). Ganglioside function in the development and repair of the nervous system. *Molecular Neurobiology*, 173(3): 173-199.

40. Sorice, M.; Garofalo, T.; Misasi, R.; Manganelli, V.; Vona, R.; Malorni, W. (2012). Ganglioside GD3 as a raft component in cell death regulation. *Anticancer Agents in Medical Chemistry*, 12(4):376-82.

41. Lopez, P.H.; Schnaar, R.L. (2009). Gangliosides in cell recognition and membrane protein regulation. *Current Opinion of Structural Biology*, 19(5):549-57.

42. Park, E.J.; Suh, M.; Ramanujam, K.; Steiner, K.; Begg, D.; Clandinin, M.T. (2005). Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *Journal of Pediatrics Gastroenterology and Nutrition*, 40(4): 487-495.

43. Ngamukote, S.; Yanagisawa, M.; Ariga, T.; Ando, S.; Yu, R.K. (2007). Developmental

changes of glycosphingolipids and expression of glycogenes in mouse brains. *Journal of Neurochemistry*, 103(6):2327-2341.

44. Dyatlovitskaya, E.V.; Bergelson, L.D. (1987). Glycosphingolipids and antitumor immunity. *Biochimica et Biophysica Acta*, 907(2):125-143.

45. Bektas, M.; Spiegel, S. (2004). Glycosphingolipids and cell death. *Glycoconjugate Journal*, 20(1):39-47.

46. Malisan, F.; Testi, R. (2002). GD3 ganglioside and apoptosis. *Biochimica et Biophysica Acta*, 1585: 179-187.

47. Morales, A.; Colell, A.; Mari, M.; Garcia-Ruiz, C.; Fernandez-Checa, J.C. (2004). Glycosphingolipids and mitochondria: role in apoptosis and disease. *Glycoconjugate Journal*, 20(9):579-588.

48. Seyfried, T.N.; Yu, R.K. (1985). Ganglioside GD3: structure, cellular distribution, and possible function. *Molecular and Cellular Biochemistry*, 68(1):3-10.

49. Sonnino S, Mauri L, Ciampa MG, Prinetti A. (2013). Gangliosides as regulators of cell signaling: ganglioside-protein interactions or ganglioside-driven membrane organization? *Journal of Neurochemistry*, 124(4):432-5.

50. Keranen A. (1975). Gangliosides of the human gastrointestinal mucosa. *Biochimica and Biophysica Acta*, 409(3):320-328.

51. Natomi, H.; Sugano, K.; Takaku, F.; Iwamori, M. (1990). Glycosphingolipid composition of the gastric mucosa. A role of sulfatides in gastrointestinal mucosal defense? *Journal of Clinical Gastroenterology*, 12 Suppl 1:S52-S57.

52. Jung, Y.J.; Lee, K.L.; Kim, B.K.; Kim, J.W.; Jeong, J.B.; Kim, S.G.; Kim, J.S.; Jung, H.C.; Song, I.S. (2006). Usefulness of NCI-N87 cell lines in Helicobacter pylori infected gastric mucosa model. *Korean Journal of Gastroenterology*, 47(5):357-362.

53. Basque, J.R.; Chénard, M.; Chailler, P.; Ménard, D. (2001). Gastric cancer cell lines as models to study human digestive functions. *Journal of Cellular Biochemistry*, 81(2):241-251.

54. Lemieux, M.; Bouchard, F.; Gosselin, P.; Paquin, J.; Mateescu, M.A. (2011). The NCI-N87 cell line as a gastric epithelial barrier model for drug permeability assay. *Biochemical and Biophysical Research Communications*, 412(3):429-434.

55. Folch, J.; Lees, M.; Sloane Stanley, G.H.(1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, 226:497-501.

56. Suzuki, K. (1964). A simple and accurate micromethod for quantitative determination of ganglioside patterns. *Life Science*, 3, 1227-1233.

57. Culp, D.J.; Forte, J.G. (1981). An enriched preparation of basal-lateral plasma membranes from gastric glandular cells. *Journal of Membrane Biology*, 59(2):135-142.

58. Optiprep Protocols. Application Sheet S31. Analysis of plasma membrane domains and apical junctional complex from polarized epithelial cells in a self-generated gradient from http://www.axis-shield-density-gradient-media.com/S31.pdf. Available: November 2014.

59. Li, X.; Donowitz, M.(2008). Fractionation of subcellular membrane vesicles of epithelial and nonepithelial cells by OptiPrep density gradient ultracentrifugation. *Methods of Molecular Biology*, 440:97-110.

60. Fantini, J.; Rognoni, J.B.; Culouscou, J.M.; Pommier, G.; Marvaldi, J. Tirard, A. (1989). Induction of polarized apical expression and vectorial release of carcinoembryonic antigen (CEA) during the process of differentiation of HT29-D4 cells. *Journal of Cellular Physiology*, 141(1):126-134.

61. Baranov, V.; Hammarström, S. (2004). Carcinoembryonic antigen (CEA) and CEA-related cell adhesion molecule 1 (CEACAM1), apically expressed on human colonic M cells, are potential receptors for microbial adhesion. *Histochemistry and Cell Biology*, 121(2):83-89.

62. Wang, J.; Ma, Yun.; Zhu, Z.H.; Situ, D.R.; Hu, Y.; Rong, T.H. (2012). Expression and prognostic relevance of tumor carcinoembryonic antigen in stage IB non-small cell lung cancer. *Journal of Touracic Disease*, 4(5): 490-496.

63. Garcia, M.; Seigner, C.; Bastid, C.; Choux, R.; Payan, M.J.; Reggio, H. (1991). Carcinoembryonic Antigen has a different molecular weight in normal colon and in cancer cells due to N-Glycosylation Differences. *Cancer Research*, 51: 5679-5686.

64. Suhail, M.; Rizvi, S.I. (1987). Red cell membrane (Na+ +K+)-ATPase in diabetes mellitus. *Biochemical and Biophysical Research Communications*, 146(1):179-186.

65. Dohi, T.; Ohta, S.; Hanai, N.; Yamaguchi, K.; Oshima, M. (1990). Sialylpentaosylceramide detected with anti-GM2 monoclonal antibody. Structural characterization and complementary expression with GM2 in gastric cancer and normal gastric mucosa. *Journal of Biological Chemistry,* 265: 7880–7885.

66. Dressman, J.B.; Berardi, R.R.; Dermentzoglou, L.C.; Russell, T.L.; Schmaltz, S.P.; Barnett, J.L.; Jarvenpaa, K.M. (1990). Upper gastrointestinal (GI) pH in young, healthy men and women. *Pharmaceutical Research*, 7(7):756-61.

67. Russell, T.L.; Berardi, R.R.; Barnett, J.L.; Dermentzoglou, L.C.; Jarvenpaa, K.M.; Schmaltz, S.P.; Dressman J.B. (1993). Upper gastrointestinal pH in seventy-nine healthy, elderly, North American men and women. *Pharmaceutical Research*. 10(2):187-196.

68. Idota, T.; Kawakami, H. (1995). Inhibitory effects of milk gangliosides on the adhesion of *Escherichia coli* to human carcinoma cells. *Bioscience, Biotechnology, and Biochemistry*, 59:69–72.

69. Schmidt, D.G.; Meijer, R.J.G.M.; Slangen, C.J.; Van Beresteijn, E.C.H. (1995). Raising the pH of the pepsin-catalysed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolysates. *Clinical & Experimental Allergy*. 1995;25:1007–1017.

70. Huang, J.; Shao, W.; Wu, L.; Yang, W.; Chen, Y. (2012). Effects of exogenous ganglioside GM1 on different stages of cell spreading studied by directly quantifying spreading rate. *Cell Communication and Adhesion,* 19(5-6):85-95.

71. Chigorno, V.; Tettamanti, G.; Sonnino, S. (1996). Metabolic processing of gangliosides by normal and Salla human fibroblasts in culture. A study performed by administering radioactive GM3 ganglioside. *Journal of Biological Chemistry*, 271(36):21738-21744.

72. Schwarzmann, G.; Hoffmann-Bleihauer, P.; Schubert, J.; Sandhoff, K.; Marsh, D. (1983). Incorporation of ganglioside analogues into fibroblast cell membranes. A spin-label study. *Biochemistry*, 22(21):5041-5048.

73. Cantú, L.; Corti, M.; Sonnino, S.; Tettamanti, G. (1986). Light scattering measurements on gangliosides: dependence of micellar properties on molecular structure and temperature. *Chemistry and Physics of Lipids*, 41(3-4):315-328.

74. Sonnino, S.; Cantu, L.; Acquotti, D.; Corti, M.; Tettamanti, G. (1990). Aggregation properties of GM3 ganglioside (II3Neu5AcLacCer) in aqueous solutions. *Chemistry and Physics of Lipids*, 52(3-4):231-241.

75. Schwarzmann, G. (2001). Uptake and metabolism of exogenous glycosphingolipids by cultured cells. *Seminars in Cell and Developmental Biology*., 12(2):163-171.

76. Pagano, R.E. (1990). Lipid traffic in eukaryotic cells: mechanisms for intracellular transport and organelle-specific enrichment of lipids. *Current Opinion in Cell Biology*, 2(4):652-263.

77. Simons, K.; Ikonen, E. (1997). Functional rafts in cell membranes. *Nature*, 387(6633):569-572.

78. Simons, K.; van Meer, G. (1988). Lipid sorting in epithelial cells. *Biochemistry*, 27(17):6197-6202.

79. Corbeil, D.; Marzesco, A.M.; Fargeas, C.A.; Huttner, W.B. (2010). Prominin-1: a distinct cholesterol-binding membrane protein and the organisation of the apical plasma membrane of epithelial cells. *Subcellular Biochemistry*, 51:399-423

80. Simons, K.; Wandinger-Ness, A. (1990). Polarized Sorting in Epithelia. *Cell*, 62: 207-210.

81. Cao, X.; Surma, M.A.; Simons, K. (2012). Polarized sorting and trafficking in epithelial cells. *Cell Research*, 22:793-805.

82. Sonnino, S.; Prinetti, A. (2010). Gangliosides as regulators of cell membrane organization and functions. *Advances in Experimental Medicine and Biology*, 688:165-184.

83. Rodriguez-Boulan, E.; Nelson, W.J. (1989). Morphogenesis the Polarized Epithelial Cell Phenotype. *Science,* 245: 718-724.

84. Olaisson, H, Mårdh S, Arvidson G. (1985). Phospholipid organization in H,K-ATPase-containing membranes from pig gastric mucosa. *Journal of Biological Chemistry*., 260(20):11262-11267.

85. Slomiany, B.L.; Piotrowski, J.; Ismail, A.; Klibaner, M.; Murty, V.L.; Slomiany, A. (1991). GM1 ganglioside protection against ethanol-induced gastric mucosal injury. Alcohol *Clinical and Experimental Research*., 15(2):196-204.

86. McJarrow, P.; Schnell, N.; Jumpsen, J.; Clandinin, T. (2009). Influence of dietary gangliosides on neonatal brain development. *Nutrition Reviews*. 67(8): 451-463.

87. Jennemann, R.; Kaden, S.; Sandhoff, R.; Nordström, V.; Wang, S.; Volz, M.; Robine, S.; Amen, N.; Rothermel, U.; Wiegandt, H.; Gröne, H.J. (2012). Glycosphingolipids are essential for intestinal endocytic function. *Journal of Biological Chemistry*, 287(39):32598-325616.

88. Van Meer, G.; Simons, K. (1988). Lipid polarity and sorting in epithelial cells. *Journal of Cell Biochemistry*, 36(1):51-58.

89. Blanco, L.P.; DiRita, V.J. (2006) Bacterial-associated cholera toxin and GM1 binding are required for transcytosis of classical biotype *Vibrio cholerae* through an in vitro M cell model system. *Cell Microbiology*, 8(6):982-998.

90. Dragsten, P.R.; Blumenthal, R.; Handler, J.S. (1981). Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? *Nature*, 294 (5843):718-722.

91. Saslowsky, D.E.; te Welscher, Y.M.; Chinnapen, D.J.; Wagner, J.S.; Wan, J.; Kern, E.; Lencer, W.I. (2013). Ganglioside GM1-mediated transcytosis of cholera toxin bypasses the retrograde pathway and depends on the structure of the ceramide domain. *Journal of Biological Chemistry*, 288(36):25804-25809.

92. te Welscher; Y.M.; Chinnapen, D.J.; Kaoutzani, L.; Mrsny, R.J.; Lencer, W.I. (2014). Unsaturated glycoceramides as molecular carriers for mucosal drug delivery of GLP-1. *Journal of Controlled Release*, 175:72-78.

93. Chinnapen, D.J.; Hsieh, W.T.; te Welscher, Y.M.; Saslowsky, D.E.; Kaoutzani, L.; Brandsma, E.; D'Auria, L.; Park, H.; Wagner, J.S.; Drake, K.R.; Kang, M.; Benjamin, T.; Ullman, M.D.; Costello, C.E.; Kenworthy, A.K.; Baumgart, T.; Massol, R.H.; Lencer, W.I. (2012). Lipid sorting by ceramide structure from plasma membrane to ER for the cholera toxin receptor ganglioside GM1. *Developmental Cell*, 23(3):573-586.

94. Mendelson, K.; Evans, T.; Hla, T. (2014). Sphingosine 1-phosphate signalling. *Development*. 141(1):5-9

95. Duan, R.D. Physiological functions and clinical implications of sphingolipids in the gut. *Journal of Digestive Diseases*, 12(2):60-70.

96. Usai, C.; Robello, M.; Gambale, F.; Marchetti, C. (1984). Effect of gangliosides on phospholipid bilayers: a study with the lipophilic ions relaxation method. *Journal of Membrane Biology*, 82(1):15-23.

97. Lichtenberger, L.M. (2013). Role of phospholipids in protection of the GI mucosa. *Digestive Diseases and Sciences*, 58(4):891-893.

98. Laine, L.; Takeuchi, K.; Tarnawski, A. (2008). Gastric mucosal defense and cytoprotection: bench to bedside. *Gastroenterology,* 135(1):41-60.

99. Bartlett, GR. (1959). Phosphorus assay in column chromatography. *Journal of Biological Chemistry*, 234:466–468.

CHAPTER V. Exogenous gangliosides decreases *H. pylori* adhesion in human gastric cells

5.1 Introduction

Approximately half of the world's population is infected with *H. pylori.*[1,2] In developing countries, between 70-90% of the population is infected, while in developed countries the infection incidence varies from 25 to 50%.[3,4] *H. pylori* infection is acquired mainly during the first years of life.[5,6] Factors of transmission are not clear but is associated with poor socioeconomic status[7,4,8] and poor hygienic conditions.[2] *H. pylori* has been recognized as the main cause of chronic gastritis and is associated with development of peptic ulcer, gastric carcinoma, and mucosa associated lymphoid tissue (MALT) lymphoma.[9] Successful colonization established during childhood may persist throughout life.[10]

Adhesion to the host's cell is a critical step to survive, colonize and initiate infection by bacterial pathogens.[11,12] Adhesion involves a stable bacteria-host interaction.[12] *Helicobacter pylori* binds complex receptors on gastric cells to colonize human stomach. These complex receptors are glycoproteins and glycolipids containing glycans as part of structure.[13] Pathogen-binding proteins are expressed for bacterial pathogens to bind sialic acids on the host cell membrane.[14] One initial study of receptors for *H. pylori* reported that GM3, GD1a, GD1b and GM1 inhibited the agglutination of erythrocytes by *H. pylori.*[15] This finding suggests that gangliosides are receptors for *H. pylori*. Asialogangliosides suppressed the inhibitory effect of hemoagglutination suggesting that sialic acids are key moieties to bind *H. pylori.*[15] Saitoh et al. (1992) also reported that *H. pylori* binds GM3.[16]

Gangliosides are glycolipids composed of a ceramide portion and a glycan portion. The ceramide portion is inserted in the plasma membrane while the glycan portion that

contains sialic acid is oriented to the exterior of the plasma membrane.[17] Gangliosides are present in mammalian cells performing different biological functions[18,19] and opportunistic bacteria bind gangliosides by the presence of sialic acid in the structure of the ganglioside.[20] Subclasses of gangliosides are differentiated by the number of sialic acids and glycan units. Different ganglioside structure can produce a different affinity of *H. pylori* for gangliosides.[15,20] Hata et al. (2004) found *H. pylori* has less affinity for GM1 than for GM3 or GD3.[20] *H. pylori* adherence to poorly differentiated gastric cells (MKN-45 cells), coming from adenocarcinoma of a 62-year old female, was inhibited by GD3 but not by GM3.[20]

In vitro models using cell lines are useful for study of *H. pylori* adhesion, but it is critical to use valid models that resemble gastric conditions. Previous studies using undifferentiated cells[20] or intestinal cells Caco2-cells[21] do not resemble the characteristics of cells of the stomach. NCI-N87 cells grow as well differentiated monolayers forming tight-junctions and produce mucus (MUC 6).[22] NCI-N87 cells are epithelial cells from gastric adenocarcinoma metastized from liver from an American man.[40] The limitation of this study is that NCI-N87 cells are an immortalized cell line.

Dietary anti-adhesion therapies[23,24] and modulation of glycosphingolipids metabolism[25] have been proposed to inhibit bacterial adhesion. Salcedo et al. (2013) pre-incubated Caco2-cells with ganglioside for one hour to evaluate *H. pylori* inhibition.[21] Ganglioside uptake for one hour might not be enough to alter ganglioside content in the plasma membrane. To determine *H. pylori* adhesion, the present study evaluated the alteration of gangloside content on the apical side in NCI-N87 cells treated for 48 h with GM3, GD3 or a ganglioside mixture to enriched media at different concentrations (5-30 μg/mL).

5.2 Materials and Methods

5.2.1 Materials. Ganglioside mix enriched powder was obtained from Fonterra (Cambridge, New Zealand; average molecular weight 1,542 g/mol, GD3 91%, GM3 4.5%, GT3 2.5%, GM1 1%, GD1 0.5%, GT2 0.5%). All cell culture flasks and plates were purchased from Costar (Cambridge, MA). Brain Heart Infusion (BHI) was purchased from Oxoid (Nepean, ON, Canada). Yeast extract (YE) was purchased from Beckton Dickinson (Mississauga, ON, Canada). Trypan blue stain (0.4%), trypsin-EDTA (0.25%), Fetal Bovine Serum (FBS), Horse Serum (HS), AB-Human serum, penicillin (10,000 units/mL), streptomycin (10,000 μg/mL), Roswell Park Memorial Institute-1640 (RPMI-1640), HEPES, sodium pyruvate were ordered from Gibco (Life Technologies, Burlington, ON, Canada). Amphotericin B, vancomycin and select agar were purchased from Sigma Aldrich (Oakville, ON, Canada). LC/MS solvents were of LC/MS grade and solvents for ganglioside extraction were ACS grade (Fisher Scientific Company, Ottawa, ON, Canada).

5.2.2 *H. pylori* strain. *H. pylori* was cultured from a gastric biopsy obtained from a resident of Aklavik, Northwest Territories, Canada in February 2008 by a member of CAN*Help* Working Group. This strain was named *H. pylori* A64. Histology detected moderate gastritis (inflammation). *H. pylori* A64 strain genotypic characteristics are *cagA* (negative), *vacA* (s2i2m2) (positive); *babA* (positive); *sabA* (negative); *oipA* (negative); *iceA1* (positive), *iceA2* (positive), *hopQ1* (negative), *dupA* (positive). *H. pylori* was grown on Brain-Heart Infusion (36 g/L) - Yeast Extract (5 g/L) - Select agar (15g/L) supplemented with horse serum (50 mL/L), amphotericin B (15 mg/L) and vancomycin (15 mg/L) at 37°C under microaerobic conditions (5% CO_2, 5% H_2, 90% N_2).

5.2.3 Human gastric cell culture. NCI-N87 [NCI-N87] (ATCC® CRL-5822™ cell line) was used for adhesion experiments. NCI-N87 cell line was chosen because it is a well-differentiated polarized cell line and forms tight monolayers secreting E-cadherin and zonula occludens-1 (ZO-1), expresses gastric zymogens, secretes pepsinogen and gastric lipase and expresses mucin-6 glycoprotein. To ensure mucus production, cells were grown to 72 h post - confluence.[22] NCI-N87 cells were grown under humidified atmosphere, 5% CO_2, at 37°C (standard conditions). Cells were grown for passages 28-41 as adherent monolayers in T75 culture flasks and cultured in RPMI-1640 supplemented with 5% (v/v), FBS heat inactivated, 2% (v/v) AB-human serum, 2.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1 mM sodium pyruvate, 1% penicillin-streptomycin. When cells were 80-90% confluent, cells were detached using 0.25% trypsin-EDTA, counted and transferred to six well plates for adhesion studies. Cell number and cell viability was estimated with a hemocytometer by the trypan blue exclusion method. Trypan blue exclusion method is based on live cells have intact plasma membranes and do not take up certain dyes such as trypan blue. In dead cells, trypan blue penetrates damaged plasma membranes and appear blue under microscope.[41]

5.2.4 Ganglioside uptake by human gastric epithelial cells. For adhesion assays, NCI-N87 cells were seeded at a density of $9x10^5$ cells /well in six well plates. Cell culture media (2 mL/well) was changed every 2-3 days until confluent. At 24 h post-confluence, cells were washed with PBS and treated with media containing gangliosides. After 24 h, media was changed and discarded at 24 h (total of 48 h treatment). This media contained a ganglioside mix enriched powder at different concentrations (0, 10, 20, 30 μg/mL); or pure gangliosides, GD3 (99% purity) or GM3 (98% purity) at different concentrations (0, 5, 10, 20, 30 μg/mL);

or a combination of GM3:GD3 at 2 and 8 μg/mL, respectively or a combination of GM3:GD3 at 8 and 2 μg/mL, respectively. Cells were incubated under standard conditions. After 48h, cells were washed three times with a solution of PBS and 10% FBS, and PBS to eliminate residual gangliosides.

5.2.5 Assessment of trypsin and cell culture media on viable *H. pylori* for cell adhesion assays. Before adhesion studies, the effect of trypsin-EDTA and RPMI-1640 media on gastric cells were tested to determine any detrimental effect on viable *H. pylori*. *H. pylori* was grown from frozen stocks in BHI-YE-HS agar plates for 48 h. A bacterial suspension was prepared in BHI-YE-HS broth to an OD_{600}=0.1 in a 15 mL sterile conical tube (Corning). Bacterial suspension (1 mL) was centrifuged in maximum speed at clinical centrifuge (IEC, Geneva Switzerland) for 15 min. The centrifuged pellet was resuspended in RPMI-1640 or trypsin-EDTA. The mixture of *H. pylori*/RPMI-1640 was incubated under microaerobic conditions at 37°C for 90 min. While, the mixture of *H. pylori*/trypsin-EDTA was incubated under microaerobic conditions for 12 min (maximum time for dissociating gastric cells). The number of viable *H. pylori* colonies was quantified by CFU counting, 1/10 Serial dilutions were prepared in triplicate in BHI-YE broth. An aliquot (50 μL) was plated on BHI-YE-HS plates in triplicate. Plates were incubated under microaerobic conditions at 37°C for 4 to 5 days. Colonies were counted and reported using equation CFU/mL = (Number of colonies x dilution factor)/volume plated.

5.2.6 Adhesion assays. *H. pylori* was grown from frozen stocks (-80°C) prepared in 20% glycerol BHI/YE broth. A few crystals of culture were inoculated onto BHI-YE-HS agar

plates and incubated 48 h at 37°C under microareobic conditions. It was necessary to test a variety of suspensions at different OD (0.01 to 0.1) at broth growth conditions to consistently obtain a $11x10^6$ CFU/mL. An *H. pylori* suspensión was prepared from BHI-YE-HS agar plates and inoculated into BHI-YE-HS broth without select agar at a variety of OD to determine the OD_{600}=0.150-0.9 and grown under microaerobic conditions with shaking at 120 rpm (Forma Orbital, Thermo Scientific shaker). After 12 h, the *H. pylori* suspension was adjusted to an OD=0.2 corresponding to approximately $11x10^9$ cells/mL. The bacterial suspension was centrifuged at 1500 xg (Rotofix 32 Hettich Zentrifugen) for 5 min. The pellet was resuspended in 2 mL RPMI-1640 media without antibiotics or serum.

H. pylori suspension was added to washed treated and washed untreated NCI-N87 cells (Chapter from section 5.2.4) at a multiplicity of infection (MOI) of 100:1, meaning 100 bacteria per gastric cell. This MOI was selected based on previous studies and to increase the probability to homogeneously infect the cell monolayer.[26,36]

To allow *H. pylori* to adhere to cells in six well plates, the plates were incubated at standard conditions from 30 to 360 min to determine the optimum incubation time. After incubation, non-adherent bacteria were removed by washing with PBS. Cells were removed using a sterile disposable cell scraper for each well. After scraping, cells were centrifuged at 1500 xg (Rotofix 32 Hettich Zentrifugen) for 5 min and resuspendend in 1 mL of BHI-YE-HS broth. Cell suspensions were vortexed for quantitative analysis of *H. pylori*. The number of viable *H. pylori* colonies were quantified by 1/10 serial dilutions and counting the number of colonies forming units (CFU). Each adhesion experiment consisted of a 0 μg/mL (untreated cells) and ganglioside treated cells at increasing concentrations. Each adhesion experiment consisted of three wells (for each concentration) and each well was quantified in duplicate.

Adhesion data was expressed as *H. pylori* bound in % of control values. Data was also expressed as the number of CFU/well.

5.2.7 Statistical Analysis Data is expressed as mean±SD. Significant differences between control and experimental groups were determined by a one-way ANOVA using SPSS statistical software version 22. Significant effects for ganglioside treatments were determined by the Games-Howell test (for non-homogeneity of variance). Differences among all treatments were considered statistically significant at $P<0.05$.

5.3 Results

5.3.1 Optimization of *H. pylori* growth conditions. Establishing a predictable number of CFU/mL is a key step to control the repeatability and MOI for *H. pylori* adhesion assays. Previous studies used a *H. pylori* suspension of OD_{600}=0.5 to inoculate cells.[21,37] Bacterial suspensions were prepared from plate cultures at OD_{600}=0.1 to 0.5. Bacterial suspensions prepared at higher ODs were unstable, resulting in inaccuracies in dilutions and plate counts. Suspensions at lower ODs provided more accurate and consistent colony counts. *H. pylori* suspensions were also prepared at OD_{600}= 0.01 to 0.1 and grown for 12 h at microaerobic conditions in shaker at 120 rpm. After 12 h, bacterial final suspensions were prepared at OD_{600}=0.150 to 0.9. The optimum OD_{600}=0.02 consistently achieved at CFU/mL for inoculating NCI-N87 cells at MOI=100:1.

5.3.2 Assessment of trypsin and cell culture media on viable *H. pylori* for cell adhesion assays. Trypsinization was the technique used to dissociate cell monolayers. After co-culture

with *H. pylori*, the effects of trypsin were tested on *H. pylori* viability. *H. pylori* viability decreased >50% after 12 min exposure to trypsin. Due to this detrimental effect, a non enzymatic method was chosen to dissociate cell monolayers after *H. pylori* inoculation. Cell scrapers were chosen to dissociate cell monolayers.

To ensure that there was no detrimental effect of RPMI-1640 media on *H. pylori* viability, *H. pylori*/RPMI-1640 mixture was incubated for 90 min and the number of CFU was determined. RPMI-1640 had no effect on *H.pylori* cell count.

5.3.3 Determination of incubation time for optimal *H. pylori* adherence to NCI-N87 cells. NCI-N87 were incubated at 30 to 360 min with *H. pylori* to evaluate the optimum incubation time for *H. pylori* adherence to gastric cells (Fig. 5.1). *H. pylori* adhesion followed a sigmoidal relationship over 90 min and reached a plateau. Incubation time for adhesion assays at 90 min provided the highest CFU/well at a MOI of 100:1.

5.3.4 Effect on *H. pylori* adherence to gastric cells treated with a ganglioside mix enriched powder. To analyze if a ganglioside mixture modifies *H. pylori* adhesion to gastric epithelial cells, gastric epithelial cells were treated with ganglioside mix enriched powder for 48 h at increasing concentrations (0-30 μg/mL). *H. pylori* adhesion was not affected when gastric cells were treated with this ganglioside mix enriched powder. Four experiments were performed in triplicate wells and each well was counted twice. Gastric epithelial cells treated with 0 μg/mL had 2.10 $x10^6$ to $6.5x10^6$ CFU/well, at 10 μg/mL had $4.90x10^5$ to $9.50x10^6$ CFU/well, at 20 μg/mL had $4.20x10^6$ to $8.10x10^6$ CFU/well, and at 30 μg/mL had $4.40x10^5$ to $9.62x10^6$ CFU/well. Control values for *H. pylori* adhesion for this treatment represent

one/third compared to control values in the other ganglioside treatments. Results are illustrated on Appendix B.

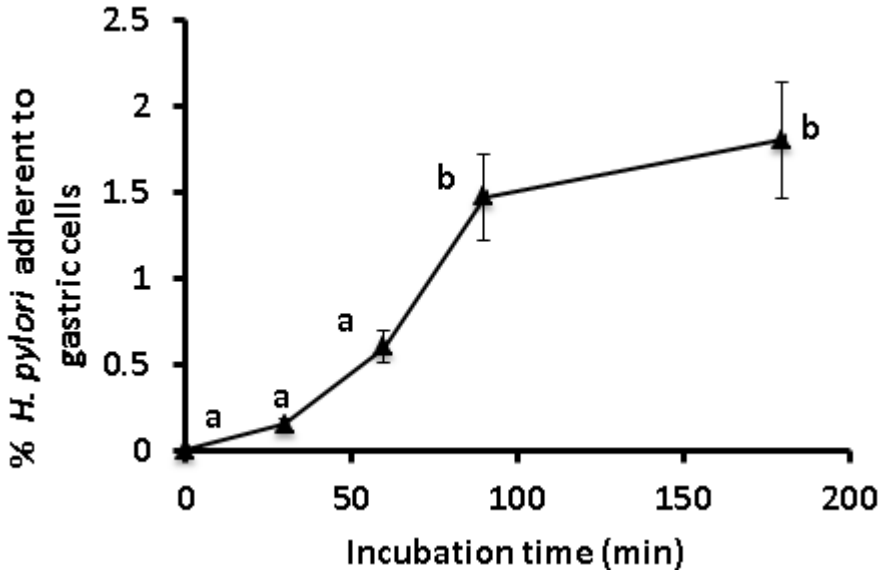


Figure 5.1 Percentage of *H. pylori* adherent to untreated gastric cells with increasing incubation time. *H. pylori* adherence increases from 0 to 90 min. Data is expressed as mean ± SD from 3-6 determinations. Different letters indicate significant difference at P<0.01.

5.3.5 GD3 or GM3 enriched treatment decreases *H. pylori* adhesion to gastric epithelial cells. To analyze if GM3 or GD3 alter *H. pylori* adhesion to gastric epithelial cells, gastric epithelial cells were treated with GM3 or GD3 enriched media for 48 h at increasing concentrations (5-30 μg/mL). Gastric cells treated with GD3 at all concentrations decreased *H. pylori* adhesion compared to untreated gastric cells (P<0.007) (Fig. 5.2). When cells were treated at 5 μg/mL, *H pylori* adherence decreased to only 17% of control values. The highest inhibition was observed when cells were treated with 20 μg/mL of GD3 since *H. pylori* adhesion decreased to only 5% but this binding was not significant lower than the GD3 concentrations. This corresponded to the number of viable *H. pylori* bound to untreated cells at $11x10^6$ to $21x10^6$ CFU/well and decreased to $1.7 x10^6$ CFU/well in treated cells (Fig. 5.3).

Gastric cells treated with GM3 at all concentrations also decreased *H. pylori* adhesion compared to untreated gastric cells (P<0.0002) (Fig. 5.4). Maximum reduction of *H. pylori* adhesion was achieved when gastric cells were treated with GM3 at 10 μg/mL were *H. pylori* binding was 8% of control values. When cells were treated at higher concentrations of GM3 there was no statistically significant difference in *H. pylori* binding. The average *H. pylori* binding in GM3 treated cells was to 20% of control values. This corresponds to the number of *H. pylori* in untreated cells at $11x10^6$ to $21x10^6$ CFU/well and decreased to an average of $2x10^6$ CFU/well (Fig. 5.5).

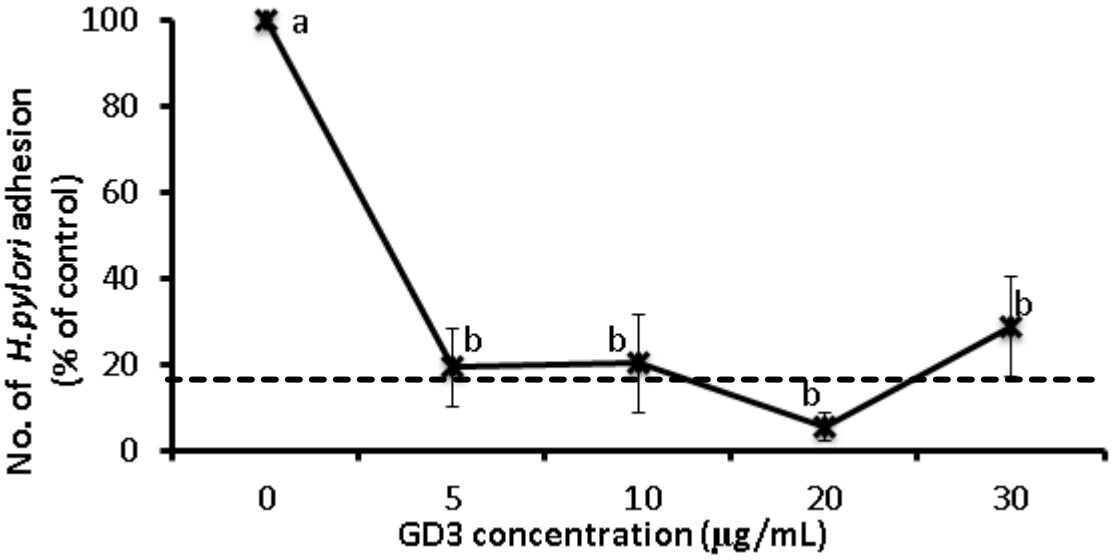


Figure 5.2 GD3 decreased *H. pylori* adhesion to human gastric cells. Human gastric cells with GD3 incorporated in the plasma membrane decreased *H. pylori* adhesion at all concentrations (5-30 μg/mL) to an average of 18% of control values (----). Data is expressed as mean ± SD from 3 experiments in triplicate (3 wells). Each well was counted in duplicate. Different letters indicate significant difference at P<0.007.

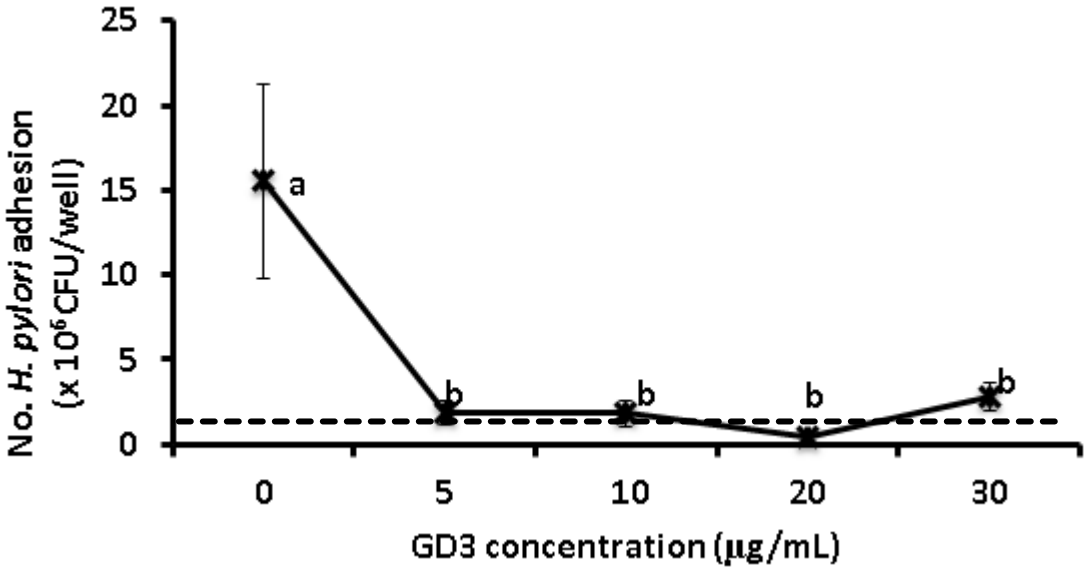


Figure 5.3 GD3 decreased number of *H. pylori* adherent to human gastric cells. Human gastric cells with GD3 incorporated in the plasma membrane decreasing *H. pylori* binding at all concentrations (5-30 µg/mL) to average of 1.71×10^6 CFU/mL (---- **).** Data is expressed as mean ± SD from 3 experiments in triplicate (3 wells). Each well was counted in duplicate. Different letters indicate significant difference at $P<0.002$

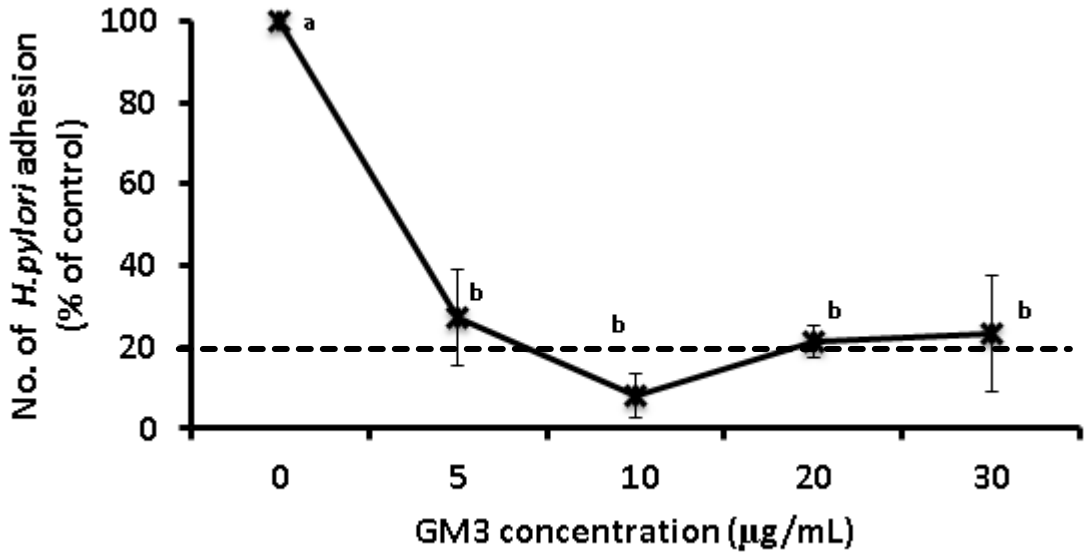


Figure 5.4 GM3 decreased *H. pylori* adhesion to human gastric cells. Human gastric cells with GM3 incorporated in the plasma membrane decreased *H. pylori* adhesion at all concentrations (5-30 µg/mL) to an average of 20% of control values (---- **).** Data is expressed as mean ± SD from 3 experiments in triplicate (3 wells). Each well was counted in duplicate. Letters indicate significant difference at $P<0.0002$.

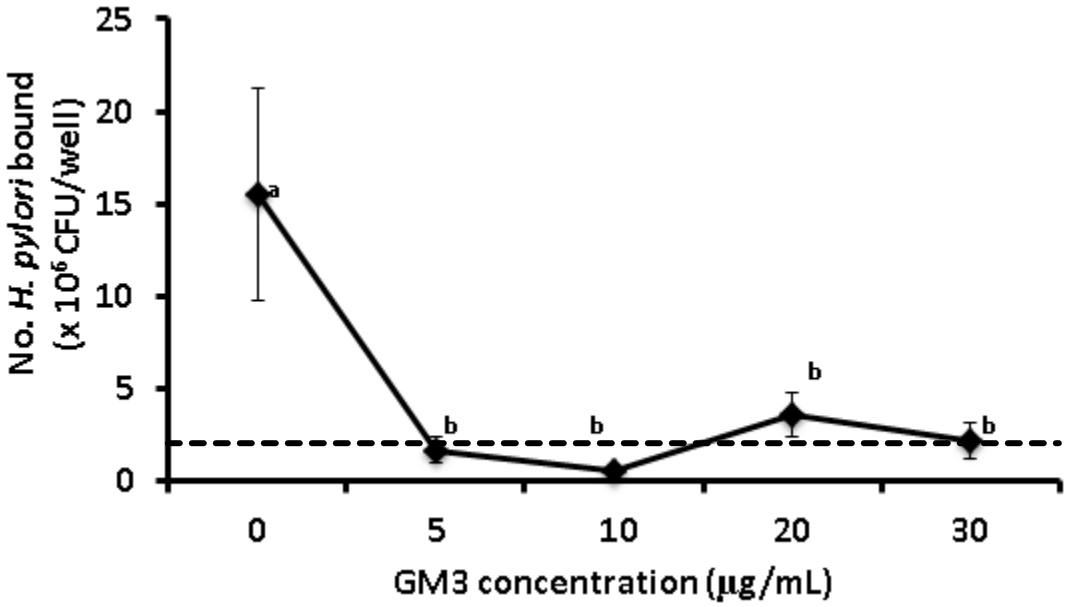


Figure 5.5 GM3 decreased *H. pylori* adherent in human gastric cells. Human gastric cells with GM3 incorporated in plasma membrane decreasing *H. pylori* binding at concentrations (5-30 µg/mL) to an average of $2x10^6$ CFU/mL (----). Data is expressed as mean ± SD from 3 experiments in triplicate (3 wells). Each well was counted in duplicate. Different letters (a,b) indicate significant difference at $P<0.002$

5.3.6 GM3:GD3 decreases *H. pylori* adhesion to human gastric epithelial cells. To evaluate the effectiveness of ganglioside treatments, two different ratios of gangliosides were tested: GM3:GD3 (2:8) or GM3:GD3 (8:2). Gastric human epithelial cells were treated at concentrations of GM3:GD3 (2 and 8 µg/mL, respectively) or GM3:GD3 (8 and 2 µg/mL, respectively) for 48 h hours (Fig. 5.6). *H. pylori* adhesion decreased after cells were treated with GM3:GD3 (2:8) and GM3:GD3 (8:2). When cells were treated with this combination of GM3:GD3 (2:8), *H. pylori* adherence decreased to 13% of control. When cells were treated with GM3:GD3 (8:2), *H. pylori* adhesion decreased to only 40% of control. This corresponds to the number of *H. pylori* in untreated cells at $10.5x10^6$ to $21x10^6$ CFU/well and decreased to an average of $3x10^6$ CFU/well for GM3:GD3 (8:2) and $1x10^6$ CFU/well for GM3:GD3 (2:8) (Fig. 5.7).

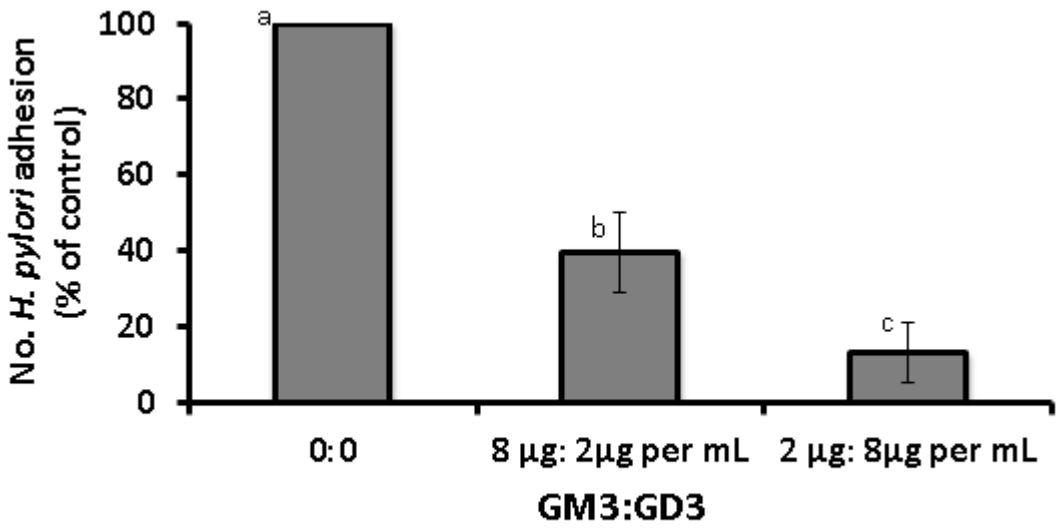


Figure 5.6 GM3:GD3 (2:8) and (8:2) decreased *H. pylori* adhesion to human gastric cells. GM3: GD3 (2:8) decreased *H. pylori* adhesion to 13% of control. Data is expressed as mean ± SD from 3 experiments in triplicate (3 wells). Each well was counted in duplicate. Different letters (a,b,c) indicate significant difference. For GM3:GD3 (2:8) treatment at $P<0.00007$ and for GM3:GD3 (8:2) treatment at $P<0.02$.

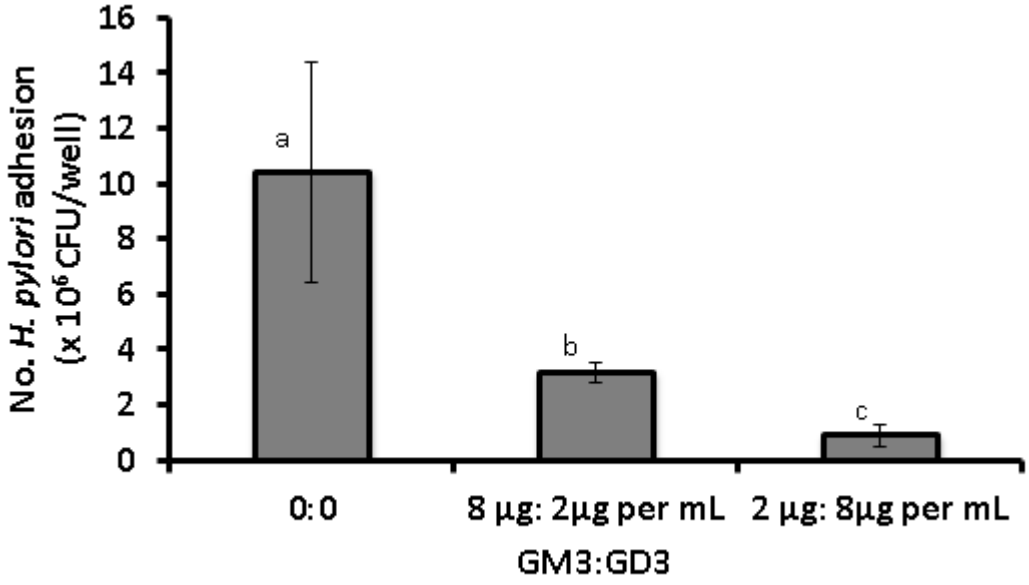


Figure 5.7 GM3:GD3 (2:8) and (8:2) decreased number of *H. pylori* adherent to human gastric cells. Data is expressed as mean ± SD from 3 experiments in triplicate (3 wells). Each well was counted in duplicate. Different letters (a,b,c) indicate significant difference. For GM3:GD3 (2:8) treatment at $P<0.00007$ and for GM3:GD3 (8:2) treatment at $P<0.02$.

5.3.7 Relationship between GD3 and GM3 content in the plasma membrane of gastric cells and *H. pylori* adhesion. Gastric cells take up gangliosides in a dose-dependent manner and are absorbed in the plasma membrane of gastric cells. Relationships between GD3 and GM3 gangliosides content in plasma membrane in gastric cells after treatments and *H. pylori* adhesion is explained. Untreated gastric cells exhibited a GD3 content of 17±4 ng of GD3/well and GM3 content of 63±22 ng of GM3/well. *H. pylori* adhesion was 16±5x10^6 CFU/well. Gastric cells treated with GM3 at concentrations of 10 μg/mL increased GD3 and GM3 content to 132±11 ng of GD3/well and GM3 increased to 284±1 ng of GM3/well, respectively. *H. pylori* adhesion was reduced to 0.6±0.15x10^6 CFU/well. Gastric cells modified with GD3, at concentration of 10 μg/mL, GD3 increased to 33±3 ng of GD3/well and GM3 to 247±13 ng of GM3/well. At 30 μg/mL, GD3 increased to 118±17 ng of GD3/well and GM3 to 316±1 ng of GM3/well. After 10 and 30 μg/mL GD3 treatments, a reduction of *H. pylori* binding was observed (2±0.8x10^6 CFU/well and 3±0.8 x10^6 CFU/well, respectively). Ganglioside concentration increasing in plasma membranes represent less *H. pylori* adhesion to gastric epithelial cells (see Appendix C).

When cells contain low concentrations of GM3 and GD3, *H. pylori* binding is greater compared to treated gastric cells. Increasing GD3 and GM3 content decreased *H. pylori* binding in both treatments. After treatment, GM3 is present in higher amounts than GD3 in plasma human gastric cells. It is not clear if GM3 may inhibit effect on *H. pylori* adhesion. GD3 and GM3 species, have different ceramide moieties, but mainly gastric cells incorporated monounsaturated and saturated ceramides (Chapter 4 Table 4.1 and 4.2). The carbohydrate moiety is essential for adhesion of *H. pylori* to gangliosides; however differences in binding due to saturation/unsaturation of ceramides may affect the conformation of the carbohydrate

and alter receptor recognition for binding *H. pylori* to gangliosides.[42] In the present study, gangliosides have saturated and unsaturated ceramide moieties. It is not clear how saturation/unsaturation of ceramides in gangliosides may contribute to the inhibition of *H. pylori* binding.

5.4 Discussion

H. pylori binds human gastric cells using adhesins. Sab A is a *H. pylori* adhesin that adheres to sialic acid moieties.[27] SabA is not present in *H. pylori* A64 strain, but other adhesins (Nap and HpaA) may adhere to sialic acids (gangliosides).[23] *H. pylori* adhesins binds different glycoproteins or glycolipids present in gastric cells. At 90 min of incubation time, 1.5% of *H. pylori* A64 was found binding NCI-N87 cells. After 90 min, binding reached a plateau, possible because the number of binding sites was saturated.

When gastric cells treated with ganglioside mix enriched powder containing different GD3 91%, GM3 4.5%, GT3 2.5%, GM1 1%, GD1 0.5%, GT2 0.5%, *H. pylori* adhesion was not affected. The ganglioside mix enriched powder contains different gangliosides that might be involved in *H. pylori* adhesion. Other components present in ganglioside mix enriched powder may influence *H. pylori* binding. *H. pylori* A64 strain might have affinity for one or more gangliosides contained in ganglioside mix enriched powder.[28] Hirmo et al. (1996) reported that *H. pylori* sialic acid dependent strain bind glycolipids on α-2,3 linked sialic acids.[29] All ganglioside species (GD3, GM3, GT3, GT2, GM1) contain α-2,3 linked bond in ganglioside structure might represent a receptor for *H. pylori* attachment. For example, Moss et al. (1976) found that NCTC 2071 cells (mice fibroblasts) do not synthesize GM1, but the uptake of exogenous GM1 made this cell susceptible to cholera toxin.[30]

When cells were treated with GD3 or GM3, *H pylori* adhesion decreased. GD3 and GM3 decrease by a 80% *H. pylori* adhesion to human gastric epithelial cells. Differences between GD3 and GM3 treatments are not significant probably because the uptake of GD3 increases GM3 synthesis by catabolism of GD3 and/or other gangliosides (see section 5.3.7). The decrease observed in *H. pylori* adhesion may due to a spatial configuration and structural arrangement of GD3 and GM3 gangliosides that block entrance and attachment by *H. pylori* to other host receptors.[24,31] Reuter et al. (1999) found that when some low affinity interactions occur between influenza virus and monomeric sialic acid. High concentrations of sialic acids might inhibit influenza virus attachment to cells. In the present study, the presence of numerous sialic acids might contribute to decrease *H. pylori* adhesion.[32] Bacteria move to a surface by the effect of physical interactions. When bacteria are proximal to the surface, these interactions can be attractive or repulsive. In the first step of colonization, if bacteria overcomes repulsive interactions binding is loose and reversible, this interaction is regularly a hydrophobic interaction.[12,33,34] Wada et al. (2010) reported that gangliosides neutralize and inhibit the internalization of VacA virulent protein of *H. pylori* to gastric cells suggesting that gangliosides are decoy receptors to impede the binding of pathogens to cells.[45]

Dietary gangliosides are found the Milk Fat Globule Membrane (MFGM) of human and bovine milk. GM3 and GD3 are the most abundant species.[43,44] Concentration and composition of GM3 and GD3 may change with state of lactation.[43,44] In the first stage of lactation, GD3 is the most abundant ganglioside in human milk (colostrum) and in late lactation, GM3 increases.[43] In bovine milk, GD3 is always the most abundant ganglioside.[35] Two combinations with different GM3:GD3 ratios were tested in the present study. GM3:GD3 (2:8) ratio resembles bovine milk or human milk during the first 8 days of lactation while

GM3:GD3 (8:2) ratio resembles human milk after 8 days of lactation. Following the hypothesis that *H. pylori* infection occurs during childhood, bovine and human milk are the source of nutrients during this period. GM3:GD3 (2:8) had a greater effect decreasing *H. pylori* adhesion by 87% while GM3:GD3 (8:2) by 60%. GM3:GD3 (2:8) showed higher inhibition compared to GM3:GD3 (8:2) suggesting that human milk may be more protective. *H. pylori* infection may be obtained in the first years of life. Few studies have evaluated *H. pylori* infection in neonates. Two studies show acquisition of *H. pylori* infection as neonates, one neonate breast-feeding and consuming formula.[38,39] Human milk has not be found to prevent *H. pylori* infection in these two reports in unknown.[38,39] In general, human and bovine milk might contribute to decrease *H. pylori* infection, but this inhibition cannot be confirmed because other gangliosides are also present in the MFGM that may alter *H. pylori* adhesion.

In summary, adhesion is an essential step to colonize to the human stomach by *H. pylori.* Decreasing *H. pylori* adhesion also decreases the possibility of colonization by this bacterium. Gangliosides are bioactive compounds that might decrease *H. pylori* infection.

5.5 References

1. Go, M.F. (2002). Review article: natural history and epidemiology of *Helicobacter pylori* infection. *Alimentary Pharmacology & Therapeutics* 16(Suppl. 1): 3-15.

2. Ahmed, K.S.; Khan, A.A.; Ahmed, I.; Tiwari, S.K.; Habeeb, A.; Ahi, J.D.; Abid, Z.; Ahmed, N.; Habibullah, C.M. (2007). Impact of household hygiene and water source on the prevalence and transmission of *Helicobacter pylori*: a South Indian perspective. *Singapore Medical Journal*. 48(6): 543-549.

3. Dunn, B.E.; Cohen, H.; Blaser M.J. (1997). *Helicobacter pylori*. *Clinical Microbiology Reviews*, 10(4):720-741.

4. Perez-Perez G.I., Rothenbacher D. and Brenner H. (2004). Epidemiology of *Helicobacter pylori* Infection. *Helicobacter*, 9 (Suppl. 1):1–6.

5. Rothenbacher, D.; Bode, G.; Berg, G.; Gommel, R.; Gonser, T.; Adlerb, G.; Brenner H. (1998). Prevalence and determinants of *Helicobacter pylori* infection in preschool children: a population-based study from Germany. *International Journal of Epidemiology*, 27:135-141.

6. Thomas JE, Dale A, Harding M, Coward WA, Cole TJ, Weaver LT. *Helicobacter pylori* colonization in early life. *Pediatric Research* 1999; 45: 218-223.

7. Bani-Hani, K.E.; Nawaf, J.S.; Qaderi, S.E.; Khader, Y.S.; Bani-Hani, B.K. (2006). Prevalence and risk factors of *Helicobacter pylori* infection in healthy schoolchildren. *Chinese Journal of Digestive Diseases*, 7:55–60.

8. Strebel, K.; Rolle-Kampczy, U.; Richter, M.; Kindler, A.; Richter, T.; Schlink, U. (2010). Arigorous small area modelling-study for the *Helicobacer pylori* epidemiology. *Science of the Total Epidemiology*, 408: 3931-3942.

9. Makola, D.; Peura, D.A.; Crowe, S.E. *Helicobacter pylori* infection and related gastrointestinal disesases. *Journal of Clinical Gastroenterology,* 2007, 41(6): 548-558.

10. Yucel, O. (2014) Prevention of *Helicobacter pylori* infection in childhood. World *Journal of Gastroenterology*, 20(30): 10348-10354.

11. Karlsson, K.A. (1989). Animal Glycosphingolipids as Membrane Attachment Sites for Bacteria. *Annual Review of Biochemistry*, 58:309-350.

12. Shoaf-Sweeney, K.D.;b Hutkins, R.W. (2009). Adherence, Anti-Adherence, and Oligosaccharides: Preventing Pathogens from Sticking to the Host. *Advances in Food and Nutrition Research*, 55: 101-161.

13. Aspholm, M.; Olfat, F.O.; Nordén, J.; Sondén, B.; Lundberg, C.; Sjöström, R.; Altraja, S.; Odenbreit, S.; Haas, R.; Wadström, T.; Engstrand, L.; Semino-Mora, C.; Liu, H.; Dubois, A.; Teneberg, S.; Arnqvist, A.; Boren, T. (2006). SabA is the *H. pylori* hemagglutinin and is polymorphic in binding to sialylated glycans. *PLoS Pathogens*, 2(10):e110.

14. Varki, A. (2008). Sialic acids in human health and disease. *Trends in Molecular Medicine*, 14(8): 351-360.

15. Slomiany, B.L.; Piotrowski, J.; Samanta, A.; VanHorn, K.; Murty, V.L.N.; Slomiany, A. (1989). *Campylobacter pylori* colonization factor shows specificity for Lactosylceramide sulfate and GM3 Ganglioside. *Biochemistry International,* 19(4): 929-936.

16. Saitoh, T.; Sugano, K.; Natomi, H.; Zhao, W.; Okuzumi, K.; Iwamori, M.; Yazaki, Y. (1992). Glycosphingolipid receptors in human gastric mucosa for *Helicobacter pylori*. *European Journal of Gastroenterology & Hepatology,* 4:S49-S53.

17. Tettamanti, G. (2004). Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconjugate Journal*, 20(5):301-317.

18. Yu, R.K.; Tsai, Y.T.; Ariga, T.; Yanagisawa, M. (2011). Structures, biosynthesis, and functions of gangliosides-an overview. *Journal of Oleo Science*, 60(10):537-544.

19. Ryan, J.M.; Rice, G.E.; Mitchell, M.D. (2013). The role of gangliosides in brain development and the potential benefits of perinatal supplementation. *Nutrition Research*, 33(11): 877-887.

20. Hata, Y; Murakami, M.; Okabe, S. (2004). Glycoconjugates with NeuAc-NeuAc-Gal-Glc are more effective at preventing adhesion of *Helicobacter pylori* to gastric epithelial cells than glycoconjugates with NeuAc-Gal-Glc. *Journal of physiology and Pharmacology*. (55) 3: 607-625.

21. Salcedo, J.; Barbera, R.; Matencio, E.; Alegría, A.; Lagarda, M.J. (2013). Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: an in vitro study. *Food Chemistry*, 136(2):726-734.

22. Horemans, T.; Kerstens, M.; Clais, S.; Struijs, K.; van den Abbeele, P.; Van Assche, T.; Maes, L.; Cos, P. (2012). Evaluation of the anti-adhesive effect of milk fat globule membrane glycoproteins on *Helicobacter pylori* in the human NCI-N87 cell line and C57BL/6 mouse model. *Helicobacter*, 17(4):312-318.

23. Lane, J.A.; Mehra, R.K.; Carrington, S.D.; Hickey, R.M. (2010). The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. *International Journal of Food Microbiology*,142(1-2):1-13.

24. Sharon, N. (2006). Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochimica et Biophysica Acta*, 1760(4):527-537.

25. Radin, N. S. (2006). Preventing the binding of pathogens to the host by controlling sphingolipid metabolism. *Microbes and Infection*, 8: 938-945.

26. Shabram, P.; Aguilar-Cordova, E. (2000). Multiplicity of Infection/Multiplicity of Confusion. *Molecular therapy*. 2(5):420-421.

27. Amieva, R.M.; El–Omar, M.E. (2008). Host-Bacterial Interactions in *Helicobacter pylori* Infection. *Gastroenterology,* 134:306–323.

28. Schengrund, C.L. (2003) ``Multivalent'' saccharides: development of new approaches for inhibiting the effects of glycosphingolipid-binding pathogens. *Biochemical Pharmacology,* 65:699-707.

29. Hirmo, S.; Kelm, S.; Schuer, R.; Nilsson, B.; Wadstrom, T. (1996). Adhesion of *Helicobacter pylori* strains to α-2,3-1inked sialic acids. *Glycoconjugate Journal*, 13: 1005-1011.

30. Moss, J.; Fishman, P.H.; Manganiello, V.C.; Vaughan, M.; Brady, R.O. (1976). Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. *Proceedings of the National Academy of Sciences of the United States of America*. 73(4):1034-1037.

31. Kelly, C.G.; Younson, J.S. (2000). Anti-adhesive strategies in the prevention of infectious disease at mucosal surfaces. *Expert Opinion on Investigational Drugs*, 9(8):1711-1721.

32. Reuter, J.D.; Myc, A.; Hayes, M.M.; Gan, Z.; Roy, R.; Qin, D.; Yin, R.; Piehler, L.T.; Esfand, R.; Tomalia, D.A.; Baker, J.R. Jr. (1999). Inhibition of viral adhesion and infection by sialic-acid-conjugated dendritic polymers. *Bioconjugate Chemistry*, 10(2):271-278.

33. Katsikogianni, M.; Missirlis, Y.F. (2004). Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *European Cells and Materials*, 8:37-57.

34. Hasty, D.L.; Ofek, I.; Courtney, H.S.; Doyle, R.J. (1992). Multiple adhesins of streptococci. *Infection and Immunology.* 60(6):2147-2152.

35. Rueda, R.; Maldonado, J.; Narbona, E.; Gil, A. (1998). Neonatal dietary gangliosides. *Early Human. Development, 53: S135-S147.*

36. Correia, M.; Michel, V.; Osorio, H.; El Ghachi, M.; Bonis, M.; Boneca, I.G.; De Reuse, H.; Matos, A.A.; Lenormand, P.; Seruca, R.; Figueiredo, C.; Machado, J.C.; Touati, E. (2013). Crosstalk between *Helicobacter pylori* and gastric epithelial cells is impaired by docosahexaenoic acid. *PLoS One*, 8(4):e60657.

37. Conlin, V.; Curtis, S.B.; Zhao, Y.; Moore, E.D.; Smith, V.C.; Meloche, R.M.; Finlay, B.B.; Buchan, A.M. (2004). *Helicobacter pylori* infection targets adherens junction regulatory proteins and results in increased rates of migration in human gastric epithelial cells. *Infection and Immunity,* 72(9):5181-5192.

38. Halabi, I.M. (2009). *Helicobacter pylori* infection in a 3-week-old. *Annals of Tropical Paediatrics*, 29:247–249.

39. Guelrud, M.; Mujica, C.; Jaen, D.; Machuca, J.; Essenfeld, H. (1994). Prevalence of *H. pylori* in Neonates and Young Infants Undergoing ERCP for Diagnosis of Neonatal Cholestasis. *Journal of Pediatric Gastroenterology*, 18:461-464.

40. Park, J.G.; Frucht, H.; LaRocca, R.V.; Bliss, D.P. Jr.; Kurita, Y.; Chen, T.R.; Henslee, J.G.; Trepel, J.B.; Jensen, R.T.; Johnson, B.E. (1990). Characteristics of cell lines established from human gastric carcinoma. *Cancer Research*, 50(9):2773-2780.

41. Strober, W. (2001). Trypan blue exclusion test of cell viability. *Current Protocol Immunology*. Appendix 3:Appendix 3B.

42. Martin, M.J.; Martin-Sosa, S.; Alonso, J.M.; Hueso, P. (2003). Enterotoxigenic Escherichia coli Strains Bind Bovine Milk Gangliosides in a Ceramide-Dependent Process. *Lipids*, 38(7):761-768

43. Rueda R, Garcia-Salmero´n JL, Maldonado J, Gil A. (1996). Changes during lactation in ganglioside distribution in human milk from mothers delivering preterm and term infants. *Biological Chemistry*, 377:599 – 601.

44. Puente R, Hueso P. (1993). Lactational changes in the N-glycolylneuraminic acid content of bovine milk gangliosides. *Biological Chemistry Hoppe-Seyler*,374:475–478.

45. Wada, A.; Hasegawa, M.; Wong, P.F.; Shirai, E.; Shirai, N.; Tan, L.J.; Llanes, R.; Hojo, H.; Yamasaki, E.; Ichinose, A.; Ichinose, Y.; Senba, M. (2010). Direct binding of gangliosides to Helicobacter pylori vacuolating cytotoxin (VacA) neutralizes its toxin activity. *Glycobiology*, 20(6):668-678.

CHAPTER VI. Conclusion and discussion

6.1 Overall conclusion

Hypotheses tested in this thesis have been addressed as follows:

Hypothesis 1. Pasteurization treatment does not affect ganglioside content measured in whole milk.

Pasteurization increases ganglioside content in whole milk. After pasteurization treatments, ganglioside content increased by 20% after LTLT pasteurization and HTST pasteurization also increased ganglioside content. Ganglioside profile was not altered by any pasteurization treatment.

This finding suggests that dietary gangliosides contained in milk products preserved under LTLT and HTST are not altered compositionally. This finding also suggests that MFGM is denatured by heating treatments which enable removal of gangliosides easily by Folch extraction. If gangliosides are released from the MFGM; this release also might improve bioavailability.

Hypothesis 2. Ganglioside profile will be different in the three different dairy products analysed (whole raw milk, colostrum, buttermilk).

Seven different mono, di and tri-sialogangliosides were found in whole milk, colostrum and buttermilk. All ganglioside species are present in different relative amounts. GT2 and GT4 were found in very small amounts and are present in colostrum. In agreement with the literature review, GD3 was found to be the most abundant ganglioside for the three products. GT3 was found to be the second most abundant ganglioside which represents a

disagreement with previous studies, since GM3 is considered the second most abundant ganglioside. This discrepancy might be due to ionization efficiency of gangliosides, extraction procedures or biological factors. Differences in ceramide saturation/unsaturation are not remarkable since monounsaturated ceramides are present in high amounts in GM3, GD3 and GT3 in the three milk products. Saturated and polyunsaturated ceramides are less abundant.

Hypothesis 3..Human gastric epithelial cell membrane will be modified by GD3, GM3 and a ganglioside mix enriched powder.

Gastric cells exposed to ganglioside enriched media containing GD3, GM3 or ganglioside mix enriched powder increased ganglioside content in human gastric cells. The content of GM3 and GD3 was modified by the three treatments. Ganglioside mix enriched powder treatment, containing both GD3 and GM3 ganglioside species along with other ganglioside species, increased content of both gangliosides, but in smaller amounts than the GD3 and GM3 treatment. The most remarkable change was when cells treated with GD3 since cells increased GM3 ganglioside content more than GD3 content. This finding might indicate that GM3 is an important ganglioside for this type of cell. During GD3 treatment, degradation of GD3 results in GM3. During GM3 treatment, GD3 increased in content possibly because GM3 also produces GD3.

Media contained different GD3 and GM3 species with variations in ceramide composition. Some ganglioside species, containing mainly saturated and monounsaturated ceramides, were taken up into gastric cells. For gastric cells treated with GD3 at 30 μg/mL, this treatment resulted in seven new ganglioside species in the cell with different ganglioside than observed

after GD3 treatment at 10 μg/mL. This finding indicates that gangliosides might be absorbed from the apical membrane side of human stomach.

Hypothesis 4. Human gastric epithelial cell membranes enriched with GD3 and GM3 will modify apical membrane domain and not the basolateral membrane domain.

In NCI-N87 cells, GM3 was the most abundant ganglioside located in the apical membrane domain, while GD3 was found in higher amounts in the basolateral membrane domain. When human gastric epithelial cells were treated with a ganglioside mix enriched powder to determine if gangliosides alter the apical and basolateral membrane domains, GD3 and GM3 gangliosides increased in content in the apical membrane domain. After treatments GD3 and GM3 were found in higher amounts in basolateral membrane domain. The mechanism for migration of gangliosides is unknown, but probably transcytotic pathway using protein carriers or vesicles may be involved. Saturation/unsaturation in the ceramide moieties present in gangliosides can have important roles. In this regard, GM3 C43:0 was found in both the apical and basolateral membrane, however GM3 C43:1 was only found in the apical membrane domain.

This finding suggests that absorption of gangliosides modify GD3/GM3 ratio in the apical membrane domains. This finding also suggests that gangliosides have varied roles and need to be transported to other sites of the cell.

Hypothesis 5. Exogenous gangliosides will modify phospholipids (PC and PE) in human gastric epithelial cells.

Gastric epithelial cells modified with exogenous gangliosides did not show statistically significant changes in the amounts of total phospholipids, PC and PE. Composition of PC and PE was also not modified. This finding suggests that any effect of exogenous gangliosides on bacterial adhesion does not involve change in the phospholipids of the membrane.

Hypothesis 6. Human gastric cells modified with a ganglioside mix enriched powder in human gastric epithelial cells will decrease *H. pylori* adhesion.

Incubating gastric cells with a complex ganglioside mixture did not produce consistent effects on *H. pylori* attachment. It is not a conclusive experiment for the variations at this study. These ganglioside mix enriched powder contains other components not quantified.

Hypothesis 7. Human gastric cells treated with purified GD3 ganglioside will reduce *H. pylori* adhesion more than human gastric epithelial cells treated with GM3.

Human gastric cells modified with purified GD3 and GM3 ganglioside decreased *H. pylori* binding to a similar degree. Cells treated with GM3 and GD3 decreased *H. pylori* adhesion to epithelial cells at all concentrations. Gastric cells modified with a mixture of GM3:GD3 (2:8) (resembles human milk or human colostrum) decreased *H. pylori* adhesion in similar proportion to GM3 and GD3 treatments; however, the mixture of GM3:GD3 (8:2) (resembles human milk) was less effective (reason is unknown). The mechanism for anti-adhesion was not examinated. This finding suggests that GM3 and GD3 can decrease *H. pylori* adhesion in human stomach. This finding also suggests that bovine and human milk may protect against *H. pylori* adhesion.

6.2 Overall thesis discussion

LC/MS is an optimum method to determine composition and content of gangliosides in milk products and biological systems. LC/MS is a very sensitive and accurate analytic method allowing saving time, sample size and use of solvents. More than 600 species of gangliosides can be scanned in less than three hours. Each ganglioside with more than 50 different ceramides can be detected in approximately 15 min. MS can be considered a rapid and routine method to assess the identity and quantity of milk gangliosides.

Gangliosides are being considered as a beneficial nutrient; and thus can be supplemented in milk products. The present study identifies that pasteurization can be continued as the main preserving method for these products because this procedure does not damage or alter ganglioside structure.

GM3 and GD3 ganglioside species can be incorporated into the plasma membrane of human gastric epithelial cells. This observation is significant because it indicates that ganglioside present in the gastric lumen may be absorbed in the human stomach.

This study demonstrates that GM3 is mainly present in the apical membrane domain and GD3 is present in the basolateral membrane domain in human gastric epithelial cells. Exogenous gangliosides altered the ganglioisde content of the apical domain, but also gangliosides in the basolateral membrane. Migration of gangliosides to basolateral membrane can be related to saturation/unsaturation of the ceramide composition of gangliosides.

Alteration of adherence to *H. pylori* by ganglioside treatments is not related to modification of composition and amount of phospholipids in the plasma membrane.

The significance of this study involves the possibility of an anti-adhesion strategy to decrease or to prevent *H. pylori* infection without use of antibiotics. GM3 and GD3 decreased

H. pylori adhesion to gastric cells. Anti-adhesion may be due to gangliosides blocking entrance and adhesion by *H. pylori* to other host receptors, repulsive interactions or multiple sialic acids inhibit attachment. This finding suggests that individual dietary ganglisiosides in the appropriate concentrations might contribute to decreased *H. pylori* adhesion in some *H. pylori* strains. Since the use of vaccines to control *H. pylori* infection is far from developed, the use of one or more alternative therapies may be an option against this bacterium.

6.3 Future studies

To improve understanding of the mechanism of ganglioside absorption and trafficking in cells, functions, physiology and anti-adhesion therapy of gangliosides, future studies must be addressed:

1. To determine sphingosine and FA carbon chain number using analytic methodologies such as Time-of-flight MS, Fourier transform ion cyclotron resonance MS. Enzymatic methods may be required to break linkages in the ceramide portion and analyze the number of carbons only present in the sphingosine portion.

2. To perform *in vivo* studies (for example using mice models) for absorbance of gangliosides in stomach cells. *In vivo* studies may show the effectiveness of taking up ganglioside in the stomach and evaluate modifications in apical membrane for anti-adhesion therapies.

3. To study different trafficking and transcytotic pathways for ganglioside species migrating to the basolateral membrane. The fates of ganglioside and functions of gangliosides specifically in gastric cells is not clear. Confocal microscopy studies with radiolabelled gangliosides might clarify these pathways.

4. To evaluate adhesion to human gastric cells using other *H. pylori* strains with different adhesins. The *H. pylori* strain used in this study did not express SabA and other adhesins that bind sialic acids were not identified. Other *H. pylori* strains must be studied to establish the role of gangliosides as inhibitors of adhesion.

5. To determine the mechanism of interaction between *H. pylori* adhesins and gangliosides. The present study did not determine the optimum ganglioside species to inhibit *H. pylori* adhesion. It is not clear why alterations in plasma membranes modified adhesion.

6. To determine the concentration of ganglioside where *H. pylori* adhesion decreased. The present study tested a minimum concentration of 5 μg/mL and found significance on *H. pylori* adhesion. Higher concentrations of 30 μg/mL to determine toxic levels need to be evaluated.

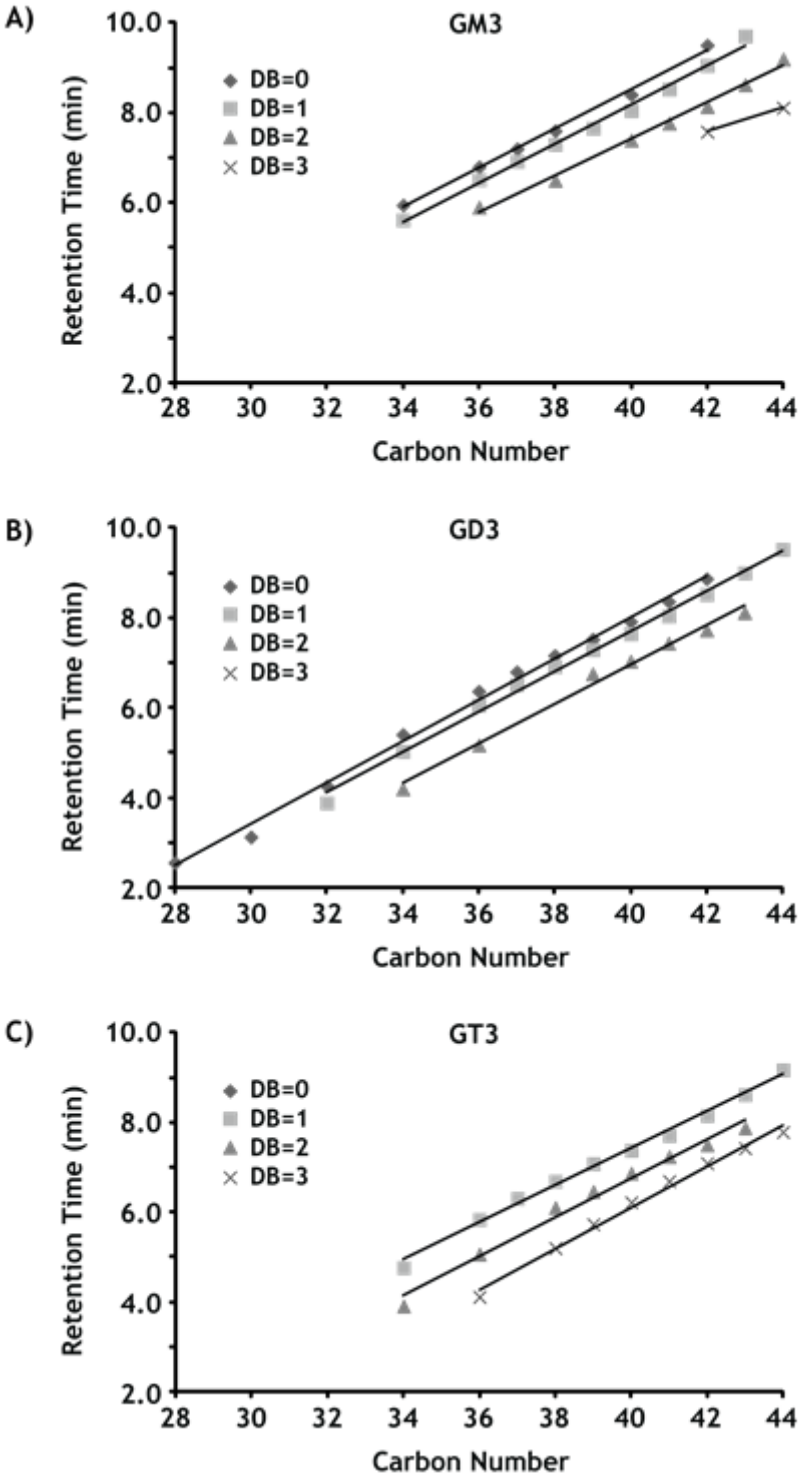
7. To evaluate if ganglioside concentrations found in milk products are bioavailable to be incorporated in gastric human membranes and decrease *H. pylori* adhesion.

8. The role of ganglioside species in human gastric cells in the apical and basolateral membrane domains is unknown and need to be studied to evaluate the effects on cell functions after ganglioside enrichment.

9. To evaluate the presence of enzymes involved in the metabolism of gangliosides in gastric human cells.

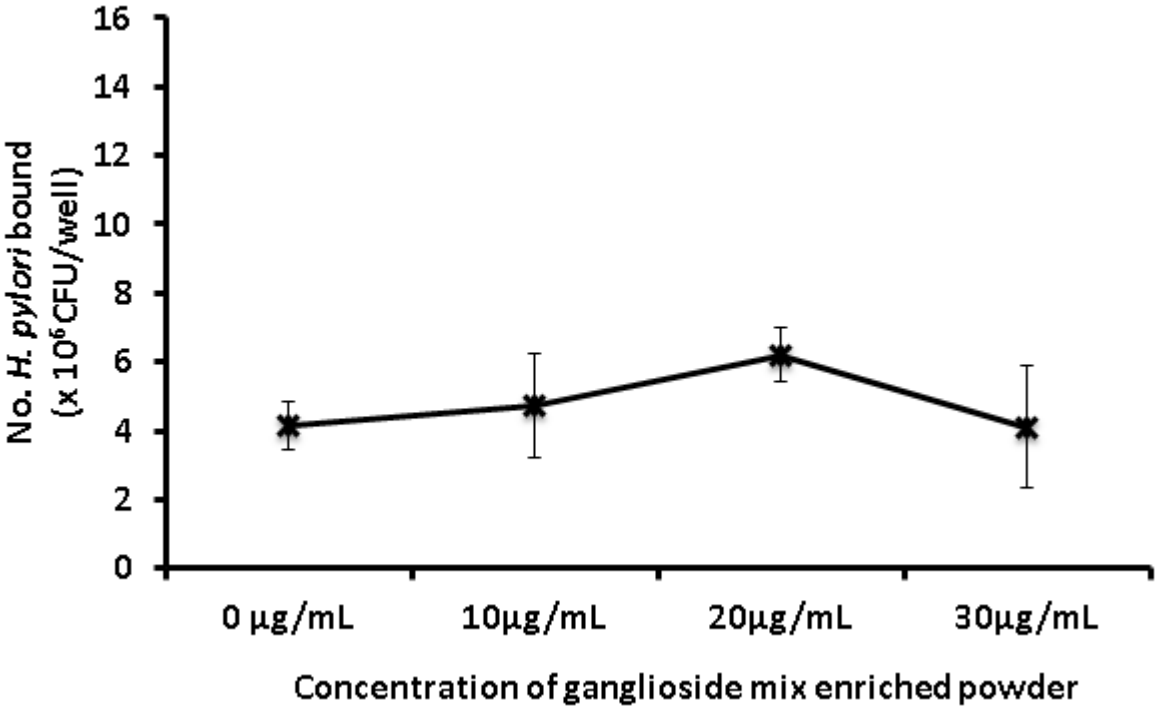
10. To evaluate the implications and functions of ceramide composition of gangliosides in human gastric cells and the role of ceramide composition in transcytotic pathways.

Appendix A



Trends in retention time for each GM3, GD3, and GT3 gangliosides. When grouped within their respective ganglioside class as well as the degree of saturation in the ceramide moiety, retention time on the C18 column was found to be linear with respect to carbon chain length. DB= double bonds.

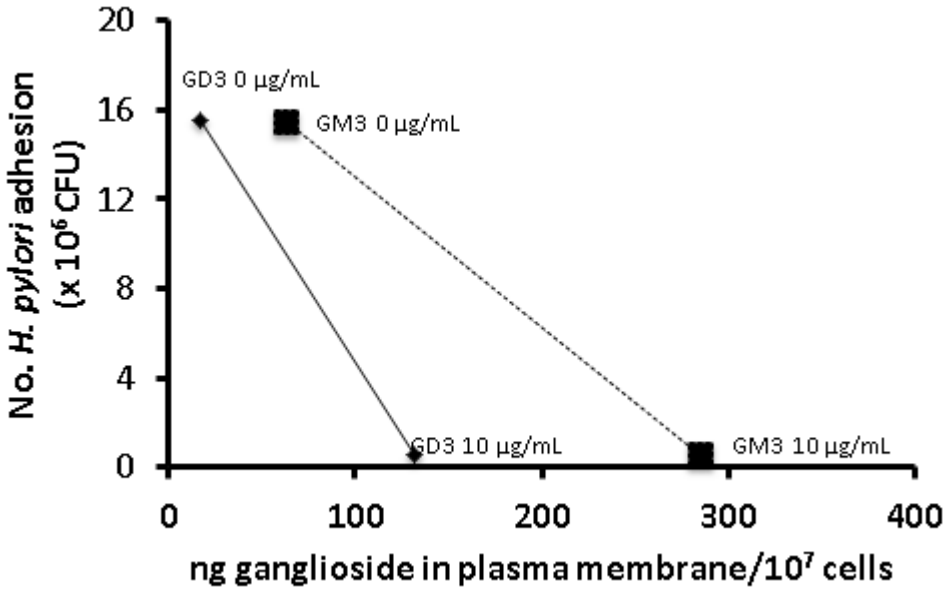
Appendix B



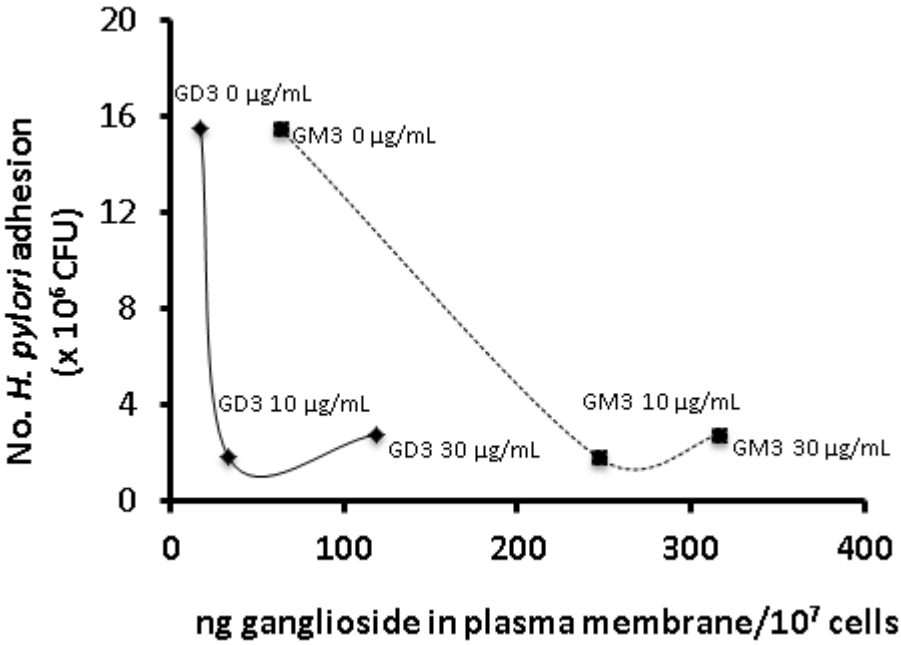
Gastric cells treated with ganglioside mix enriched powder and *H. pylori* binding to human gastric cells. Standard deviations are too high to stablish a relationship when cells are treated with ganglioside mix enriched powder. Data is expressed as mean±SD from 4 experiments in triplicate (3 wells). Each well was counted in duplicate.

Appendix C

A)



B)



Representation of the relationship between ganglioside content in plasma membrane and *H. pylori* binding. A) GD3 and GM3 content in plasma membrane relate to *H. pylori* binding after GM3 treatment (at 10 µg/mL). B) GD3 and GM3 content in plasma membrane relate to *H. pylori* binding after GD3 treatment (at 10 and 30 µg/mL). Each well contained 10^7 gastric cells. Low ganglioside content high *H. pylori* binding. When cells treated with gangliosides increased GM3 and GD3 content and decreased *H. pylori* adhesion.