Early infant gut flora and neutral oligosaccharides in colostrum in relation to allergy development in children.

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“If you always must know what’s right you will end up among the ones who always are wrong” – Ola Salo
SUMMARY

Today, atopic allergy is the most common chronic disease among children in the developed world. The increase in allergy prevalence during the past decades in these countries might be associated with lower microbial exposure. The gut flora, consisting of approximately 800 different species of bacteria, has been postulated to be important for the development of a fully functional immune system. Essentially, these bacteria are in constant contact with the gut flora associated lymphoid tissue, the largest lymphoid tissue of the human body. Following birth, the sterile gut of the newborn is immediately colonised by various bacterial species. Actually, alterations in the infant gut flora have been associated with allergy development.

Human milk is the major food in infancy and could thus influence the composition of the infant gut flora. Immunomodulatory components in human milk might differ between mothers and could therefore explain the contradictory results seen regarding breastfeeding and allergy development. Oligosaccharides, the third most abundant solid component in human milk, survive the passage through the stomach and are utilised by the gut microbiota. We analysed nine abundant neutral oligosaccharides in colostrum samples from allergic and non-allergic women and related to subsequent allergy development in their children. We found a considerable variation in the concentration of neutral oligosaccharides in colostrum, which was not to be explained by the allergic status of the women. Neither was the consumption of neutral colostrum oligosaccharides related to the allergy development in children.

Relevant bacterial species in early faecal samples were analysed, with Real-time PCR, and related to allergy development in children followed up to five years of age. Infants who harboured Lactobacilli (L.) group I (L. rhamnosus, L. paracasei, L. casei) at 1 week of age and Bifidobacterium adolescentis at 1 month of age developed allergic disease less frequently during their first five years than infants who did not harbour these bacteria at the same time (p=0.004 and p=0.008 respectively).

In conclusion, the work presented in this thesis implies the importance of a diverse gut flora early in life for the development of a fully functional immune system. However, consumption of colostrum with high amounts of neutral oligosaccharides does not protect against early allergy development.
LIST OF PAPERS

This thesis is based on the following original articles, referred to in the text by their Roman numerals:


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ABBREVIATIONS

2’-FL  2’-Fucosyllactose
3-FL   3-Fucosyllactose
APC    Antigen presenting cell
B.     Bifidobacterium
CBMC   Cord blood mononuclear cell
CD     Cluster of differentiation
Cl.    Clostridium
DC     Dendritic cell
E.     Escherichia
GALT   Gut associated lymphoid tissue
GF     Germ-free
HMO    Human milk oligosaccharides
HPLC   High Performance Liquid Chromatography
IFNγ   Interferon γ
Ig     Immunoglobulin
IL     Interleukin
ILF    Isolated lymphoid follicle
L.     Lactobacilli
LDFT   Lactodifucotetraose
LNDFH I Lacto-N-difucohexaose I
LNFP I  Lacto-N-fucotetraose I
LNFP II Lacto-N-fucotetraose II
LNFP III Lacto-N-fucotetraose III
LNnT   Lacto-N-neotetraose
LNT    Lacto-N-tetraose
LPS    Lipopolysaccharide
MAMP   Microbial associated molecular pattern
M cell  Microfold cell
MLN    Mesenteric lymph node
PCR    Polymerase chain reaction
PP     Peyer’s patches
PPR    Pathogen recognition receptor
PBMC   Peripheral blood mononuclear cell
RA     Retinoic acid
SCFA   Short chain fatty acids
TFAN   4-Trifluoroacetamidoaniline
TGFβ   Transforming growth factor β
TLR    Toll-like receptor
Th     T helper cell
TNF    Tumor necrosis factor
Treg   T regulatory cell
INTRODUCTION

INNATE AND ADAPTIVE IMMUNITY

The immune system in mammals is characterised by innate and adaptive immune responses. The innate immune response is the first defence against pathogens and involves anatomical and physiological barriers, like the skin and temperature, as well as innate immune cells\(^1\). These cells respond rapidly by phagocytosing pathogens and by secreting substances like chemokines and cytokines which attract and activate other cells. The adaptive immune system is slower, reacts to specific antigens and includes immunological memory. Adaptive immunity is characterised by T and B lymphocytes as well as antibodies produced by the B cells. There is a constant collaboration between the innate and the adaptive immune system.

Antigen presenting cells (APCs), like macrophages, dendritic cells (DCs) and B cells recognize microbial associated molecular patterns (MAMPs) via pathogen recognition receptors (PRRs)\(^2\). Common PRRs are Toll like receptors (TLRs) and 10 different TLRs have been found in man. A putative ligand for TLR2 is bacterial peptidoglycan, whereas TLR4 recognises lipopolysaccharide (LPS) and TLR 9 recognises unmethylated CpG motifs in bacterial DNA. APCs instruct naïve CD4\(^+\) cells to differentiate into either T helper (Th) 1, Th2 or T regulatory cells (T regs). The different CD4\(^+\) T cells are characterised by their cytokine production with Th1 cells producing mainly IFN\(\gamma\), Th2 cells secrete IL-4, IL-5 and IL-13 and different Tregs produce regulatory cytokines like IL-10 and TGF\(\beta\).

T and B cell clones have receptors which are specific for diverse antigens\(^1\). The B cell presents the antigen to the activated CD4\(^+\) T cell which in turn produces cytokines activating the B cell. The B cell thereby differentiates into an antibody secreting plasma cell. Apart from being the antigen receptors on B cells, antibodies can bind antigens facilitating clearance, complement activation and phagocytoses by cells like macrophages and neutrophils.
THE GUT ASSOCIATED IMMUNE SYSTEM

Mucosal epithelia are primary sites for antigen entry. Therefore mucosal associated lymphoid tissue (MALT) is of vast importance for mounting immune responses towards foreign antigens. There are more lymphocytes in the Gut Associated Lymphoid Tissue (GALT) than in all other secondary lymphoid tissues combined. The GALT is an immune privileged organ with the difficult task to mount immune responses towards antigens (pathogens) while at the same time being non-responsive to commensal bacteria and food antigens. The primary barrier of the gut mucosa is the single layer of epithelial cells with the underlying connective tissue, lamina propria. In the lamina propria the GALT has its main site. The GALT consists of Peyer’s Patches (PP) in the small intestine and of Isolated Lymphoid Follicles (ILF) in the colon. Both PP and ILF are aggregates of B-cell follicles with intervening T-cells. PP and ILF are the inductive sites where activation occurs whereas the diffuse tissues of the lamina propria are effector sites where high amounts of IgA are produced.

There are mainly two types of cells in the gut mucosa which are able to present antigens to cells in PP and ILF; DCs and Microfold (M) cells. The M cells are integrated in the epithelial layer and transport live microbes and microbial material from the lumen. DCs, present in the lamina propria, are able to open tight junctions in the epithelium making it possible for the DC to send their dendrites into the gut lumen and sample antigens. The DCs can then present antigens to lymphocytes in PP and MLNs, but appear to never stray beyond this lymphoid tissue and thus systemic infection is prevented.

Secretory IgA (sIgA) is of immense importance for mucosal immunity. It agglutinates bacteria (and other pathogens) which facilitates the clearance of the bacteria and thereby prevents invasion of the body. As shown in antibody deficient mice, sIgA produced by GALT B cells prevent the gut flora from breaching the gut mucosal barrier. Large amounts of sIgA, 40-60 mg/kg, is produced in the gut lumen every day. The classical way of activating IgA-producing B cells is T cell dependent and starts in PP where DCs present the antigens to CD4+Th2 cells. These antigen-primed CD4+Th2 cells then produce IL-4 and TGFβ which make the B cells undergo μ→ α switching. IgA+ B cells differentiate further in the mesenteric lymph nodes before they enter the blood stream. It appears to be the ability of
GALT DCs to produce retinoic acid (RA) (which up regulate gut homing receptors, $\alpha_4\beta_7$ and CCR9, on the IgA primed B cells) that facilitates the B cell-trafficking to the lamina propria$^8$. There, the IgA primed B cells finally become IgA secreting plasma cells by the help of IL-5 and IL-6 produced by CD4$^+$Th2 cells$^8$. Lately a T cell independent pathway for the activation of IgA producing B cells has been postulated$^8$. Work by Mora and colleagues show that the production of IL-5, IL-6 and RA by DCs in the PP provide a milieu for the B cells to start producing IgA as well as up regulating gut homing receptors$^9$. Peritoneal B1 cells have also been shown to produce IgA without T cell help$^6, 8$. It has previously been shown that the production of commensal specific IgA is independent of T cell activity$^5$.

![Fig 1. The structure of the gut associated lymphoid tissue (GALT).](image)

**Oral tolerance**

The intestinal immune system encounters more antigens than any other part of the body$^{10}$. Antigens from pathogens should mount a strong immune response but on the other hand food and commensal antigens should be tolerated. When not tolerated, diseases like inflammatory bowel disease, coeliac disease and food allergy could occur. As mentioned above,
lymphocytes that are primed in the PP migrate to the MLN for further differentiation, then enter the bloodstream and further accumulate in the mucosa. As with B lymphocytes, T lymphocytes up-regulate the adhesion molecules CCR9 and α4β7, which facilitates the migration to the gut mucosa. Both CD4+ and CD8+ T cells inhabit the lamina propria but it is primarily CD8+ T cells that migrate to the epithelium. Of importance for oral tolerance are clonal deletion and clonal anergy of antigen-specific CD4+ T cells. This appears to happen after high-dose feeding. Many intestinal CD4+ T cells have been postulated to be regulatory T cells and thus important for maintaining local tolerance towards environmental antigens. When IL-10 and TGFβ were depleted from lamina propria T cells, they lost their unresponsiveness towards commensal bacteria indicating that T regulatory cells are involved in the tolerance towards commensal antigens. The Th3 cells that produce TGFβ have been isolated from mice MLNs after feeding small doses of antigen for tolerance induction. Tr1 which produces IL-10 and CD4+ CD25+ T cells could also be important for tolerance induction in the gut.

Mice undergoing mesenteric lymphadenectomy cannot induce oral tolerance which postulates a central role for MLNs in oral tolerance induction. It is not known whether tolerance to commensals and food antigens is acquired in the same way. Food antigens can be found in systemic peripheral lymph organs, however when the MLNs are intact commensals never stray beyond the MLNs. The commensal flora can also, in contrast to food antigens, signal through TLRs which might be important for tolerance induction.

DCs are important regarding antigen presentation to T cells. Several different subtypes of DCs have been identified in the different compartments of the GALT. The most abundant DC subset in PP produces the anti-inflammatory cytokine IL-10. Furthermore PP DCs induce antigen specific T cells to produce IL-10 and Th2 cytokines. Lately some DCs that resemble the plasmacytoid DC have been identified. Plasmacytoid DCs have been described to be tolerogenic in mice. Interestingly, it has been proposed that DCs only partially mature when encountering commensal and food antigens leading to T cells with T regulatory properties and further local IgA production. However, when pathogens are encountered DCs fully mature with the help of signals from macrophages, mesenchymal cells and epithelial cells. This leads to Th1 and Th2 activation with inflammatory responses both locally and systemically as well as local IgA production. A study by Braat and colleagues showed that receptors involved in Th1 activation, e.g. CD40, are induced more frequently when DCs are matured in the
presence of the pathogen *Klebsiella pneumoniae* than when DCs are matured together with the commensal *Lactobacillus (L.) rhamnosus*.

The gastrointestinal tract also constitutes the largest reservoir of macrophages in the body (reviewed in 13). These macrophages appear to be blood monocyte-derived but they lose several receptors and adhesion molecules when they mature into intestinal macrophages. Therefore they are non-responding to inflammatory stimuli like LPS. Also the intestinal macrophages do not seem to present antigens to T cells. Instead, this is done by DCs. Still, intestinal macrophages are good at phagocytosis and scavenging of bacteria.

**ALLERGY**

Today, atopic allergy is the most common chronic disease among children in the developed world. In some countries the prevalence is as high as 20-25%14. The symptoms of atopic allergy are hay fever, allergic rhinitis, gastrointestinal disturbances, asthma and eczema1. Exposure to common environmental antigens such as plant pollen, animal proteins and house dust can lead to an allergic response. It is not known why some individuals develop allergic disorders. Genetic factors seem to be of importance (see below). Some people have a hereditary predisposition, termed *atopy*, to the development of allergies. Atopic allergies are IgE mediated and also called hypersensitivity type 1 or immediate hypersensitivity reactions. Since the prevalence of atopic allergy has increased during the last decades genetics alone cannot explain the development of the disorder15. The current view about atopic allergy is that both genetic and environmental factors seem to interact with each other, leading to the production of interleukin-4 (IL-4)16. IL-4 is one of the cytokines which play an important role in developing naive T cells into CD4+ Th2 cells. The Th2 cells secrete IL-4 and IL-5 when activated by an allergen. This induces the B cells to secrete IgE. Mast cells, eosinophils and basophils have receptors for IgE1. The immediate allergic response occurs when the allergen crosslinks IgE Fce receptors on the mast cells. This leads to degranulation followed by the release of histamine, heparin and proteases within minutes, all leading to the clinical symptoms. The clinical symptoms arise from the contraction of intestinal and bronchial smooth muscles, increased mucus production, vasodilatation and increased vascular permeability. The eosinophils, the neutrophils and the basophils are responsible for the late allergic response. This response occurs 6-24 hours after contact with the allergen and the
mediator’s, leukotrienes and prostaglandines, effects are more pronounced and long-lasting than those of histamine.

Figure 2. The allergic reaction. The naïve Th cell is presented to an allergen. In a IL-4 rich milieu the Th cell will develop into a Th2 cell and produce IL-4, IL-5 and IL-13. When a B cell encounters the allergen it will produce IgE antibodies in this milieu. The IgE antibodies will attach to Fcε receptors on B cells and eosinophils. Once the individual encounters an allergen once more this will lead to crosslinking of the Fcε receptors and release of inflammatory mediators causing the allergic response. Th1 cytokines like IL-12 and IFNγ dampen the Th2 response and the IgE production from B cells. Adopted from Wills-Karp M, Santeliz J, Karp CL. Nat Rev Immunol. 2001;1(1):69-75.16

Factors influencing allergy development

Parental allergy is a strong risk factor for allergy development17. The allergy status of the mother is a stronger predictor for allergy development in the child suggesting that the in utero environment might be of relevance. Polymorphisms in several genes are linked to allergic disease18. They include e.g. pro- and anti-inflammatory cytokine genes, receptor genes and HLA alleles. The many genes linked to atopic allergy reflect the complexity of the disease.

The dose, timing and route of allergen exposure seem to influence allergy development. The foetus can be exposed to allergens already in utero18. It has been shown that maternal exposure to high levels of birch pollen during pregnancy tends to increase the risk of birch allergy in the children19. Also, the exposure to high birch pollen levels during the first months of life significantly increases the risk for allergic asthma and positive skin prick test20, postulating that the exposure in infancy might be of further importance. In addition, high exposure to tobacco smoke is associated with childhood asthma and higher IgE production from cord blood18.
**The Hygiene hypothesis**

In 1989 David Strachan developed the hygiene hypothesis. The base of this hypothesis was epidemiological studies showing that children with older siblings developed atopic allergy to a lower extent\textsuperscript{21}. It was postulated that the lower incidence of atopic disease among these children was due to higher microbial stimulation in these children. Matricardi et al showed that the seropositivity for food and orofecal pathogens reduced the risk of developing atopic allergy by 60\%\textsuperscript{22}. The association of respiratory infections and allergy is less clear. In one study no association was seen between seropositivity against respiratory viruses and IgE sensitization\textsuperscript{23}. However, seropositivity towards the herpesvirus Epstein Barr Virus (EBV) was negatively associated with IgE sensitization and this was further strengthened if the subjects simultaneously were seropositive against cytomegalovirus\textsuperscript{23}. Also, a recent study showed that the presence of few IgG antibodies, against several infectious pathogens, increases the risk of different atopic disorders\textsuperscript{24}. Nevertheless, several other studies cannot confirm that childhood infections protect from atopic disease (reviewed in\textsuperscript{25}).

There is a lower prevalence of atopic allergy among children growing up on farm\textsuperscript{26}. In this environment children are more exposed to microbial products like endotoxin and bacterial DNA\textsuperscript{27}. Consequently, it might be the microbial products and not the infections in per se that prevent allergic disease. Children exposed to higher levels of endotoxin are less frequently allergic\textsuperscript{28}. Interestingly, it was shown that mice with a deficient TLR4 receptor respond with high IgE responses and anaphylactic shock when a food allergen coupled to an adjuvant was administered\textsuperscript{29}. However, if the mice simultaneously were given CpG oligodeoxynucleotides (mimicking bacterial DNA) no such responses were seen.

There appears to be an imbalance between Th1 and Th2 cytokines in individuals developing atopic disorders (reviewed in\textsuperscript{30}). The immune system of the neonate is somewhat Th2 skewed, partly to achieve a successful pregnancy. The Th2 cytokine IL-4 is important for IgE production in B cells. It is believed that a lower load of microbial products early in life leads to a lower activation of TLRs on e.g. innate cells and subsequently to a poorer development of Th1 cells. These cells secrete pro-inflammatory cytokines i.e. IFN\gamma which are able to down regulate Th2 cytokines. Lower amount of Th1 cytokines therefore creates a milieu for allergic sensitisation. It has been shown in vitro that substances mimicking microbial products switch the allergen-specific response from Th2 to Th1 by increasing the production of Th1
cytokines\textsuperscript{31}. Studies from our group further indicate an early immaturity of anti-microbial immune responses by monocytes in children with allergic mothers, an impairment which seems to persist during the first 2 years of life\textsuperscript{32,33}.

Peripheral blood mononuclear cells (PBMC) from already allergic subjects appear to produce high amounts of IL-4, IL-5, IL-9 and IL-13 after stimulation with both allergen and polyclonal activators\textsuperscript{34,35}. However, the amount of Th1 cytokines produced by PBMC from allergic subjects appears to differ depending on the stimuli. Polyclonal activators appear to induce a lower production of Th1 cytokines by PBMC from allergic subjects than PBMC from non-allergic individuals. Allergens might instead induce increased Th1 cytokine responses\textsuperscript{34}. Allergic subjects also appears to have an enhanced expression of the Th2 transcription factor GATA-3 and instead a decreased production of T-bet which is involved in Th1 differentiation\textsuperscript{30}.

There might be an imbalance in cytokine production in children developing allergic disease already prenatally. One study showed a significantly lower proportion of IL-12 producing cord blood mononuclear cells (CBMC) following allergen stimulation in children who became IgE-sensitized at two years of age when compared to CBMC from non-sensitized children\textsuperscript{36}. However, others have shown that allergen stimulated Th1 cytokine production from CBMC, does not correlate with sensitization at two years of age\textsuperscript{37}. This study instead postulates a role for the postnatal priming of T cells since sensitisation correlates with significantly higher amounts of Th2 cytokines produced by PBMC from 6 month year old infants.

T regulatory cells appear to dampen both Th1 and Th2 responses (reviewed in\textsuperscript{38}). Cytokines secreted by Tregs are anti-inflammatory cytokines like IL-10 and TGFβ. Treg cells producing IL-10 have been shown to switch antibody production away from IgE towards IgG4. It has been postulated that factors leading to poorer development of Treg cells, like insufficient microbial stimulation, are involved in allergy development.
THE COMMENSAL GUT FLORA

The gut harbours around 800 different species of bacteria\textsuperscript{38}. These species belong to nine different phyla of bacteria with Firmicutes and Bacteroides being the most predominant. The total amount of bacteria is estimated to weigh approximately one kg and outnumbers the number of cells in the human body by a factor of ten\textsuperscript{39, 40}. The bacteria are differently distributed along the gastrointestinal tract, with few species in the acidic stomach. The bacterial density increases in the small intestine and the colon harbours the highest number of bacteria, $10^{12}$ colony forming units (CFU)/ml\textsuperscript{38}. Both anaerobic and aerobic genera of bacteria harbour the gut\textsuperscript{38, 40} however the majority is strict anaerobes\textsuperscript{41}. Bifidobacterium, Clostridium, Bacteroides, Lactobacilli and Eubacterium are among the most common anaerobic genera of bacteria\textsuperscript{40, 41} in the gut. Streptococcus, Enterococcus and Escherichia (E.), together with other Enterobacter, are common aerobic genera of bacteria.

A polysaccharide rich mucus gel layer is overlying the gut epithelium. This mucus layer provides carbohydrate motifs that enable microbes to attach to the mucus and form biofilms\textsuperscript{42}. The biofilm promotes several of the functions exerted by the microflora\textsuperscript{42}. It also makes the microbes resist the forces of gut peristalsis\textsuperscript{4}. The gut microbiota is important regarding several metabolic functions like synthesis of vitamins e.g. vitamin K and biotin. Furthermore, the commensal flora ferments non-digestible carbohydrates (see below). Apart from being important for immune system development (see below) the commensal flora also protects the host from pathogens in additional ways. Lactic acid producing bacteria e.g. Lactobacilli and Bifidobacteria lower the pH in the gut and create a milieu less friendly for pathogens. The commensal flora also competes with pathogens for nutrients and receptors. It has been postulated that the commensal flora can induce the production of antimicrobial peptides\textsuperscript{4}. The antimicrobial peptides shape the composition of the gut flora in the small intestine\textsuperscript{43}. These cationic peptides are produced by paneth cells in the crypts of the small intestinal mucosa and disrupt the membrane of microbes. Dependent on different cationic activity different antimicrobial peptides have bactericidal activity against diverse bacteria.
Factors influencing the composition of the gut flora

The composition of the intestinal flora is rather stable in healthy individuals but can be altered by several factors like drugs, diet, stress, disease and aging. Mitsouka has studied the gut flora in relation to age and show that Bifidobacteria, *E. coli* and Streptococcus are most numerous in infancy (see below for further discussion on gut flora in infancy). Bacteroides and Eubacterium appear thereafter and the numbers of *E. coli* and Streptococcus decreases. In elderly people Bifidobacteria numbers are decreased, however other bacteria like Lactobacilli, Clostridia, *E. coli* and Streptococcus increase in numbers. The consumption of various antibiotics alters the gut flora. Numbers of *Bifidobacterium* and *Bacteroides* have been shown to decrease after antibiotic consumption. *Clostridium (Cl.) difficile* is regarded as a pathogen but can persist in the lumen without causing disease. However, when the existing community is changed e.g. by antibiotics it can give rise to pseudomembranous colitis.

The host genotype also appears to influence the diversity of the gut flora. Differing genotypes could give rise to differing attachment sites for microbes. Also the major histocompatibility complex genotype has been reported to influence the murine fecal microbiota. Interestingly, sIgA has been proposed to be a mediator of bacterial selection. sIgA does not only prevent microbes from increasing in numbers but might also be able to anchor microbes to enterocytes. This was demonstrated with a subset of sIgA that was able to anchor cultured human fecal bacteria and *E. coli* to an enterocyte-like cell line. Microbes that are responsible for the de-glycosylation on enterocytes could be creating binding sites for sIgA which makes this anchoring possible. The possibility that there is a host selection for specific bacteria indicates cooperation between the host and the bacteria. The term
commensal, which means that one part benefits while the other part is unaffected, is therefore misleading and one would rather refer to the gut bacteria as mutualistic\textsuperscript{48}.

\textbf{Infant gut flora}

The foetus is sterile \textit{in utero} but the colonisation starts immediately after birth\textsuperscript{49}. It has been shown that the type of delivery influences the gut flora. Neonates born with caesarean section often have less Bifidobacteria and Bacteroides\textsuperscript{45} and are colonised later with \textit{E. coli}, Lactobacilli, \textit{Bacteroides} and \textit{Bifidobacterium (B.)} species\textsuperscript{50, 51}. It can take up to over one month before infants born with caesarean section have similar levels of these bacteria compared to those of vaginally delivered infants\textsuperscript{51}. A long vaginal delivery appears to increase the chance of finding live bacteria in the mouth and stomach of newborn infants\textsuperscript{49}. Neonates born with caesarean section are initially mainly exposed to bacteria in the hospital environment and of nursing staff. Studies have been performed to investigate infant gut flora in developing countries compared to developed countries. A higher prevalence of Ethiopian and Estonian infants harbour Lactobacilli than Swedish infants\textsuperscript{52, 53}. Adlerbeth et al showed that Pakistani infants were colonised earlier than Swedish infants and also harboured a more diverse enterobacterial flora\textsuperscript{54}. The hygienic procedures in developed countries might be responsible for this difference which also might have unfavourable consequences (see below).

The early colonisers \textit{E. coli} and Streptococcus followed by Bifidobacteria and Bacteroides appear to be influenced by the diet. In breast-fed infants higher numbers of Bifidobacteria can be detected\textsuperscript{55} whereas Clostridia have been found in higher numbers in formula-fed infants\textsuperscript{49}. Streptococcus, Bacteroides and enterobacteria might also be higher in formula-fed infants\textsuperscript{49}. Microflora associated characteristics also appear to differ between breast-fed and formula-fed infants\textsuperscript{56}. During weaning the gut flora of previously breast-fed infants assumes the phenotype of formula-fed infants and when only solid food is given the gut flora resemble the adult gut flora.

\textbf{Bacterial fermentation, prebiotics and probiotics}

Several attempts have been made to affect the composition of the gut flora. As mentioned above the diet appears to influence the gut flora. Non-digested carbohydrates like dietary
fibers, resistant starch and oligosaccharides are main fermentable dietary substrates\textsuperscript{57}. The end products produced following this fermentation are mainly short chained fatty acids (SCFA) and several gases\textsuperscript{58}. Bifidobacteria and Lactobacilli are producers of lactate and therefore often called lactic acid producing bacteria\textsuperscript{44}. Bifidobacteria also produce acetate. Acetate and lactate are common fermentation products in breast-fed infants\textsuperscript{58}. Genes involved in poly/oligosaccharide metabolism have been shown to account for 8\% of the genome of \textit{B. longum} however \textit{Bacteroides thetaiotaomicron} have even more genes responsible for polysaccharide breakdown\textsuperscript{59}. The diversity of these genes is important for the substrate utilization of different bacteria.

Prebiotics are defined as “non-digestible food ingredients that beneficially affects the host by selectively stimulating one or a limited number of bacterial species already resident in the human colon”\textsuperscript{57}. Prebiotics such as inulin and fructo-oligosaccharides have been shown to function as substrates for Bifidobacteria and Lactobacilli and thereby increasing their growth, but investigations on how the whole gut microbiota is affected by prebiotics are lacking. Nevertheless prebiotics have been shown to improve gut health by increasing faecal weight and thereby increase gut peristalsis counteracting constipation\textsuperscript{60}.

Probiotic bacteria are live orally administered bacteria with potential health benefits\textsuperscript{61}. Mainly, species of Bifidobacteria and Lactobacilli have been used as probiotics. Probiotics have been postulated to have many effects but are so far only concluded to have effects on lactose intolerance (since they increase the production of lactase) and some types of diarrhoea. Still, there is no conclusive evidence that probiotics actually colonise the gut thus further research is needed\textsuperscript{62}.

\textit{Gut flora and the immune system}

To investigate the role of the commensal flora germ-free (GF) animals have been used. These animals have alterations in their intestinal morphology and thicker muscle cell wall compared to animals reared conventionally\textsuperscript{40, 63}. The large intestine of these animals accumulates mucus due to the lack of mucus-degrading enzymes and they therefore have a larger caecum. The GALT of GF animals is poorly developed\textsuperscript{11} and these animals are more susceptible to infections\textsuperscript{40}. Also other peripheral lymphoid organs in GF mice contain structural defects\textsuperscript{4}.
The CD4⁺CD25⁺ T cells of GF mice elicits lower suppressor activity than CD4⁺CD25⁺ T cells from conventional mice. Neonatal and GF mice have very few IgA⁺ B cells. However, after colonisation increased populations of IgA⁺ B cells can be detected. Also increases in IgA, both total and commensal specific, can be seen after colonisation. Several species like sheep, cattle, rabbits and pigs are dependent on the GALT for the final somatic diversification of the antibody genes. In rabbits it is only in the presence of intestinal microbiota that the B-lymphocytes proliferate and produce diverse antibodies. It has been shown that the biofilm formation of commensal bacteria is important for the maturation of B cells in the GALT of rabbits.

Several studies have investigated the role of the commensal flora regarding intestinal homestasis and oral tolerance. Following gut injury, mice with a disturbed gut flora produce less cytokines compared to mice with a normal gut flora. Also, in the same study it was shown that mice deficient in MyD88, an adaptor molecule essential for TLR-mediated induction of inflammatory cytokines, produce significantly less cytokines than wild type mice after gut injury. This postulates a role for the commensal flora in intestinal homestasis and demonstrates that the activation of TLRs is important for the recognition of the commensal flora. Germ-free mice have also been shown to produce low amounts of IFNγ and IgG2a after tolerogenic doses of ovalbumin (OVA) followed by a systemic challenge with OVA. Instead, these mice produce high amounts of IgE, IgG1 and IL-4. When Bifidobacterium infantis was introduced to GF mice the IFNγ and IgG2a response was restored in neonatal mice but not in adult mice. This postulates a role for the commensal flora in early tolerance induction. Interestingly, also administration of LPS to germ-free mice has been shown to restore the induction of oral tolerance. The proportion of CD4⁺ T cells in GF mice appears to be affected systemically. However, a polysaccharide from Bacteroides fragilis was able to restore the proportion of CD4⁺ T cells in the spleen of GF mice. GF mice are also somewhat Th2 skewed and this polysaccharide is able to drive the T cell development towards a Th1 phenotype.

**Gut flora and allergy**

The gut flora could have changed according to increased hygiene conditions. There appears to be an early colonisation with “skin bacteria” like Staphylococcus today compared to decades ago. Also, differences in gut flora between children from affluent countries compared to
developing countries postulates a role for increased hygiene as a modulator of the gut flora\textsuperscript{52-54}. Differences in gut flora have also been shown between children raised with an anthroposophic lifestyle with more fermented food and less use of antibiotics\textsuperscript{46}. Anthroposophic children less often develop allergies compared to children raised during conventional conditions.

In the late 1990s studies postulated a role for the gut flora in relation to allergy development. Children who were allergic at two years of age were less often colonised with Bifidobacteria and Lactobacilli whereas they had higher counts of coliforms and \textit{Staphylococcus aureus}\textsuperscript{70}. Also, species of Bifidobacteria appear to differ between allergic and non-allergic children. Colonisation with \textit{B. adolescentis}, was more common in children with atopic allergies as compared to non-allergic children who were colonised with \textit{B. infantis}, \textit{B. bifidum} and \textit{B. breve} to a higher extent\textsuperscript{71}. However, Murray et al did not see any differences, regarding species of Bifidobacteria, when they compared the faecal microbiota between sensitized wheezy and non-sensitised non-wheezy children\textsuperscript{72}. Yet, they found that children with eczema had lower amounts of Bifidobacteria. These studies indicate that there might be a difference in the gut flora between allergic and non-allergic children.

Nevertheless, these studies do not explain whether it is potential differences in the infant gut flora that drives development of atopic disease or if atopic disease might influence the gut flora. Therefore it is also relevant to study the infant gut flora and relate to subsequent allergy development. Such prospective studies have mainly been focusing on genera of bacteria and show interesting but not quite concurrent results. One study showed that children who had become allergic at the age of two years were less often colonised with enterococci and Bifidobacteria but more frequently colonised with \textit{Staphylococcus aureus} and had higher counts of Clostridia already as infants\textsuperscript{39}. Kalliomäki et al did also show that children who had become allergic at 12 months of age had higher counts of Clostridia at three weeks of age\textsuperscript{73}. No other genera of bacteria appeared to differ but the fatty acid profile of bacteria were different between the two groups suggesting that children who develop allergic disease have a different gut flora composition. A large study by Adlerbeth et al showed that increased total IgE in serum at 18 months correlated with later colonisation by Lactobacilli species\textsuperscript{50}. Lately, the KOALA study has shown alterations in \textit{Clostridium difficile} and \textit{E. coli} colonisation at 1 month in children who develop allergy during their first 2 years\textsuperscript{74}. However, no alterations in total Bifidobacteria\textsuperscript{74} or at \textit{Bifidobacterium} species level\textsuperscript{75} were seen between infants who developed allergy compared to infants who did not. Evaluation of allergic outcome was
performed early in all of these studies (at 12, 18 or 24 months). Therefore, later development of allergic disease is not considered.

**IMMUNOLOGY OF HUMAN MILK**

Human milk is the ideal nutrition for infants. It contains optimal proportions of the macronutrients; carbohydrates, lipids and proteins, as well as micronutrients such as vitamins and minerals. Apart from containing nutrients important for the growth of the infant, human milk also contains several immunomodulatory components. The immune system of the infant is far from developed at birth and therefore human milk IgA, lactoferrin, oligosaccharides, leukocytes, cytokines and lysozyme play a substantial role in protecting the infant from pathogens. The production of Ig isotypes is impaired at birth and IgG present in the circulation is mainly of maternal origin. Maternal IgG acquired via the placenta is mainly catabolised two months after birth making human milk sIgA even more important against mucosal infections at this time. Since the lactating mammary glands belong to the mucosal immune system, the human milk antibodies reflect the antigens the MALT of the mother has been stimulated with. Therefore, the sIgA in human milk protects the infant from the antigens the mother has been exposed to. The infants own production of sIgA is rare during the first ten days after birth indicating the importance of microbial stimulation of the GALT for sIgA production. Thereafter, numbers of sIgA producing plasma cells increases and peak around 12 month after birth.

Several different leukocytes are present in human milk (reviewed in ). They are most predominant in colostrum decreasing during the course of lactation. Macrophages and neutrophils are most common but also lymphocytes, mainly T cells, are present. The human milk macrophages express activation markers and appear to activate the infant’s lymphocytes, while the role of the neutrophils in human milk is not known.

Both anti-inflammatory (e.g. IL-10, TGFβ) and pro-inflammatory (e.g. IL-1β, IL-6, IL-8, TNFα) cytokines are present in human milk. They mainly arise from production in the mammary gland, although the leukocytes in human milk contribute to the production of cytokines. The physiological role of these cytokines is not completely understood since it is not known to which extent the cytokines survive the passage through the stomach. Still the
cytokines in human milk could be of importance for the development of the immune system in infants (see below).

Lactoferrin is a major protein in human milk. It chelates free iron leading to increased absorption of iron for the infant as well as making this nutrient unavailable for bacteria. Another antimicrobial peptide in human milk is lysozyme which is able to break bonds in peptidoglycan, a component in the cell wall of bacteria.

Exosomes have also been found in human milk. These human milk exosomes have been found to increase the number of Foxp3+CD4+CD25+ T regulatory cells and also to regulate the production of several pro-inflammatory cytokines.

Breast feeding in relation to allergy development

It has been estimated that 13% of the world deaths of children below five could be prevented if the World Health Organisation’s breast feeding guidelines (exclusive breastfeeding during the first 6 months) were followed. However, the role of breastfeeding in allergy prevention is controversial. One meta-analysis, regarding exclusive breastfeeding for the first 3 months, showed a decreased risk of breastfeeding on development of allergic rhinitis. The effect was however not that pronounced for children with atopic heredity. Kull et al showed that exclusive breastfeeding for 4 months or more decreased the risk of developing eczema and asthma. However, another large Swedish study showed that breastfeeding did not protect children from atopic dermatitis during their first year. Therefore, it is debated whether the different immunomodulatory components in human milk differ between mothers. Indeed, some studies show that low levels of sIgA is associated with increased risk of cow’s milk allergy in infants, however not all studies confirm this. Levels of antigens like casein, egg proteins and peanut proteins have been detected in human milk though it is not clear if they have any role in allergy development. The composition of polyunsaturated fatty acids in milk might differ between allergic and non-allergic mothers and could also be related to allergy outcome in children. The levels of cytokines might vary between allergic and non-allergic mothers with significantly higher IL-4 concentrations in the milk from allergic mothers. A recent study from our group indicates that mothers with higher exposure to
infectious, before the age of ten, have increased levels of TGFβ1, IL-2 and IL-8 in their breast milk.\textsuperscript{89}

\textit{Human milk oligosaccharides}

Human milk oligosaccharides (HMOs) are the third most abundant solid component in human milk after lactose and lipids.\textsuperscript{90} Colostrum contains roughly 20g HMOs/L while the concentration in mature milk is lower, 12-14g/L.\textsuperscript{91} HMOs are complex carbohydrate structures (3-10 monosaccharide units) that are synthesised in the mammary gland by glycosyl- and fucosyltransferases.\textsuperscript{92} HMOs contain lactose in the reducing end.\textsuperscript{91, 93} Lactose consists of the two monomers D-glucose and D-galactose. Other monomers of HMOs are N-acetylglucosamine, L-fucose and sialic acid. Different enzymes combine these monomers into manifold structures. Approximately 130 different HMOs have been characterised so far.\textsuperscript{94} Of these are roughly 90 % neutral and the rest acidic. It is the secretor status and the Lewis blood group of the mother that determines the pattern of oligosaccharides in the breast milk. Therefore, at least four different HMO patterns exist.\textsuperscript{94} Approximately 77% of Caucasians are secretors meaning they have the enzyme α1-2 fucosyltransferase.\textsuperscript{93} This enzyme is needed for the formation of 2-fucosyllactose (2-FL) and lacto-N-fucopentaose I (LNFP I). Non-secretors therefore lack these HMOs. Other common HMOs are (Lacto-N-tetraose (LNT) and Lacto-N-neo-tetraose (LNnT) which form core structures important for longer HMOs like LNFP I, II, III and Lacto-N-difuco-hexaose I (LNDFH I). 2-FL together with 3-Fucosyllactose (3-FL) and Lactodifucotetraose (LDFT) are shorter oligosaccharides.

The HMOs have been shown to be resistant towards digestive enzymes and therefore reaches the intestines intact.\textsuperscript{95} The high concentrations of HMOs combined with the fact that they are resistant to digestion have raised the question of the function of these glycans. As yet the biological function of HMOs is not fully understood.\textsuperscript{94} However several studies postulate two important functions of these oligosaccharides.\textsuperscript{80, 94} The HMOs are assumed to be the major components responsible for the differences seen between breast fed and formula fed infants (see above).\textsuperscript{94} HMOs function as the first prebiotics since they are fermented by bacteria like \textit{B. infantis}.\textsuperscript{96} The N-acetyl-glucosamine containing oligosaccharides have been shown to be essential for the growth of a subspecies of \textit{B. bifidum}.\textsuperscript{62} Also Coppa and collegues have
shown that high concentrations of oligosaccharides in milk correlate with a higher diversity of *Bifidobacterium* species.\(^{92}\)

Interestingly, HMOs also appear to act as anti-adhesion agents inhibiting pathogens from binding to gut epithelial surfaces.\(^{31, 90}\) As mentioned above the HMOs are synthesised by glycosyl- and fucosyltranferases. These enzymes are also responsible for the formation of the glycans present on different cell types. Therefore HMOs resemble glycans on human cells. Pathogens use these glycans to adhere to epithelial cells. When free HMOs are present in the gut lumen the pathogens bind to these and therefore HMOs decrease the infection load of orofecal pathogens. Indeed, HMOs have been shown to protect infants from infectious diarrhoea.\(^{97}\) High concentrations of \(\alpha1-2\) linked HMOs have been shown to be most protective.

**PRESENT STUDY**

**AIMS**

Several studies postulate a role for the infant gut flora in relation to the development of the immune system and subsequent allergy development. Also, the role of breast feeding in allergy prevention is debated. The overall aim of this thesis was therefore to investigate the infant gut flora and the consumption of human milk oligosaccharides, which stimulate species of the gut flora, and relate to allergy development in children.

The specific aims of each study were to:

Study I: Investigate whether the colostrum from allergic and non-allergic mothers differ in composition regarding neutral oligosaccharides and if there is a difference in the consumption of colostrum oligosaccharides between neonates who at the age of 18 months had developed allergic disease compared to neonates who had not developed allergy at 18 months.

Study II: Study the presence and amounts of four *Bifidobacterium* species, 2 groups of Lactobacilli, *Cl. difficile* and *Bacteroides fragilis* in infant faecal samples collected at 1 week, 1 month and 2 months of age and relate to the development of allergic disease in these children at five years of age.
MATERIAL AND METHODS

The methods of the studies are further discussed in each paper. The different study populations in each study are described below.

Study population study I

Mothers attending the Antenatal Health Care Centres in Linköping August 1993 to March 1996 and in the beginning of 2004 were invited to participate in a prospective study of the development of atopic symptoms in relation to environmental factors and maternal immunity. The children were born between January 1994 to July 1997 and June to October 2004. All the children were delivered at term and they had an uncomplicated perinatal period. Mothers who breast-fed their babies for less than 3 months were excluded.

Clinical examinations and skin prick tests (SPT) against fresh hen’s egg, milk and extracts from cat and peanut (ALK, Denmark) were carried out at 6, 12 and 18 months of age, and whenever allergic symptoms were suspected. At these appointments the parents also completed a questionnaire regarding clinical symptoms, nutrition and allergen exposure to pets of their babies.

The diagnosis of atopy in the parents was based on a convincing clinical history of bronchial asthma, allergic rhinoconjunctivitis, atopic eczema and food allergy. The mothers were classified as allergic if they showed clinical symptoms of allergic disease and had circulating IgE antibodies against a panel of common allergens, measured with allergy screen test (Magic Lite™, ALK, Hørsholm, Denmark) or Phadiatop (Pharmacia CAP System RAST FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden). Mothers not reporting clinical symptoms of allergy and having a negative allergy screen test or Phadiatop were classified as non-allergic. Children were classified as non-allergic when they did not show any symptoms of allergy and had a negative SPT. Children were classified as allergic if they showed clinical symptoms of allergic disease.

The SPT was considered to be positive if the mean diameter of the wheal reaction was \( \geq 3 \) mm. Atopic eczema was defined as pruritic chronic or chronically relapsing dermatitis with typical morphology and distribution. Food allergy was defined as a positive skin prick test, combined with a positive clinical history of immediate skin and/or gastrointestinal reactions or atopic symptoms upon exposure to a certain food and clinical remission of atopic
symptoms on an exclusion diet. Breast-feeding was defined as exclusive when all cows milk formula, except for extensively hydrolysed formula (i.e. Nutramigen™, Bristol Meyers), were avoided.

From the original study population, we randomly selected 20 women (11 allergic and 9 non-allergic) and 20 children. Eleven of these children were non-allergic and nine showed symptoms of atopic eczema, food allergy or both (table 1).

The local Ethical Committee at the University Hospital, Linköping, Sweden, approved the study.

**Study population study II**

This study population has been described in further detail by Voor et al. Pregnant women and their families attending maternity clinics in Linköping, Sweden were invited to participate in a prospective study of the development of atopic symptoms in relation to environmental factors. The children were born during March 1996 to October 1999. All the children were delivered at term and they had an uncomplicated perinatal period.

A clinical examination of the babies was made at 3, 6, 12, 24 and 60 months of age. At these occasions questionnaires were completed regarding symptoms of allergy, use of antibiotics and incidence of infections. Atopic eczema was defined as pruritic chronic or chronically relapsing dermatitis with typical morphology and distribution. Asthma was defined as three or more episodes of bronchial obstruction during the last 12-month period, of which at least one should be verified by a physician. Allergic rhinitis/conjunctivitis was defined as rhinitis and/or conjunctivitis following at least twice in one hour after allergen exposure and not in relation to infection. Urticaria was defined as allergic if it appeared at least twice in one hour after allergen exposure.

Also SPT were performed at the follow-ups. These included standardized allergen extracts with cow’s milk- and egg-, cat-, dog-, timothy- and birch allergen (ALK, Horsholm, Denmark). The SPT was considered to be positive if the mean diameter of the wheal reaction was ≥3 mm.

Children regarded as allergic had shown symptoms of allergic disease during their first five years as well as positive SPT. Non-allergic children had not shown any symptoms of allergic disease nor a positive SPT during their first five years. Inclusion in this study was based on availability of faecal samples and allergic status during the first 60 months. Thirty seven
children were included in total. Fourteen of these children had developed allergy during their first five years while 23 had not. None of the children included in this study had received oral antibiotics during their first 3 months. All children, except two in each group, were exclusively breastfed for at least 3 months. Two children in the non-allergic group were delivered with caesarean section. The rest of the children were delivered vaginally.

The study was approved by the Regional Ethics Committee for Human Research at Linköping University. The parents of all children gave their informed consent in writing.

RESULTS AND DISCUSSION

Study I

Some components in human milk appear to differ between allergic and non-allergic mothers\textsuperscript{87, 88}. Oligosaccharides are the third most abundant solid component in human milk\textsuperscript{90}. In order to investigate whether maternal allergy influences the content of these, the concentration of the neutral oligosaccharides LNDFH I, LNFP I, LNFP II, LNFP III, LNT, LNnT, 3-FL, 2´-FL and LDFT were analysed in colostrum samples from allergic (n=11) and non-allergic (n=9) women. The colostrum samples were collected at the same time of the day and at the same time in the lactation period, 2-4 days post partum. In accordance with previous studies\textsuperscript{97, 100, 101}, 2´-FL and LNFP I were the most abundant neutral oligosaccharides. There was no difference in the amount of neutral oligosaccharides in the colostrum from allergic compared to non-allergic mothers. This suggests that the amount of neutral oligosaccharides in human milk is not influenced by the allergy status of the mother.

The variation in oligosaccharide concentration in milk from different women was immense. This variation is in accordance with previous studies and varies according to the secretor status, the ABO blood group type, and the Lewis blood group type\textsuperscript{102}. It is the same enzymes, glycosyltransferases including fucosyltransferases, which are necessary for the production of breast milk oligosaccharides that are involved in the production of the cell surface glycolipids that determine blood group type in erythrocytes\textsuperscript{102}. Also, these enzymes are responsible for the production of cell surface glycoconjugates, which can bind particles and pathogens. Consequently, blood group phenotype and secretor status have been associated with certain
diseases i.e. urinary tract infection and asthma. Additionally, high amounts of α1,2-linked fucosylated oligosaccharides appears to protect the infant from several types of diarrhoea. Consequently, the variation in HMOs could be of medical importance.

The oligosaccharides in human milk have been shown to be resistant toward digestive enzymes and therefore reach the intestines intact. This in combination with the fact that they occur in high concentrations postulate a significant role for the HMOs in the intestine. They might function as the first prebiotics by increasing the growth certain commensal gut bacteria. Also, as mentioned above, HMOs might prevent the binding of certain pathogens to the mucosa and thus lowering the infection rate by these pathogens. These two functions postulate a role for the oligosaccharides in early immunity making it relevant to study oligosaccharide consumption by infants in relation to allergy development in these children. Our results indicate that children who had developed allergic symptoms at the age of 18 months tended to have consumed colostrum with higher concentrations of neutral oligosaccharides in total, although the difference did not reach statistical significance (p=0.12). This tendency could however not be attributed to any specific neutral oligosaccharide. The fact that oligosaccharides in human milk act as receptor homologues and thus prevent pathogens from binding to the cells in the mucosa implies that a high amount of neutral oligosaccharides protects against orofecal pathogens. Interestingly, it has been shown that infections with food and orofecal pathogens were able to reduce the risk of atopy with 60%. Accordingly, the ability of human milk oligosaccharides to bind pathogens and serve as anti-infective agents might influence the development of the immune system more than their ability to stimulate gut microbiota-immune interactions.

Study II

The commensal gut flora appears to be important for oral tolerance induction and gut homeostasis. Previous studies have shown differences in the infant gut flora between children who develop allergic disease compared to children who do not. However, this has not been confirmed to such an extent by others. By using Real-time PCR we investigated the presence and amounts of four different Bifidobacterium species, two groups of Lactobacilli, Cl. difficile and Bacteroides fragilis in faecal samples collected from infants 1 week, 1 month and 2 months old. This was related to the allergy outcome in these children up
to five years of age. Interestingly, infants who harboured Lactobacilli group I (L. rhamnosus, L. paracasei, L. casei) at 1 week of age significantly less frequently developed allergic disease than infants who did not harbour these bacteria at the same time (p=0.004). Also, the presence of these bacteria at several time points was more common among non-allergic children suggesting that a persistent colonisation with these species of Lactobacilli might be of importance. Children that developed allergic disease also significantly less commonly harboured B. adolescentis as one month old infants (p=0.008). In comparison, B. adolescentis has been shown to be more common among children with an already established allergic disease compared to non-allergic children71.

Mainly genera of bacteria in infant faecal samples have been studied in relationship to the development of allergy in children previously39, 73, 74. This might explain why some studies show an association with certain gut bacteria and allergy development while other studies do not show this difference. We showed differences at species level namely that Lactobacilli group I (L. rhamnosus, L. paracasei, L. casei) and B. adolescentis were less common in faeces of allergic infants at one week and one month respectively. Indeed different species of these bacteria might elicit differing cytokine patterns. Several species of Bifidobacteria, e.g. B. bifidum and B. infantis, have been shown to be good inducers of IL-10 production by macrophages104. B. adolescentis and B. longum did instead appear to induce TNF, IL-6 and IL-12 production. Diverse Bifidobacterium species have also been shown to induce activation markers (CD83, CD86) on DCs generated from PBMCs. Regarding the DCs it was only B. bifidum, B. longum and B. pseudocatenulatum that induced IL-10 production105. A balanced Bifidobacterium flora might therefore induce appropriate cytokine responses. Lactobacillus rhamnosus stimulated DCs have been shown to induce hypo-responsive T cells106. The study by Smiths et al show that some lactobacilli species (L. reuteri, L. casei) bind to the PRR DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) on DCs leading to priming of IL-10 producing Tregs107. Taken together these studies postulate that different commensal bacteria are responsible for various cytokine responses. A diverse gut flora might therefore be of importance for a balance in cytokine production which could be important for the development of a fully functional immune system.

It is important to consider that we do not gain full insight into the entire gut microbiota when analysing bacteria from faecal samples. Different bacteria might adhere at various sites within the diverse compartments of the gut. These bacteria might therefore exert different functions.
throughout the gut. Also, aerobe bacteria might appear to exist in higher numbers when faeces are studied instead of biopsies from the more anaerobe gut\textsuperscript{108}. Another useful tool, for studying differences in the gut flora between two populations, is to use microflora-associated characteristics. This method measures the function of the microbes since metabolic products, like short chained fatty acids, are studied. Böttcher et al have investigated gut flora associated characteristics and indeed found differences between already allergic and non-allergic children\textsuperscript{109}.

**Clinical relevance**

By studying the infant gut flora in relation to allergy development we might gain insight into which bacteria that might be of importance in allergy prevention and thus appropriate probiotic candidates. Studies have investigated the role of giving probiotics from birth (or prenataley to pregnant mothers) and subsequent development of allergic disease\textsuperscript{110, 111}. Interestingly a strain of *L. rhamnosus*, given to pregnant mothers and their infants, reduced the frequency of eczema in the children indicating that this species might be important in eczema prevention\textsuperscript{110}. However, total IgE or positive skin prick test did not differ between the probiotic or the placebo treated group. A more recent double-blind randomised placebo-controlled trial investigated the role of *Lactobacillus reuteri* administration to mothers prenataley and to their infants for their first 12 months\textsuperscript{111}. The prevalence of eczema outcome and asthma did not differ between the two groups however the probiotic group had significantly lower proportion of IgE associated eczema. Studies showing no association with probiotics have also been completed\textsuperscript{112}. Today there is therefore still not enough data to recommend probiotics in the prevention of allergic disease\textsuperscript{113}.

Importantly, since new research is constantly emerging new insights into the complexity of the gut flora the correct way might not be to administer one strain of probiotic bacteria but to increase the diversity of the gut flora by several means like promoting breast feeding, vaginal delivery and even prebiotics. Indeed one double-blind randomised placebo-controlled trial study where several probiotics and prebiotic galacto-oligosaccharides were administered showed promising results regarding eczema and atopic eczema outcome at two years of age, however the cumulative incidence of atopic disease was not decreased\textsuperscript{114}. Our study regarding consumption of colostrum oligosaccharides did not show any effect on allergy development at
18 months of age. However this pilot study was relatively small and measured the allergy outcome at a relatively early age. Additional studies might be of importance for studying the impact of HMOs on the gut flora and subsequent allergy development. Human milk has also been shown to modulate the TLR responses which are of importance in the recognition of microbes. Additional substances in human milk might also stimulate species of the gut flora\textsuperscript{91}. Therefore, it might be of relevance to promote breastfeeding also for its ability to stimulate gut flora immune interactions.
CONCLUDING REMARKS

- The content of neutral oligosaccharides in colostrum does not depend on maternal allergy, nor does consumption of colostrum with high amounts of oligosaccharides protect children against allergy development.
- Infants who harbour Lactobacilli group I (L. rhamnosus, L. paracasei, L. casei) at 1 week of age and B adolescentis at 1 month of age significantly less frequently develop allergic disease compared to infants who do not harbour these bacteria at the same time. This might imply the importance of a diverse gut flora early in life for the development of a fully functional immune system.

ONGOING AND FUTURE PROJECTS

STIMULATION OF CORD BLOOD MONONUCLEAR CELLS AND PERIPHERAL BLOOD MONONUCLEAR CELLS WITH COMMENSAL BACTERIAL DNA.

The intracellular pathogen recognition receptor TLR 9 is expressed in B cells and pDCs\textsuperscript{115}. Following TLR 9 activation with e.g. bacterial DNA and synthetic CpG oligonucleotides, mimicking bacterial DNA, these cells produce cytokines like IL-6, IL-10 and type 1 interferons. This further activates other cells like NK cells, monocytes and neutrophils resulting mainly in production of Th1 cytokines\textsuperscript{115}. CpG oligonucleotides have been shown to directly inhibit IgE and IgG1 synthesis in B cells\textsuperscript{116}. Some trials, investigating the use of synthetic CpG oligonucleotides as allergy vaccine, have been performed with promising results\textsuperscript{117}. The unmethylated DNA from different bacterial species appears to lead to different cytokine responses largely depending on the different frequencies of CG dinucleotide content\textsuperscript{118}. We have investigated the presence and amount of bacterial DNA in faeces from infants developing and not developing allergies and indeed found differences. We are therefore interested in studying the cytokine response following activation of PBMC and CBMC with DNA from different commensal bacterial species. We have previously shown that PBMC and CBMC produce IL-6 and IL-10, after incubation for 24 hours, when stimulated with at least 5µg bacterial DNA/ml. DNA from different bacterial species appears to induce production of different amounts of cytokines, however an increase in concentration might be needed to further investigate this. We will therefore continue this work and use higher concentrations of DNA from different species, incubate for several time points and
measure IL-2, 4, 6, 10, TNF and IFNγ with cytometric bead array. We also have the possibility to measure the cytokine production with Luminex to detect additional cytokines. We will evaluate whether bacterial DNA from various commensal species induces varying cytokine responses and whether PBMC and CBMC respond differently towards the stimuli. Human intestinal epithelia express TLR9 mRNA119. However, whether intestinal epithelia respond to CpG oligonucleotides is not quite clear and might differ between primary epithelia and cell lines119. Therefore we will also stimulate epithelial cell lines, foetal and adult, with commensal bacterial DNA. Synthetic CpG oligonucleotides will be used as positive reference control.

**HUMAN MILK OLIGOSACCHARIDES IN RELATION TO INFANT GUT FLORA AND ALLERGY DEVELOPMENT IN CHILDREN AT FIVE YEARS OF AGE.**

Our study regarding consumption of colostrum oligosaccharides did not show any effect on allergy development up to 18 months of age. However this pilot study was relatively small and measured the allergy outcome at a relatively early age. In our second study we investigated the early infant gut flora in relation to allergy development up to five years of age and found differences at species level. Since human milk is the only food during the first two months it might influence the early gut flora. Interestingly, also breast milk consumed by the children in study II has been collected. Therefore, using methods described in paper I, we will measure the neutral oligosaccharide content in colostrum and mature milk consumed by these children. These results will then be related to the early gut flora and to the allergy development up to five years of age in these children.
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