

**THE RELATIONSHIP BETWEEN THE PREVALENCE OF RESPIRATORY
ILLNESS AND DERMATITIS AND INFANT DIET IN THE FIRST YEAR OF
LIFE**

By

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ABSTRACT

The progression from dermatitis in infancy to asthma and allergy in childhood has been termed the “atopic march”. Upper respiratory infections (URIs) in infancy are also linked to allergy and asthma. Human milk feeding and higher intakes of docosahexaenoic acid (DHA) may be protective. For the study, URIs and dermatitis in infancy were recorded and compared to human milk and DHA intake for 50 healthy term infants born to women enrolled in a study of DHA supplementation during pregnancy. All had 24-hour dietary recalls at 6 wk, 4, 6, 9, and 12 months, first year medical records, and information about environmental factors related to allergy/asthma. Forty-one of 50 received at least some human milk. DHA intake was estimated from individual milk (determined by chromatography) and formula DHA concentrations. Most infants had one or more episodes of dermatitis (26/50) and URI (39/50). Duration of human-milk feeding in infancy was associated with fewer dermatitis episodes ($R = -0.289$, $p = 0.044$). Breast feeding for >16 wks vs. <16 wks appeared to result in later onset but not statistically fewer total first year URIs ($p = 0.611$). With approximately 25% of the expected final sample complete, we find evidence already that longer human milk feeding may offer some protection against the “atopic march”.

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GLOSSARY

Allergen: antigen which produces an allergic response

Antibodies: proteins produced by B-cells after stimulation by an antigen that act against the foreign substance in an immune response

Antigen: any foreign substance that is bound by a specific antibody or lymphocytes

Atopic Dermatitis: chronic inflammation of the skin that may be related to other atopic diseases

Atopy: term used to describe an immunoglobulin E mediated allergic response

B-lymphocytes: precursors of antibody-forming plasma cells

Cytokines: substances secreted by cells that have effects on other cells

Eczema: an inflammatory condition of the skin causing redness and itching and oozing lesions which become scaly and crusted

Dermatitis: general term to describe inflammation of the skin

Helper T-cells: subset of T-cells that stimulate B-cells (plasma cells) to make antibodies against thymus-dependent antigens

Hypersensitivity: greater than normal immune response, same as allergy

Immunoglobulin: term used for antibodies

Interleukins: glycoproteins secreted by leukocytes that have effects on other leukocytes

Lymphocytes: cells in blood and tissues that contains antigen-specific receptors

Lymphokines: substances secreted by lymphocytes

Macrophages: phagocytic cell

Phagocytosis: engulfment of a particle by leukocytes

Upper Respiratory Illness: illnesses caused by an acute infection which involves the upper respiratory tract

CHAPTER 1: INTRODUCTION

What is immunity?

The immune system is a complex body system that begins to develop in utero and continues to mature throughout childhood (1). Immunity is important for protecting organisms from any foreign substances that attempt to invade. The immune system is composed of innate immunity (present at birth) and adapted or acquired immunity. Innate immunity is always present and available on short notice to protect against nonspecific foreign agents. Inflammation is part of the body's immediate response and is part of innate immunity. Acquired immunity is more specialized and develops after the body has already been exposed to a specific antigen. A normal immune response fights common illnesses and infections and they resolve after a period of time.

Abnormal immune responses can occur as well and lead to autoimmune diseases, chronic inflammation, allergies and asthma. An allergy is a common example of an abnormal immune response. Allergy occurs when there is an overreaction of the immune response, which then becomes more problematic than protective. An imbalance of T-helper-1 (Th-1) cytokine production (interferon-gamma and interleukin-2) and T-helper-2 (Th-2) cytokines (interleukin-4 and interleukin-5) may be the cause of certain allergies. Th-2 lymphocyte cytokines stimulate antibody production, especially immunoglobulin E, which is important in an allergic response. Normal and abnormal immune function is discussed in detail in

chapter 2. Immunity in utero, infancy and early childhood has also been examined. It is important to understand early childhood illnesses and their relationship to the development of chronic illnesses later in life.

Upper Respiratory Illness, Dermatitis and Allergy

The term “atopic march” has been used for some time to describe the progression of allergic manifestations in early childhood. Many times the first signs of allergic disease are atopic dermatitis and food allergy presenting within the first two years of life (2). Acute respiratory illnesses (ARI) and dermatitis are the most frequently diagnosed clinical pediatric illness (3-5). ARIs and dermatitis occurring in an underdeveloped immune system in early infancy may increase the risk of developing asthma and allergy symptoms later in life (3, 5, 6). According to Hahn et al. (2), children with atopic dermatitis within the first two years of life were more likely to have wheezing during that same time than children without atopic dermatitis. As well, the children with atopic dermatitis and wheezing were at a threefold greater risk of wheezing at 7 years of age compared to children without an early onset of atopic dermatitis.

The prevalence of allergic manifestations in infants is increasing worldwide (5). Infants’ immune function is not fully mature at birth because acquired immunity develops with exposure to new antigens. Neonates’ immune function relies on antibodies (immunoglobulins), cells, cytokines and other proteins reviewed in this paper from their mothers during intrauterine life for protection. Research now is

examining the relationship between the duration of breast feeding and infant immune function. It is known that maternal antibodies passed to the infant in utero and in human milk decrease in the first 6-12 months of life. Discovering ways to enhance maturation of the infant immune system is important in order to prevent illnesses in early childhood (4).

Protective Factors of Human Milk

Research has also examined the relationship of breast feeding compared to formula feeding in the development of illnesses early in life. Results of several studies suggest that breast-fed infants have a more mature immune system and fewer respiratory illnesses than formula-fed infants due to the immunoprotective factors in human milk (6-8).

Secretory IgA is one such immunoprotective component of human milk that is passed to the infant and survives in the infant respiratory and GI tract. Secretory IgA may be one of the main sources of antibodies that protects against respiratory and gastrointestinal illnesses (4). Several studies have shown a relationship between human milk consumption and lower incidence of respiratory illness (7-10). Results from a study conducted by Chantry et al. (7) found that infants who were breast fed for greater than 6 months had a lower incidence of respiratory illness than infants who were exclusively formula fed or breast fed for less than 6 months. Oddy et al. (8) also looked at infant feeding to determine if human milk for a longer duration was protective. These researchers found a strong relationship between any breast feeding

and protection against respiratory morbidity in infants compared to no exposure to human milk. Another study conducted by Sinha et al. (10) found that breast feeding was inversely associated with neonatal respiratory tract infections among female infants but not among male infants. The author speculated that the discrepancy between the genders was due to more heterogeneous respiratory conditions in male infants that were diagnosed as respiratory tract infections.

In contrast, Kramer et al. (11) did not find exclusive breast feeding for greater than 6 months protective against asthma or allergic manifestations in infancy. However, based on the researchers' methods, there was a lower degree of confidence in the results because they had to exclude 6 polyclinics for exceedingly high rates of positive skin pricks suggesting a systematic error. The effect of human milk versus formula feeding on the maturation of the infant immune function deserves further study.

Long Chain Polyunsaturated Fatty Acids (PUFAs) and the Infant Immune System

More recently, research has focused on long chain polyunsaturated fatty acids (LCPUFAs) and their influence on immune function. Human milk contains both arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3). AA and DHA were added to US infant formulas in 2002. AA synthesizes proinflammatory eicosanoids (prostaglandins, leukotrienes, and thromboxanes) that promote inflammation in an immune response. DHA has also been studied for potentially

positive immunomodulatory effects on the adult and infant immune system. Long-chain n-3 PUFAs decrease the production of proinflammatory eicosanoids, cytokines and reactive oxygen species, making them potential anti-inflammatory agents (12). A study by Kew et al. (13) demonstrated that supplementation with 3 grams of DHA for 4 weeks in healthy adults could reduce the expression of an early marker of T-lymphocyte activation (this is explained in more detail in chapter 2). Damsgaard et al. (14) supplemented healthy 9-month-old infants with a teaspoonful of fish oil per day for 3 months and increased red blood cell (RBC) n-3 PUFAs and reduced n-6 PUFAs. N-3 PUFA supplementation decreased interleukin-10 (IL-10) production, evidence of lower Th-2 cell response. In theory, a lower Th-2 response could decrease infant allergy and enhance maturation of the immune system.

Fish oil contains DHA and eicosapentaenoic acid (EPA, 20:5n-3) and fish oil supplementation will increase milk DHA and EPA. Dunstan et al. (15) measured secretory immunoglobulin A (sIgA) concentration in milk of mothers who were breast feeding with and without a fish oil supplement and showed sIgA was positively associated with milk DHA (22:6n-3) ($p= 0.046$) and DPA (22:5n-3) ($p= 0.003$) and inversely associated with linoleic acid ($p= 0.034$). Soluble CD14 is a component of innate immunity and has been associated with protection against many diseases, including allergic diseases. This topic is discussed in detail in chapter 2. Breast milk levels of soluble CD14 (sCD14) were correlated with 22:5n-3, and IgA concentration was correlated with milk IL-10, IL-6, IL-13 and sCD14 concentration. Overall, the study provided evidence that an increase in n-3 concentration in human milk could

increase IgA and sCD14 concentrations in human milk. The high concentrations in milk can be passed to the infant and enhance the infant's immune system.

Minns, et al. (16) conducted a study in US toddlers that measured RBC DHA before and after consumption of a milk-based beverage for 60 days containing various amounts of DHA. A secondary measure involved the collection of adverse events. The results from the adverse events showed that in 82 children, a difference was detected in the number of incidences of URIs. The infants who consumed the beverage containing 130 mg of DHA for two months had a significantly lower incidence of respiratory events than the group who consumed the control beverage without DHA (17% vs 45%, respectively; $p = 0.024$). The results suggested that US toddlers could benefit from higher DHA intake.

There has been limited research on the relationship between maternal milk fatty acid composition and the prevalence of URIs, dermatitis and allergic manifestations in infants. Duchon, et al. (17) conducted a study including 63 atopic and 57 non-atopic mothers and their infants. They observed clinical symptoms of allergic disease in 22 out of 63 (35%) children of atopic mothers and 22 out of 57 (39%) non-atopic mothers. The concentration of linoleic acid (LA) in milk increased from transitional milk to mature milk in atopic mothers but not in non-atopic mothers. The non-atopic mothers already had a high concentration of LA in transitional milk. The total n-6 to n-3 ratio was higher in milk from mothers of atopic children than in those of non-atopic children. At 3 months postpartum, the total SIgA concentration

was lower in milk from atopic compared to non-atopic mothers. A high AA to EPA ratio in milk and serum was related to the development of allergic symptoms in these children by 18 months of age. Because milk fatty acids reflect dietary fatty acid intake, it would be easy to manipulate milk fatty acid composition and examine the development of allergy and asthma experimentally.

In another study conducted by Dunstan et al. (18), 83 atopic mothers were supplemented with 4 1-gram fish oil capsules per day in order to examine the influence of supplemented n-3 PUFAs on allergic manifestations in their infants. All neonatal cytokines to all allergens were lower in the fish oil supplemented group than in the control group. Infants of mothers given supplements had less severe atopic disease at one year of age than infants in the control group.

To our knowledge, there have not been any studies comparing immune function in infants fed human milk and formula since DHA and AA were added to US infant formulas in 2002.

Environmental Factors Affecting Infants' Immune Function

It is important to take into account other factors associated with asthma and allergy in order to control for any effects of these potentially confounding variables. Among these factors, smoking during pregnancy, smoking in the child's home, daycare attendance, multiple children in the home and furred pets in the home have been found to influence the development of URIs, dermatitis and allergic manifestations in infancy. The factors that seem to protect against the development of

URIs, dermatitis, allergy and asthma include multiple siblings in the home, daycare attendance and a household pet exposure since birth. Those factors believed to adversely influence the immune system include smoking during pregnancy, exposure to second-hand smoke and a maternal history of allergy and asthma.

A study by Gern et al. (19) suggested that early exposure to furred pets in the home may protect against the development of allergic sensitization by increasing IL-10 and IL-13 cytokine secretions. Hugg et al. (20) found that early exposure to dogs in the home protected against allergies, while exposure to cats in the home increased the risk of developing asthma in childhood. de Meer et al. (21) observed atopic sensitization less frequently in children who had attended a daycare facility or who had a household pet before 2 years of age (OR= 0.73, 95% CI; 0.55-0.98, OR= 0.78, 95% CI; 0.61-0.99, respectively). Having an older sibling in the home was not associated with atopic symptoms nor was having more than one older sibling in the home (OR= 0.88, 95% CI; 0.70-1.11). Rothers et al. (22) found that daycare exposure by 3 months of age was associated with decreased IgE levels in the first year of life compared with no daycare exposure or daycare exposure after 3 months of age. Only children with atopic or asthmatic mothers had lower IgE when in daycare compared to no daycare.

Justification for Further Investigation

Significant gaps exist in the literature. Currently, there are limited and mixed results from studies of the relationship of fatty acid composition in human milk to the

prevalence of URIs, dermatitis and allergic manifestations in the first year of life. Therefore, it is important to further examine human milk compared to infant formula consumption in the protection against URIs, dermatitis and allergic manifestations. It is equally as important to examine the relationship between fatty acid composition of human milk and infant formula and the prevalence of URIs, dermatitis, allergy and asthma. As evidenced by the studies noted above, there may be potential benefits with exclusive breast feeding for a longer duration, DHA supplementation in the neonate, and DHA supplementation in pregnant and lactating women for the maturation of the infant immune system. By enhancing our knowledge of different components affecting immune function, we may find new ways to protect infants from developing URIs, dermatitis, allergy or asthma, or to delay the onset decrease of these conditions.

Statement of Purpose

The purpose of this study is to a) compare the prevalence of upper respiratory illness, dermatitis and manifestations of allergy and asthma in human milk-fed infants and formula-fed infants during the first year of life and b) to determine if the 1st year fatty acid intake of DHA from mother's milk and formula is associated with lower prevalence of upper respiratory illness, dermatitis and allergic manifestations during the first year of life.

Research Questions

Primary Question:

1. Does longer duration human milk feeding result in a difference of upper respiratory illness, dermatitis and allergic manifestations in the first year of life?

Secondary Question:

1. Is there a relationship between docosahexanoic acid (DHA) or arachidonic acid (AA) intake from maternal milk and formula and the prevalence of upper respiratory illness, dermatitis and allergic manifestations in the first year of life?

CHAPTER 2: REVIEW OF LITERATURE

What is Immunity?

The immune system is a body system designed to protect the organism from all manner of foreign agents that can result in infection (microorganisms) or allergy (food, pet dander and hair, plant pollen, environmental chemicals, etc.) (1). The immune system includes both innate and acquired immunity and begins to develop in utero and continues to mature in early childhood into adulthood.

Innate immunity is present at birth and responds to any nonspecific foreign agent that attempts to enter the body (1). The skin and mucous membranes are the first barriers that respond to foreign substances immediately upon entry. When a foreign substance passes the first barriers of protection, it is engulfed by leukocytes in a process called phagocytosis. Lysosomes in the leukocytes release enzymes to destroy the foreign substance.

The second line of defense is adaptive immunity also known as acquired immunity. Adaptive immunity differs from innate immunity in that it is highly specific to antigens (1). Antigen specificity occurs only after a previous exposure to a particular substance. There are three major cell types required for acquired immunity: thymus or T-cells, bone marrow or B-cells, and macrophages. Acquired immunity has both a cellular (cell-mediated) and humoral (antibody-mediated) component. Humoral immunity (antibody-mediated) consists of B lymphocytes (plasma cells), which secrete antibodies specific to an antigen. Antibodies are made up of serum globulins, and activated globulins are called immunoglobulins (Ig). Table 1 indicates

the types and functions of specific immunoglobulins as well as their placental passage and presence in human milk.

TABLE 1 Properties of Immunoglobulins

Immunoglobulins and Immune Function				
Name	Where present	Function	Placental Passage	Present in milk
Immunoglobulin G (IgG)	Serum	1. Activates the complement system 2. Neutralizes toxins 3. Immobilizes mobile bacteria 4. Neutralizes viruses	Yes	yes
Immunoglobulin A (IgA)	tears, saliva, human milk, sweat	1. Primary defense against local infections 2. Prevents viruses from entering the host cell	No	yes
Immunoglobulin M (IgM)	Serum	1. Activates the complement system 2. Important provider of early immunological defense	No	trace
Immunoglobulin E (IgE)	Serum	1. Important in an allergy response 2. Releases histamine, heparin, leukotrienes when activated	No	no

T-lymphocytes are responsible for cell-mediated immunity and includes both helper T-cells and cytotoxic T-cells (1). Cell-mediated immunity involves signaling in response to cytokines. The helper T-cells include T-helper type 1 and T-helper type 2 (Th-1 and Th-2) that produce different cytokines. Th-1 cells produce the cytokines interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α). Th-1 plays a role

in the cellular immune system and maximizes macrophages killing ability of foreign substances. Th-2 cells are partnered with B-cells and play a role in the humoral immune function stimulating B-cell proliferation and increasing antibody production. Th-2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-13 (IL-13). Innate and acquired immunity may both involve an inflammatory response.

Helper T-cells (Th-cells) release lymphokines that activate B cells (plasma cells). The types and functions of lymphokines are listed in table 2. During an inflammatory response, lymphokines released by T-cells induce activation of monocytes and macrophages that contain lysosomes increasing the destruction of foreign organisms or cells.

In cell-mediated immunity, the foreign substance invading the body is broken down and appears on the surface of macrophages complexed with class II major histocompatibility complex (MHC) proteins (23). This protein complex then interacts with an antigen-specific receptor on a helper T-lymphocyte leading to production of interleukins: interleukin-1 (IL-1) by macrophages and interleukin-2 (IL-2) by lymphocytes. This causes the activation and proliferation of specific helper- T cells and the end result is a delayed hypersensitivity reaction to the antigen.

In antibody-mediated immunity, the MHC protein complex binds to receptors on the Th cell, which produces IL-2, IL-4 and IL-5. The interleukin proteins then activate the B-cells capable of producing antibodies specific for the antigen. Activated B-cells proliferate to form plasma cells that secrete immunoglobulins

(antibodies). When bacterial polysaccharides can activate B-cells directly, only immunoglobulin M (IgM) is produced. Interleukins 4 and 5 are then able to produce IgG, IgA, and IgE antibodies. B-cells are important for recognizing the invading microorganism with the surface IgM. IgM antigen receptor can then activate B-cells to produce antibodies against the majority of molecules known.

CD4⁺ is a glycoprotein that plays a role in adhesion of T-cells and non-T-cells (1). It is encoded by the CD4 gene and expressed on the surface of T-cells during various stages of development in the thymus. CD4⁺ interacts with the MHC Class II molecule. CD4⁺ is also expressed by monocytes (24, 25) and is detectable in blood (24). CD4⁺ T-cells are essential for both cell-mediated immunity and antibody-mediated immunity (26). In cell-mediated immunity, CD4⁺ cells bind to an antigen presented by macrophages and the T-cells release lymphokines that attract other cells to the area as part of the inflammatory response (1). In antibody mediated immunity, the CD4⁺ cells bind to an antigen presented by B cells and produce clones of these B cells that secrete antibodies against the foreign substance. CD14⁺, on the other hand, is part of the innate immune system and encoded by the CD14 gene. It exists in two forms and is either attached to the membrane or found in the soluble form (sCD14) (25). The liver and monocytes secrete the soluble form of CD14 as does the mammary gland. One role of CD14 in human milk is to regulate beneficial microbial growth in the infant gut (27).

Table 2 Types and functions of cytokines in the immune response.

Cytokines in the Immune Response		
Name	Produced/Secreted By	Function
Interleukin-1 (IL-1)	monocytes and macrophages	<ol style="list-style-type: none"> 1. Stimulates acute-phase protein synthesis 2. Promotes T-cell proliferation 3. Fever-producing substance
Interleukin-2 (IL-2)	CD4 ⁺ Th-1 cells	<ol style="list-style-type: none"> 1. Required for growth of some T-cells 2. Activates B-cells to secrete immunoglobulins
Interleukin-3 (IL-3)	Primarily by T-cells	<ol style="list-style-type: none"> 1. Growth factor for hematopoietic stem cells and for mast cells
Interleukin-4 (IL-4)	CD4 ⁺ Th-2 cells	<ol style="list-style-type: none"> 1. Growth factor for B-cells and some T-cells 2. Enhances expression of MHC class II molecules on B-cells 3. Promotes production of IgE and IgG₁
Interleukin-5 (IL-5)	CD4 ⁺ Th-2 cells	<ol style="list-style-type: none"> 1. Stimulates B-cell growth 2. Stimulates immunoglobulin secretion 3. Constitutes a growth and differentiation factor for eosinophils
Interleukin-6 (IL-6)	Many cells, including T-cells	<ol style="list-style-type: none"> 1. Induces acute phase protein synthesis 2. Promotes T-cell activation 3. Promotes IL-2 production 4. Stimulates secretion of immunoglobulins by B-cells
Interleukin-7 (IL-7)	Fibroblasts, endothelial cells, some T-cells	<ol style="list-style-type: none"> 1. Growth factor for pre-B and pre-T cells
Interleukin-8 (IL-8)	many cells	<ol style="list-style-type: none"> 1. Draws neutrophils and granulocytes into the area where its being secreted
Interleukin-10 (IL-10)	monocytes and some lymphocytes	<ol style="list-style-type: none"> 1. Effects in regulation of the immune system and inflammation 2. Down-regulates expression of Th-1 cytokines, MHC class II antigens

		3. Enhances B-cell proliferation and antibody production 4. Can inhibit synthesis of pro-inflammatory cytokines
Interleukin-12 (IL-12)	Macrophages	1. Involved in differentiation of T-cells into Th-1 cells 2. Stimulates growth and function of T-cells 3. Stimulates production of Interferon-gamma and tumor necrosis factor
Interferon-gamma (IFN- γ)	Activated CD4 ⁺ Th-1 cells	1. Activates killer T-cells, natural killer cells, and macrophages 2. Induces expression of MHC class II molecules
Tumor Necrosis Factor (TNF)	TNF α - monocytes, TNF- β -T-cells	1. Activate many different cells 2. Cytotoxic to some tumor cells in vitro

Abnormal Immune Responses: Allergy and Atopy

Abnormal immune responses include hypersensitivity, an overreaction of the immune response to an antigen. Allergy is defined as hypersensitivity to an antigen in response to a first exposure while asthma is defined as a chronic lung disorder triggered by hyper-reactivity to stimuli such as allergens (28). Atopy is defined as an allergy, most likely hereditary, that occurs upon exposure of an allergen and is characterized by symptoms such as hay fever, hives, and asthma. Asthma and allergy can occur independently; however, atopy is often associated with asthma as evidenced by increasing prevalence of asthma and skin-testing reactivity in the United Kingdom and Australia (29).

Hypersensitivity reactions occur during an allergic reaction which is discussed in more detail later. It is generally understood that increased risk of developing allergy and allergic inflammation may occur in response to an exaggerated expression of Th-2 activity or failure of Th-1 or T-regulatory function (9, 30). The inflammatory response becomes out of control in a hypersensitivity reaction and begins to damage tissues and organs. An allergic response is caused by the expression of Th-2 lymphocytes. Th-2 lymphocytes release interleukins (as noted above) that result in the production of IgE which in turn stimulates inflammatory cells (31).

There are different classes of hypersensitivity reactions (1). Type I hypersensitivity reactions are mediated by IgE antibodies and cause anaphylactic reactions. This occurs only minutes after exposure to an antigen. Because Th-2 cells produce IL-4 and IL-13, Th-2 cells play an important role in this type of hypersensitivity. Type 1 hypersensitivity is believed to underly food allergy, autoimmunity, and inflammatory bowel diseases. Type IV hypersensitivity is a cell-mediated immune reaction and is mediated by T-cells rather than antibodies. Local damage to the cell occurs when T-cells release lymphokines that cause accumulation and activation of macrophages resulting in chronic inflammation. This type of reaction is also called delayed hypersensitivity because it does not occur immediately after exposure to an antigen. Rather it may occur 1-2 days after exposure to an antigen.

The mechanism of atopic allergy is the Type I hypersensitivity reaction, which involves Th-2 lymphocytes. Contact with an allergen results in processing by an

antigen presenting cell followed by presentation to Th-2 helper cells, which help B cells make IgE antibody. Production of IgE is protective when in response to stimuli such as parasitic worms. However, it may cause allergic rhinitis, eczema, hives or anaphylaxis when production is in response to harmless airborne or ingested allergens. Only 10% of the general population develops clinical symptoms from these airborne pathogens such as plant pollens, mold spores, and animal dander.

Allergists have experimented with treatment for asthma and allergic disease since the 12th century when a Jewish physician and philosopher, Maimonides, produced a remedy of Rhazes to “clear the lungs of moisture, ease respiration and eliminate the cough” (32). The relationship of the environment to asthma and allergies was inferred by Maimonides who noted: “the first thing to consider is the provision of fresh air...” The air is then illustrated as, “city air is stagnant, turbid, and thick, the natural result of its big buildings, narrow streets, the refuse of its inhabitants...The air should be kept dry at all times by sweet scents, fumigation and drying agents. The concern for clean air is the foremost rule in preserving the health of one’s body and soul...”

Although the general link of some environmental conditions to asthma and allergic disease was made by the 12th century, it was left to modern scientists to define the specific environmental agents associated with allergic disease and asthma and the mechanisms of action described earlier. It is not known why the prevalence of allergic disease and asthma has increased dramatically in the last four decades (29).

As of 2003, nearly five million children in the United States and 155 million individuals worldwide were afflicted with asthma (6).

Normal and Abnormal Immunity in Development

As noted above, immunity begins to develop during gestation and continues to mature into childhood. Neonates are first protected by their mothers' IgG antibodies passed through the placenta and in human milk, but neonatal IgG antibodies decline by 3 to 6 months of age (1, 23, 33). Human milk sIgA is also immunoprotective for the duration of breast feeding. The fetus begins to synthesize IgM and IgA antibodies in the fifth month of gestation.

Acute respiratory illnesses (ARI) are a leading cause of morbidity and mortality in infants and are the most frequently diagnosed clinical pediatric illness (3, 30). URIs can be chronic or acute and are now recognized as increasing the risk of allergy and atopy. Rhinoviruses (4) and respiratory syncytial virus (RSV) (6) are the two most common viruses that are detected in URIs during infancy and both appear to increase the risk of allergy and atopic diseases in infancy. The mean number of episodes of upper respiratory illnesses (URI) during infancy was 4.08 with a range of 0 to 11 episodes (3). Furthermore, neonates have limited ability to produce interleukin-2 (IL-2) from Th-1 cells (34), increasing their vulnerability to developing allergy or atopy.

According to the Centers for Disease Control and Prevention (35), the prevalence of asthma in US children younger than 4 years of age increased from 5.8 percent in 2002 to 6.5 percent in 2005. The first manifestation of the "atopic march"

leading to asthma and allergy typically occurs in infancy and early childhood (29-31, 36, 37) as food allergies and atopic dermatitis (5). Otitis media and allergic rhinoconjunctivitis are other clinical symptoms of early allergic disease (30).

Because it is understood that the first allergic manifestations may begin early in life, the immune function of neonates has been studied in the hope of learning how the developing immune system is programmed for protection. As well, the factors that influence normal and abnormal development of the immune system can be elucidated.

Relationship between Intrauterine Environment and Later Allergy or Atopy

The fetal intestinal tract is one route of exposure to allergens in the mother's environment that end up in amniotic fluid and are swallowed. Placental transfer of an allergen complexed with the immunoglobulin- G subclass antibodies has been identified as a second route of allergen exposure (31). Research in the last 12 years has shown that specific allergen-induced responses can occur as early as 22 weeks' gestation (38).

IgG is the only antibody that moves across the placental barrier to provide protection for the fetus (4, 39, 40). IgG has four subclasses (1) : IgG₁ constitutes 70%, IgG₂ 20% and IgG₃ and IgG₄ approximately 10% of total IgG. Subclasses IgG₁ and IgG₃ bind strongly to monocytes, while IgG₃ functions in complement binding. The complement system results in cell lysis if the antibody is bound to an antigen. There is a rapid increase in fetal IgG in the third or fourth month of pregnancy and IgG levels are found in the fetus until birth. Because the fetus is unable to synthesize immunoglobulins, the fetal IgG must be from the mother. The half-life of IgG is 23

days and maternal levels of IgG begin to be replaced by neonate IgG beginning around 3 or 4 months of age.

IgG₂ is the only subclass of IgG that does not pass through the placenta. Hay et al. found that concentrations of IgG₂ in the fetus were low compared to concentration of IgG₂ in maternal serum, which was several fold greater (39).

There is evidence that maternal IgG may have a role in enhancing the fetal immune response to an allergen (31) and in protection against upper respiratory illnesses (41). A study done in Germany by Stiller et al. (42) found that low cord blood concentrations of IgG and IgG₁ to cat dander were associated with atopic symptoms in early childhood while high concentrations of IgG to cat dander and birch pollen were associated with less atopic disease in children in this cohort. A study done in Britain by Warner (33) showed a similar relationship between high concentrations of cord blood IgG antibodies to cat dander and the major allergen of birch pollen and less atopy in children during the first 8 years of life.

As noted earlier, it is generally understood that increased risk of developing allergy and allergic inflammation may occur in response to an exaggerated expression of Th-2 activity or failure of Th-1 or T-regulatory function (9, 30). As well, it has been noted that neonates have limited ability to produce the Th-1 interleukin-2 (IL-2) and T-helper cells that are important in the response to an allergen (34). This could increase their vulnerability to allergy or atopy depending upon maturity of their immune system at the time of exposure.

Much like the upper respiratory distress response to an allergen, during RSV infection, monocytes and macrophages play a role in the acute inflammatory process by producing cytokines IL-12 and IL-10 (6). IL-12 induces T-cells to produce IFN- γ and IL-2. IL-10 down-regulates cytokine production and produces the “asthma-inducing” immune response. RSV also produces an increased release of proinflammatory markers such as TNF- α , IL-1, IL-6, IL-8, platelet-activating factor, and prostaglandin E2. Many prostaglandins cause vasoconstriction of the bronchial tubes making it harder to breathe. Prostaglandins are also explained in greater detail later.

Grzela et al. (43) examined a cohort of 43 infants in order to analyze the relationship of Th-2 IL-4 expression on monocytes during the first year of life and the occurrence of allergy symptoms in neonates. After one year of observation, 10 of 29 children that expressed an increased IL-4 concentration developed severe atopic dermatitis. In 8 of the 10 children who developed severe atopic dermatitis, the cord blood monocytes and Th-lymphocytes contained high concentrations of IL-4 suggesting that increased concentrations of IL-4 may be related to atopic dermatitis. Moreover, 6 of the 10 children who had atopic dermatitis showed a significant decrease in IL-12 after one year of observation ($p= 0.02$). They found evidence that higher expression of IL-4 was associated with suppressed expression of IL-12. This study may suggest that Th-2 compared to Th-1 predominance even before birth is associated with allergic and atopic symptoms in childhood.

Research has concentrated primarily on environmental factors rather than genetic factors because of the dramatic increase in prevalence of allergy and asthma, however, family history of atopy clearly plays a role in the development of allergy and asthma in children who are at increased risk from environmental exposures (30). Maternal atopy is associated with a higher risk of development of atopic disease in the offspring than is paternal atopy. It has been suggested that an atopic mother generates a more susceptible placental environment to allergens. Even though IgE antibodies do not cross the placental barrier, the fetus is still exposed to higher serum levels of IgE in atopic mothers versus non-atopic mothers because IgE in amniotic fluid resembles the mother's IgE serum levels (44).

Other protective molecules such as IgM are present at birth. IgM is found in the intestine and is the first immunoglobulin to be made by the fetus. IgM is the first immunoglobulin to bind to an antigen and initiate the complement sequence. The complement sequence is a series of serum proteins involved in the mediation of immune reactions (1). Mucosal IgA antibodies from the mother are needed for protection against foreign agents in the environment (4, 45). Secretory IgA is passed to the infant in human milk and can be found in the respiratory and gastrointestinal tract of human milk-fed infants, where it provides protection against microorganisms in the mother's environment (4).

Soluble and membrane CD14 monocytes may help program a healthy neonatal immune system, and reduce the Th-2 preference associated with IgE production and atopic disease (25). The hereditary factors for allergic manifestations

and asthma have been studied in relation to single nucleotide polymorphisms (SNPs) of CD14 (24, 27, 46, 47) and show lower concentrations in children with atopic family history. Zdolsek et al. (47) examined a cohort of 76 children and found that children with a double atopic family history had lower soluble CD14 (sCD14) levels than children with no atopic family history at 3, 12, and 18 months, and at 7 years of age. Children with or without maternal history of atopic disease had similar sCD14 levels at birth, 3, 6, and 12 months, but at 7 years of age sCD14 levels were lower in children with a maternal history of atopic disease than in children with non-atopic mothers.

In human milk, sCD14 concentrations are approximately 10-fold higher than in maternal plasma, suggesting that milk sCD14 could be of sufficient quantity to program the developing immune system (24). Infant formulas do not provide sCD14.

Protective Factors of Human Milk

The role of human milk immunoglobulins in maturation of the neonatal immune function has been extensively studied (4, 7-11, 34, 37, 48-52). In addition to sCD14 just discussed, there are other molecules and chemicals that contribute to the immunomodulatory effects of human milk feeding, including leukocytes (B-lymphocytes, macrophages, neutrophils, and T-lymphocytes), oligosaccharides, bifidus factor, lysozyme, lactoferrin, IFN- γ , nucleotides, and cytokines (49). Human milk feeding may also stimulate the development of the neonate's immune system by achieving a beneficial T-helper 1 / T-helper 2 ratio earlier than in formula-fed infants (53).

Human milk also contains an anti-inflammatory cytokine interleukin-10 (IL-10) and proinflammatory cytokines (IL-1- β , IL-6, IL-8, and TNF- α) in variable amounts (34). IL-10 decreases the Th-1 response and inhibits pro-inflammatory cytokine release. This is important because increased inflammatory response may result in reduced intake, illness, and gut damage.

The results of several observational studies suggest that human milk protects infants in developing countries against URIs (54-56), but the relationship between human milk consumption and the prevalence of URIs, asthma, and atopic dermatitis still remains controversial (8-11, 37, 48). Because URIs and other illnesses are common among all infants, researchers have examined the duration of feeding human milk in relationship to the prevalence of respiratory illness.

Oddy et al. (8) conducted a study in Australia examining the relationship between breast feeding and respiratory morbidity in infancy. The prospective study included 2,602 infants cared for in antenatal clinics in Perth, Australia. Parents were asked to complete a diary card recording feeding history and illnesses. The illnesses recorded were those that required a visit to the physician or hospital. At the end of the first year, parents completed a questionnaire and a trained child health nurse at the clinic assessed the children for growth and development. Responses from the questionnaire were then coded using ICD 9 codes for illnesses.

Respiratory illness and infection included diagnosis of unspecified URIs, tonsillitis, otitis media, otitis media with effusion, and wheezing, while lower respiratory tract infections included wheezing associated with respiratory illness,

bronchiolitis, bronchospasm, or asthma. Non-wheezing lower respiratory infections included chest infection, pneumonia, whooping cough, chronic cough, or croup. As a check for reliability and validity of parental reporting of illnesses, the investigators compared 100 hospital medical records against the parents' notes and found agreement in 99 out of 100 cases suggesting that parents' records were highly reliable.

Feeding was coded as either predominantly breast feeding, meaning that human milk was given as well as water or water based fluids, or partial breast feeding. Partial breast feeding was described as other milk or formula had been given, but breast feeding had not yet stopped. The statistical analysis models adjusted for gender, gestational age, smoking in pregnancy, older siblings, maternal education, and maternal age at the time of birth. Other covariates considered but not found significant were birth weight, daycare attendance, father's occupation, family income, concurrent parental smoking and parental history of asthma.

By 4 months of age, 48% of the cohort stopped predominant breast feeding or any breast feeding. By 6 months of age, 61 percent of the mothers had stopped predominant breast feeding. By 8 months of age 58 percent had stopped breast feeding entirely. At 12 months of age 5 percent of the cohort was still breast feeding predominately, and 24 percent of the cohort were still partially breast feeding. Infants fed human milk for less than 2 months had 4 or more hospital and doctor visits because of a URI ($p= 0.041$). Those partially breast feeding for less than 6 months also had significantly more URIs ($p= 0.018$) compared to the rest of the cohort. If

infants were weaned from breast feeding before 8 months of age they had an increased risk of 2 or more hospital, doctor, or clinic visits because of wheezing lower respiratory infections compared to those breast fed for greater than 8 months ($p=0.001$) (8). Overall the results of the study provided evidence for benefits of longer duration breast feeding with regard to the number of hospital, doctor, and clinic visits for respiratory illnesses.

Another study by Chantry et al.(7) looked at the relationship between duration of full breast feeding and respiratory tract infection in 2277 US children. Data for the study came from NHANES III and included 6 to 24-month-old children who had been breast fed in infancy. Breast-fed infants were categorized as fully breast fed less than one month, fully breast fed for one to four months, fully breast fed for four to six months, and fully breast fed for greater than or equal to six months.

Pneumonia, colds or influenza, and wheezing were identified and recorded as respiratory tract infections. The number of episodes and age of first occurrence of otitis media were also recorded. Infants fully breast fed for greater than 6 months had less pneumonia than those fully breast fed for 4 to 6 months ($p= 0.017$). The study also found that infants who were breast fed for only 1 to 3 months were more likely to have the first episode of otitis media before 1 year of age than those breast fed for longer (61.7 percent versus 47.2 percent, $p= 0.016$). If full breast feeding stopped between 4 and 6 months of age, the risk of having greater than or equal to 3 episodes of otitis media was increased compared to full breast feeding for 6 months or longer. The results suggested that full breast feeding for greater than or equal to 6 months of

age provided greater protection against respiratory tract infection than did breast feeding for less than 6 months (7).

Although the studies above are evidence that longer duration of breast feeding is protective against URIs, not all studies have found benefits of human milk versus formula feeding. Cushing et al.(51) examined the relationship between breast feeding and the risk of respiratory illness in 1,202 infants born in New Mexico between 1998 and 1990. Parents recorded signs and symptoms of any illness displayed by the infant on a daily calendar. Symptoms included: runny nose, stuffy nose, fever, dry cough, wet cough, wheezing, difficulty breathing, loss of appetite, rash and the mother's perception of illness. Even though the researchers had data until 18 months of age, the study focused on the first 6 months of the infant's life when evidence has shown breast feeding to be most protective. Rates were expressed on an annualized basis reflecting 365 days at risk so that the rates could be compared across the breast feeding categories. The annualized rates for respiratory tract infections in infants 0 to 2 months of age were 8.3 in infants who were not breast fed, 7.8 in infants who were partially breast fed, and 8.1 in infants who were fully breast fed. These numbers included upper and lower respiratory infections and did not demonstrate any benefit of human milk. It was noted that full breast feeding was associated with a shorter duration of all respiratory illnesses combined during the first 6 months of life (OR = 0.90, CI; 0.83-0.98) compared to partial human milk feeding and formula feeding.

A prospective study conducted in Copenhagen, Denmark by Rubin et al. (52) demonstrated a relationship between infant feeding and infectious illnesses during the

first year of life in a largely middle class population including 500 infants.

Investigators collected demographic information and information about the plan for infant feeding in an interview conducted with mothers 3 to 4 days postpartum.

Monthly mailed questionnaires obtained information about feeding, infectious illnesses, and covariates. Inclusion criteria included: birth weight greater than 2,000 grams, gestation greater than 36 weeks and absence of underlying diseases. After controlling for covariates, incidence density ratios were used to determine the prevalence of illness between breast fed and formula fed infants. The mothers did not have information about the hypothesis of the relationship between the type of feeding and illnesses or about the illnesses examined.

The illnesses examined in the study were gastroenteritis, upper respiratory illness, otitis media, and lower respiratory illness. If not diagnosed by a physician, a diagnosis of upper respiratory infection required at least 2 of the following symptoms of the following criteria: a temperature greater than 38.5 °C, rhinorrhea, cough or fast breathing for 2 to 20 days. Three of the symptoms were required in cases where duration of symptoms was not known.

Feeding was categorized according to the classification by the World Health Organization and included: 100 percent human milk-fed, human milk-fed greater than formula-fed, human milk-fed equal to formula-fed, human milk-fed less than formula-fed, and 100 percent formula-fed. The ratio of incidence density was calculated in order to compare the rates of illnesses in the human milk-fed and formula-fed infants. A ratio significantly greater than one meant that the rate among

formula-fed infants was higher than among those fed human milk, evidence that human milk-feeding was protective against infectious illness.

After adjusting for birth weight, social class, number of children in the family, daycare and other illnesses in the family, breast feeding had a protective effect on otitis media but not against other common infectious diseases in infancy (52). The results were similar to those reported by Kramer et al. (11) and Cushing et al (51).

Thirty-four atopic mothers and their infants were recruited at 35 to 36 weeks' gestation for an infant follow up study conducted in Finland by Hoppu et al. (48). At the first study visit a questionnaire regarding family history of atopic disease was completed. The extent of maternal atopic sensitization was measured with skin prick tests for milk, egg, wheat, cod, soy bean, peanut, hazelnut, birch pollen, alder, mugwort, a mixture of six local grasses, cat, dog, house dust mite, and latex. Sensitization to three other foods was tested by a different technique. The diagnosis of atopic dermatitis was based on the Hanifin criteria, which requires the presence of 3 of 4 major criteria and 3 of 23 minor criteria established by experienced dermatologists. The infants of the mothers were then examined at 1, 3, 6 and 12 months of age. Four consecutive days of maternal food records were collected at the time the infant was one month old, and nutrient intakes were calculated using Nutrica software (version 3.0, Research Centre of Social Insurance Institution, Turku, Finland.) If the mothers predominately or exclusively breast fed when the infant was

one month old, the fatty acid composition in the milk was analyzed by gas chromatography.

By 12 months of age, 38 percent (13 of 34) of infants had been diagnosed with atopic dermatitis and of the 13 infants, seven had positive skin prick reactions to at least one of the allergens tested. Infants who developed atopic dermatitis had mothers with a significantly higher proportion of stearic acid in their milk compared to infants without atopic dermatitis ($p= 0.0009$) and the proportion of total n-3 PUFAs, specifically EPA, was lower in the milk of mothers whose infants developed atopic dermatitis than those whose infants did not develop atopic dermatitis. It is not clear how to interpret these results because differences, while statistically significant, were small, and there was a lot of overlap between the groups.

Long Chain Polyunsaturated Fatty Acids (PUFAs) and the Infant Immune System

Many PUFAs have been examined for their role in immune function including AA, linoleic acid, DPA and DHA. AA is a 20 carbon chain unsaturated at carbons 4, 8, 11, and 14, and is oxygenated by cyclooxygenase to give prostaglandins and thromboxanes (1). Many prostaglandins and thromboxanes from AA are vasoconstrictive and can cause constriction of the bronchial tubes. Prostaglandins are also involved in immune regulation. Specifically, prostaglandin E2 synthesized from AA increases the production of IL-4, IL-5 and IL-10 (12). As noted before, IL-4 in turn stimulates IgE production and plays a role in allergy (57).

Calder (12) reviewed the relationship between long-chain n-3 fatty acids and the incidence of asthma. Eicosanoids derived from AA are produced by mast cells that mediate pulmonary inflammation in asthma. He cited two epidemiologic studies that linked asthma to a high intake of n-6 polyunsaturated fatty acids and a low intake of n-3 PUFAs (58, 59). However, a systematic review conducted on the relationship between fish oil supplementation and the prevalence of asthma symptoms conclude there was not a benefit of supplementation based on all available reports (12).

A study conducted by Kew et al.(13) sought to find effects of oils rich in EPA and DHA on immune cell composition and function. The researchers reported that fatty acid composition of plasma phospholipids was altered by supplementation of EPA and DHA, however, the effects of these two fatty acids on T-lymphocyte activation were different. The results suggested that supplementation with DHA, but not EPA, suppressed T-lymphocyte activation.

Damsgaard et al. (14) provided healthy 9-month-old infants for 3 months with a teaspoonful of fish oil and increased the n-3 PUFAs of their red blood cell, but did not affect any of the markers for inflammation measured. They did observe higher IFN- γ production and a tendency for lower IL-10 production compared to controls.

Dunstan et al. (60) provided 83 atopic women at 20 weeks gestation with 4 grams per day of fish oil capsules (n=40) (56% DHA and 27.7% EPA) or a placebo (n=43) and looked at neonatal allergen-specific immune responses in their infants. Although it did not reach statistical significance, the fish oil supplemented group had decreased IL-5, IL-13, IL-10, and IFN - γ responses to house dust mite, ovalbumin

(OVA), cat, and mitogen allergens compared to the group without fish oil supplementation. The fish- oil supplemented group also had lower neonatal in vitro IL-10 response to cat allergen compared with the control group ($p= 0.046$). When the groups were analyzed using a linear-regression model, IL-5, IL-10, IL-13, and IFN- γ cytokine responses to allergens and mitogens were correlated inversely with PUFA composition of cord blood red blood cells. As well, the control group was 3 times more likely to have a positive skin prick test to egg allergen compared to the fish oil supplemented group at one year of age (60). Both groups had similar amounts of dermatitis ($p= 0.167$), however, the fish-oil group were one-tenth as likely to have severe atopic dermatitis (OR= 0.09, 95% CI; 0.01-0.94) compared to the control group. The results suggested that fish oil supplemented during pregnancy could potentially reduce the severity of atopic disease in infants.

A randomized, double-blinded, placebo-controlled clinical trial conducted by Krauss-Etschmann et al. (61) examined the effects of fish oil supplementation on altering the allergy-related immune parameters among mothers and their offspring. Beginning at 22 weeks gestation, 311 women were assigned to one of 4 groups: daily fish oil supplements containing either 0.5 grams of DHA and 0.15 grams of EPA, or 400 micrograms of methyl-tetrahydrofolic acid, both supplements, or a placebo without either supplement.

When compared to the placebo, fish oil supplementation was associated with increased maternal mRNA expression of IFN- γ and IL-1. In cord blood samples, fish oil supplementation compared to the placebo was associated with decreased mRNA

levels of the Th-2- associated IL-13 and IL-4. The results of the study are similar to those of Dunstan et al. (60) showing a decrease in IL-13 in offspring of mothers supplemented with n-3 PUFA, however the dose used was considerably lower. A follow up to this study may reveal if the decrease of Th-2 molecules decreases the incidence of allergic disease in offspring supplemented with fish oil.

DHA and EPA Supplementation in Human Milk

The evidence that n-3 fatty acid or a decrease in the ratio of n-6/n-3 fatty acids may play a role in maturation of the infant immune system provide the basis for examining the relationship of fatty acid composition of human milk and the infant immune system (48). Human milk contains long chain n-3 PUFAs, predominately EPA and DHA (57). Recent evidence suggests that DHA and AA, as well as conjugated linoleic acid in human milk may enhance the neonates' immune function (48, 53). DHA and EPA supplemented during gestation and lactation is passed to the infant in utero and in human milk altering cytokine production toward a more tolerable balance of Th-2/Th-1. There have not been studies of the effects of fish oil supplementation on human milk immunoglobulins and therefore more research is needed to examine if there is a positive trend.

In 2004, Dunstan et al. (15) reported that fish oil supplementation during pregnancy increased the concentrations of DHA and EPA in RBC ($p < 0.001$) and decrease AA ($p = 0.045$) compared with the control group. IgA concentration were further positively associated with milk DHA ($R = 0.235$, $p = 0.046$) and DPA (22:5n-3) ($R = 0.344$, $p = 0.003$), and associated inversely with linoleic acid ($R = -0.249$, $p =$

0.034) in both groups. IgA concentrations were also positively correlated with levels of IL-10 ($p < 0.001$), IL-6 ($p = 0.013$), IL-13 ($p = 0.001$), and sCD14 ($p = 0.004$). The concentration of sCD14 in human milk was positively correlated with DPA ($R = 0.306$, $p = 0.009$) in both groups. Despite the fact that there was no significant difference between the control group and the fish oil group in the concentration of any of the immunological parameters studied, higher n-3 PUFA levels correlated with higher levels of IgA and sCD14. The results suggested that IgA levels in human milk could be increased by increasing the 22 carbon n-3 PUFA and decreasing the 20 carbon n-6 PUFA concentrations. Higher IgA concentrations could have clinical benefits for the infant.

Duchen et al. (17) compared the composition of PUFAs and secretory IgA levels in mother's milk of children allergic or not to different food proteins (Ovalbumin and β -lactoglobulin) and an inhalant cat allergen. Blood samples were obtained from 120 children at birth and at 3 months of age. Skin prick tests were performed at 6, 12, and 18 months of age to assess allergic responses in the children. Maternal milk samples were collected at birth and monthly during the lactation period and PUFAs were measured by gas chromatography.

The researchers found that EPA, DPA (22:5n-3) and DHA concentrations were lower in milk from mothers of allergic children compared to mothers with non-allergic children ($p < 0.05$). At 3 months of age, total IgA antibodies were lower in the allergic mothers' milk, however, IgA antibody levels against β -LG, OVA, and cat were similar in atopic and non-atopic mothers' milk. The study results agree with

other reports that found lower levels of n-6 and n-3 LCPUFAs in plasma phospholipids and membranes of atopic persons compared with non-atopic persons. The study also confirmed results of a previous study by Yu G et al. (62) that concentration of linoleic acid and linolenic acid are lower in early transitional milk from atopic mothers compared to non-atopic mothers.

Environmental Factors Affecting Infants Immune Function

Environmental exposure to various pathogens and life-style exposures have been linked to early URIs and may be related to increased allergy and asthma prevalence in childhood compared to the past. However, we could not find any evidence related to changing environmental exposures in the years when allergy and asthma have increased. As well, we now understand that environmental exposures begin in utero. Consequently there is much work to be done to understand exposures that might increase susceptibility to URIs leading to allergy and asthma (29). Smoking during pregnancy, smoking in the child's home, daycare attendance, the number of children in the home, and exposure to furred pets have all been shown to influence the development of URIs, allergy and asthma in early childhood (20, 21, 63-65).

A study by Ball et al. (66) demonstrated that children who attended a daycare with many other children early in life had higher rates of URI in early childhood, but by age 8 these children were significantly less likely to have asthma than those children who did not attend a daycare or who did not attend a daycare with many other children.

Similar to that study, Rothers et al. (22) conducted a study to determine if the influence of early daycare exposure affected IgE levels in 434 children during the first 3 years of life. Blood levels of IgE were obtained at 3 months, 1 year, 2 years, and 3 years of age. Information on daycare attendance was gathered monthly through 4 months of age, and again at 6 and 9 months of age. The researchers found that total IgE levels increased with age (1.3 IU/mL at 3 months, 8.9 IU/mL at 12 months, 15.0 IU/mL at 24 months, and 16.4 IU/mL at 36 months.) Children attending daycare of any kind by 3 months of age were found to have marginally lower concentrations of IgE antibodies at each age compared to children who did not attend daycare ($p= 0.007$ at 3 months, $p= 0.004$ at 1 year, $p= 0.005$ at 2 years, and $p= 0.009$ at 3 years). Maternal smoking either during pregnancy or postpartum, dogs or cats in the home and paternal atopy were unrelated to total IgE concentration in the cohort as a whole. By 3 years of age, daycare attendance outside the home without other children, and daycare attendance outside the home with other children were associated with lower IgE concentrations ($p= 0.023$ and $p= 0.006$, respectively) compared to children without daycare exposure. In relation to food allergen-specific IgE levels, the risk for having a food-allergy response was decreased through 3 years with any daycare exposure compared to without daycare exposure. However, lower IgE concentrations were not found for inhalant-specific IgE levels.

Between 1987 and 1994, Jaakkola et al. (65) investigated the relationship between maternal smoking in pregnancy and the risk of childhood asthma through the first 7 years of life in 58, 841 Finnish children. The investigators examined whether

childhood asthma was enhanced by reduced fetal growth due to smoking during pregnancy. Maternal smoking during pregnancy was categorized as: nonsmoking, smoking less than 10 cigarettes per day, and smoking greater than 10 cigarettes per day. Asthma was defined as a child having at least one hospitalization due to asthma, at least one entitlement to free medication due to asthma or at least one entitlement to special care support due to asthma before 7 years of age. Adjustment was made for infant gender, birth order, low birth weight and pre-term delivery, and for maternal age, marital status and maternal occupation.

The investigators found that low birth weight and preterm delivery increased the risk of asthma (OR = 1.83, CI; 1.50-2.24, and OR = 1.64, CI; 1.38-1.95, respectively) compared to a normal birth weight and term delivery. The researchers also found that smoking during pregnancy increased the risk of asthma by 7 years of age (OR = 1.23, CI; 1.07- 1.42 for children of women who smoked less than 10 cigarettes per day; OR 1.35, CI; 1.13-1.62 for children of women who smoked more than 10 cigarettes per day). After adjusting for fetal growth and preterm delivery, the effect of maternal smoking on asthma remained respectively: OR = 1.20, CI; 1.04-1.38 and OR = 1.31, CI; 1.09-1.58. Evidence that maternal smoking during pregnancy increases the risk of childhood asthma (65).

Other researchers have investigated the effects of second-hand smoke in relation to the development of allergy and asthma in young children (63, 67). A meta-analysis of 53 studies reported between 1970 and 2005 examined exposure to second-hand smoke and the development of childhood asthma (67). Researchers reviewed 53

studies, half case-controlled and half cross-sectional. Outcome search terms included asthma, wheezy bronchitis, asthmatic bronchitis, and reactive airway disease while exposure search terms included passive smoking, environmental tobacco smoke, second-hand smoke, and cigarette smoke.

Among the 53 studies, 200,000 children less than 18 years of age were examined. After controlling for family history of asthma and atopy, there was a positive association between household second-hand smoke exposure and the development of asthma. The meta-analysis found the relative risk to be 1.51 with a 95% confidence interval of 1.31-1.75 ($p < 0.001$) for household second-hand smoking after adjusting for covariates. The study suggested even in the absence of family history of atopy and asthma that second-hand smoke exposure increases the likelihood of developing asthma before 18 years of age.

With regard to risk of developing an allergic disease and asthma, early exposure to household pets has also been controversial. Pohlabein et al. (68) examined 1,881 children during the first two years of life for development of allergy in relation to exposure to household pets. The mothers of these children completed the International Study of Asthma and Allergies in Childhood (ISAAC). Twenty-three percent of the children showed symptoms of allergic disease with the majority being eczema (69.4 percent). Forty-one percent of the families with atopic children had household pets compared to 48 percent of the families without atopic children ($p = 0.006$). There was a non-significant trend towards less allergic symptoms in children without family history of asthma if they had a pet in the home since birth. In

children with a family history of allergic disease there was a trend toward an increased risk among those exposed to dogs since birth. Children without a family history of allergic disease had a lower risk of developing eczema and asthma in the first two years of life than children with a family history of allergy who were exposed to a dog in the home. The study suggested there is a protective factor of dog ownership in the development of atopic dermatitis and asthma in children.

Zirngibl et al. (64) found a protective effect of dog ownership in a study of 4,578 children with and without a family history of allergic disease (OR= 0.63, 95% CI; 0.40-0.98). This study agreed with the hygiene hypothesis that early exposure to microbial substances is associated with decreased production of IgE antibodies, thus decreasing the risk for allergic disease (68). A recent study by Hugg et al. (20) also found that in 1,106 children, exposure to dogs early in childhood reduced the risk of developing allergies and asthma (OR= 0.35, 95% CI; 0.13-0.95).

A variety of factors in the environment may influence the development of URIs, dermatitis and allergic manifestations. A family history of allergies and asthma increases the risk for offspring to develop allergies and asthma when exposed to an environmental allergen with adverse effects. The findings noted above provide guidance for protection against developing these chronic illnesses in infancy. Infants with a family history of allergies and asthma may gain added protection by pet exposure and attending daycare early in life.

CHAPTER 3: METHODS

This is an exploratory study of a sample of convenience, of infants of women who participated in a double-blinded, randomized, controlled trial. The purpose of the study was to compare infant diet, including DHA intake to URIs, dermatitis and allergy in the first year of life. Data had been collected in the parent trial on feeding history, including food sources of DHA in the first year of life and maternal DHA status at entry into the study. The primary objective of the parent study was to determine if a dietary supplement of 600 mg/day of docosahexaenoic acid (DHA) during the last half of pregnancy would increase gestation duration and enhance the infants' visual acuity, attention span and decrease distractibility in the first 18 months of life.

Overview

Women between 8 and 20 weeks gestation were recruited from University of Kansas Medical Center and Truman Medical Center for the parent study. Each of these women received either 600 mg DHA in 3 capsules or a placebo in 3 capsules per day from enrollment to the birth of her baby. Plasma and total RBC phospholipid fatty acid composition was collected at the time the women enrolled for the parent study and at delivery. Healthy infants born at term continued to be followed to 18 months of age. Maternal milk samples were collected at the 6 week postpartum study visit from those mothers who were breast feeding at that time. Medical records of infants were obtained at one year of age. A 24-hour dietary recall and anthropometric

data were obtained at 6 weeks, 4 months, 6 months, 9 months, and 12 months of age. Other information regarding maternal allergies and asthma, smoking during pregnancy, smoking in the home, number of children in the home, daycare attendance and pet exposure were obtained at each of the follow up study visits until the infant reached 12 months of age.

Setting

All study subjects reported to the University of Kansas Medical Center Infant Nutrition Clinic for parent-study appointments at 6 weeks, 4 months, 6 months, 9 months, and at 12 months of age. Anthropometric data and descriptive data were collected at each of these 5 visits.

Ethics

This study was approved by KU Medical Center Human Subjects Committee under the parent trial 1R01 HD047315 entitled Kansas University DHA Outcomes Study or KUDOS. The current study used data collected under that study to explore relationships of DHA intake and human milk intake in the first year of life on URIs, dermatitis and allergy development. These relationships were not planned as study outcomes; however, all data were collected for the purpose of including infant illnesses as part of adverse event monitoring.

Subject Selection

The mothers of subjects were recruited primarily through University of Kansas Medical Center and Truman Medical Center, as well as through flyers, brochures, my KUMC e-mail system and word of mouth. The inclusion and exclusion criteria for the parent study included women between 8 and 20 weeks gestation, women who were 16 to 36 years of age, healthy women with a BMI less than 40 and a blood pressure less than 140/90 and women with no preexisting diabetes or medical conditions that would affect the outcome of their pregnancy. All subjects in the subordinate study met the following inclusion criteria:

1. Offspring from mothers who consented in parent study and were assigned to 600 mg of DHA or placebo in the beginning of the second half of pregnancy,
2. Infants healthy (without a congenital disease) and born at term (at least 37 weeks gestation),
3. Infants at least 12 months of age,
4. Availability of a milk sample in mothers who breast fed for at least 6 weeks postpartum of known DHA content,
5. Available medical records through 12 months of age.

The first infant study visit was in July of 2006. Data collection for the subordinate study began in October 2008 and continued until February 2009. All primary descriptive, anthropometric, and dietary data as well as human milk samples

were collected between July 2006 and January 2009. Secondary descriptive data related to maternal allergies and asthma, smoking during pregnancy, smoking in the home, number of siblings in the home, daycare attendance and pet exposure was collected between the months of October 2008 and January 2009 after receiving permission from the HSC for an amendment to the approved protocol.

Data Collection

Anthropometric Data

At each study visit (6 weeks, 4 months, 6 months, 9 months and 12 months of age), body weight was obtained using a standard calibrated scale to the nearest gram, and body length was determined recumbent on a length board measuring to the nearest centimeter.

Dietary Intake Data

A 24-hour dietary recall was obtained at each study visit by registered dietitians trained in the Multiple Pass Methodology of interviewing. To determine breast feeding, the mother was asked to indicate time of breast feeding and any formula feeding. For formula-fed infants, questions were asked regarding when infant formulas were consumed and the amount and type of infant formula. By the end of the exploratory study, the 24-hour dietary recalls were more detailed and included the time of breast-feeding as well as how infant formula was prepared and how much of the infant formula the child actually consumed.

Medical Records

Medical records through 12 months of age were obtained from the study subjects' primary care physician as part of the parent study collection of adverse events. URIs and allergic manifestations documented in the infants' medical records were recorded as URI, otitis media, croup, allergy and asthma. If 2 or more symptoms were noted for duration of at least 3 days, i.e. rhinorrhea, congestion and cough, these were recorded as one URI episode. Dermatitis was also recorded as atopic dermatitis, eczema and rash of unknown origin for duration greater than 3 days.

Primary Descriptive and Anthropometric Data

Primary descriptive and anthropometric data were obtained from the delivery medical records and included the infant's gender, race, birth length and birth weight as well as maternal cigarette and alcohol use during and prior to pregnancy (Appendix A).

Descriptive Data Obtained for Covariate Analysis with Upper Respiratory Illness and Allergic Manifestation Status

The infants' parent(s) or caregiver(s) answered questions at the 12 month parent-study follow up visit about factors believed to influence the development of URI, allergy and asthma. The questions included if the mother smoked during pregnancy, the number of people who smoked in the home, number of children under

the age of 13 in the home, maternal history of asthma and allergies, number and type of furred pets in the home and daycare attendance. (Appendix B).

Laboratory Methods

Human Milk Analysis

Lactating women were sent a sterile container which they returned at the 6 week parent-study follow up visit with a milk sample. The sample was logged and kept frozen at -80°C until analysis. Methanol containing BHT (4 mL) was added to 15 mL extraction tubes containing 0.5 mL of human milk. Eight milliliters of chloroform was added and vortexed for 15 minutes. Next, 1.6 mL of 0.05M KCl was added and centrifuged for 5 minutes at 750 r.p.m. The upper phase was discarded, and the lower phase was evaporated in a 35°C water bath. The sample was then transmethylated with 0.25 mL BF_3 14% solution in methanol, 0.2 mL Benzene, and 0.55 mL methanol. After this was layered with N_2 , it was placed on ice until all samples were finished. The samples were then placed in a dry bath at 100°C for 30 minutes. Next, they were placed on ice and 1 mL of distilled H_2O and 2 mL of pentane were added. Samples were vortexed in order to extract fatty acid methyl esters into the pentane phase and centrifuged for 5 minutes at 800 r.p.m. When the samples were completely dry under nitrogen dissolved in 10 μl dichloromethane, they were analyzed by gas chromatography using an autoanalyzer to determine the concentration of each fatty acid methyl ester or the weight (g/100g) of each fatty acid. (Appendix C.)

Plasma and Red Blood Cell Total Phospholipid Fatty Acid Analysis

For each woman, plasma and red blood cell total phospholipid fatty acid composition was analyzed at enrollment and at the time of delivery. Whole blood was collected in a sterile tube containing sodium heparin and centrifuged at 3000 g at 4°C to separate plasma and red blood cells. Four mL of methanol and BHT were pipeted into sterile 15 mL extraction tubes. Five hundred microliters of blood or plasma was added and vortexed. Eight mL of chloroform was then added and vortexed; 15 minutes for red blood cells and 10 minutes for plasma. The contents were then added to a clean extraction tube and 1.6 mL of 0.05M KCl was added and vortexed for 10 seconds. This was then centrifuged for 5 minutes at 750 r.p.m. and the upper phase was discarded. The lower phase was evaporated in a 35°C water bath under nitrogen. Total phospholipids were separated and identified using the Smuts et al. (69) technique. For transmethylation, each sample was layered with N₂ and placed in a dry bath at 100°C for 10 minutes. Once transmethylation was complete, samples were cooled on ice and 1mL of distilled H₂O and 2 mL of pentane were added. The samples were then vortexed for 1-2 minutes in order to extract fatty acid methyl esters into the pentane phase and then centrifuged for 5 minutes at 800 r.p.m. Next, the samples were evaporated under nitrogen dissolved in 10 µl dichloromethane and analyzed by gas chromatography using an autoanalyzer. (Appendix D).

Statistical Analysis

Complete data were obtained for 50 infants. Forty-one of the 50 infants were fed human milk for some duration of time, consequently most of the analyses used duration of human milk feeding as the independent variable. There were 25 subjects fed human milk greater than 16 weeks and 25 subjects fed human milk for less than 16 weeks, including 9 infants fed exclusively formula. Of the 50 original subjects, only 8 infants (16%) were exclusively fed human milk until at least 52 weeks of age. Most subjects (33 of 50, or 66%) were fed both human milk and infant formula for some duration of time in infancy.

For some statistical analyses, subjects were evaluated using a continuous variable, i.e. weeks of human milk feeding, or by comparing those fed human milk for greater than 16 weeks compared to less than 16 weeks or greater than 26 weeks compared to less than 26 weeks. Greater than and less than 16 weeks and greater than or less than 26 weeks was used for statistical analyses based on previous evidence that human milk feeding is immunoprotective if fed for 4 to 6 months compared to less than 4 months. Those fed human milk for 16 weeks or more were recorded as receiving human milk for the exact number of weeks fed human milk up to 52 weeks of age because outcomes were not measured beyond 52 weeks.

Descriptive Data, URIs and Dermatitis

Means, standard deviations, and ranges were calculated for descriptive data for the 50 infants. The total number of URI and dermatitis episodes diagnosed for the

group fed human milk less than 16 weeks and greater than 16 weeks were recorded before 6 months of age and between 6 and 12 months of age. The total number of infants in each group who experienced a URI and/or dermatitis was recorded before 6 months of age and between 6 and 12 months of age. The number of infants and total number of URI and dermatitis episodes between 0 and 3, 3 and 6, 6 and 10 and 10 and 12 months are tabulated by duration of human milk feeding (greater than or less than 16 weeks) in Appendix E.

The duration of human milk feeding in relation to respiratory illness and dermatitis was also evaluated up to 6 months (26 weeks) using an independent samples *t*-test. This was done in order to test if human milk feeding greater than 16 weeks results in a statistically significant difference in relation to URIs and dermatitis. The median for the age of the first diagnosed URI and first diagnosed dermatitis episode was measured for infants fed human milk less than or greater than 16 weeks. Pearson's Product Moment correlations were used for simple correlation analysis for descriptive data, number of weeks the infants were fed human milk and the number of URI and dermatitis episodes diagnosed, as well as the age of the first occurrence of an URI and/or dermatitis episode.

DHA Concentration and Calculated Relative DHA Exposure

The weight percent for plasma RBC total phospholipids for DHA and AA at enrollment were available for all 50 subjects. Weight percent for plasma RBC total phospholipids DHA and AA at delivery were available for 48 of the 50 original

subjects (96%). Milk DHA and AA intake at 6 weeks lactation was available for 35 infants; 25 of the 25 human milk-feeders (100%) and 10 of those fed human milk less than 16 weeks but greater than 6 weeks (40%) as they had milk samples available for the 6-week follow up visit. Means, standard deviations and ranges were calculated for fatty acid composition data of the total RBC plasma phospholipids at enrollment and delivery, and human milk fatty acid composition for the groups fed human milk for greater than or less than 16 weeks.

Because 66% of our subjects were “mixed” feeders (fed both formula and human milk for some duration of time), we attempted to calculate infant relative DHA exposure from formula and human milk by multiplying the number of weeks the infant was fed human milk by the weight percent of DHA samples. The number of weeks the infant consumed formula was multiplied by the DHA concentration in the infant formula that they received. If the infant received both milk and formula for some duration of time, the two DHA exposure concentrations were added together. We tested for statistical differences for the number of URI and dermatitis episodes diagnosed in the formula-fed group and the human milk-fed group with total DHA exposure in the first 6 months of life as well as for 0-12 months. Pearson’s Product Moment correlations were used to test whether or not there was a statistically significant difference in relative DHA exposure and the number of URI and dermatitis episodes diagnosed in either group.

Covariate Analysis

Covariates considered included if the mother smoked during pregnancy, smoking in the home, maternal allergies and asthma, pet exposure, daycare attendance and number of children in the home under the age of 13. However, only one was related to URIs or dermatitis for either group. As well, an independent samples t-test was also used to test differences in the covariates with duration of less than or greater than 26 weeks.

All statistical procedures were done using Microsoft Office Excel 2007[®] for Windows (Microsoft Corp., Seattle, WA) and SPSS[®] version 16.0 (SPSS Inc., Chicago, IL). Significance was determined by alpha less than 0.05 and all p-values were based on two-sided tests.

CHAPTER 4: RESULTS

The objective of this study was to a) determine the relationship between duration of human milk feeding and the prevalence of URI, dermatitis, allergies and asthma in the first year of life and b) to determine if there is a relationship between fatty acid composition, particularly DHA, in maternal milk and in infant formula and the prevalence of URI, dermatitis, asthma and allergies.

All available subjects (n=50) were used to generate summary statistics (Table 3). For the two groups of infants defined by milk feeding (breast feeding greater than or less than 16 weeks), gestational age, birth weight, birth length, head circumference and duration of human milk feeding were obtained from the parent study. Nine of the 50 original subjects (18%) were fed only formula for 52 or more weeks. Eight of the 50 original subjects (16%) were fed only human milk for 52 or more weeks. The remaining 33 subjects (66%) were fed human milk and infant formula for some duration of time. The formula-fed group (n=25) included infants who were fed exclusively or predominately formula and were fed human milk for less than 16 weeks. The human milk-fed group (n=25) included infants who were fed exclusively or predominately human milk for greater than 16 weeks, although they may have also received formula for some duration of time.

TABLE 3 Summary statistics of study population

Characteristic	Formula-Fed (n=25)	Breast-Fed (n=25)
Gender		
<i>Male</i>	14	9
<i>Female</i>	11	16
Gestational Age (wks)		
<i>Mean ± SD</i>	39.2 ± 1.3	39.9 ± 0.8
<i>Range</i>	37-41.2	38.4-41.3
Birth Weight (g)		
<i>Mean ± SD</i>	3306 ± 429.9	3523 ± 486.2
<i>Range</i>	2043-4075	2615-4190
Birth Length (cm)		
<i>Mean ± SD</i>	50.1 ± 1.5	50.3 ± 2.4
<i>Range</i>	43.2-53.8	47-55.8
Head Circumference (cm)		
<i>Mean ± SD</i>	34.1 ± 1.5	34.6 ± 1.4
<i>Range</i>	29-36	32-37.5
Duration of Human Milk Feeding (wks)		
<i>Mean ± SD</i>	5.6 ± 5.7	44.8 ± 14.6
<i>Range</i>	0-15.9	17.6-52.0

There were no differences in birth weight, length and head circumference or gestational age between the two defined feeding groups.

Prevalence of URI

In the sample as a whole, 39 of 50 infants (78%) experienced at least one URI in the first 12 months of life. Of the 11 infants who did not experience a URI in the first 12 months of life, 5 of those infants (45%) were in the formula-fed group (fed human milk less than 16 weeks) and 6 of those 11 infants (54%) were in the human

milk-fed group. Of the 39 infants who experienced a URI in the first 12 months of life, 17 (43%) experienced 1 URI, 11 (28%) experienced 2 URIs, 7 (18%) experienced 3 URIs, 2 (5%) experienced 4 URIs, 1 experienced 5 URIs (3%), and 1 infant (3%) experienced 6 URIs. Figure 1 shows the frequency of URIs experienced in the first 12 months of life in the formula-fed group and the human milk-fed group.

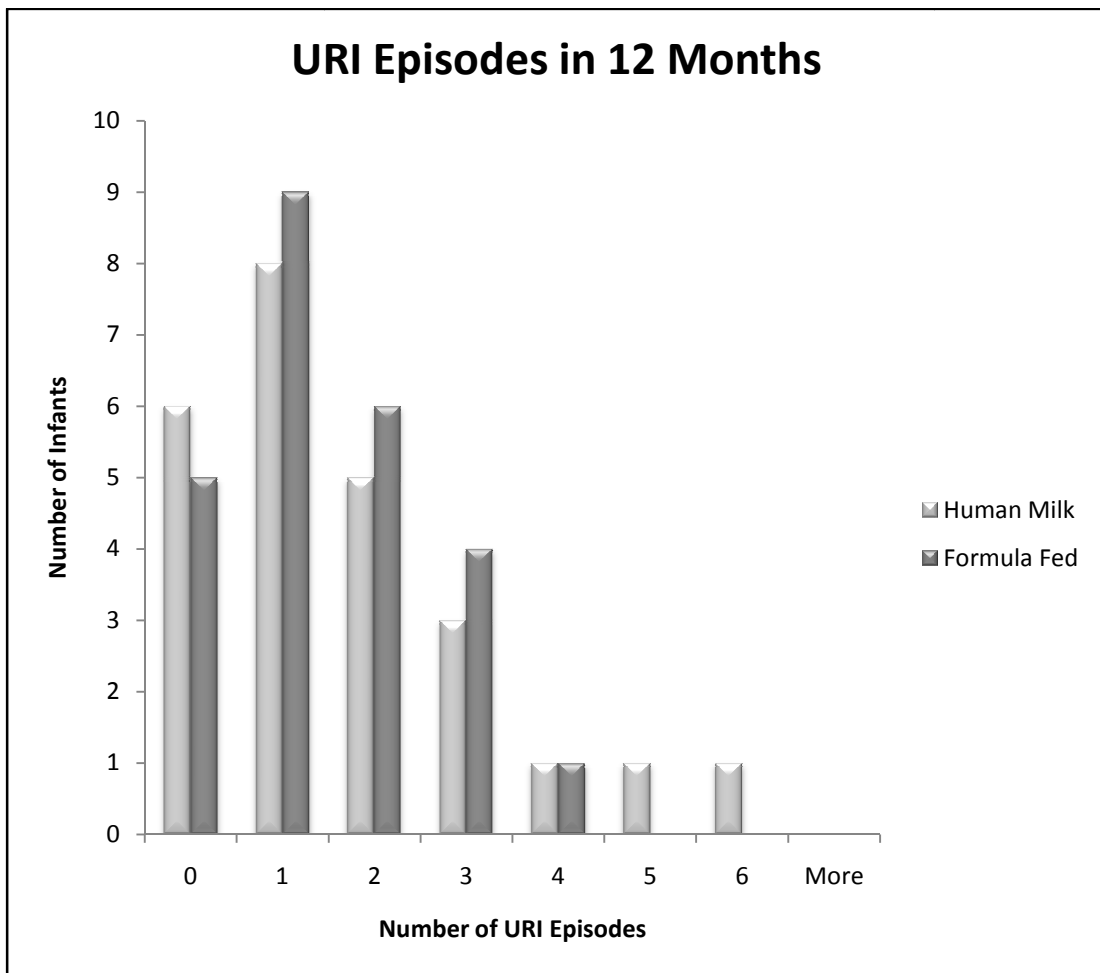


FIGURE 1 Frequency of URI for 39 of 50 infants who experienced a URI in the first 12 months of life.

Of the 50 infants, the range in age for the first URI diagnosis was from 2.3 weeks to greater than 52 weeks of age. Infants fed human milk for greater than 16

weeks experienced their first URI approximately 7 weeks later compared to infants fed human milk less than 16 weeks. The median age for the first diagnosed URI of the 25 infants in the human milk-fed group was 26.71 weeks of age while the median age for the 25 infants fed human milk less than 16 weeks was 20.0 weeks. Figure 2 shows the relationship between the age of the first diagnosed URI and the duration of human milk feeding in the first 12 months of life.

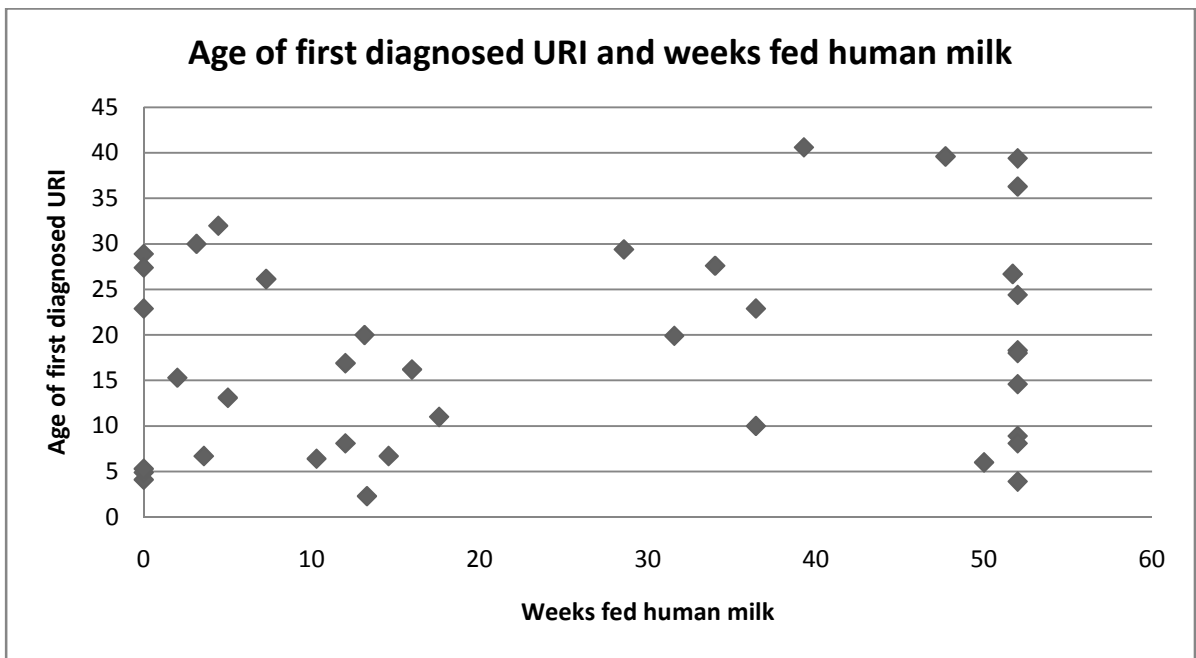


FIGURE 2 Age of first URI compared to weeks fed human milk in the 39 infants who experienced a URI in the first 12 months of life.

Fourteen of 25 (56%) infants fed human milk less than 16 weeks were diagnosed with at least one URI before 6 months of age with the total number of URI episodes before 6 months of age being 18. Thirteen of 25 (52%) infants fed human milk less than 16 weeks experienced at least one URI between 6 and 12 months of age with a total of 19 URI episodes in this group between 6 and 12 months of age. Six

of the 13 (46%) infants who experienced a URI between 6 and 12 months of age, did not experience a URI before 6 months of age.

In the group of infants fed human milk greater than 16 weeks, 12 of 25 (48%) infants were diagnosed with a URI before 6 months of age and there were a total of 20 URI episodes experienced in this group before 6 months of age. Between 6 and 12 months of age, 15 of 25 human milk-fed infants (60%) were diagnosed with a URI and there were a total of 22 URI episodes between 6 and 12 months of age. Seven of these 15 (47%) infants were not diagnosed with a URI before 6 months of age. A positive trend was found in infants fed human milk for at least 26 weeks compared to human milk-fed for 16 weeks for fewer URI episodes ($p=0.519$, $p=0.767$). Table 4 shows the number of infants and number of URI episodes in each group before 6 months of age and between 6 and 12 months of age. The age of the first diagnosed URI was unrelated to the diagnosis of allergy or asthma in the first year of life ($p=0.740$, $p=0.888$, respectively). Similarly, the number of URIs before 6 months of age was unrelated to the diagnosis of allergy and asthma ($p=0.745$, $p=0.419$, respectively), as was the number of URIs between 6 and 12 months of age ($p=0.321$, $p=0.175$ respectively). Appendix E shows the tabulation of URI episodes by 3 month intervals (0-3 months, 3-6, 6-10, and 10-12 months).

TABLE 4 Number of Infants with URI and number of URI episodes

	Formula Fed (n=25)	Human Milk (n=25)
Mean Duration of Human Milk (wks) (n=25)	5.6	45.07
Infants with URI before 6 months	14	12
Number of URI Episodes before 6 months	18	20
Infants with URI 6-12 Months of Age	13	15
Number of URI Episodes 6-12 Months	19	22

Prevalence of Dermatitis

Dermatitis occurred in 11 of 25 (44%) human milk-fed infants and in 15 of 25 (60%) formula-fed infants. A statistically significant inverse correlation was found for longer duration of human milk feeding and number of dermatitis episodes before 6 months of age ($R = -0.289$, $p = 0.044$). This is shown in Figure 3. Based on this R , 8.3% of the dermatitis episodes before 6 months of age can be explained by shorter duration of human milk feeding. Infants fed human milk for at least 26 weeks exhibited an even greater significant reduction in dermatitis episodes ($R = -0.329$, $p = 0.020$) with an $\alpha = 0.05$. Figure 4 shows the number of dermatitis episodes in relation to the weeks fed human milk before 6 months of age.

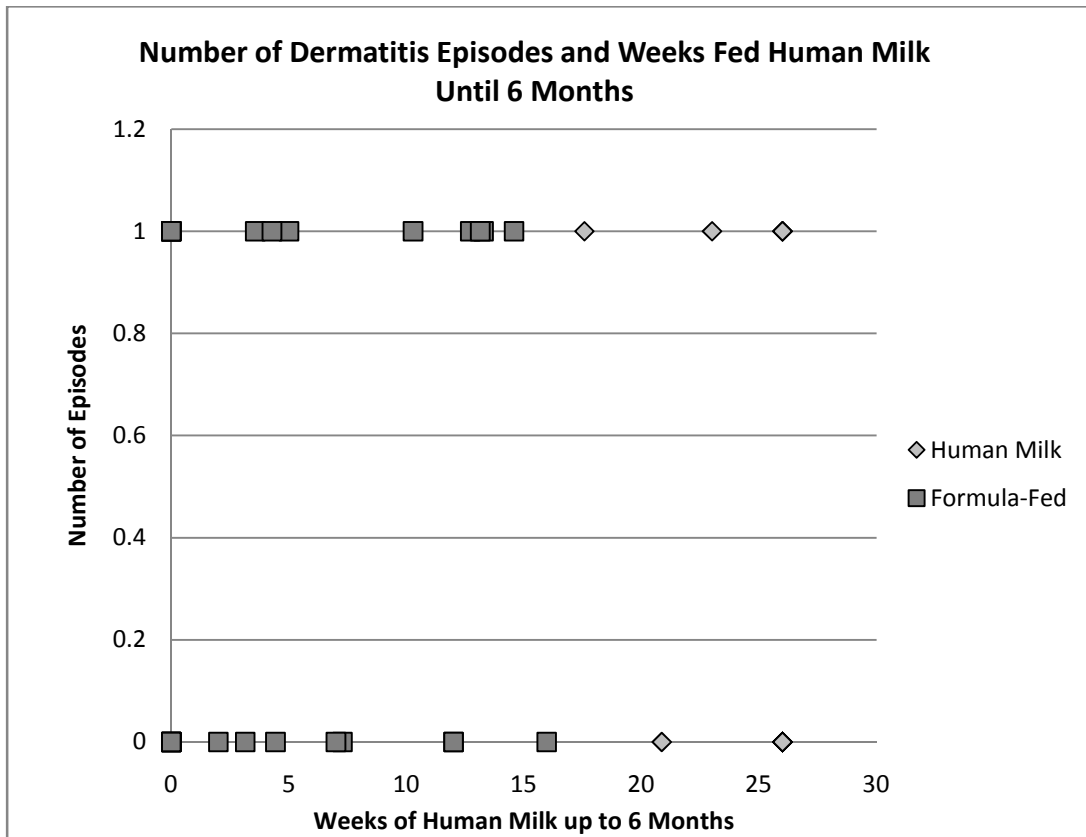


FIGURE 4 Number of dermatitis episodes and weeks fed human milk to 6 months of age (26 weeks) ($R = -0.329$; $p = 0.020$).

In the human milk-fed group, 8 of the 11 (73%) infants who experienced a dermatitis episode in the first year of life experienced just one episode, 2 of 11 (18%) infants experienced 2 episodes, and 1 of 11 (9%) infants experienced 3 episodes. In the formula-fed group, 11 of 15 (73%) infants that experienced a dermatitis episode in the first 12 months of life experienced one episode and 4 of 15 (27%) infants experienced 2 dermatitis episodes. Table 5 shows the number of infants who experienced a dermatitis episode and the number of dermatitis episodes that occurred

in each group by 6 month intervals. Figure 5 shows the frequency of dermatitis episodes in the first 12 months of life in the two groups.

Of the 25 infants fed human milk longer than 16 weeks, the median age for the first dermatitis was greater than 52 weeks with a range from 4.43 to greater than 52 weeks of age. The median age for the first diagnosed dermatitis episode was 32.28 weeks with a range from 1.43 to greater than 52 weeks of age. The age of the first diagnosed episode of dermatitis did not show a statistically significant correlation with the diagnosis of allergy or asthma in either group ($p= 0.230$, $p= 0.984$, respectively). The number of dermatitis episodes before 6 months of age did not statistically correlate with the diagnosis of allergy and asthma in either group ($p= 0.184$, $p= 0.185$, respectively). The number of dermatitis episodes diagnosed between 6 and 12 months of age and the diagnosis of allergy and asthma in the infant also were not statistically significant ($p= 0.562$, $p= 0.216$, respectively).

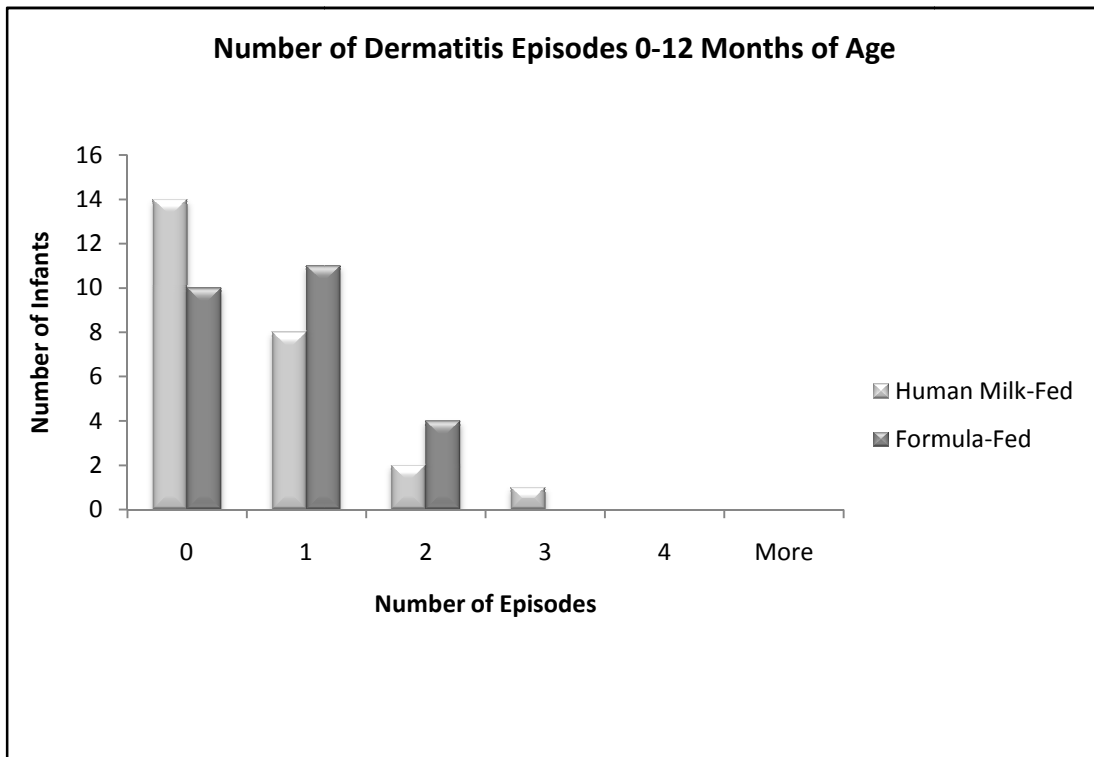


FIGURE 5 Frequency of Dermatitis Episodes in the first 12 months of life in 26 of the 50 original subjects.

TABLE 5 Number of Infants who Experienced Dermatitis and Number of Dermatitis Episodes

	Formula Fed (n=25)	Human Milk (n=25)
Mean Duration of Human Milk (wks) (n=25)	5.6	45.07
Infants with Dermatitis Before 6 Months	12	6
Number of Dermatitis Episodes before 6 mo.	12	6
Infants with Dermatitis 6-12 Months	7	7
Number of Dermatitis Episodes 6-12 Months	7	9

Fatty Acid Composition of RBC

The weight percent of plasma and red blood cell (RBC) total phospholipid fatty acid composition for AA and DHA at enrollment was available for all 50 subjects. The weight percent for plasma and RBC total phospholipid fatty acid composition for AA and DHA at delivery was available for 48 of the 50 subjects (96%). Data were missing for the weight percent AA and DHA concentrations at delivery from one mother in the human milk-fed group and one mother in the formula-fed group. When the groups of mothers were measured as a whole, the mean weight percent AA concentration at enrollment was 15.65 with a range of 8.20-23.90. The mean weight percent AA concentration at delivery was 12.93 with a range of 2.58-19.06. The mean weight percent DHA concentration at enrollment was 4.59 with a range of 1.73 to 8.60. The mean corrected weight percent DHA concentration at delivery was 5.92 with a range of 1.48 to 10.61. The AA and DHA fatty acid composition separated in to the two groups is displayed in Table 6.

TABLE 6 Weight percent AA and DHA Concentration

	Human Milk-Fed		Formula-Fed	
	Enrollment (n=25)	Delivery (n=24)	Enrollment (n=25)	Delivery (n=24)
DHA (%)				
<i>Mean</i>	5.05	6.7	4.12	5.14
<i>Range</i>	2.82-8.6	3.02-10.61	1.73-7.01	1.48-8.30
<i>SD</i>	1.48	2.42	1.4	1.7
AA (%)				
<i>Mean</i>	16.05	12.31	15.25	13.53
<i>Range</i>	11.02-23.9	8.18-15.9	8.19-20.42	2.58-19.06
<i>SD</i>	2.76	1.86	2.53	3.25

No statistically significant differences were found in the total phospholipid weight percent of DHA or AA in mother's who fed their infants human milk for greater than 16 weeks compared to mothers who fed their infants human milk less than 16 weeks.

Fatty Acid Composition of Human Milk

The fatty acid composition for human milk was available for 35 of the 50 (70%) sub-study subjects at 6-weeks postpartum. The mean weight percent DHA concentration in the human milk was 0.28 and the mean weight percent for AA in human milk was 0.52. The mean relative DHA exposure for the amount of human milk and infant formula intake before 6 months of age for the group fed human milk less than 16 weeks was 7.6 with a range of 3.6 to 10.86 and for 12 months in the infants fed human milk less than 16 weeks was 14.8 with a range from 7.8 to 17.5. The mean relative DHA exposure before 6 months of age for the group of infants fed human milk greater than 16 weeks was 9.26 with a range of 3.2 to 21.8 and for 12 months the mean relative DHA was 20.3 with a range from 11.6 to 48. DHA exposure did not correlate with the number of URIs or dermatitis episodes, the age of the first URI or first dermatitis episode for either group or by interval of infancy (before 6 months of age or between 6 and 12 months of age). Figure 6 shows the total relative first year DHA exposure by group.

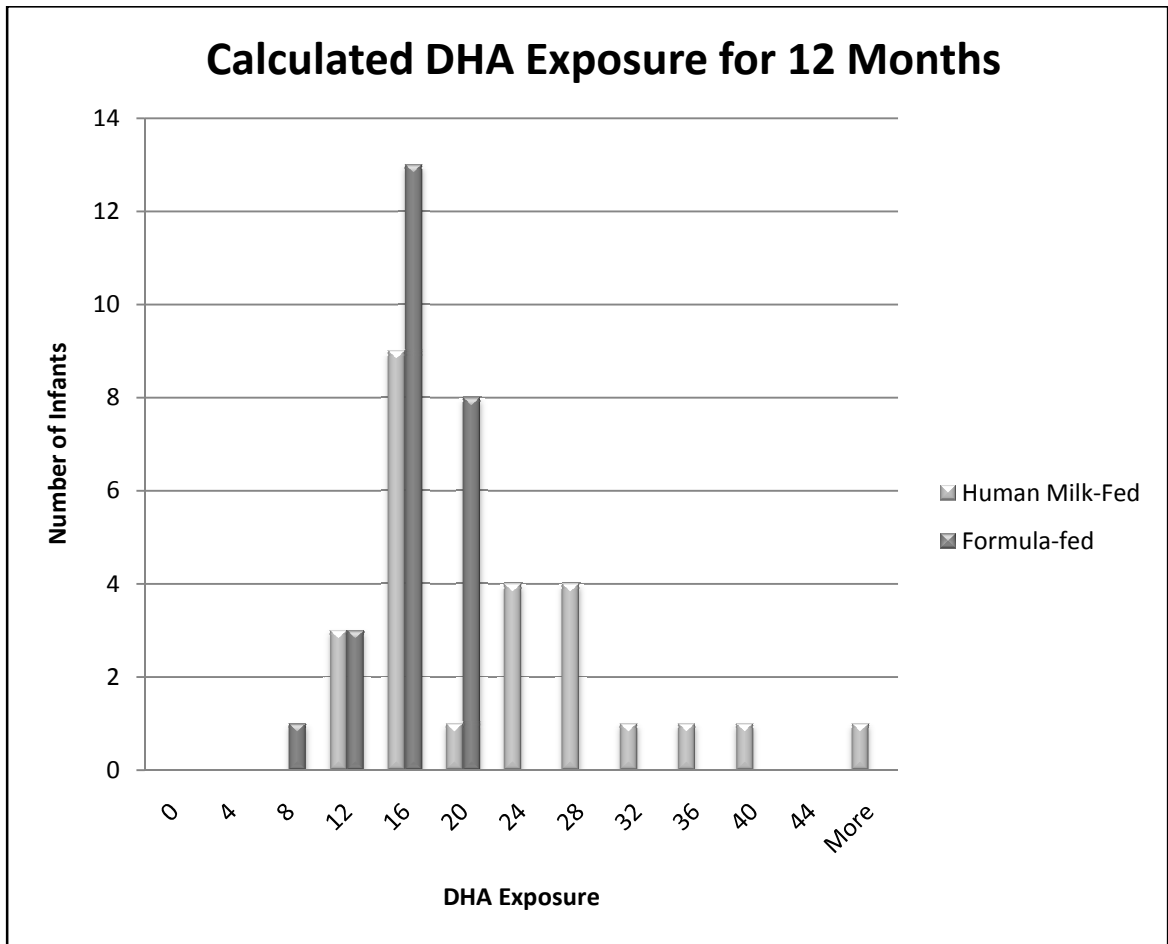


FIGURE 6 Mean relative DHA exposure in infancy.

Potential Influential Factors in the Development of URIs, Dermatitis, Allergy and Asthma

The presence of potentially influential factors for developing URIs, dermatitis, allergy and asthma are shown for each group in Table 7. The human milk-fed group and the formula-fed group appeared to differ, however, none was significantly different when groups fed human milk greater than or less than 16 weeks were compared including smoking during pregnancy, number of other children in the home, daycare attendance, number of furred pets in the home, atopic mothers, and

maternal history of asthma. The absence of a statistical difference does not mean that these groups are similar in regard to several risk factors like maternal history of asthma. In one case, a statistically significant inverse correlation was found between the number of weeks of human milk feeding and exposure to smoking in the home ($R = -0.281, p = 0.048$).

When the data for influential factors in relation to human milk-feeding for greater than or less than 26 weeks was analyzed, significant trends were found with diagnosis of maternal asthma ($p=0.041$) and smoke exposure in the home ($p= 0.009$). Three women who breast fed their infants to 16 weeks did not feed human milk up to 26 weeks. Two of these women were atopic and smoked during pregnancy, one of the women had asthma, and all three of the women's infants were exposed to smoke in the home. Table 8 shows summary statistics for influential factors when comparing infants fed human milk for greater than 26 weeks compared to infants fed human milk for less than 26 weeks.

TABLE 7 Summary statistics of influential factors for human milk-fed greater than or less than 16 weeks

	Human milk-fed < 16 weeks	Human milk-fed > 16 weeks
Characteristic	(n=25)	(n=25)
Smoking During Pregnancy	8 (32%)	5 (20%)
Smoking in the Home	6 (24%)	4 (16%)
Other Children in the Home	16 (64%)	10 (40%)
Daycare Attendance	11 (44%)	11 (44%)
Furred Pets in the Home	9 (36%)	11 (44%)
Atopic Mothers	12 (48%)	15 (60%)
Maternal History of Asthma	8 (32%)	3 (12%)

TABLE 8 Summary Statistics of influential factors for human milk-fed greater than or less than 26 weeks.

	Human milk-fed < 26 weeks	Human milk-fed > 26 weeks
Characteristic	(n=22)	(n=28)
Smoking During Pregnancy	10 (45%)	3 (11%)
Smoking in the Home	9 (41%)	1 (4%) **
Other Children in the Home	16 (73%)	10 (36%)
Daycare Attendance	13 (59%)	9 (32%)
Furred Pets in the Home	11 (50%)	9 (32%)
Atopic Mothers	14 (64%)	13 (46%)
Maternal History of Asthma	9 (41%)	2 (7%)*

*P<0.05; **P<0.01

CHAPTER 5: DISCUSSION

URIs, atopic dermatitis, allergy and asthma are frequent clinical pediatric diagnoses. Finding a way to protect infants early in life from these illnesses is important for their immune function maturation, which is related to the “atopic march.” Immunoprotective agents in human milk and the development of these illnesses have been extensively studied with mixed results (7-11). It has been argued that longer duration of human milk feeding may lead to decreased risk or delayed occurrence of developing URIs (7, 8), dermatitis (48, 50), allergy and asthma (4, 50, 70). More recently, the relationships between fatty acid composition in human milk and the prevalence of URIs, dermatitis, allergy and asthma have been explored (14, 18, 59, 60).

Prevalence of Upper Respiratory Illnesses

The total number of infants in our study who experienced at least one URI episode in the first 12 months of life (39 out of 50 or 78%) is consistent with previous findings (7, 8). As well, Sinha et al. (10) found a gender difference in infants who experienced URIs before 12 months of age with male infants having a greater number of URIs compared to female infants. Sinha et al. did not find a significant difference in the prevalence of URIs in the first 12 months of life in infants fed human milk less than 16 weeks compared to infants fed human milk greater than 16 weeks. Our data is in agreement with this finding. We did find a later onset of first URI among infants who consumed human milk for greater than 16 weeks compared to infants who

consumed human milk for less than 16 weeks (26.71 weeks vs. 20 weeks of age). This could be clinically significant in that a later onset of an illness may indicate a more mature immune system and/or later illness may protect against or slow the atopic march later in childhood.

It is important to keep in mind that this is an exploratory evaluation and that it will eventually be applied to a sample of approximately 200 children. Non-significant trends would be hypothesized to be statistically significant at the completion of the study. As well, we will be able to include the amount of DHA provided in utero as a covariate in the mixed model analysis for relationships between DHA exposure and illnesses in infancy and beyond as these children will be followed until 5 years of age. Accordingly, we should be able to evaluate the effects of human milk and DHA on both early and late illnesses.

We examined the number of URI episodes diagnosed before 6 months of age and the number of URI episodes diagnosed between 6 and 12 months of age in infants fed human milk greater and less than 16 weeks and did not find any statistically significant differences. This could be for several reasons. Many infants (78% in our study) experience a runny nose, congestion or a cough before 12 months of age as we have discovered, but we do not know if all parent(s) or caregiver(s) took their child to their primary physician if they considered the illness minor or if the duration of the illness was short. Because only physician-diagnosed medical records were used in our study, there may have been more infants in either group who experienced a URI but

did not see a doctor. On the other hand, we are not sure this is a true limitation of the study as parent(s) or caregiver(s) were asked about recent illnesses at each visit and the study coordinator thought these reports correlated well (100%) with the medical records.

Another reason for not detecting a difference in the number of URIs in the infants fed human milk greater than 16 weeks and those fed human milk less than 16 weeks may be due to our small sample size with only approximately 25% of our study completed. Although we had an equal number of infants who were fed human milk less than 16 weeks and who were fed human milk greater than 16 weeks, a larger sample size may detect a difference in the number of infants who experienced a URI as well as the number of URI episodes diagnosed in each group. Statistically speaking, with an alpha set at 0.05 and a power of 0.80, 146 subjects per group would be needed to detect a decrease in the number of URI episodes by a half in the first year of life.

Prevalence of Dermatitis

We found significantly fewer infants diagnosed with dermatitis before 6 months of age in the group of infants fed human milk less than 16 weeks compared with the infants fed human milk greater than 16 weeks. This finding is consistent with previous findings in that human milk has immunoprotective factors against developing dermatitis early in life (2, 50, 54, 70). This finding is important to continue exploring with a larger sample size in order to determine if longer duration

of human milk feeding eventually protects against the atopic dermatitis link in the “atopic march.”

Our finding that longer duration of human milk feeding appears to protect against the development of atopic dermatitis in the first year of life agrees with Saaeinen et al. (71). The investigators found that human milk feeding for greater than 6 months resulted in lowest prevalence of eczema at one and 3 years of age and slightly lower rates of atopy at 17 years of age compared to human milk feeding less than 6 months. These researchers also found the prevalence of eczema peaked at one year of age. Their results suggest that a short duration of human milk feeding does not protect against eczema compared to a longer duration of human milk feeding. We found that duration of human milk feeding was related to lower numbers of dermatitis episodes in infancy.

Although it did not reach statistical significance, the onset of the first dermatitis episode was later in the infants fed human milk greater than 16 weeks compared to those infants fed human milk less than 16 weeks (greater than 52 weeks versus 32.28 weeks). Our results suggest that duration of human milk feeding reduces dermatitis episodes before 6 months of age and delays appearances of dermatitis. This outcome is clinically important as studies have suggested earlier incidences of atopic dermatitis put the infant at a greater risk for developing allergy or asthma in childhood (6, 41). For example, Gustafsson et al. (72) found that onset of eczema

before 4 months of age was a significant risk factor for inhalant allergens at 36 months or later (OR = 3.0, 95% CI; 1.0-8.6).

DHA Concentration and Early Illnesses

Several recent studies have examined the relationship between DHA and immune function including the prevalence of URIs, dermatitis, allergy and asthma. Results are mixed. While we did not find a relationship to DHA exposure, it is important to keep in mind that all infants received DHA throughout infancy whether from human milk or formula. However, most infant formulas did not contain DHA only a few years back. If an infant in our study was fed human milk along with infant formula for some duration of time, this would further increase the amount of DHA consumed.

We found that the mean DHA concentration in human milk (0.28 %) was similar to the DHA concentration found in the majority of infant formulas (0.35%). However, higher milk DHA is likely related to maternal DHA intake particularly as half of the women in each group would be expected to have received a DHA supplement during pregnancy. Accordingly, in utero and postnatal exposure to DHA in this subset of the parent study was highly variable. Although not statistically significant, the mean corrected weight percent DHA concentration for the milk samples we analyzed was 0.07 less than what is found in some infant formulas.

The DHA exposure before 6 months of age and for 0 to 12 months of age was greater for infants fed human milk greater than 16 weeks compared to those fed

human milk less than 16 weeks. This was the case despite the fact that the DHA concentration in most infant formulas consumed was higher than the mean DHA concentration in human milk from the mothers in our study. Again this apparent difference could have been due to small sample size and outliers in the groups. One mother in our study who breast fed her infant for greater than 16 weeks had a very high DHA concentration in her milk (0.76%), then also fed her child infant formula for the same duration of time as the human milk. Consequently, this infant had a much higher DHA exposure than the other infants in our study. On the other hand, one infant who received no human milk and was fed a lower DHA infant formula than most other infant formulas for the whole 52 weeks (Similac Advance®) had a lower DHA concentration exposure than most of the other infants in our study.

No significant differences were found in the weight percent DHA concentration and the number infants who experienced a URI. As well, no significant differences were found with DHA concentration and the number of URIs diagnosed before 6 months and between 6 and 12 months in either group. However, with a larger sample size, the clinical and statistical significance may change. A limitation to the calculation for the DHA exposure is that we did not have enough information to determine exactly how much formula compared to human milk the infant was receiving on a day to day basis in all cases. This is discussed further in the limitations section.

Confounding Variables

When smoking during pregnancy, smoke exposure in the home, the number of children in the home, daycare attendance and pet exposure were considered, only one significant difference was found. Infants fed human milk were less likely to be exposed to smoke in the home. In regard to interpretation of the lower number of dermatitis episodes, it could be argued that lower smoke exposure was actually the important factor. As well, women in the human milk-fed group had much less asthma than those in the formula-fed group so genetic differences could also account for apparent effect of human milk to reduce dermatitis. Even though no statistically significant evidence was found with other environmental exposures and the development of URIs, dermatitis, allergy and asthma, environmental exposures are important to continue investigating and some of the apparent limitations should be accounted for when the entire cohort can be studied. There have been mixed results as noted above in the relationship of daycare and pet exposure to the development of URIs, dermatitis, allergy and asthma. It would be interesting to explore intrauterine environmental exposures to IgE levels in cord blood in infants as a risk factor for allergic sensitization, but we do not have these samples available.

Limitations

One key limitation of this study is the current sample size. This is exploratory data for the larger study, so we anticipated this limitation from the beginning. In our statistical analysis, with a sample size of only 50 subjects, when the true rho was

equal to 0.3 we had a 59% power to reject the null hypothesis of no differences found in infants fed human milk greater than 16 weeks compared to infants fed human milk less than 16 weeks. Based on the results we obtained with 50 children, a larger sample size would be able to detect more statistically and clinically significant differences in URIs and dermatitis in relation to human milk feeding.

A second limitation to this study was the difficulty in defining distinct feeding groups. The lack of specificity between the groups was due to the fact that 66% of the sample received both human milk and formula while only 8 infants were fed human milk exclusively during infancy. Defining the groups as infants fed human milk greater than or less than 16 weeks was based on previous reports that suggested human milk feeding for 4-6 months may be more protective against illnesses than terminating human milk feeding before 4 months (4, 7, 50). Analysis using a 16-week cutoff for human milk did reveal significance for lower dermatitis and a trend to later first URI. With a small sample size, it was difficult to have a large enough number of subjects who were fed human milk longer than 26 weeks (6 months) in order to compare with the subjects who were fed human milk less than 26 weeks. Therefore, we used 16 weeks or 4 months. The smaller subset fed human milk for 26 weeks or more (n=22) did reveal evidence of additional protection against URIs and dermatitis compared to less than 6 months human milk feeding.

In regard to calculating DHA exposure of the infants in our study, we did not have adequate records to determine how many ounces per day the “mixed feeders”

consumed of infant formula and human milk. Furthermore, if an infant in our study was not consuming human milk from a bottle, it was difficult to gauge how many ounces of human milk the infant was consuming. Therefore this made it difficult to calculate the amount of DHA concentration each infant was consuming. The relative DHA exposure was calculated by the number of weeks the infant consumed infant formula and/or human milk. We have since begun asking the parent(s) or caregiver(s) the type of formula used, how much formula is used in each bottle, how many bottles are given in a day, and how many ounces the infant is drinking per bottle per day. For the infants receiving exclusively human milk, we now ask questions regarding how long the infant feeds on each breast. This information will make the future calculation of relative DHA exposure more reliable. The data analysis for this study was based on the assumption that the formula was made as instructed and that the infant consumed 6 feedings per day before they were started on solid foods at approximately 4 to 6 months of age.

We are aware that intrauterine exposure to DHA concentration was also variable. The odds are that half of the mothers in our study who breast fed for greater than 16 weeks and half of the mothers who breast fed for less than 16 weeks received the DHA supplement during pregnancy. Until the study is complete and we know who received the DHA supplement or the placebo, we cannot analyze the intrauterine DHA exposure and truly determine the effect of DHA on infant illness. At the completion of the study, we can account for intrauterine DHA exposure along with postnatal DHA exposure from mothers' milk and infant formula.

Another limitation to this study was recording data from the medical records. One medical record in particular was not clear as to what was being diagnosed at one doctor's visit. The physician's progress note had a diagnosis of URI and allergic rhinitis at the same visit, which made it difficult to determine if it really was a URI or if it was allergic rhinitis. In this case, it was coded as allergic rhinitis as that seemed to be the final diagnosis made by the physician.

The diagnosis for dermatitis was also not clearly indicated in the medical records. Atopic dermatitis is not explored as well in infants as it is in young children, adolescents and adults. Infant rashes appear and disappear and there is a limitation of detecting if the rash is from a delayed hypersensitivity reaction or something less relevant. The severity of the rash and sometimes the record of the duration of the rash were also not clearly indicated. Research cited in a review by Hahn et al. (72) suggested that the severity of dermatitis is more importantly linked to the "atopic march" than just the presence of atopic dermatitis.

Exploratory Data for Larger Study

The exploratory data provides excellent guidance for how to analyze the larger sample size when all infants from the parent study reach 12 months of age. It will also be possible in future research to examine infants beyond one year of age because the intent is to follow children to the age of 5. The ability to record data from medical records to school age offers an opportunity for obtaining much more convincing evidence of later allergy. The "atopic march" postulates that the earlier an

infant is diagnosed with atopic dermatitis the greater the risk that the infant will develop allergies and/or asthma by the age of 3 (2). With a larger sample size from our parent study, and as the children in our study age, we may be able to determine if there is a clinical and statistical difference with an earlier onset of atopic dermatitis and the development of allergy and asthma in early childhood, following the pattern of the “atopic march.”

For statistical analysis, the data suggest an analysis by duration of human milk feeding, rather than an analysis by human milk or formula because of the prevalence of mixed feeding in the population.

Equally as important is examining DHA supplement intake during pregnancy to distinguish intrauterine exposure to DHA. We already know that women in our population have a low consumption of DHA at approximately 50 mg/day. Some of the intrauterine changes may have occurred through diet alone rather than supplementation. We will be able to determine antecedent and intrauterine DHA exposure from the pre-study and post-study blood samples. The diets vary greatly among the women in our study, and exposure to certain foods or non-exposure to certain foods may also have immunomodulatory effects in the fetus, however, we do not have 24-hour dietary recalls on women who enrolled in the study.

The exploratory study also identified problems with recording of infant dietary intakes before solid food was added and provided guidance on how to gather data for more accurate analysis. By recording the nutrient information from dietary

recalls in a nutrient assessment program, NDSR (version 2008), we will have more accurate information regarding DHA intake for the remaining sample of approximately 150 infants. It may be beneficial to examine the n-6 to n-3 fatty acid ratio in human milk and in blood serum of infants rather than examining the DHA concentration alone as suggested in previous research (48, 73).

New data suggests that vitamin D may play a role in immune function. The investigators who continue research with this population of women and children may want to look at the infants' vitamin D status as a possible covariate when comparing human milk-fed infants to formula-fed infants in relation to the development of URIs, atopic dermatitis and allergy and asthma. Infants who receive exclusively human milk for a long duration of time may not be getting adequate vitamin D, especially if they are not given a vitamin D supplement as recommended by the American Academy of Pediatrics (74). Recent research has suggested that vitamin D may play a role in protecting infants from developing respiratory infections and enhance maturation of their immune function (75). In this scenario, formula-fed infants receiving vitamin D fortified infant formula may have an advantage compared to human milk-fed infants in protection from respiratory infections.

CHAPTER 6: SUMMARY

The purpose of our study was to a) determine if the duration of human milk-feeding made a difference in the prevalence of URI, dermatitis, allergy and asthma in the first year of life and to b) determine if there was a relationship between intake from human milk and infant formula and the prevalence of URI and dermatitis in the first year of life.

The term “atopic march” has been used to describe the progression of allergy manifestation and asthma during early childhood and has been debated for its mixed results. Our results suggest that longer duration of human milk feeding decreases the risk of developing atopic dermatitis during the first 6 months of life. Our results also suggest that human milk may be more influential than infant formula in delaying the onset of illness. This could protect the infant from the “atopic march” by allowing earlier maturation of the immune system.

Although our results did not show a statistically significant difference in DHA concentration exposure to infants and the prevalence of URIs and dermatitis, other studies suggest high DHA concentration exposure may lead to fewer incidences of URIs and dermatitis in early childhood (18). With approximately 25% of our study completed, further examination of DHA concentration in serum and human milk and prevalence of URIs and dermatitis is needed with a larger cohort than the 50 subjects in our preliminary study. We now have a better understanding for the importance of recording more accurate data on the fatty acid composition consumption in human

milk-fed and formula-fed infants. This more accurate data may show a relationship in the fatty acid composition consumption in both human milk-fed and formula-fed infants and the prevalence of URI and dermatitis episodes.

In conclusion, this cohort of infants will be followed until completion of the parent study. It is also planned to follow them throughout early childhood to determine if significant differences in infants who develop atopic dermatitis before 6 months of age are more likely to develop allergy and asthma manifestations in early childhood. Our findings suggest feeding human milk for a longer duration of time (greater than 16 weeks) may protect infants from developing atopic dermatitis in the first 6 months of life and offer protection from the atopic march.

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Appendix A

Sample Anthropometric Data Form

INVESTIGATOR PROTOCOL

RANDOM CODE

ID

CARLSON

HSC #10186

F

ANTHROPOMETRICS

Study Visit 1

Weight

 g

(42 days of age*)

MO DA YEAR

Length

 cm

Head circumference

 cm

Study Visit 2

Weight

 g

(120 days of age*)

MO DA YEAR

Length

 cm

Head circumference

 cm

Study Visit 3

Weight

 g

(180 days of age*)

MO DA YEAR

Length

 cm

Head circumference cm

Study Visit 4
(275 days of age*)

MO DA YEAR

Weight g

Length cm

Head circumference cm

Study Visit 6
(365 days of age*)

MO DA YEAR

Weight g

Length cm

Head circumference cm

Study Visit 7
(550 days of age*)

MO DA YEAR

Weight g

Length cm

Head circumference cm

Appendix B

Sample of Descriptive Data Form

INVESTIGATOR PROTOCOL

RANDOM CODE

ID

CARLSON

HSC #10186

[Empty box for Random Code]

E

INFANT FEEDING

Visit 1 (42 days of

Breast

age*)

Feeding

Date started

[Date selection box with columns for MO, DA, YEAR]

Stopped

Frequency

MO DA YEAR

Formula

Feeding

Date started

Stopped

Frequency

Name

Visit 2 (120 days

Breast

of age*)

Feeding

Date started

[Date selection box with columns for MO, DA, YEAR]

Stopped

Frequency

MO DA YEAR

Formula

Feeding

Date started

Stopped

Frequency

Name

Visit 3 (180 days

Breast

of age*)

Feeding

Date started

Stopped

Frequency

--	--	--

MO DA YEAR

Formula

Feeding

Date started

Stopped

Frequency

Name

Visit 4 (275 days

Breast

of age*)

Feeding

Date started

Stopped

Frequency

--	--	--

MO DA YEAR

Formula

Feeding

Date started

Stopped

Frequency

Name

Visit 5 (300 days

Breast

of age*)

Feeding

Date started

Stopped

Frequency

--	--	--

MO DA YEAR

Formula

Feeding

Date started

Stopped

Frequency

Name

Visit 6 (365 days

Breast

of age*)

Feeding

Date started

Stopped

--	--	--

MO	DA	YEAR

Frequency

Formula

Feeding

Date started

Stopped

Frequency

Name

Visit 7 (550 days

Breast

of age*)

Feeding

Date started

Stopped

Frequency

MO	DA	YEAR

Formula

Feeding

Date started

Stopped

Frequency

Name

* age adjusted based on Estimated Date of Confinement

(EDC)

INVESTIGATOR	PROTOCOL	RANDOM CODE	FORM ID
CARLSON	HSC #10186		C

DEMOGRAPHICS

Maternal Education

Paternal Education

Maternal

Smoking

Before

pregnancy

Packs per day

Date/Year started

If stopped,

date

During

pregnancy

Packs per
day

Date started

If stopped,
date

After

pregnancy

Packs per
day

Date started

If stopped,
date

Does anyone living in the baby's home smoke?

If yes, how many people smoke & how many ppd?

No Yes _____

List any maternal allergies:

Including the baby enrolled in this study, how many children 13 years of age or younger live in your house?

- 1 2 3 4 5 6 or more

Do any pets live in the baby's home?

No If yes, how many pets?

- Yes _____

What kind?

—

Do you plan to take your baby to a daycare (facility or homecare) with other infants and children?

No

- Yes, with 1 to 5 children
- Yes, with 6 to 10 children
- Yes, with more than 10 children

Appendix C

Human Milk Fatty Acid Analysis

Breast milk Fatty Acid Analysis

KUDOS

Revised September 2008

Reagents:

0.05 M KCl

Methanol

Methanol + butylated hydroxytoluene (BHT) (0.05% v/v)

Chloroform

Internal Standard: 17:0PE (52 μ g/100 μ L)

Glass distilled water

Pentane

Hexane

Diethyl ether

Benzene

Transesterification (methylation)-reagent: boron trifluoride-methanol or BF₃-M (14% w/w)

Fatty methyl ester standards- Supelco 37 Component FAME Mix (Sigma#47885-U)

Equipment and Supplies:

Extraction tubes

Spotting tubes

Varian 2mL screw top vials (clear vials, black caps, PTFE/Silicon liners, 12x32mm)

200 μ L, 12x32mm, flat bottom glass inserts

Filter paper (Whatman #1: 15cm circles and 46x57 sheets)

Glass funnels

Pasteur pipettes

Automatic pipettes in range of volumes

Centrifuges

Nitrogen evaporator with water bath that can be temperature controlled

Gas chromatograph

Developing tank

Heating block (dry bath)

Hamilton syringes (100 μ L for spotting and concentrating FAME's and 10 μ L for injecting into GC)

Ice bucket

Vortexer

EXTRACTION (Smuts et. al, 2003):

1. Pipet 4mL of methanol+ BHT into clean 15mL extraction tubes.
2. Add 500µL breast milk cap, and immediately vortex for a few seconds.
3. Add 100µL internal standard (17:0 PE) and vortex.
4. Add 8mL Chloroform and vortex 15 minutes.
5. Transfer contents through a funnel lined with filter paper (Whatman #1) into a clean extraction tube.
6. Add 1.6mL 0.05M KCl and vortex for 10 seconds.
7. Centrifuge for 5 minutes at 750 r.p.m. and then using a clean Pasteur pipette discard upper phase into appropriate hazardous waste bottle.
8. Evaporate the lower phase in a water bath at 35°C under nitrogen.
9. [Add 1-2mL benzene and re-evaporate if small amounts of water remain in sample].

TRANSMETHYLATION

10. When extract is completely dry, add 0.25mL BF_3 14% solution in methanol, 0.2mL Benzene, and 0.55mL Methanol.
11. Layer each tube with N_2 and place on ice until all samples are finished.
12. Tighten caps and place in dry bath at 100°C for 30 minutes.
13. After 1-2 minutes retighten caps as they tend to loosen during heating.

14. After transmethylation is complete, immediately place tubes on ice. When tubes are very cool, open and add 1 mL of distilled H₂O and 2 mL pentane.
15. Vortex for 1-2 minutes to extract fatty acid methyl esters (FAME) into the pentane phase, then centrifuge for 5 min. at 800 r.p.m.
16. Transfer the upper phase (pentane) with a Pasteur pipet to a Varian 2 mL vial.
17. Concentrate the FAME under a stream of N₂.
18. When completely dry, add 150 μL of dichloromethane to vial. Swirl tube to catch FAME on all sides and then transfer with Hamilton syringe to a 200 μL, 12x32mm, flat bottom glass insert. Place the glass insert inside of the 2 mL vial and cap with a Teflon-lined cap.
19. Place sample in GC auto-sampler tray to inject or in freezer until it can be analyzed.

Appendix D

Plasma and Red Blood Cell Fatty Acid Analysis

Plasma and Red Blood Cell Fatty Acid Analysis

(Total Phospholipids): KUDOS

Revised February 2009

Reagents:

0.05 M KCl

Methanol

Methanol + butylated hydroxytoluene (BHT) (0.05% v/v)

Chloroform

Internal Standard: 17:0PE (52µg/100µL)

Glass distilled water

Pentane

Hexane

Diethyl ether

Benzene

Transesterification (methylation)-reagent: boron trifluoride-methanol or BF₃-M (14% w/w)

Fatty methyl ester standards- Supelco 37 Component FAME Mix (Sigma#47885-U)

Equipment and Supplies:

Extraction tubes

Spotting tubes

Varian 2mL screw top vials (clear vials, black caps, PTFE/Silicon liners, 12x32mm)

200 μ L, 12x32mm, flat bottom glass inserts

Filter paper (Whatman #1: 15cm circles and 46x57 sheets)

Glass funnels

Pasteur pipettes

Automatic pipettes in range of volumes

Centrifuges

Nitrogen evaporator with water bath that can be temperature controlled

Gas chromatograph

Developing tank

Heating block (dry bath)

Hamilton syringes (100 μ L for spotting and concentrating FAME's and 10 μ L for injecting into GC)

Ice bucket

Vortexer

BLOOD PREP PROCEDURE (Dennis Hoffman):

1. Centrifuge blood (4°C) at 30,000g-min [3000 x g x 10 minutes i.e., 3,600 RPM] in Ependorf 5804 R refrigerated bench-top centrifuge with an A-4-44 swing-bucket rotor.
2. Pipet plasma to a 1.5mL, 10.8x 40.6mm plastic micro centrifuge tube (Fisher #05-408-129).
3. Pipet buffy coat to a 1.5mL, 10.8x 40.6mm plastic micro centrifuge tube (Fisher #05-408-129).
4. Pipet RBC to a 1.5mL, 10.8x 40.6mm plastic micro centrifuge tube (Fisher #05-408-129).
5. Flush tubes with N₂, cap, and store in (-80°C).

EXTRACTION (Smuts et. al, 2003):

1. Pipet 4mL of methanol+ BHT into clean 15mL extraction tubes.
2. Add 500 μ L RBC's or Plasma, cap, and immediately vortex for a few seconds.
3. Add 100 μ L internal standard (17:0 PE) and vortex.
4. Add 8mL Chloroform and vortex. Vortex 15 minutes for RBC's and 10 minutes for Plasma.
5. Transfer contents through a funnel lined with filter paper (Whatman #1) into a clean extraction tube.
6. Add 1.6mL 0.05M KCl and vortex for 10 seconds.
7. Centrifuge for 5 minutes at 750 r.p.m. and then using a clean Pasteur pipette discard upper phase into appropriate hazardous waste bottle.
8. Evaporate the lower phase in a water bath at 35°C under nitrogen.
9. [Add 1-2mL benzene and re-evaporate if small amounts of water remain in sample].

SEPARATION OF TOTAL PHOSPHOLIPIDS (Smuts et. al, 2003):

10. When extract is completely dry, dissolve in 100 μ L cold dichloromethane and place immediately on ice until ready to spot plates.
11. When plates have been heated for at least 20 minutes at 120°C, take out of oven and cool. Spot 100 μ L for analysis of total phospholipids.

12. Place the spotted plate immediately in the TLC chamber containing the developing solvent 80:20:1 Hexane: Ether: Acetic Acid and that has been lined with filter paper.
13. Allow the solvent front to run to the top of the plate and then remove from TLC chamber to dry.
14. Identify phospholipid line (it is the same line as the spotting line). Remove the gel containing the lipid fraction of interest with a single edge razor blade onto weighing paper. Carefully transfer the gel to a clean 15mL extraction tubes containing 1 mL cold BF_3 14% solution in methanol (Sigma # B1252).

TRANSMETHYLATION

15. Layer each tube with N_2 and place on ice until all samples are finished spotting.
16. Tighten caps and place in dry bath at 100°C for 10 minutes.
17. After 1-2 minutes retighten caps as they tend to loosen during heating.
18. After transmethylation is complete, immediately place tubes on ice. When tubes are very cool, open and add 1mL of distilled H_2O and 2 mL pentane.
19. Vortex for 1-2 minutes to extract fatty acid methyl esters (FAME) into the pentane phase, then centrifuge for 5 min. at 800 r.p.m.
20. Transfer the upper phase (pentane) with a Pasteur pipet to a Varian 2mL vial.
21. Concentrate the FAME under a stream of N_2 .
22. When completely dry, add $70\mu\text{L}$ of dichloromethane to vial. Swirl tube to catch FAME on all sides and then transfer with Hamilton syringe to a $200\mu\text{L}$,

12x32mm, flat bottom glass insert. Place the glass insert inside of the 2 mL vial and cap with a Teflon-lined cap.

23. Place sample in GC auto-sampler tray to inject or in freezer until it can be analyzed.

Appendix E

URI and Dermatitis Episodes by 3 Month Interv;als by Duration of Human Milk Feeding

URI episodes diagnosed between 0 and 3 months of age.

	Number of URI Episodes 0-3 Months of Age			
	0	1	2	Total
Fed Human Milk <16 weeks	18	6	1	25
Fed Human Milk >16 weeks	19	6	0	25
Total	37	12	1	50

URI episodes diagnosed between 3 and 6 months of age.

	Number of URI Episodes 3-6 Months of Age				
	0	1	2	3	Total
Fed Human Milk <16 weeks	16	8	1	0	25
Fed Human Milk >16 weeks	16	5	2	2	25
Total	32	13	3	2	50

URI episodes diagnosed between 6 and 10 months of age.

	Number of URI Episodes 6-10 Months of Age			
	0	1	2	Total
Fed Human Milk <16 weeks	13	10	2	25
Fed Human Milk >16 weeks	10	13	2	25
Total	23	23	4	50

URI episodes diagnosed between 10 and 12 months of age.

	Number of URI Episodes 10-12 Months of Age		
	0	1	Total
Fed Human Milk <16 weeks	21	4	25
Fed Human Milk >16 weeks	21	4	25
Total	42	8	50

Dermatitis by 3 month Intervals by Duration of Human Milk Feeding

Dermatitis episodes diagnosed between 0 and 3 months of age.

	Number of Dermatitis Episodes 0-3 Months of Age		
	0	1	Total
Fed Human Milk <16 weeks	21	4	25
Fed Human Milk >16 weeks	23	2	25
Total	44	6	50

Dermatitis episodes diagnosed between 3 and 6 months of age.

	Number of Dermatitis Episodes 3-6 Months of Age			
	0	1	2	Total
Fed Human Milk <16 weeks	18	7	0	25
Fed Human Milk >16 weeks	21	3	1	25
Total	39	10	1	50

Dermatitis episodes diagnosed between 6 and 10 months of age.

	Number of Dermatitis Episodes 6-10 Months of Age			
	0	1	2	Total
Fed Human Milk <16 weeks	20	5	0	25
Fed Human Milk >16 weeks	20	3	2	25
Total	40	8	2	50

Dermatitis episodes diagnosed between 10 and 12 months of age.

	Number of Dermatitis Episodes 10-12 Months of Age		
	0	1	Total
Fed Human Milk <16 weeks	23	2	25
Fed Human Milk >16 weeks	23	2	25
Total	46	4	50