Combined dietary supplementation with *Lactobacillus reuteri* and omega-3 PUFA during pregnancy and postnatally in relation to development of IgE-associated disease during infancy.

Background

The incidence of allergic disease has increased worldwide during the last decades (1). Initially, a lot of effort has been put in elucidating witch of the known risk factors commonly associated to the development of allergic disease early in life was the cause of this increase. Studies showing a reduced incidence of allergic disease in the former socialist countries in comparison to countries with a "Western lifestyle" have shown that risk factors as allergen exposure, environmental pollution and tobacco exposure are also present in societies with a less affluent lifestyle (2-5). This suggests the disappearance of factor protecting against the development of allergic diseases in affluent environment (6).

The development of allergic diseases begins during the first year of life with eczema, both non-IgE- and IgE-associated, and food allergy, progressing during childhood with the development of asthma bronchiale, also both non-IgE- and IgE-associated, and later development of allergic rhinoconjuctivitis, *i.e.* the atopic march (7). Allergic disease, particularly IgE-associated, is related to a Th2-skewed immune response mediated by IL-4, IL-5, IL-13 and other Th2-related cytokines, as well as PGE2 (8). Th2-skewing may also be required for a successful pregnancy, in order to prevent Th1-mediated rejection of the semi-allogeneic foetus (9). Although controversial regarding the importance for later development of allergy in infancy (10), antigen specific T-cells responsiveness to common food and inhalant allergens has been suggested to occur in the foetus already at 25 weeks of gestation (11). The newborn child is born from a "Th2-like" environment, with an immune system pre-destined to Th2-skewed responses to foreign antigens. Genetic predisposition to the development of allergic disease early in infancy seems to be related to sustained Th2-skewed immunity during infancy (8).

The immune system of the neonate is influenced by maternal immunity, both via the placenta and breast milk (12, 13). Thus, the immunological interaction between the mother and her offspring is close during pregnancy and lactation. The association of cord blood IgE levels {Round, 2009 #1013}with maternal but not paternal atopic heredity (14), may depend on a possibly stronger placental Th2 shift in atopic mothers. We recently observed that maternal IgE correlated with cord blood IgE and CCL22 (MDC), a Th2-associated chemokine (15). Interestingly, high CCL22 levels and high ratios of CCL22 to the Th1-induced chemokine CXCL10 (IP-10) in cord blood were associated to development of sensitisation and allergic disease, possibly particularly asthma, during the first two years of life (16).

Thus, factors influencing/protecting against the development of allergic disease early in life, would be important already during pregnancy, birth and early postnatal life. Two major hypotheses have been assessed during the last decade: Proper microbial stimulation, including the establishment of the gut flora in infancy (17) and the relationship between low omega 3-polyunsatureated fatty acids in the western diet and the incidence of allergic disease (18).

The gut flora and allergic disease

The gut flora is quantitatively the most important source of microbial stimulation and may provide a primary signal for the maturation of a balanced postnatal immune system (19). Estonian and Swedish infants have a different gut microbiota, e g less Lactobacilli colonisation in Sweden (20). The gut flora differs during the first months of life in children who later do or do not develop allergic disease (21-23). Furthermore, administration of Lactobacillus GG to mothers 2-4 weeks before delivery and during breast-feeding, and then to the babies when weaned up to 6 months of age, halved the cumulative incidence of atopic eczema at 2 and 4 years of age *vs.* placebo, although sensitisation or serum IgE levels were not affected (24, 25). In contrast, we showed that supplementation of *Lactobacillus reuteri* to mothers from gestational week 36 and then to the children during the first year of life reduced IgE-associated eczema at two years of age, but had no effect on eczema alone (26). Two other studies showed less atopic eczema, both with and without sensitisation, after administration of probiotics *vs* placebo from gestational week 36 and for the first 6 months of life (27, 28) while no effect of 6-month postnatal Lactobacillus acidophilus vs. placebo administration was shown in an Australian study (29). Thus, supplementation to the mother during pregnancy may be important for preventive effects, and the maternal influences during foetal life could be particularly important for immune development, possibly via epigenetic mechanisms (30). No allergy prevention study giving probiotic supplementation to the mother earlier than gestational week 36 has yet been presented, however.

In this cohort, we could confirm our previous findings (15) that sensitisation was preceded by elevated CCL22 levels (Abrahamsson *et al*, under revision). The Th2-associated chemokine levels were as highest at birth and then decreased, whereas the Th1 chemokines increased with age. Low Th1- and high Th2-associated chemokine levels were observed in children developing allergic disease. *Lactobacillus reuteri* colonisation was associated with low Th2-related CCL17 and CCL22 and increased Th1-related CXCL11 levels (Abrahamsson *et al*, under revision).

Long chain polyunsaturated fatty acids and allergic disease

The polyunsaturated fatty acids LA (linoleic acid, C18:2 ω-6) and LNA (a-linolenic acid, C18:3 ω-3) are essential fatty acids, *i.e.* they cannot be synthesized by humans and thus must be provided by food. They compete for the same enzyme systems for desaturation/elongation of the carbon chain and are thus precursors to the LCPUFAs (long chain polyunsuturated fatty acids) of the ω-6 and the ω-3 series (31).

Allergic responses are related to the production of preferential Th-lymphocyte production of IL-4, IL-5 and other cytokines related to Th2-cell differentiation and proliferation, eosinophilia and IgE antibody production (32, 33). The eicosanoid PGE_2 enhances the Th2-lymphocyte production of IL-4 (34) probably by the inhibiting on the production of IL-12 by the antigen presenting cell (APC) (35). Prostaglandin E_2 also potentiates the IL-4 induced IgE antibody production (36, 37), while the eicosanoid LTB_4 could influence allergic responses by modulating the expression of the low affinity IgE antibody receptor (CD23) on monocytes and macrophages (38).

We have previously reported a relationship between levels of PUFA in milk from atopic and non atopic mothers during lactation and the development of atopic disease in early childhood (39-42). Low levels of LA, LNA, ω-6 LCP and ω-3 LCP in transitional and mature human

milk were related to maternal atopy. Particularly, low levels of LNA and EPA, expressed as higher LA/LNA and AA/EPA ratios were found in milk from atopic as compared to non atopic mothers (42). Furthermore, low levels of ω-3 PUFA, particularly LNA and EPA, and a high AA/EPA ratio in transitional and mature human milk seemed to be associated with the development of sensitisation and allergic disease in the children (39, 40).

Both ω-6 and ω-3 LCPUFA are delivered to the baby through the placenta and after delivery, through breastfeeding (43). Thus, in a double blind prospective and randomised study, we supplemented 145 mothers with 2.6 g EPA+ DHA (n=70) or soy oil (n=75) during the last trimester of pregnancy and the first 3 months of lactation (44). The cumulative incidence of food allergy was less frequent in the ω-3 group (1/52, 2%) compared to the placebo group (10/65, 15 %, p=0.01) as well as the incidence of IgE associated eczema (ω-3 group: 4/52, 8%, placebo group 15/63, 24% p=0.02). Furthermore, the risk of developing food allergy was reduced ten times in the ω-3 group compared to the placebo group (OR: 0.1, p=0.04). Our results suggest that maternal ω-3 fatty acid supplementation may decrease the risk of food allergy and IgE associated eczema during the first year of life in infants with a family history of allergic disease. This is supported by a previous study where healthy women were supplemented with EPA (0.15g/d) and DHA (0.5g/d) during the second half of pregnancy and yielding decreased mRNA levels of Th2 related molecules in the foetus (n=311) (45). Omega-3 supplementation with 1.5g ω-3 LCPUFA daily during lactation has also been associated with increased in vitro IFNg production in children which may reflect a faster maturation of the immune system (46).

Possible mechanisms

Interestingly, the preventive effect of supplementation with *L. reuteri* on the development of allergy in infancy was more pronounced in children to atopic mothers (26) while the preventive the effect of omega-3 PUFA maternal supplementation during pregnancy and lactation on the development of IgE associated allergic disease in infancy seemed to be more significant among children to healthy mothers (44). These findings suggest that the mechanisms leading to sustained IgE antibody production early in life may be inhibited by dietary supplementation with *L. reuteri* and the ω-3 fatty acids EPA and DHA.

The maturation of the immune system in the infant is closely related to the development of the early gut flora (47). Early immune Th1 and Th2 related immune response may be influence by the early bacterial colonization M (47) and different gut flora has been reported in infants developing allergic disease early in infancy (21). Precense of *L. reuteri* in faeces at 1 week of age was associated to decreased levels of Th2 related chemokines as CCL22 (48, 49)). Furthermore, *L. reuteri* supplementation decreased allergen-induced Th2 cytokine responses *in vitro* (in manuscript). Thus suggests that *L. reuteri* supplementation induces an attenuation of Th2-skewed responses by the gut flora, but the exact mechanism is not well understood. In a murine model, the *L. reuteri*-induced protection against allergic airway responses was mediated via regulatory T cells, attenuating Th2 responses (50).

DHA and EPA compete with AA for enzymes and spaces in the cell membranes. Experimental studies in human fibroblasts show that DHA increases membrane permeability and decreases AA level in membrane phospholipids while EPA only decreases AA levels in human fibroblast membranes (51). The composition of LCPUFA:s in cellular membranes

influences several physiological functions, as membrane fluidity, permeability, receptor and enzyme activity as well as other interactions between lipids and membrane protein. Changes in immune cell membrane fluidity and decreasing the content of AA may influence cell responsiveness to exogenous stimuli (52). Arachidonic acid is a precursor of the eicosanoids PGE_2 and LTB_4 and EPA competitively inhibit the metabolism of AA which in turn limits the production of PGE_2 (53) in favour of less inflammatory eicosanoids as PGE3 and LTB4 (54). Thus, the decreased AA/EPA ratio we have reported in maternal plasma phospholipids may explain the decreased maternal PGE2 production (55). During the last decade, novel pathways in the synthesis of eicosanoids from AA, EPA and DHA have been reported. Lipoxin (AA-metabolite), Resolvin E1 (EPA – metabolite) and Protectin D1 (DHA-metabolite) are synthesised during the resolution of inflammatory activity (56). These novel substances control the magnitude and duration of inflammation by regulating cellular traffic into inflammatory sites and by removing inflammatory chemokines ferried by T-cells and neutrophils (56). The detected levels are at nanomolar range suggesting potent anti-inflammatory properties theoretically able to balance early Th2 skewed immune responses in genetically predisposed children. Furthermore, as the ω-3 and ω-6 PUFA modulate Th1 and Th2 responses, have antibiotic-like actions and can influence microbial adhesion to the mucosal surface, the ω-3/ω-6 ratio in the diet may affect gut microbiota establishment and enhance potentially beneficial action of probiotics (57).

Probiotics and gastrointestinal function

In a review of the effect of probiotics on functional constipation, it has been reported that Bifidobacteria and Lactobacilli strains improve bowel function in adults and children (58). Constipation and irritable bowel symptoms are common problems during pregnancy (59). This symptoms have been studied in a normal Swedish population with validated questionaires (60).

Primary and secondary aims in this study

Thus, the primary aim of this study is to assess the preventive effect of a combined maternal supplementation with two supplements, which by their own have previously shown to prevent the development of IgE associated disease in childhood. We also hypothesize that the preventive effect may be more effective as the combination of both the supplements, *L. reuteri* and omega-3 PUFA, may potentiate the effect on children from both atopic and healthy mothers. Also, ω−3 PUFA and probiotics may induce anti-inflammatory responses by divergent, possibly synergistic, pathways, *e g* via resolvins and regulatory T cells (50, 61).

As a secondary aim, we will assess the effect of supplementation with *L. reuteri* on maternal bowel function during pregnancy.

Material and methods

We have previously performed two intervention studies in order to prevent the development of allergic disease in childhood (26, 44). In the placebo treated groups, the cumulative incidence of any IgE associated disease during the first two years of life among children with family heredity of allergic disease was 31% and 35%. To detect a 50% decrease in the cumulative incidence of any IgE associated disease at two years of life (35%) with 80%

power and a probability of 5%, 99 children are needed in each group. Four study groups are needed and with an expected drop out frequency of 20%, 495 children are needed in all in this study.

The frequency of constipation and irritable bowel symptoms in pregnancy has been reported in 24% and 19% of the mothers (59). In order to detect a 50% decrease in the frequency of these symptoms in pregnant mothers, with an 80% power at a significance level of 0.05, 160 and 151 mothers respectively are needed in each group. In this study, 220 mothers will receive *L. reuteri* and 220 will receive placebo.

In order to invite enough mothers within a feasible period of time, the study will be performed within the South East region in Sweden (Linköping, Norrköping, Motala, Jönköping). We will also seek cooperation with Prof Gunnar Lilja and Prof Magnus Wickman, Karolinska Institutet in Stockholm and Prof Göran Wennergren The Queen Silvias Children´s Hospital in Gothenburg.

This is a double blind randomized study. Families with at least one parent/sibling with clinical symptoms/history of allergic disease will be invited to participate in this study. Pregnant mothers will be included in the study at the 20^{th} week of gestation according to the protocol below. They will be randomized to 4 study groups, one will receive placebo capsules, the second will receive ω–3 PUFA supplementation and placebo regarding *L. reuteri*, the third will receive *L. reuteri* and placebo regarding ω-3 PUFA and the fourth group will receive both ω-3 PUFA and *L. reuteri* supplementation. Omega-3 supplementation will be given to mothers from pregnancy and lactation while *L. reuteri* will be given to the mothers during pregnancy and later to the children during the first year of life (Table 1).

Omega-3 PUFA treatment comprises of maternal supplementation of 3 capsules of Pikasol® (1g capsules containing 640 mg ω-3 PUFA) 2 times daily during pregnancy and lactation, the placebo capsules contain similar amounts of olive oil. The *L. reuteri* supplementation comprises of *L. reuteri* suspension 10^9 colony forming units (CFU) in oil (refined coconut and peanut oil) (20 droplets x 2 daily) to the mothers during pregnancy and 10^8 CFU (5 droplets x 1) to the children during the first years of life. The placebo comprises similar amounts of oil without *L. reuteri* to the mother and child.

Mothers with current or previous allergy to fish, and mothers previously/currently using omega-3 PUFA or probiotic dietary supplementation will not be included in the study. *Children born before the end of the 33^{rd} gestational week or with serious illness during the neonatal period and children feed parentally for more than 3 days will be excluded from the study.* Breastfeeding for at least 3 months is mandatory for inclusion in the statistical assessment in the study.

The children will be clinically followed by an allergy nurse regularly. Questionnaires regarding data on environment, siblings, pets, breast feeding, smoking exposure, upper respiratory and other infections and clinical symptoms of allergic disease will be filled regularly. Skin prick tests (SPTs) will be performed in the children at 6 and 12 months with milk, egg, wheat, peanut and cat. At 24 months, timothy and birch allergen extracts will be added (ALK-ABELLÓ, Hørsholm, Denmark, Soluprick®). A pediatrician will assess the children at 24 months of life and whenever it is needed during the study period. Dietary habits will be assessed during pregnancy (25^{th} gestational week) and 6 months after child birth.

Blood samples in the children will be taken from cord blood (CB) and at 6, 12 and 24 moths of life. Maternal blood samples will be taken at 20th weeks of gestation and at child birth. Milk samples will be collected 1-4 days after partus (colostrum) and monthly during the first 4 months of lactation. Maternal gastrointestinal function will be addressed by validated diary cards (60). Saliva from the children and fecal samples from mother and child will also be collected according to the following protocol.

Study protocol

Combined *L reuteri* /ω - 3 PUFA supplementation during pregnancy and lactation in relation to allergy in children

	20th week	**Partus**	**1 m**	**2 m**	**3 m**	**4 m**	**6 m**	**12 m**	**24 m**
Clinical examination	nurse	nurse	nur	nur	nur	nur	nurse	nurse	Pediatrician
Clin History Family	**x**								
Questionnaire									
Allergy	**x**				**x**		**x**	**x**	**x**
Dietary/ Bowel function*	**x** **x**						**x**		
SPT							**x**	**x**	**x**
Milk samples		**1-4 d**	**x**	**x**	**x**	**x**			
Blod samples									
Maternal	**x**	**x**							
Children		**CB**					**x**	**x**	**x**
Saliva							**x**	**x**	**x**
Faeces									
Maternal	**x**	**x (1-7 d)**							
Children		**x (5-7 d)**	**x**		**x**			**x**	**x**
Supplementation ω - 3									
Supplementation L reuteri									
Maternal									
Children									

*at 20th and 32th weeks of gestation.
Blood sample from mothers at 20th and 32nd weeks of gestation.

Clinical definitions

A food reaction is defined as gastrointestinal symptoms, hives, aggravated eczema or wheezing following ingestion of a certain food with recovery after food elimination from the diet and reoccurrence of symptoms after ingestion of the particular food. If food specific positive SPT or serum IgE antibodies is present, the food reaction is considered as IgE associated. Eczema is characterized as reoccurring, itching eczematous and lichenified or nummular dermatitis (according to Oranje (62)). If detectable IgE antibodies or a positive SPT are present, it is defined as IgE associated eczema. Doctor diagnosed wheezing at least three times during the first two years is required for the diagnosis of asthma. Together with

allergic sensitisation (circulating IgE antibodies to allergens or positive SPT), asthma is defined as IgE associated asthma bronchiale. Rhinoconjunctivitis is very uncommon at this age but when present it is defined as itching and running eyes and nose in the spring or at allergen exposure (pets) and it is considered allergic if there is corresponding positive SPT or detectable specific IgE antibodies. A child with only clinical manifestations of eczema, food reaction, asthma or rhino-conjunctivitis is diagnosed with allergic disease. Similarly, concomitant sensitisation defines the disease as IgE associated.

Dietary Habits

A 3 day-recall of food intake diary and questions related to fish intake will be filled by the mothers at the 25th week of gestation and 6 months after delivery, A certified dietician will assessed the maternal intake of energy and fat using the software `Dietist XP` (produced by Kost- och näringsdata in Bromma, Sweden, www.kostdata.se) used in clinical routine.

Maternal gastrointestinal function

Maternal gastrointestinal function will be addressed by validated diary cards (60). The mothers will record every single stool, stool consistency, and corresponding defecatory symptoms (urgency, straining, and feeling of incomplete evacuation) for seven days at gestational week 25 and 35. Stool consistency will be defined by the Bristol Stool Form Scale. The mothers will also record every meal, and episodes (start and ending time) of abdominal pain and bloating.

SPT and IgE antibodies

Allergic sensitization is defined as a positive prick-to prick skin prick test (SPT) with a wheel diameter ≥ 3 mm and/or the detection of allergen specific IgE antibodies in serum. SPTs will be performed in the children at 6 and 12 months with milk, egg, wheat and cat. At 24 months timothy and birch allergen extracts will be added (ALK-ABELLÓ, Hørsholm, Denmark, Soluprick®). Specific IgE antibodies towards egg, milk, wheat and cat will be analyzed in serum samples from the infants at 12 and 24 months. At 24 months timothy and birch were added to the analysis (UniCap® Pharmacia CAP System™, Phadia, Uppsala, Sweden). Maternal serum IgE antibodies to a panel of inhalant antigens (Phadiatop®) will also be analysed at the research laboratory, Division of Paediatrics, Linköping University.

Fatty acid analysis

Polyunsaturated fatty acids from the ω-6 (LA, GLA, DHGLA, AA, 22:4 n-6) and ω-3 (LNA, EPA, DPA and DHA) metabolic chains will be analyzed in maternal and child serum phospholipids ant maternal total milk lipids by gas chromatography according to a method originally described by Kaluzny et al (63). This method has been established at the research laboratory, Division of Paediatrics, Linköping University

Gut microbiota analysis

The vast complexity of the gut microbiome of the mothers and children will be addressed by novel powerful DNA sequencing technologies in collaboration with Prof Dusko Ehrlich, coordinator of the EC-funded Metagenomics of the Human Intestinal Tract (MetaHIT) project at INRA, Jouy en Josas, France (64) and Prof Lars Engstrand, Dept of Microbiology, Tumor and Cell Biology, KI (65).

Chemokines

Circulating chemokines associated with Th1-like (CXCL10 & CXCL11), Th2-like (CCL17 & CCL22) and Th17-like (CCL20, CXCL8) responses will be analysed by Luminex in matrenal and infant plasma samples as immune balance biomarkers.

Whole blood cell culture and PGE_2 synthesis

Whole blood cultures will be established from heparinised blood with equal parts of blood and RPMI-1640 (VWR, Stockholm, Sweden) supplemented with 2mM L-glutamine (VWR) and 250 µg/ml gentamycin (Sigma, Sigma-Aldrich, Stockholm, Sweden) and stimulated with 100 ng/ml lipopolysaccaride (LPS) (E.Coli 026:B6, Sigma) for 1h for LTB4 secretion and for 24h for PGE2 and cytokine secretion in 37°C with 5% CO2. Eicosanoids in cell supernatants will be analyzed with commercial ELISA kits from R&D Systems (Abingdon, UK) according to the manufacturer's instructions (55).

PGE_2, TGF-ß2 and SIgA in breast milk

Prostaglandin E_2 will be analyzed with Prostaglandin E_2 Direct Biotrak™ Assay (GE Healthcare, Stockholm, Sweden) according to the manufacturer's instructions. TGF-ß2 and sIgA will be performed according to Böttcher, *et al* (12).

Cytokine production in vitro

Maternal and infant immune regulation will be addressed by analysing Th1, Th2, Th17 and T regulatory PBMC responses to allergens, vaccine antigens and mitogens *in vitro* by Luminex or ELISA. Pro- and anti-inflammatory cytokine responses and innate signalling after TLR2, TLR4 and TLR9 ligand stimulation will also be characterised.

Maternal immune regulatory function

The proportion of "true" resting and activated maternal regulatory T cells will be analysed based on their expression levels of CD4, CD25, Foxp3, CD45RA and CD45R0, according to established methodology (66). Maternal Treg immunoregulatory function will be addressed by evaluating suppressive function of FACS sorted T regulatory cells *in vitro* according to established methodology regarding cytokine secretion (66) and using CFSE labelling for proliferative responsiveness.

Epigenetic regulation

Epigenetic modifications in immunoregulatory loci in Th-cells of the children at birth and later during childhood will be characterised in relation to maternal w−3 and probiotic supplementation. We have established cooperation with Prof Ola Winqvist, KI, to establish methodology in our laboratory to study epigenetic regulation of regulatory T cells and Th1-, Th2- and Th17-like immunity (67, 68). Genomic DNA is extracted from sorted CD4+ CD45RA+ naïve and CD45 RO+ memory cells and then bisulphite converted (68). After performing PCR reactions targeting *e g FOXP3*, *IFN-G*, *IL-13* and *IL-17* loci, methylation sensitive Single NUcleotide Primer Extension (Ms-SNUPE) is performed with the SNaPshot Multiplex kit, using SNaPshot primers of different lengths to distinguish between the different loci [Janson *et al*, in manuscript]. Products from the Ms-SNUPE reactions is then analysed by capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems).

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