

Thesis BSc Biomedical Sciences Rijksuniversiteit Groningen

# Do artificial oligosaccharides in infant formula reduce the risk of atopic disease and allergies similar to human milk oligosaccharides?

In comparison to infant formula without prebiotic supplementation and to human milk

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28-7-2012

## Summary

Allergies and atopic diseases constantly increase over the last decades. Genetic predisposition alone does not explain this epidemic rise. Consequently environmental factors have to play an important role. Especially early infant nutrition is thought to have a great impact on atopy development and its clinical manifestations. Human milk is broadly recommended as the best nutrition for infants. This indicates that in cases where breast-feeding is not an option infant formula should match human milk as close as possible. Therefore beneficial components of human milk should not be absent in infant formula. As important factors among, human milk oligosaccharides (HMO) have been identified. HMO are almost absent in bovine milk and therefore in bovine milk based infant formula. Broadly accepted are the prebiotic functions of HMO. Increasing evidence suggests various other beneficial functions of HMO like inhibition of pathogens upon competitive binding. The specific functions of HMO are thought to be determined by their various structures. HMOs are promising modulators of allergy and atopic disease development. Currently artificial synthesized oligosaccharides (GOS/FOS) are added to infant formula in order to mimic properties of HMOs. The molecular structures of enzymatically synthesized GOS and plant-derived FOS are much simpler and differ significantly from the highly complex structures of HMOs thus, it is unlikely that they can mimic all of the structure dependent functions of HMOs.

The aim of this thesis is to investigate if and how artificially synthesized oligosaccharides can mimic functions of naturally occurring HMO especially in regard to allergy and atopic disease development. General factors that possibly contribute to the atopy epidemic, mechanisms of the pathophysiology of atopy and its clinical manifestations as well as the role of some human milk components herein will be discussed first.

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## Introduction

Atopic diseases and allergies have increased significantly over the last decades. The exact etiology of this epidemic, is still unclear. Genetic predisposition alone does not explain the inter-individual differences in development of these complex conditions. This indicates that environmental factors like nutrition play an important role. Especially early infant nutrition seems to have a great impact and has therefore been subject to a lot of research. (Mišák Z. 2011, Gordon BR. 2011)

Recommendations about infant nutrition reach far back in time and are already found in ancient civilizations like the ancient Greek (4000-1000 BC) and ancient Egypt (1500 BC). Recommendations show great differences and are strongly influenced by culture, family, economy and even religion. Breastfeeding though is almost solely recommended as the best nutrition for infants. More or less specific recommendations vary for as well the recommended start and duration of (exclusive) breastfeeding as the time and kind of the introduction of complementary feeding. (Turck D. 2010) Today World Health Organization (WHO) recommends starting breastfeeding within an hour after birth to provide the newborn with colostrum which is according to the WHO the perfect nutrition for the newborn. Colostrum composition is adapted to the higher (immunological) needs of a newborn by containing much more of certain immune modulatory and protective components than human milk in later stadia. (Iyengar SR. 2012) Furthermore exclusive breastfeeding is recommended for six month and in combination with complementary feeding up to two years of age. WHO also provides a list with acceptable medical reasons for the use of breast-milk substitutes. Which points out how important they believe breastfeeding is for an healthy development of children. To give just one example: without development of defense against pathogens, and thus infection, the cytokines released during an infectious response lead to release of the satiety hormone leptin and consequently loss of appetite. A reduced and even delayed intake of mother milk can lead to a vicious circle of infection, inflammation and loss of appetite with finally death from starvation. (Edmond KM. 2007) Human milk obviously contains many health protecting and –promoting components. This includes components for the development of the newborn gastrointestinal microbiome, immune system and protection against allergic disease. A lot of research is done to investigate the immune modulatory properties of human milk in order to identify responsible components and mechanisms. Presently various components are identified including immunoglobulins, lactoferrin, lysozyme, bioactive lipids, leukocytes, and various milk glycans (glycolipids, glycoproteins, and free oligosaccharides) among others (Newburg DS. 2005). A lot of evidence has shown that human milk has protective properties against numerous of conditions including atopic diseases like asthma, and allergies. (Mišák Z. 2010, González J. 2010, Brew BK. 2012, Iyengar SR. 2012) Human milk shapes the development of an intestinal flora that may protect against atopic disease possibly upon stimulation of tolerogenic cytokines, like TGF-beta, or upon stimulation of a Th1 response. (Friedman NJ. 2005) Factors such as immunoglobulins (e.g., secretory IgA), anti-microbial proteins, cytokines and fatty acids provide protection against infections that could promote atopy (Iyengar SR. 2012) and will be discussed in more detail later.

Still there remains a lot to be investigated because the exact role and the mechanisms of human milk in the development of allergic diseases is still not fully elucidated and in addition controversial. The controversy results from methodological differences and flaws and biases in performed studies as well as variability in duration and frequency of breast feeding, use of supplemental formulas, recall of feeding practices, type of allergic disease being studied, and maternal atopic background. Controversy is also influenced by the complexity of interactions between human milk and the infant's

intestinal environment and immune system and possibly genetic differences among individuals which could affect whether human milk protects or sensitizes for development of atopic manifestations. (Friedman NJ. 2005, Matheson MC. 2012, Iyengar SR. 2012) In addition many studies focus on the sensitization of particular antigens (i.e. IgE) rather than clinical disease. Sensitization to particular antigens however does not necessarily result in clinical symptoms. (Iyengar SR. 2012)

Nowadays bovine milk based infant nutrition is very common as a breast- milk substitute. Bovine milk differs from human milk in as well the ratio and quantity as the kind of fatty acids, proteins and carbohydrates. The form, type and composition of infant nutrition has changed in time and has been subject to a lot of research but it remains an unequal substitute and researchers seek identification and synthesis of beneficial components of human milk as possible supplements for infant formula. (Bode L & Jantscher-Krenn E. 2012) Because randomized control trials with breastfeeding would be considered unethical, collected data are from observational studies, where participation and recall bias can severely affect the objectivity and thus outcome of the research. (Matheson MC. 2012) A delayed maturation of the immune system has been associated with a higher risk of allergy development in children and despite the ongoing controversy around the role of human milk, knowledge about the latter seems to be inevitable for the further development of infant formula. (van Hoffen E. 2009)

Among other components, human milk oligosaccharides (HMOs) are thought to have an important influence on the development of the immune system of infants and therefore promising candidates as possible inhibitors of the onset of atopy and allergy. (Boehm G. 2003, van Hoffen E. 2009, Iyengar SR. 2012) Galacto- Oligo- Saccharides (GOS) and Fructo- Oligo- Saccharides (FOS) are artificial synthesized oligosaccharides, that are thought to have immune modulatory properties similar to HMOs, are currently added to infant formulas. They are however structurally different from naturally occurring HMOs (Sela DA. 2010) and it thus questionable if they are able to mimic the structure-specific effects of HMO.

The aim of this thesis is to investigate the effect of infant formula supplementation with certain oligosaccharides (GOS and FOS). Differences on allergy and atopic disease development in breastfed infants in comparison to infants fed infant formula with and without prebiotic supplementation are especially subjected. The hygiene hypothesis, the atopic march and other mechanisms and components that are suggested to participate in the pathophysiology and/or prevention of allergy and atopic disease development and that are thought to be possibly altered by HMOs and therefore possibly by artificial prebiotic oligosaccharides are discussed first.

## Pathophysiology of Atopy and Atopic Manifestations

### The Hygiene Hypothesis as possible explanation for the atopy epidemic

Next to ambiguities about the mechanisms that lead to the clinical manifestations of atopy, the enigma of the general cause of this epidemic remains unsolved as well. An explicable attempt to this problem is referred to as the hygiene hypothesis. According to this hypothesis early exposure to microbial stimuli like lipopolysaccharide (LPS, also called endotoxin), (1–3)- $\beta$ -D-glucan, muramic acid, and naturally occurring bacteria decreases the risk of atopy. A modern lifestyle with less exposure to microbial stimuli and more vaccination is thus postulated to cause the rise in allergic disorders. (Iyengar SR, 2012, Spergel JM. 2010, Alfvén T. 2006) After birth development of an appropriate immune response is essential for neonates to survive. Like mentioned earlier this immune response needs to be established in the absence of inflammation. Breast-fed infants are protected against inflammation by certain factors (e.g., lactoferrin) in human milk and can therefore develop an adapted immune response to pathogenic stimuli without undesired inflammation. (Edmond KM. 2007) The innate immune system of neonates thrives on germ line-encoded pattern-recognition receptors (PRRs) which enable neonates to distinguish between harmless and harmful organisms by selective adherence to certain components of pathogenic structures called pathogen-associated molecular structures (PAMPs). PAMPs include LPS from the cell wall of gram-negative bacteria, peptidoglycans, teichoic acids and lipoteichoic acid from gram-positive bacteria, lipoglycan from *Mycobacteria tuberculosis*, and mannans from yeast and the outer surface proteins of viruses. (Hallman M. 2001) PRRs include membrane bound C-type lectin receptors (CLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) and Rig-I-like receptors (RLRs). As important signal-transducing PRRs different Toll-like receptors (TLRs) have been identified. TLRs that control acute inflammatory responses are suggested to be essential for the development of acquired adaptive immunity. TLR4 is activated by LPS which results activation of the inflammatory cascade

through binding of the transcription factor NF- $\kappa$ B to DNA. (Fig.1 & Fig.2) (Heine H. 2011)

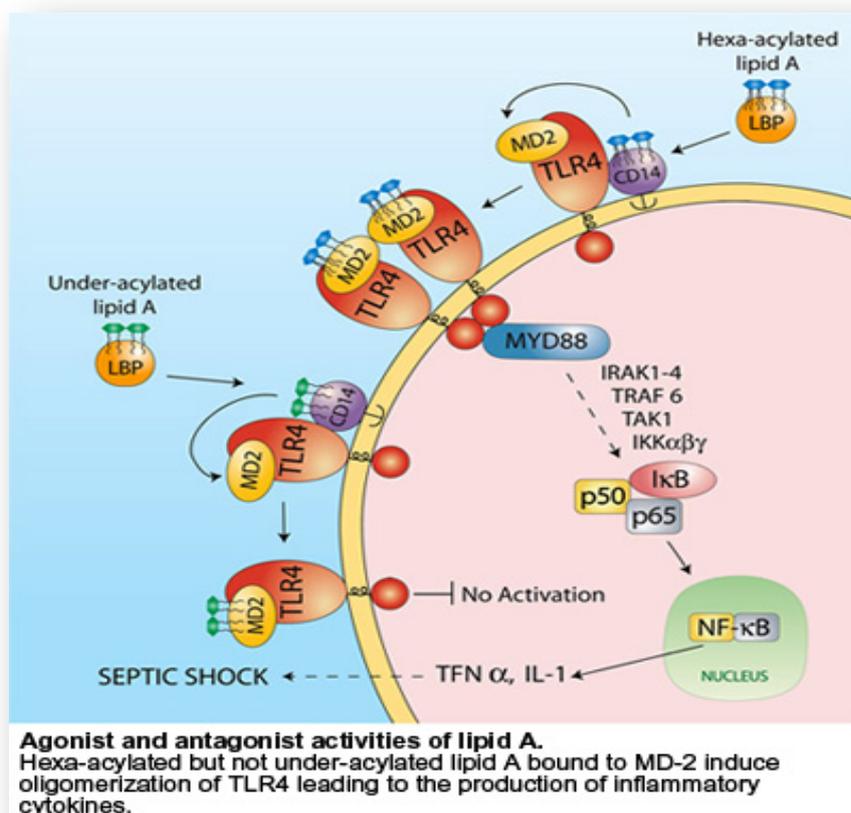


Fig.1 LPS consists of a polysaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A. Lipid A, also known as endotoxin, is responsible for the immunostimulatory activity of LPS. Lipid A is a glucosamine disaccharide linked to hydroxy fatty acids that are further substituted by non-hydroxylated fatty acids. The number of fatty acids is a major determinant of the immunogenicity of endotoxin. (Fig. and citation: InvivoGen 2007)

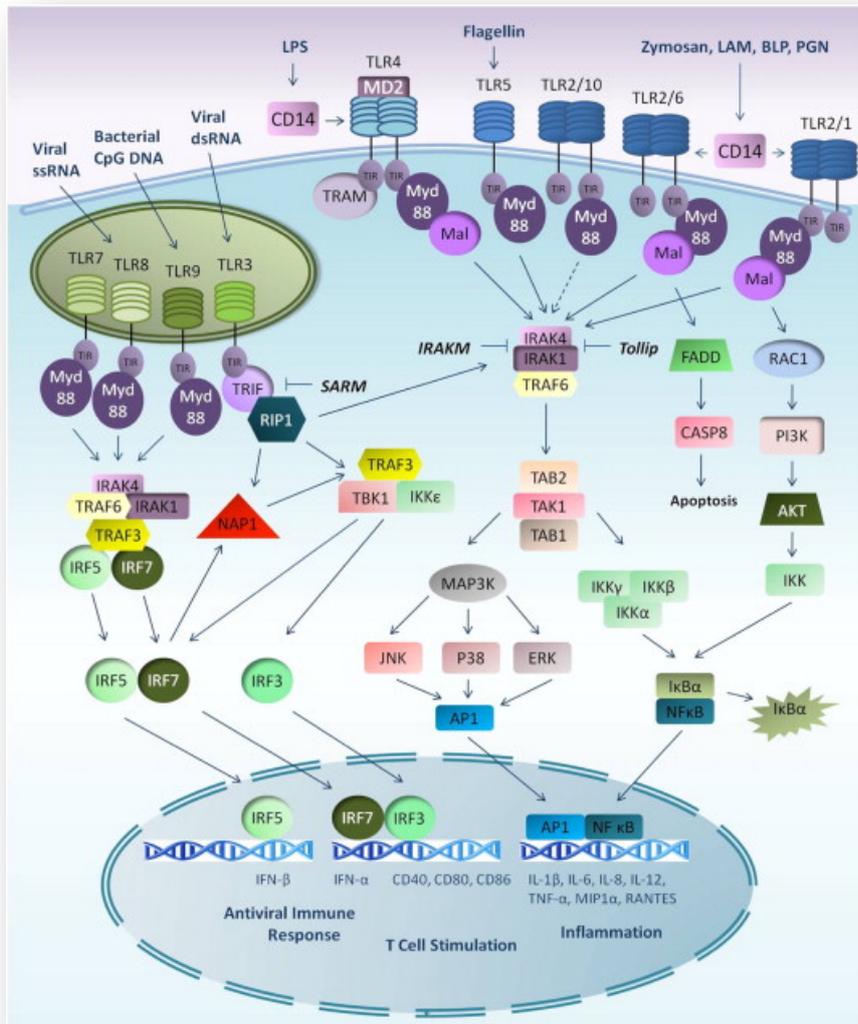


Fig. 2 TLRs and their ligands with key signaling intermediates. Agonism of all TLRs by their PAMP and/or co-receptor complexes results in the recruitment of a specific repertoire of adaptor proteins to BB loops within the TIR domain of the receptor. Once these adaptors, Mal, MyD88, TRAM and TRIF, have been recruited this sets up a chain of signaling events that include recruitment and phosphorylation of IRAKs and ubiquitination of TRAF6. These signaling events result in the activation of a number of transcription factors, which include NF-κB, IRF3, IRF5, IRF7 and AP-1. Binding of these transcription factors to their response elements within certain genes results in the de novo protein synthesis of a number of pro-inflammatory and T-cell stimulatory mediators. Apart from the positive signaling molecules

induced by PAMPs, some stimuli also induce negative signaling regulators such as SARM, IRAKM and Tollip. The dashed arrow for TLR2/10 assumes a classical MyD88 signaling will occur after agonism of this receptor. (Fig. and citation: Paul-Clark MJ. 2012)

It is suggested that variations in PRR expression may influence the predisposition to infections thereby contributing to the susceptibility to atopy, allergies, autoimmune and other inflammatory diseases. (Hallman M. 2001)

The underlying mechanism of the hygiene hypothesis is furthermore generally explained by a shift toward Th2-like cytokines. In the presence of antigens naïve T cells are exposed to either interleukin (IL) 12 and IL-18 or IL-4, which are then differentiated to either Th1 or Th2 cells respectively. (Fig. 3) Th1 cells downregulate Th2 cells, synthesize interferon (IFN)  $\gamma$  and IL-2 and promote clearance of intracellular pathogens. Th2 cells downregulate Th1 responses and produce IL-4, IL-5 and IL-13. (Spergel JM. 2010) The exact mechanisms by which reduced exposure to microbial stimuli leads to increased Th2 response and allergy are however still controversial. One explanation arises from the fact that innate immunity cells are stimulated by bacterial products via their Toll-like receptors (TLRs). Decreased exposure to bacterial products results in reduced production of IL-12 and IFNs resulting in decreased Th1 responses and therefore increased Th2 responses. (Fig. 4) (Romagnani S. 2004)

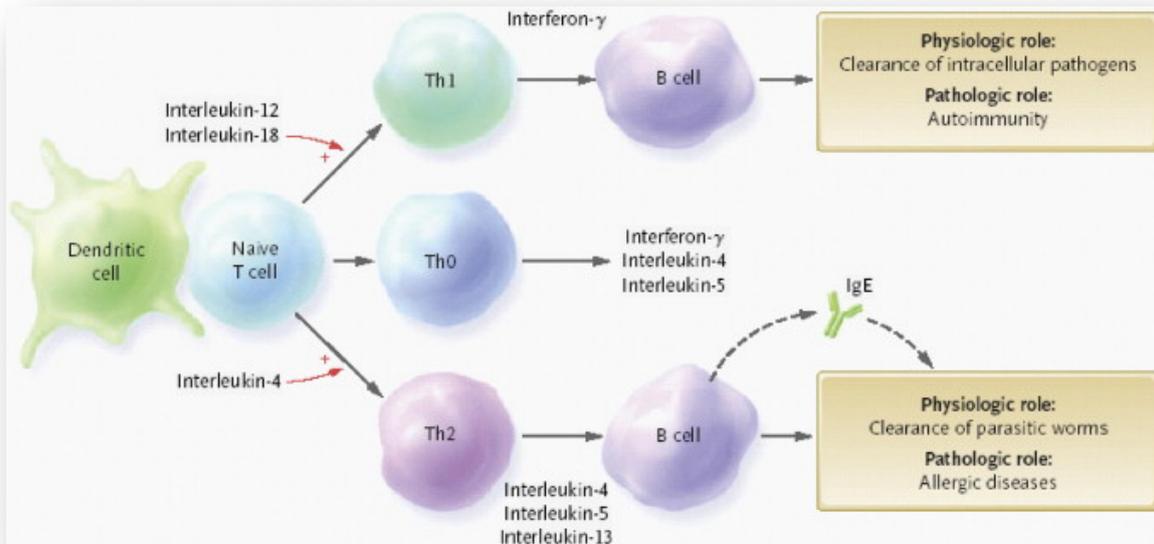


Fig. 3 Role of TH2-like cytokines, B cells, and IgE in the development of allergy. (Fig. and citation: Spergel JM. 2010)

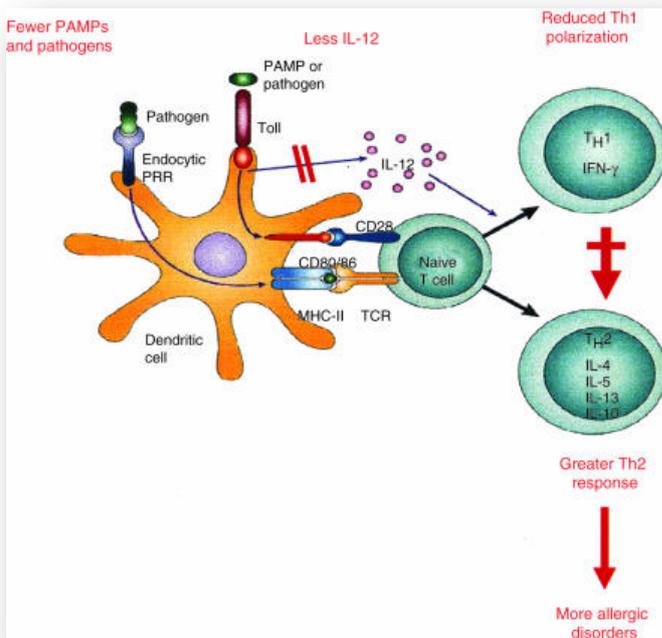


Fig.4 (Romagnani S. 2004)

Currently altered function of regulatory T (Treg) cells is thought to be responsible as well for the drive toward a Th2 immune response to allergens. Treg cells prevent immune responses against harmless environmental triggers and provide peripheral tolerance to self- antigens. Reduced microbial exposure is thought to decrease stimulation of Treg cells resulting in altered counts or impaired function thereby reducing their immune regulatory properties.

Consequently this would increase

activity of both Th1 and Th2 responses which are responsible for the increased prevalence of autoimmune and allergic disorders, respectively. (Fig. 5) (Romagnani S. 2004, Spergel JM. 2010) An integrated explanation to immunological events that can occur as a consequence of the reduced microbial exposure during childhood could result from the fact that on the one hand decreased IL-12 production leads to reduced Th1 polarization and a lack of IFN-  $\gamma$  production. This on the other hand could be counteracted by the reduced stimulation of Tregs resulting in enhanced Th1 activation. Both of this may favor Th2 responses to allergens. Reduced microbial exposure would therefore much more likely result in Th2 activation which may explain the allergy epidemic in industrialized countries. (Romagnani S. 2004)

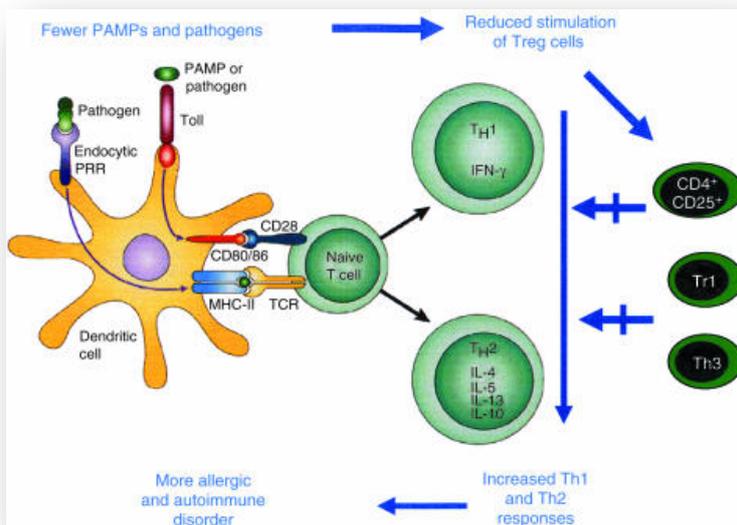


Fig.5 (Romagnani S. 2004)

It is however suggested that any alteration in the complex components and mechanisms of innate immunity may predispose to allergic responses. (Eisenbarth SC. 2004) Various components are found to have essential functions in the complicated interactions between the innate and adaptive immune system

that may lead to the development of allergic and inflammatory diseases. (Vandenbulcke L. 2006) Those components include for example, CD14 and collectins next to TLRs on dendritic cells(DCs), macrophages and in body fluids. (Kaur S. 2006) In the following some of the components and mechanisms that are assumed to result in atopy and its subsequent clinical manifestations will be discussed.

### The Atopic March

The progression from atopic dermatitis (AD) in infants to allergic rhinitis and asthma during the first years of live is generally characterized as the atopic march. (Spergel JM. 2010, Zheng T. 2011) Atopy is defined as the (genetic) tendency (personal or familial) to become sensitized and produce specific IgE antibodies in response to ordinary allergens commonly occurring in the environment. (Johansson

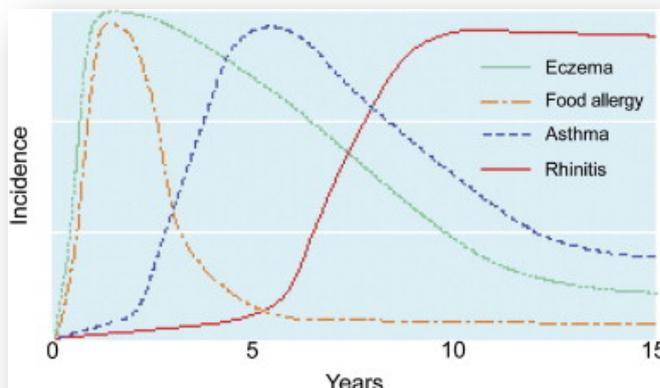


Fig. 6 Progression of the atopic march. (Fig. and citation: Spergel 2010)

SG. 2003) AD (also called eczema) is considered as the first clinical manifestation of atopy, possibly followed by other atopic diseases like previously mentioned and shown in Figure 6. (Spergel JM. 2010) Apparently more than 50% of young children with severe atopic dermatitis develop asthma and about 75% develop allergic rhinitis subsequent to AD. (Kulig M. 1999) There are different cross-sectional and longitudinal studies that provide evidence for the atopic march but nevertheless there is still controversy not only because the underlying mechanism remain largely unknown. (Zheng T. 2011, Spergel JM. 2010) There are however various genetic and environmental factors that are thought to contribute to the onset of atopy and subsequent development of clinical

manifestations in the atopic march. By discussing those factors emphasis will first be placed on the pathogenesis of AD since its considered the first clinical manifestation of atopy.

### Atopic Dermatitis and some genetic evidence for the atopic march

AD is a chronic inflammatory skin disease that arises through complex interactions of multiple genetic and environmental factors that is characterized by xerosis (dry skin), pruritus (itch), and erythematous lesions with increased epidermal water loss (TEWL). (Cork MJ. 2009) Defects in the epidermal barrier of the skin is probably the threshold event in the pathogenesis of AD. (Fig. 7) Possible subsequent sensitization of the airways may result in atopic manifestations like asthma and rhinoconjunctivitis. (Spergel JM. 2010) The epidermis is built from keratinized stratified squamous epithelial cells. The most superficial layer of the epidermis is called the stratum corneum (SC). The keratinized cells of the SC protect the body against environmental insults and water loss. (Fig.8) Multiple abnormalities contribute to the impaired epidermal barrier function. (Zheng T. 2011) Predisposition to epidermal barrier defects arises from at least three groups of genes encoding structural proteins (filaggrin), epidermal proteases and protease inhibitors (Spink5). Increase of proteolytic activity and reduced generation of lipid lamellae result in aggravated breakdown of the epidermal barrier. Loss-of-function mutations in the filaggrin gene predispose most significantly for development of epidermal barrier defects. The filaggrin gene encodes profilaggrin a highly phosphorylated protein which is dephosphorylated into 10 to 12 copies of the filaggrin protein. Filaggrin is necessary for the generation of natural moisturizing factor (NMF) essential for protection against water loss and hydration of the epidermis. (Cork MJ. 2009) Filaggrin furthermore aggregates keratin filaments which is an essential process for the formation of a functional epidermal barrier. Filaggrin gene mutations lead to reduction or loss of filaggrin and consequently to impaired keratinization and aberrant cornification. (Kezic S. 2008) This leads to erroneous responses of keratinocytes to environmental triggers resulting in reduced mechanic defense mechanisms that facilitate entry of pathogens, allergens and other environmental factors through the epidermis (Fig.9) and the ability to produce factors (e.g., IL-13, thymic stromal lymphopoietin (TSLP)) that trigger mast cells to produce Th2 cytokines thus, promote Th2 predominant inflammatory responses in acute AD lesions followed by chronic AD characterized by prominent Th1 inflammation. (Leung DY.2004, Miyata M. 2008, Zheng T. 2011) Up to know the exact mechanism of this switch are not well understood but loss-of-function mutations in the filaggrin gene are strongly associated with development of AD, other inflammatory conditions (e.g., ichthyosis vulgaris) and subsequent asthma. (Zheng T. 2011, Palmer CN. 2006) Asthma is found only in filaggrin mutation carriers who are also affected with AD which supports the hypothesis that asthma develops secondary to AD. The exact mechanisms of the indicated process remain to be investigated. (Spergel JM. 2010) However about 40% of filaggrin gene mutation carriers do not develop AD (Zheng T. 2011) thus, filaggrin gene mutations do not necessarily lead to AD development. On the other hand none of the individuals without this mutation developed AD neither (Spergel JM. 2010) which means that other factors interact directly or indirectly with filaggrin alterations in the pathogenesis of AD. (Zheng T. 2011)

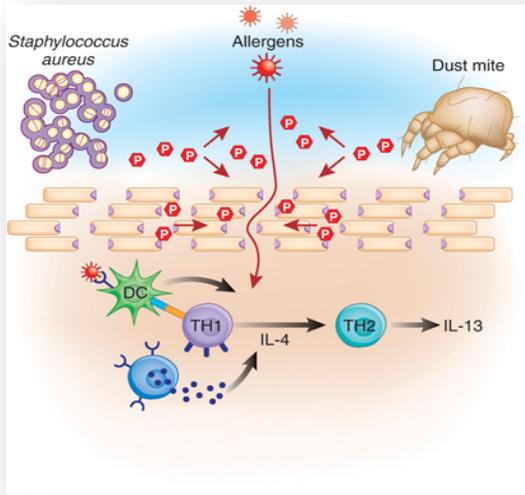


Fig.7 There is a defective epidermal barrier in individuals with atopic dermatitis. The epidermal barrier is found in the lower layers of the stratum corneum, and is composed of differentiated keratinocytes, termed corneocytes (beige rectangles), held together with corneodesmosomes (purple spheres). The hyperactivity of degradatory proteases (red hexagons) found within the epidermis, and contributed to by exogenous proteases (red hexagons), from house dust mites and *Staphylococcus aureus*, for example, facilitate the cleavage of the corneodesmosome junctions. This is just one event in the breakdown of the epidermal barrier that permits the penetration of allergens. Dendritic cells (DC) (green) found in the dermis take up and present these allergens (red stars) to helper T (TH) cells and recruit CD4+ T cells (blue). Activated DC and IL-4, expressed by CD4+ T cells, promote TH1 to TH2 switching with the subsequent release of pro-inflammatory cytokines and elevation of IgE levels). The clinical outcome of this type of response is atopy and asthma. (Fig. and citation: Cork MJ. 2009)

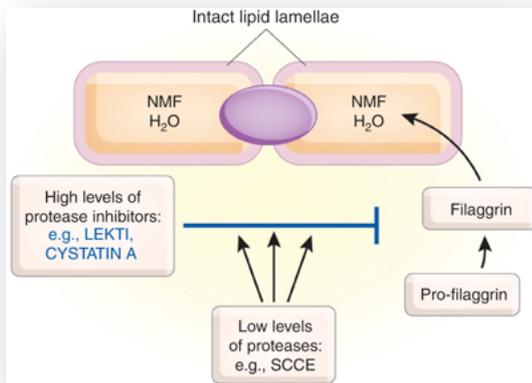


Fig.8 The structure of the epidermal barrier located in the lower part of the stratum corneum (SC). Highly differentiated flattened keratinocytes, referred to as corneocytes (beige rectangles), are the building blocks of the epidermal barrier. They contain natural moisturizing factor (NMF), derived from pro-filaggrin, a mix of hygroscopic compounds, which help maintain skin hydration. A water resistant layer of lipid lamellae (pink) encases the corneocytes preventing water loss and impeding barrier permeability. The corneocytes are held together by corneodesmosomes (purple spheres), the integrity of which is dependent on a cocktail of proteases and protease inhibitors. The balance between the expression and activity of proteases, such as KLK7 (SCCE), and protease inhibitors, such as LEKTI and cystatin A, determines the rate of desquamation (corneocytes shedding) and thereby the thickness of the barrier. Under normal conditions, the barrier is only degraded in the upper layers of the SC providing a resilient permeability barrier that prevents the penetration of allergens. (Fig. and citation: Cork MJ. 2009)

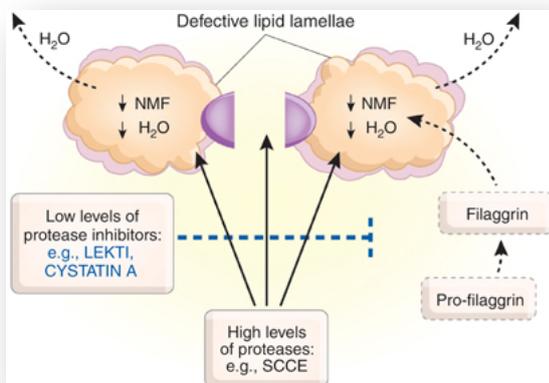


Fig.9 A defective epidermal barrier is a poor permeability barrier, which permits the entry of allergens and the loss of moisture. Changes in the FLG gene encoding pro-filaggrin result in reduced, or absent, expression of filaggrin thereby adversely affecting the structure of the corneocytes (beige)—the "bricks". The levels of natural moisturizing factor (NMF), derived from filaggrin, are also adversely affected, resulting in a decreased ability of the corneocytes to hold water and a concomitant elevation of pH. Elevated pH favors serine protease activity and inhibits enzymes involved in the synthesis of lipid lamellae (pink)—the "mortar". Genetic changes in the genes encoding SCCE (KLK7), LEKTI (SPINK5), and cystatin

A (CSTA) all lead to elevated protease activity involved in desquamation—cleavage of the corneodesmosome junctions (purple spheres) between the corneocytes analogous to "rusting" of the "iron rods". (Fig. and citation: Cork MJ. 2009)

## The Gastrointestinal Tract as a major site of immune regulation

A major site of exposure to health challenges is the gastrointestinal tract (GIT) that harbors 80% of the total immune system (Buddington RK. 2002). Except for microbial exposure necessary for development of an intact intestinal flora the intestinal microbiome is found to play an important role in immune regulation. (Iyengar SR. 2012) Results show that infants with atopic dermatitis have an altered microbiome compared to non-allergic children. (Penders J. 2007, Watanabe S. 2003) For example it is postulated that early colonization with bifidobacteria and lactobacilli protects against allergy while clostridia might sensitize for atopy. (Sjögren YM. 2009) Infants who develop allergy show less often colonization with enterococci during the first month of life and with bifidobacteria during the first year of life. Differences in the intestinal microflora were evident before any clinical symptoms of atopy could be found. (Björkstén B. 2001) Thus, for the development of the immune system the intestinal microflora is an important physiological factor and can therefore be used as an indicator for the development and functioning of parts of the immune system. The effects of different diets on bacterial metabolism are often studied by measuring fecal pH and short chain fatty acid production. (Moreno Villares JM. 2008) The intestine of neonates develops and is colonized in a stepwise way and is subjected to various different factors including environmental factors and bacterial interactions. (Boehm G. 2003) For example, prior to birth the fetal small intestine consists of immature epithelium and sparse lymphoid cells. By examination of the same section in the breastfed neonate, mature epithelium with enterocyte differentiation and abundant lymphoid tissue is found. (Iyengar SR. 2012) Human milk, containing important health and immune shaping components, plays an important role in development of the microbiome especially since its composition seems to be synchronized with the changing needs of the neonate. (Chaturvedi P. 2001, Iyengar SR. 2012) Therefore disturbances of this interaction are thought to contribute to disease development. (Iyengar SR. 2012) To identify the factors and mechanisms contributing to the advantages of human milk is thus of great importance for the development of infant formulas.

## Roles of Mast Cells and IgE in Allergy Development

There is recently found evidence that suggests that mast cells and immunoglobulin E (IgE) which are closely associated with various acute allergic reactions do also play key roles in the development of chronic allergic inflammation (i.e. long-term pathophysiological changes and tissue remodeling). (Galli SJ. 2012, Gilfillan AM. 2011) Mast cells are multifunctional cells that have a central role in the pathogenesis of allergic diseases. (Brown JM. 2008) As initiators of IgE- dependent allergic diseases (Gilfillan AM. 2011) mast cells express the high affinity IgE receptor (Fc $\epsilon$ RI). Exposed to allergen-specific IgE aggregation of Fc $\epsilon$ RI occurs and mast cells produce and release mediators which are involved in various aspects of allergic inflammation. (Fig.10) (Brown JM. 2008) Cross-bridging of mast cells with the IgE receptor is their most acknowledged mechanism of allergic activation. (Hershko A. 2012) However, IgE and mast cells can act probably as well interdependent as independent from each other in the divers complex pathophysiological processes that lead to various allergic manifestations. (Galli SJ. 2012) Recently mast cells are found to also play a fundamental role in innate and adaptive immune response to microbial infection (Gilfillan AM. 2011) upon pattern recognition receptors in innate immune responses. (Brown JM. 2008) The same mediators that are produced by mast cells for protection can have adverse effects that can lead to allergic disorders. (Gilfillan AM 2011) About the exact role and mechanisms of mast cells and IgE in the long- term tissue remodeling

that is associated with the development of allergic manifestations is however little agreement up to now. (Galli SJ. 2012)

Recently immunoglobulin free light-chain (Ig-fLC) concentrations were found to be increased under several inflammatory conditions including allergic diseases. (Kraneveld AD. 2005, Schouten B. 2011). In patients suffering from AD, cow's milk allergy, allergic rhinitis, or asthma Ig-fLC is found in increased levels which suggests a role for Ig-fLC in the pathophysiology of AD in infants at risk for allergy development. (Schouten, B, 2011) Ig-fLCs are produced by B cells and bind to mast cells which causes an immediate type of allergic response through degranulation after a second exposure to antigens. (Redegeld FA. 2002).

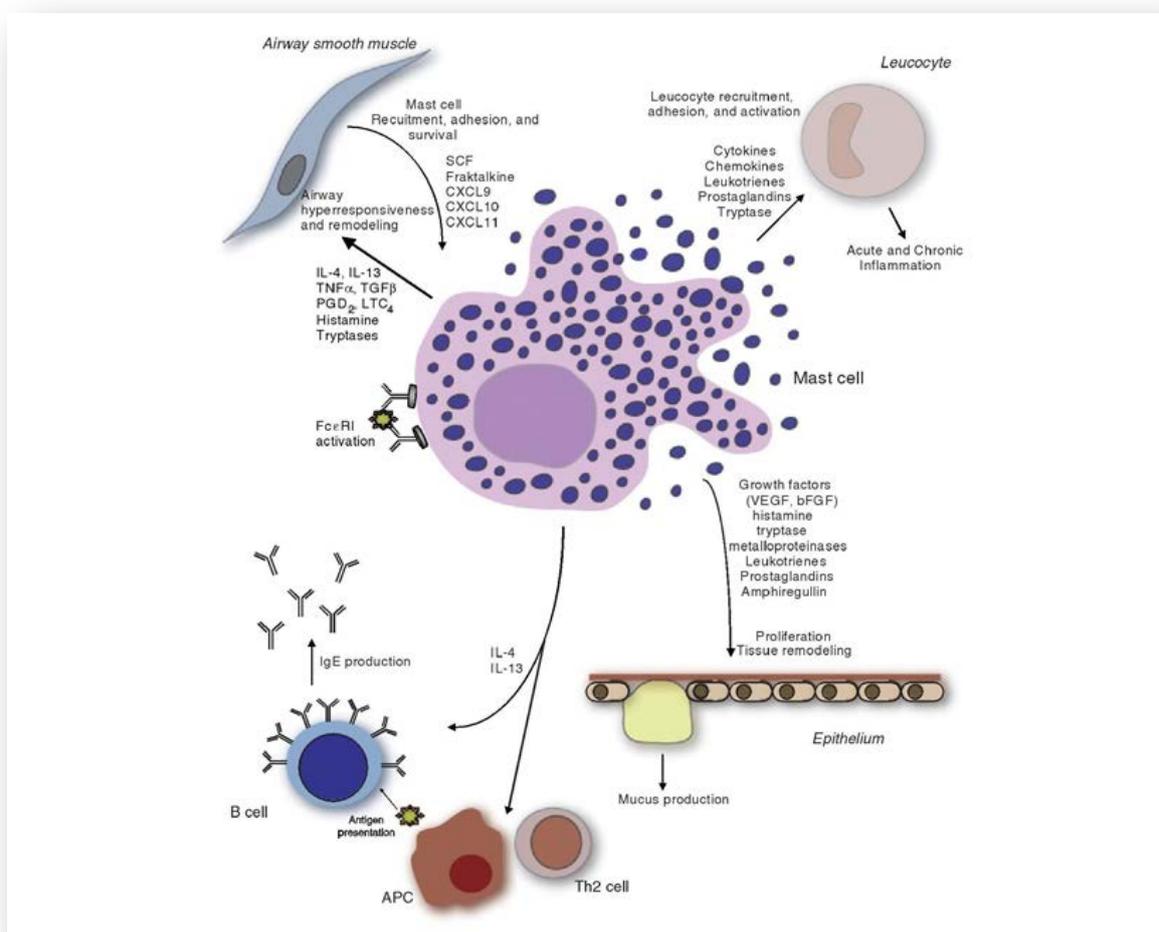


Fig.10 Role of mast cells and mast cell products in mediating effects on airway smooth muscle, leucocytes and epithelium. In asthmatics, airway smooth muscle cells can lead to recruitment, adhesion and survival of mast cells by production of stem cell factor (SCF) and fraktalkine as examples. Mast cells contribute to airway hyper-responsiveness and remodeling through production of lipid mediators, cytokines, histamine and tryptase, which influence airway smooth muscle cells. Additionally, activated mast cells and their products have effects on proliferation and remodeling of epithelium. Production of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), as well as proteases, histamine, metalloproteinases, and lipid mediators, all have effects on the epithelium, including the promotion of excess mucus production. Mast cell derived mediators produced following Fc $\epsilon$ RI crosslinking with allergen lead to leucocyte recruitment, adhesion, and activation. Mast cell-derived IL-4, IL-9 and IL-13 lead to T helper 2 (Th2) differentiation, which promotes production of allergen-specific IgE by B cells. (Fig. and citation: Brown JM 2008)

## Human milk components in the development of atopic disease

### Soluble IgA and soluble CD14

An important component of human milk is secretory IgA (sIgA) which is an immunoglobulin also present in the human gut and specifically directed against antigens in the mother's environment. Until about 30 days postpartum neonates are unable to produce protective levels of their own (Iyengar SR. 2012) and therefore depend on their mothers sIgA. In the colostrum and milk of mothers with allergic disease altered and lower sIgA is found which has also been associated with an increased risk of bovine milk allergy (Casas R. 2000) while other studies do not show predictive values for allergy development. (Duchén K. 2000)

An immunoregulatory protein which is (like sIgA) found in mature intestines and in high amounts in colostrum is soluble CD14 (sCD14). sCD14 is a soluble component of TLR4 and important in innate immunity. It binds to LPS of gram-negative bacteria and intestinal enterocytes which eventually activates the adaptive immune system. LPS elicits pro-inflammatory responses in absence of CD14 the effectiveness of the response of TLR4 to LPS is reduced. (Fig.1 & Fig.2) CD14 is linked to allergic diseases through the hygiene hypothesis. As mentioned previously this hypothesis states that early exposure to LPS reduces the risk of developing allergic disease. (Liu AH. 2002, Yang X. 2012, Amre DK. 2006, Vassallo MF. 2008) CD14 may increase early exposure of the infants immune system to LPS on gram-negative bacteria which are normally located in the neonates intestine. (Iyengar SR. 2012) In the colostrum of mothers whose children develop atopic symptoms and IgE sensitization lower levels of soluble CD14 were found compared to those whose children did not develop symptoms at four years of age. It also was significantly reduced at three month postpartum in human milk of mothers whose children developed eczema by six month of age. (Jones CA. 2002) Results of a large birth-cohort study also associates higher levels of sCD14 in human milk with a decreased incidence of AD and asthma especially in children without a history of atopic disease. (Rothenbacher D. 2005)

### Cytokines and Chemokines

Some cytokines and chemokines abundant in human milk have as well been associated with development of atopic disease. Th2 cytokines like IL-4, IL-5 and IL-13 which are involved in the production of IgE from B-cells are reduced in human milk of allergic mothers. (Bottcher MF. 2000) The predominant cytokines of human milk are transforming growth factor beta (TGF- $\beta$ ) and IL-10. They are tolerogenic cytokines involved in suppressing inflammatory responses. It has been suggested that TGF- $\beta$  is able to attenuate the inflammatory response in fetal human enterocyte cell lines (Rautava S. 2012) that recapitulate the infant gut response in vitro. TGF- $\beta$  also was found to protect against sensitization to aeroallergens transferred through human milk from mother to neonate. (Verhasselt V. 2008, Iyengar SR. 2012)

### Human Milk Oligosaccharides and Fatty Acids

In 1954 György and coworkers first identified N-acetyl-glucosamine (GlcNAc)-containing oligosaccharides, generally known as Human milk oligosaccharides (HMOs), as the bifidus factor responsible for the enrichment of bifidobacteria (György P. 1954). HMOs and Fatty Acids both stimulate the proliferation of colonizing bacteria necessary to activate the infant's immune system.

HMOs constitute the third most abundant class of molecules in human milk and are believed to have many roles in a developing infant in addition to putative prebiotic functions (Kunz C. 2000, Chichlowski M. 2011). Putative functions include protection against pathogens, development of the neonate's intestinal flora and immune system and inhibition of allergy onset. (van Hoffen E. 2009) HMOs are a family of structurally diverse complex unconjugated carbohydrates that are highly abundant in human milk but almost absent and less complex in bovine milk and consequently in infant formula (Bode L. 2006, Jantscher- Krenn E. 2012, Bode L. 2012) which explains part of the difference in (immunologic) properties between bovine and human milk. (Bode L. 2006) HMOs cannot be digested or absorbed through the small intestine and are fermented by microbiota in the colon. Among other products this process generates short chain fatty acids which selectively stimulate the growth of a complex microflora containing health- promoting components like bifidobacteria and lactobacilli but no other bacterial strains.(Fig.11) (Kunz C. 2000, Stoney RM. 2004, Moreno Villares JM. 2008)

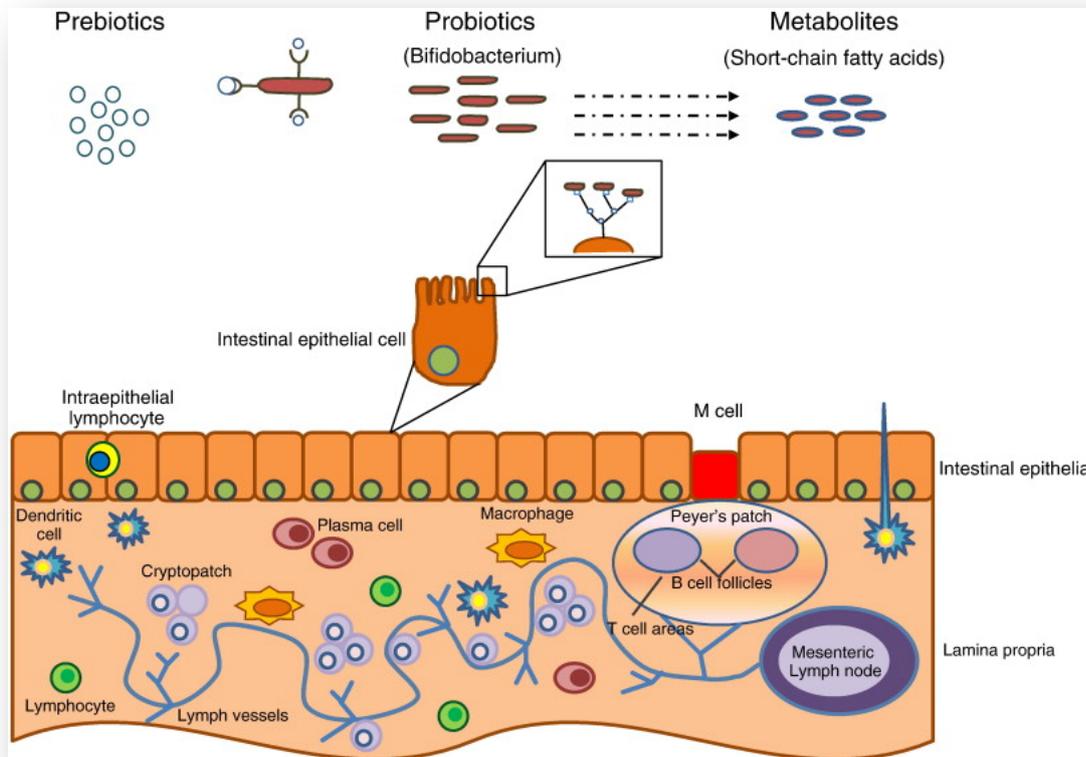


Fig. 11 Interactions of prebiotics, probiotics and immune system in the intestinal mucosa. Prebiotics promote the growth of healthy microorganisms in intestinal environment, which display immune modulatory functions, and inhibit the adherence and invasion of pathogens in the colonic epithelia. (Extracted from Choque Delgado GT. 2011)

It is suggested that the fatty acid composition of human milk may determine whether human milk promotes or protects against allergic disease (Iyengar SR. 2012) and some fatty acids have been directly associated with development of atopy in children. (Duchén K. 2000) A possible causal relationship between omega6- polyunsaturated fatty acids (PUFA) intake and allergy development has been found. Biological mechanisms that could explain this involve eicosanoid mediators of the omega6-PUFA arachidonic acid. (Kremmyda LS. 2011) Omega6 eicosanoids are generally pro-inflammatory whereas omega3 eicosanoids are much less so. Dietary intake of omega3- PUFA (e.g., from fish and fish oils) changes the production of eicosanoids, counteracts omega6- PUFA actions

thereby protecting against atopic sensitization and its clinical manifestations like chronic inflammatory and autoimmune diseases. (Kremmyda LS. 2011, Dumlao DS. 2012) Levels of omega-6 where found to be higher and that of omega-3 lower in milk of atopic mothers (Lauritzen L. 2006). A disturbed metabolism and variations in levels of PUFAs is also found to be possibly associated to AD (Duchén K. 1998) Epidemiologic investigations of associations between fatty acid (fish) intake in pregnancy and lactation, and atopic outcomes in children provides evidence to this considerations. (Kunitsugu I. 2012, Kremmyda LS. 2011) There are however conflicting outcomes in human studies regarding the relationship between fatty acid content and allergy development. (Duchén K. 1998, Stoney RM. 2004) Results from a high risk clinical birth cohort study for example, concluded no significant influence of fatty acid composition in human milk on disease development. (Giwerzman C. 2010) There are various factors that probably contribute to those and other contrasting results, like different dietary and genetic backgrounds, infection and environmental circumstances which are not taken into account in those studies. Moreover results the enormous structural diversity of HMOs combined with different fatty acid subtypes in various complex interactions and therefore various effects in the intestinal microbiome and related inflammation. (Iyengar SR. 2012, Chaturvedi P. 2001, Bode L. 2006) For example low intake of omega-3 fatty acids increase certain immune functions whereas high intake inhibits various functions like antigen presentation and proinflammatory cytokine production. Omega-6 fatty acids effect on immune function depends on their levels in human milk, which in addition is a function of dietary intake. (Harbig LS. 2003)

### Some HMO Structure-Function Relationships

Up to now more than 200 different molecular structures of HMOs are identified (Chichlowski M. 2011, Bode L. 2012) and it's becoming evident that the specific functions of HMOs are determined by their various structures. (Jantscher-Krenn E. 2012) HMOs are built from five monosaccharides: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-Fucose (Fuc), and sialic acid (N-acetyl neuraminic acid (Neu5Ac) in humans and both Neu5Ac and N-glucocyl neuraminic acid (Neu5Gc) in all other species). The reducing end is formed by Lactose (Galb1-4Glc) and can be elongated by up to 15 N-acetyllactosamine repeat units (Galb1-3/4GlcNAc). Sialylation (in  $\alpha$ 2-3 and/or  $\alpha$ 2-6 linkages) and/or fucosylation ( $\alpha$ 1-2,  $\alpha$ 1-3, and/or  $\alpha$ 1-4 linkages) can occur at the polyactosamine backbone or lactose. (Bode L. 2006) Some chemical structures of HMOs are homologues to the carbohydrate units of glycoconjugates (glycans), especially glycolipids, on cell surfaces of mammalian epithelial cells. (Fig.12) (Kunz C. 2000, Chichlowski M. 2011) Many key biological processes involve glycan participation. The various biological functions of glycans include cell adhesion (Fig.13), molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis (Ohtsubo K. 2006) and can be divided into two general categories: those based primarily upon structural and physical effects of the molecules, and those involving recognition of specific oligosaccharides by related receptors. The latter comprises self-recognition and non-self recognition. Self-recognition involves lectin receptors within the same organism whereas non-self recognition receptors are mainly of microbial or parasitic origin (hemagglutinins, adhesins, toxins, etc.) but could also be involved in interactions with symbionts (e.g., gut flora). (Gagneux P. 1999) Cell surface attached glycans exert functions in many different important biological processes (Gagneux P. 1999) and virulence of most pathogenic microorganisms requires adhesion to epithelial surfaces. (Bode L.2006) Those pathogens use cell surface glycans to identify and adhere to their target cells (Fig.14 –HMO) which is the critical first step of the pathogenesis. (Newburg DS. 2005)

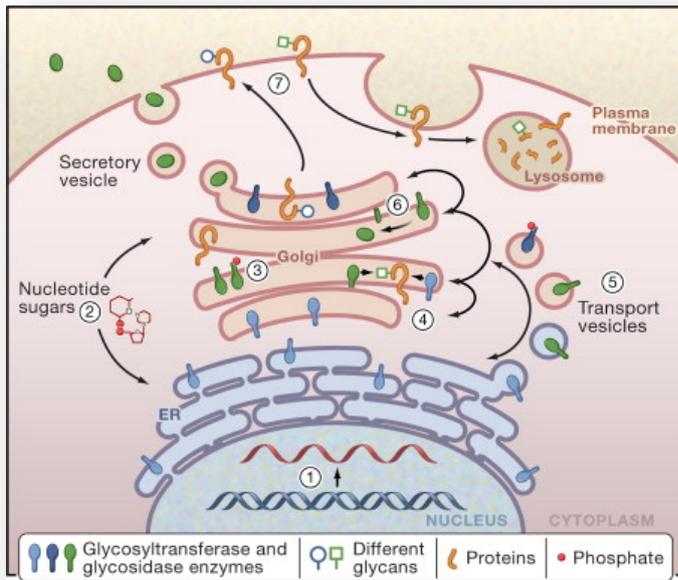


Fig.12 Cellular Regulation of Glycan Expression Representation of multiple mechanisms that alter cellular glycosyltransferase or glycosidase expression, structure, and activity, which can thereby regulate the formation of glycans. These include (1) control of glycosyltransferase and glycosidase gene transcription, (2) synthesis and transport of nucleotide sugar donors to the ER and Golgi (sugar transporters not depicted), (3) modulation of enzymatic structure through phosphorylation, (4) relative amounts of enzymes that compete for identical substrates, (5) intracellular enzyme trafficking and altered access to substrates, (6) proteolysis within the lumen of the Golgi resulting in secretion of catalytic domains, and (7) glycan turnover at the cell surface by endocytosis coincident with expression of different glycans from altered glycan synthesis. Effects of glycosyltransferase and glycosidase cytoplasmic tail

phosphorylation (3) and intraluminal proteolysis (6) on cellular glycosylation remain to be established. (Fig. and citation: Ohtsubo K. 2006)

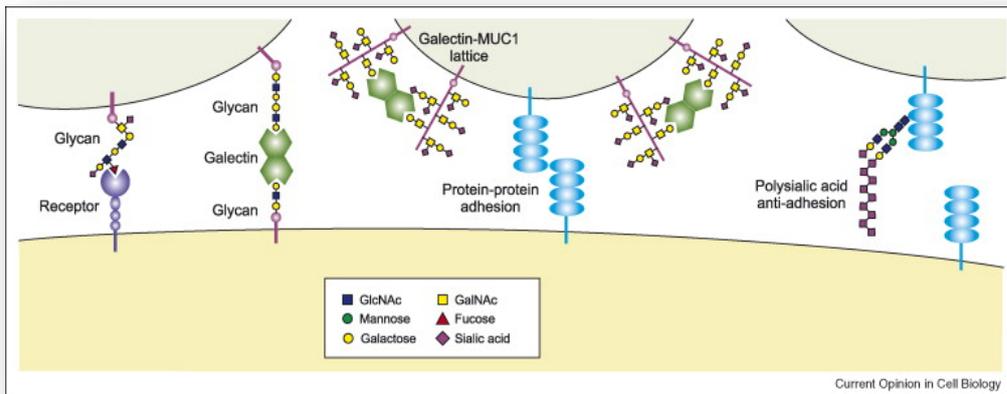


Fig. 13 Glycan-binding receptors in cell- adhesion (Taylor ME. 2007)

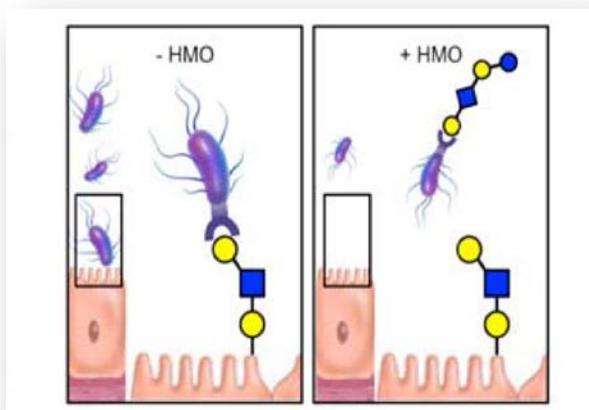


Fig. 14 Antiadhesive antimicrobials (Bode L. 2012)

Human milk glycoconjugates are suggested to express epitopes that bind to specific pathogens and therefore compete with the actual pathogen binding sites. (Fig.14 +HMO) This would forestall adhesion of pathogens to their receptors in the host mucosa and therefore prevent onset of pathogenesis in the very beginning. (Kunz C. 2000, Newburg DS. 2005) Thus, competitive inhibition at the

binding sites of pathogens by soluble glycans from human milk may protect against onset of pathogenesis. (Newburg DS. 2005) Binding of various pathogens were indeed found to be inhibited by the glycans present in HMOs (e.g., 2-fucosyllactosamine) (Bode L. 2012, Newburg DS. 2005, Ruiz-Palacios GM. 2003). Carbohydrate-binding proteins often function as adhesion-related virulence factors as well. For example lectins are such carbohydrate-binding proteins which bind to oligosaccharides on the epithelial surface. (Sharon N. 1996) Collectins comprise a family of PRRs that interact with glycoconjugates and/or lipid units present on the surface of a great variety of microorganisms and allergens. Different collectins have been identified including the protein mannan-binding-lectin (MBL) which is known to be a key player in innate immunity. MBL is able to bind a variety of different pathogens exerting different immune defense mechanisms, like direct opsonization, complement activation and clearance of apoptotic cells. It is considered an anti-antibody because it defends the body before any antibody generation occurs. (Kaur S. 2006) Because carbohydrate-binding determinants also are often expressed as parts of HMOs, HMOs can function as soluble ligand analogs and therefore as soluble decoy receptors This results in reduced or inhibited adhesion of microbial pathogens to infant mucosal surfaces (colonocytes which lowers the risk for viral, bacterial and protozoan infection, thus protects children from infections and diarrhea. (Jantscher-Krenn E. 2012, Bode L. 2012, Newburg DS. 2005)

HMOs may also have glycome- modifying effects through changing the expression of intestinal epithelial cell surface glycans. (Fig.15) (Chichowski M. 2011)

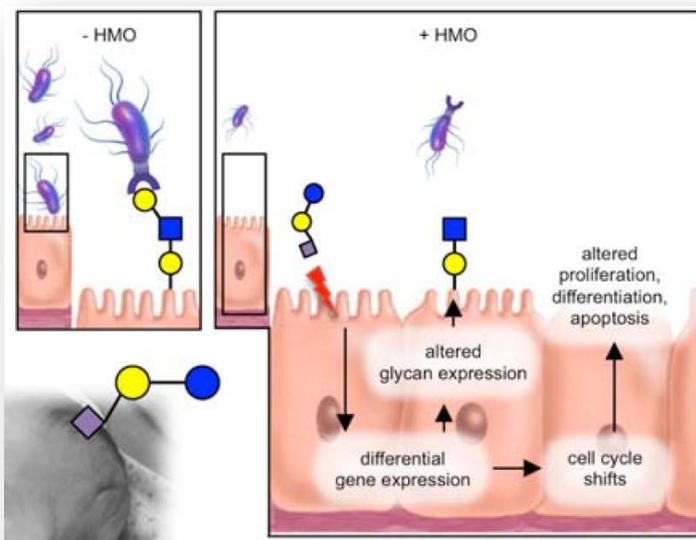


Fig.15 HMO as intestinal epithelial cell modulators (Bode L. 2012)

After exposure to 3'sialyllactose (3'SL), a constituent of HMOs, the expression of  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acid residues in Caco-2 cells was significantly downregulated. 3'SL decreases expression of various glycosyltransferases, resulting in reduced cell surface sialic acid, fucose, and galactose (Angeloni S.2005). Those are glycocalyx modifications that

may alter the glycan profile of epithelial cell surfaces and receptor sites for some pathogens. There is evidence that adhesion of certain pathogens (e.g. enteropathogenic E. coli (EPEC)) is significantly reduced by those alterations. (Angeloni S. 2005, Bode L. 2006, Chichowski M. 2011)

### Some Functions of HMO in the Immune System

HMOs are found to have direct effects on the immune system. Interactions with selectins, integrins, toll-like receptors and effects on leukocyte-endothelial cell and leukocyte-platelet have been demonstrated. The latter interactions maybe blocked by different sialylated and fucolyated HMOs that significantly affect the progression of inflammatory processes. HMOs are furthermore able to

inhibit the transfer of HIV-1 virus to CD4+ lymphocytes (fig.16) and to induce intracellular processes, including differentiation and apoptosis of intestinal epithelial cells. (Hong P. 2009, Chichlowski M. 2011)

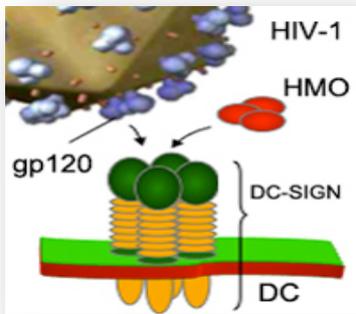


Fig.16 Human milk oligosaccharides can competitively inhibit HIV-1 gp120 binding to dendritic cell DC-SIGN (Hong P. 2009)

Since certain neutral HMO structures (e.g. LNFP III and LNnT) affect murine IL-10 production it is suggested that HMO might be involved in the production of anti-inflammatory mediators that suppress pro-inflammatory Th1 response in mice. Maturation and activation of human cord blood-derived T cells as well as Th1/Th2 skewing via production of cytokines was found to be affected by HMOs. More recent results demonstrated direct

immunomodulatory effects of the acidic fraction of HMOs in comparison to the same fraction from bovine milk. Those HMOs stimulate the production of IFN- $\gamma$  and IL-10 which direct the neonatal Th2-type T-cell phenotype toward a Th0-profile in cord blood-derived mononuclear cells and also affects Th2-type immune response of allergen-specific T cells (from peanut allergic individuals). Both outcomes offer support to the suggestion of anti-allergic properties of certain acidic HMOs. (Chichlowski M. 2011) Furthermore IgA secretion can be increased by bifidobacteria (Hiramatsu Y. 2007) which like previously mentioned are enhanced by HMOs. Bifidobacteria in the presence of selective probiotics produce factors that enhance T cell regulatory cell activity thereby promoting intestinal mucosa tolerance and prevention of allergic response. (Fujie H. 2011)

There are many more studies with specific results that support the postulated numerous functions of HMOs upon various processes. To discuss all of them in this thesis is due to quantitative limitations not possible. Nevertheless, the numerous findings of HMO functions indicate that manipulation of infants intestinal microbiota with certain HMO-like structures of synthetic oligosaccharides provides possibilities for the inhibition of allergic disease development. (Iyengar SR. 2012)

## HMO-like structures for infant formula

### GOS and FOS

Galacto- Oligo- Saccharides (GOS) and Fructo- Oligo- Saccharides (FOS) are prebiotic oligosaccharides that are suggested to have some properties similar to that of certain HMOs. They are especially known to be broadly bifidogenic and were therefore recently introduced in infant formulas. The molecular structures of enzymatically synthesized GOS and plant-derived FOS are much simpler and differ significantly from the highly complex structures of HMOs. (Fig.17) (Fanaro S. 2005, Chichlowski M. 2011) The basic structure of GOS incorporates lactose at the reducing end that is mostly elongated with up to six Gal residues (can contain different branching [Gal( $\beta$ 1-3/4/6)]1-6Gal( $\beta$ 1-4)Glc). Artificial GOS is mostly synthesized by enzymatic treatment of lactose with  $\beta$ -galactosidases from different sources, such as fungi, yeast, or bacteria which results in a mixture of oligomers with different chain lengths. FOS are linear fructose polymers and can be synthesized through reverse

reaction of fructanases and sucrases or by enzymatic hydrolysis of inulin. The first method produces short chain (sc) FOS (i.e. FOS that lacks a reducing end, contains one Glc residue and two or more fructose groups) whereas hydrolysis of inulin produces long chain (lc) FOS (free anomeric carbons containing one fructose). Presently it is not yet possible to reproduce the variable oligosaccharide content of human milk on a large scale for supplementation of infant formulas (Chichlowski M. 2011) Certain mixtures of synthetic oligosaccharides however are found to stimulate the growth of bifidobacteria and lactobacilli thereby reducing the growth of pathogens. The most studied mixture, composed to mimic the molecule size distribution of HMOs is probably a mixture of 90% scGOS and 10% lcFOS. (Boehm G. 2003, Fanaro S. 2005)

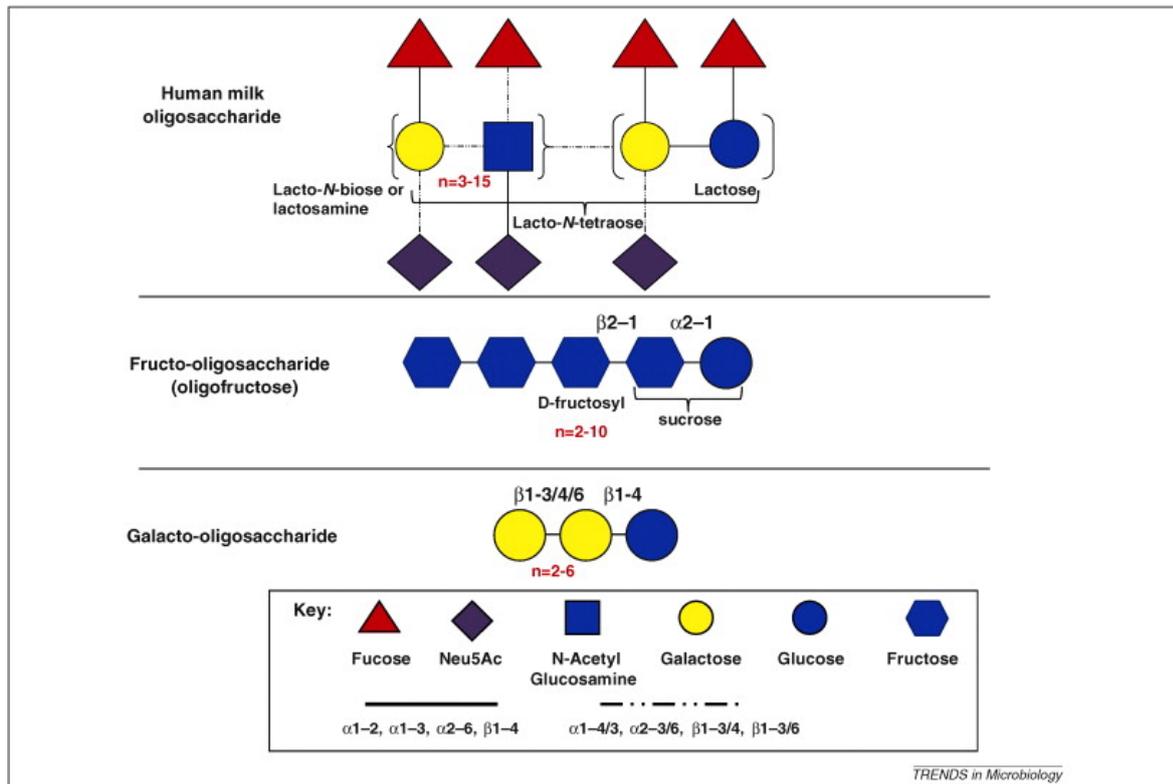


Fig.17 Schematic molecular structures of HMO, FOS and GOS(extracted from Sela DA. 2010)

## Comparisons of infant nutrition

### Comparison between human milk and formula without supplementation

No evidence supports that hydrolyzed formula without supplementation prevents allergy in comparison to human milk. Limited evidence suggests that in high risk infants who cannot be completely breastfed prolonged feeding with a hydrolyzed formula compared to a not hydrolyzed formula could protect against allergy development. Due to conflicting results through methodological inconsistencies and other (environmental) differences there is need for further large, well designed trials comparing formulas containing partially hydrolyzed whey, or extensively hydrolyzed casein to not hydrolyzed bovine milk formulas. (Osborn DA. 2006) Because this paper focuses on the possibilities of immune modulation with GOS/FOS supplementation in comparisons to other infant formula and human milk, mere comparison between infant formula without supplementation and human milk will not be further discussed.

## Comparison between human milk and GOS/FOS supplemented formula

Certain prebiotic mixtures added to infant formula are able to stimulate the development of a microbial flora similar to that of human milk fed infants. Studied mixtures enhanced the growth of certain biota who represent important factors in the postnatal development of the immune system. It is therefore suggested that prebiotics may play a role as modulators of the infants immune system. (Boehm G. 2004) More results regarding this comparison are discussed in subsequent parts.

## Comparison between formula with and without GOS/FOS supplementation

In 2006 for the first time a beneficial effect of prebiotics on the development of AD was found in a high risk population of infants. (Moro G. 2006) Up to now multiple studies showed that supplementation with certain mixtures of GOS and FOS can reduce the risk of AD in high risk infants. (van Hoffen E. 2009, Schouten B., 2011, Moreno Villares JM. 2008) There is evidence that a specific mixture of short-chain galacto- oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) induces a beneficial antibody profile and reduces the incidence of AD at 6 months of age in infants at risk for allergy. (van Hoffen E. 2009) Compared to formula without supplementation the mentioned mixture resulted in a significant reduction in the plasma level of total IgE, IgG1, IgG2 and IgG3, whereas no effect on IgG4 was observed. Cow's milk protein (CMP)-specific IgG1 was significantly decreased. In addition vaccination (DTP) – specific Ig levels were not affected. So oral GOS/FOS supplementation reduces the total Ig response and modulates the immune response towards cow's milk protein (CMP), while leaving the response to vaccination intact. (van Hoffen E. 2009) Immunofortis® is another prebiotic mixture (scGOS/lcFOS in a 9:1 ratio) that also was found to reduce the cumulative incidence of AD in infants at risk for allergy as determined using the AD symptom score (SCORAD). (Schouten B. 2011) In addition a reduced incidence of intestinal as well as upper airway infections in the first year of life was found. (Moreno Villares JM. 2008) Observational and follow- up studies that evaluated if the protective effects were lasting beyond the intervention period confirmed protective effects of the supplementation against AD and infectious episodes during the first two years of live. (Arslanoglu S. 2008, Bruzzese E. 2009). An adapted prebiotic mixture (scGOS/lcFOS and a small fraction of pectin-derived acidic oligosaccharides) was found to significantly reduce the incidence of AD of infants in the general population. (Grüber C. 2010) Significantly lower plasma levels of Ig-fLC were furthermore found in infants fed a scGOS/lcFOS supplemented formula in comparison to infants fed formula without supplementation indicating that by this formula decreases the risk of atopy in high risk infants also by decreasing levels of Ig-fLC. (Schouten B. 2011)

## Comparison between human milk and formula with and without GOS/FOS supplementation

Fecal flora (pH values, short-chain fatty acid pattern, consistency) of healthy infants fed with formula supplemented with a prebiotic mixture from galacto-oligo-saccharides and fructo-oligosaccharides (GOS/ FOS) were more similar to breastfed infants and showed a significant increase in abundance and proportion of bifidobacteria (and in some studies lactobacillus) and reduced number of pathogens in comparison to infants fed formula without supplementation. (Fanaro S. 2005, Holscher HD. 2012, Moreno Villares JM. 2008, Veereman-Wauters G. 2011) Animal models show improved response to vaccination as well as reduced allergic reaction. (Fanaro S. 2005) Reduced allergic reaction (plasma IgE/Ig4 ratio) was also found in clinical trials as well as less upper airway infections during the first year of life. Prebiotic mixtures used in different studies seem to be able to stimulate

the development of the intestinal microflora similar to that of breastfed infants which suggests that special prebiotic mixtures may function as modulators of the development of infant 's immune system and therefore are probably essential for the improvement of protective and immune modulatory properties of infant formulas. (Boehm G. 2004, Fanaro S. 2005, Moreno Villares JM. 2008) Whey-predominant starter formula containing long-chain polyunsaturated fatty acids (LCPUFAs) and galacto-oligo-saccharides and fructo-oligosaccharides (GOS/ FOS) effects the gastrointestinal comfort in infants more like breastfed infants than infants that are fed with a control casein-predominant formula without additional ingredients. (Vivatvakin B. 2010) When administered to pregnant women and then to newborn probiotics reduce the frequency and severity of AD (atopic eczema) along with other conditions. (Vanderhoof JA. 2008)

## Discussion

A lot of research in different areas is done addressing the etiological enigma of the epidemic increase as well as the pathophysiological mechanisms of atopy and its clinical manifestations in order to prevent developing allergies and atopic diseases in the future. While there is still no conclusive agreement there are some theories and hypotheses about the general cause of the epidemic as well as various specific findings of possible factors and molecular mechanisms.

The hygiene hypothesis does explain at least part of the rise in atopy and allergic diseases because of the fact that obviously after birth the adaptive immune system needs to be developed, which means it needs to establish an appropriate response to different environmental stimuli and in the absence of those stimuli naturally an immune response cannot or anyhow more difficultly be developed. Moreover due to standard vaccinations against childhood diseases, which are suggested to be important as well for the development of healthy immune system, childhood diseases decreasing and becoming quite rare. Results from a big cross sectional study confirm that both decreased exposure to microbial stimuli and vaccination against childhood diseases are associated with an increased risk for atopy and allergic disease development. (Alfvén T. 2006) Consequently for successful allergy management those environmental factors need to be considered. Atopic dermatitis is seen as the first clinical manifestation after allergic sensitization and in the allergic march. The fact that filaggrin mutation carriers develop asthma and allergic rhinitis only subsequently to AD supports the assumption of atopic manifestation following the atopic march. However 40% of mutation carriers do not develop any clinical symptoms of atopy at all. Moreover allergic rhinitis and asthma can be developed through allergic sensitization without earlier clinical manifestations of AD. Consequently this gene mutation can be seen as a genetic risk factor that predisposes but not necessarily leads to the development of clinical symptoms of atopy. Figure 6 shows in fact that different symptoms of atopy become manifest at different ages, which does not necessarily mean that they have to be developed subsequent to each other. More likely is therefore the idea of a "march" that starts with an allergic sensitization that can become manifest in various clinical symptoms. If atopic sensitization occurs and if and how sensitization will become manifest would be subject to a great range of different genetic and environmental factors and their complex interactions. And more specific as well as epidemiologic research is needed to reveal the individual and general causes, and underlying mechanisms of those processes.

One of the major sites of health challenges is the gastrointestinal tract since it harbors 80% of the total immune system. Altered microbiomes were evident in infants before any signs of atopy were, indicating how important a healthy microflora is for a healthy immune development. Consequently, nutrition has to play an important role as well. While the role of human milk is still controversial, a lot of protective components in human milk are nevertheless identified and many studies confirm that human milk does in fact contain multiple factors that modulate and promote the development of the infant's immune system. This includes the establishment of a healthy intestinal microbiome as well as a potential protective role against infection and other mechanisms that might promote atopy and subsequent clinical manifestations. That there is still controversy about the protective function of human milk is possibly due to heterogeneity in study design as well as flaws and biases in different studies. Since it is suggested that the composition of certain components (e.g., PUFAs) in human milk is associated with disease and because the composition of human milk is also a subject of maternal nutrition, it is very well possible that the controversial findings about the protective effects of human milk are also due to different maternal diets. However, the majority of studies agrees about the benefits of human milk and recommends it as the preferable and sometimes even perfect infant nutrition. (Iyengar SR. 2012) Assuming the latter is true, infant formula should match human milk as close as possible.

There is indeed a lot of research done to investigate the health-contributing components in human milk. Among those components, HMOs are identified. Artificial oligosaccharides, especially certain mixtures of GOS and FOS, are designed to mimic the prebiotic properties attributed to HMOs. Results from studies investigating the effects of GOS/FOS supplementation in infant formula show gastrointestinal microflora more similar to that of breast-fed infants than infants fed formula without supplementation. Next to the known prebiotic functions, there are findings suggesting that HMOs have a much broader range of immunological functions. For example, because parts of certain HMOs have structural similarity with certain glycans that are needed for pathogen adhesion and virulence, they can possibly inhibit disease development through competitive adhesion and by acting as soluble decoy receptors. Assumed biological functions of HMOs have also been credited to glycoproteins, glycolipids, and other milk components and numerous other glycoconjugates might share the structural features with HMOs, thus outcomes of studies with human milk have to be interpreted carefully. (Chichlowski M. 2011). Synthetic-derived oligosaccharides are furthermore structurally different from naturally occurring HMOs and despite recent advances in glycan synthesis and isolation, it is not yet possible to synthetically resemble the great variety of HMOs, especially not in industrial quantities. Thus, it is unlikely that they can mimic all of the complex structure-dependent effects of HMOs. Into account needs to be furthermore taken the very complex pathophysiology of atopy and related diseases as well as the fact that the composition of human milk changes in time in synchronization with the developing needs of the growing infant. Inter-individual genetic and environmental differences also influence the composition of human milk, which makes it even more difficult to mimic. Artificially synthesized oligosaccharides in infant formula, however, do show some immune-modulatory effects similar to HMO-attributed effects. Studies showed that special prebiotic mixtures with GOS and FOS induce beneficial antibody profiles and lower the risk of developing atopy and subsequent clinical manifestations, especially in high-risk infants. Thus, GOS/FOS supplementation are very promising components for infant formula to restrain atopy development and subsequent manifestations. Even though GOS/FOS supplementation do not fully resemble effects of human milk, it does prevent atopic developments more than infant formula without

supplementation. Findings regarding immune modulatory properties of GOS/FOS supplementation support beneficial effects through use of non-digestible carbohydrates in allergy management. Mother milk should be the first choice, but if for medical or other reasons breastfeeding is impossible GOS/FOS supplemented formula is the better choice.

Atopic manifestations unfortunately still increase especially among infants. The reason for this increase remains just partly solved and is very probably due to lots of different factors. Possible factors include individual differences like genetic predisposition, nutrition and lifestyle and more general factors like changes in climate and as well lifestyle and nutrition that function general factors since they have also been changing generally in time. For example the hygiene hypothesis is based on the idea of a general change in life style. Possibly in different individuals different factors contribute differently to disease development. Environmental influences have probably other impacts on a high risk infant than on an infant without any predisposition. However, there remains much to investigate regarding the allergy epidemic, underlying mechanisms and related factors.

Because of the inconsistencies in a lot of findings and the complexity of interactions between the various genetic and environmental factors its plausible to give much more attention to individual genetic predisposition as well as environmental circumstances in future study design. Future studies should investigate on the one hand how the composition of human milk is influenced by maternal factors like maternal atopy and diet and how this and the individual and also in time changing compositions of human milk components is related to development of the immune system, atopy and different diseases. On the other hand interactions between general environmental factors with various individual circumstances should be taken into account and be interpreted in detail. Furthermore advances need to be driven forward regarding artificial synthesis of oligosaccharides and other factors to maybe being able to resemble the great variety of HMOs and other human milk components.

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